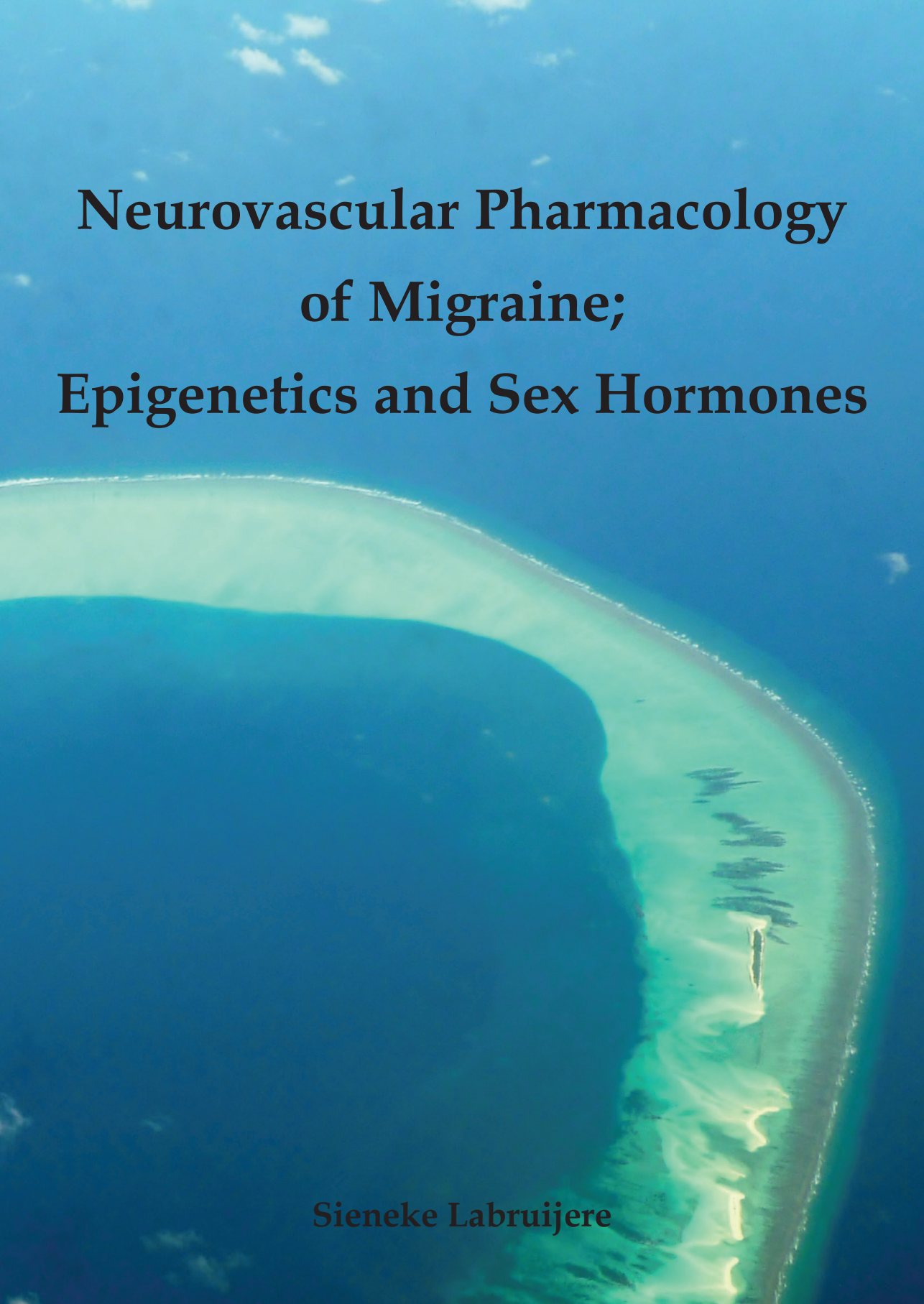
Neurovascular Pharmacology of Migraine; Epigenetics and Sex Hormones



Sieneke Labruijere

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epigenetica en geslachtshormonen

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Part 1 - Introduction

Chapter 1 – A short Introduction. Neurovascular Pharmacology of Migraine; Epigenetics and Sex Hormones.

1

Migraine is a neurovascular disorder characterized by a unilateral throbbing headache that lasts 4 to 72 hours and is often accompanied by nausea, vomiting, photo- and phonophobia [1]. It has a prevalence of 8% in men and 20-25% in women [2-4]. The exact mechanisms behind a migraine attack are not clear, but the trigeminovascular system seems to play a key role in the pathophysiology of migraine [5,6].

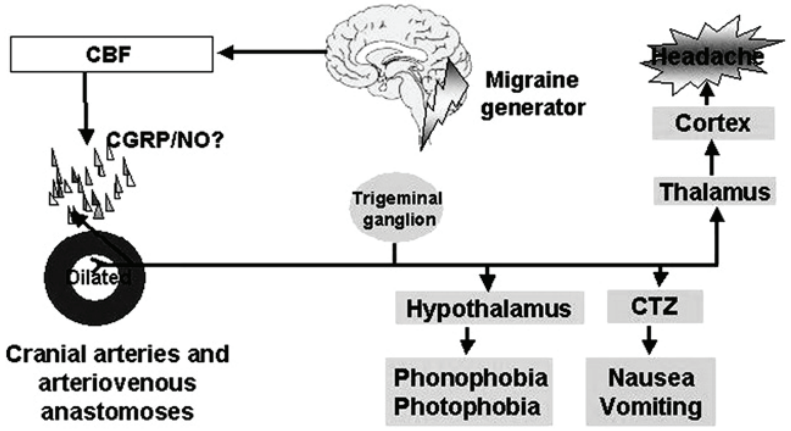

Figure 1. Schematic representation of the pathophysiology of migraine. The pathophysiologic changes in migraine putatively stem from ion leakage through channels in the brain stem, leading to a decreased cerebral blood flow (CBF), possibly owing to cortical spreading depression and, subsequently, neuropeptide release and dilatation of cranial extracerebral blood vessels. The increased pulsation in these blood vessels stimulates the trigeminovascular system, setting in peripheral and central sensitization and leading to headache and associated symptoms (nausea, vomiting, phono- and/or photophobia) [7]. As will be discussed in this thesis, sex hormones, CGRP-ergic and 5-HT-ergic ligands may affect this system at different levels, possibly also via epigenetic mechanisms. CTZ: chemoreceptor trigger zone, NO: nitric oxide.

The increased prevalence of migraine in women is thought to be related to changing estradiol levels, especially by the sudden drop in estradiol levels just before the start of menstruation [8,9]. It still needs to be elucidated how estradiol can cause this increased prevalence of migraine in women, but it has been demonstrated that estradiol can directly affect vascular sensitivity to calcitonin gene-related peptide (CGRP), a key peptide in migraine pathophysiology [10]. Furthermore, estradiol can increase neuronal excitability [11]. On the other hand, it should be kept in mind that, besides female hormones, also male sex hormones may influence neuronal and vascular responses to

different triggers and might therefore also be involved in the difference in migraine prevalence between men and women [10,12,13]. Men have a higher blood pressure than women and suffer more often from hypertension and cardiovascular diseases. Long term exposure to the male hormone testosterone, as present in women suffering from polycystic ovary syndrome as well as healthy men, is thought to cause decreased endothelial function leading to impaired vasodilatation [12,14], while increased vasodilatation is involved in migraine pathophysiology [12,13,15]. These vascular differences, caused by sex hormones might thus be involved in the sex differences in migraine prevalence.

Although twin studies show an inheritance of approximately 50% for migraine [16], a mono-genetic inheritance is only found for a rare form of migraine called familial hemiplegic migraine (FHM) [17]. A contribution of environmental factors, such a fluctuations in hormone levels, is thus likely, although admittedly these factors may also have a genetic component. A possible mechanism inducible by environmental factors is DNA methylation. Changes in DNA methylation of certain regions on the DNA can cause changes in accessibility and consequent changes in gene expression [18]. The methylene tetrahydrofolate reductase (MTHFR) gene, an important enzyme for the DNA methylation cycle, has been suggested to be involved in migraine [19-21], but genome wide significance could not be demonstrated with genome wide association studies (GWAS). However, GWAS on migraine did find a number of genes involved in epigenetic mechanisms [22-26]. Estradiol can change the vascular response to CGRP [10], a key peptide in migraine pathophysiology, and both CGRP and estradiol are involved in epigenetic mechanisms [27,28]. Furthermore, the migraine prophylactic, valproate, can inhibit the two most common epigenetic mechanisms, DNA methylation and histone modifications [29].

There are a number of drugs on the market that can successfully stop or prevent a migraine attack, but these drugs are not effective in all patients. The most used specific anti-migraine drugs are the triptans, which are specific 5-HT1B/1D receptor agonists, but also the less specific ergot alkaloids ergotamine and dihydroergotamine are still prescribed [30]. Both anti-migraine drugs are potent vasoconstrictors. Next to the fact that these drugs are not effective in all patients, ergot alkaloids and triptans can have side effects, due to their vasoconstrictive properties, which can be particularly serious in patients with cardiovascular disease [31]. Therefore, there is a need for new

or improved anti-migraine medication with fewer side effects. Recently, new delivery methods were developed for dihydroergotamine, with lower peak plasma concentrations causing fewer side effects, thus providing a better alternative when triptans are not effective [32]. Furthermore, a new class of drugs was discovered recently, the 'gepants', which are CGRP receptor antagonists [33]. These potentially novel antimigraine drugs prevent CGRP from binding to its receptor. Blockade of the CGRP receptor is shown to be effective in abolishing migraine attacks [34,35]. CGRP receptor antagonists can inhibit vasodilatation without a direct vasoconstrictor effect [31]. Due to liver toxicity and pharmacokinetic problems the development of these compounds is delayed, but the search for a less toxic compound is still ongoing and it most likely will be found in the near future. Obviously, before such a compound can be used as a drug, a detailed effect and side effect profile still needs to be made. In this thesis studies are described that investigate the role of epigenetics and hormones in migraine pathophysiology and vascular function. Furthermore, different studies investigating effects and side effects of existing and potential new anti-migraine drugs are described.

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Chapter 2 - **Aims of the Thesis.**

2 Aims of the Thesis

- The female hormone estradiol is thought to be responsible for the differences in migraine prevalence between men and women, and especially during puberty, pregnancy and menopause, when estradiol levels change dramatically, migraine attack appearance and frequencies increase. In **Chapter 3** an overview is given of the effect of hormones on migraine.

- The exact mechanism behind a migraine attack still need to be elucidated. Because genetic explains only 50% of the likelihood to get migraine, there might be a major contribution of environmental factors. The prophylactic activity of valproate, a DNA methylation inhibitor, points towards the involvement of epigenetic mechanisms. Therefore in **Chapter 4** we investigated DNA methylation of migraine-related genes in different tissues of the rat and we studied whether this methylation is the same in human and rat leukocytes. Furthermore we studied the effect of 17β-estradiol on DNA methylation.

- The female hormone 17β-estradiol is thought to cause the difference of migraine prevalence between men and women. In healthy women estradiol levels fluctuate during the menstrual cycle, but in women with PCOS, androgen levels are increased, causing estradiol levels to become low and stable, comparable to male. In **Chapter 5** we studied vascular function of mice with PCOS and compared this to normal animals.

- The antimigraine drugs ergotamine, dihydoergotamine and the different triptans are all 5-HT receptor agonists. Activation of different isoforms of the 5-HT receptor are thought to be involved in the antimigraine effect of these drugs. This is described in **Chapter 6.** The antimigraine drugs

dihydroergotamine and sumatriptan are contra-indicated in patients with cardiovascular disease because of their vasoconstricting properties, which have been assessed in proximal coronary arteries but never in distal coronary arteries. In **Chapter 7** we investigate the effects of sumatriptan and DHE in proximal and distal coronary arteries, middle meningeal arteries and saphenous veins and in **Chapter 8** the cranioselectivity of sumatriptan is further investigated.

- Calcitonin gene-related peptide (CGRP) is a neuropeptide and potent vasodilator and it is thought to play a key role in migraine pathophysiology. An introduction into existing experimental models to study the effects of newly developed CGRP receptor antagonists, is given in **Chapter 9**. CGRP has shown to terminate the vascular response to endothelin-1, a potent vasoconstrictor. In **Chapter 10** the role of CGRP in this mechanism is further investigated.

- As the most common used antimigraine drugs have a major effect on the vasculature, these effects are thought to play an important role in their antimigraine effect. In **Chapter 11** we studied vascular effects of different substances that may artificially induce migraine-like attacks, affecting the CGRP-ergic as well as other migraine involved mechanisms, in both human and rat middle meningeal arteries.

Part 2 - **Epigenetics and Sex Hormones**

Chapter 3 - Headache and Hormones, Including Pregnancy and Breast Feeding

Based on: Chapter 58. Headache and Hormones, Including Pregnancy and Breast Feeding. S. Labruijere, E. Couturier, A. MaassenVanDenBrink. Written on Invitation, Submitted April 2014, Oxford Textbook of Headache Syndromes.

Characteristics and Prevalence

Migraine is much more common in women than in men, which is attributable to the ovarian female sex hormones. At very young age, there is a slightly higher migraine prevalence in boys, but this difference disappears and switches to an increased prevalence in girls in the years around puberty [1,2]. Already before menarche, a cycling pattern of female hormones is seen in girls, as well as a monthly pattern of migraine attacks [3].

After Puberty

Migraine prevalence is highest during the fertile part of a woman's life when ~25% of all women experience migraine attacks, compared to ~8% of all men (Figure 1) [4,5]. Within women, differences exist in type and amount of migraine attacks. The attacks are often related to the menstrual cycle and these menstrually related attacks are almost always without aura [6]. The different hormone-related migraines in women are divided in four types according to the International Classification of Headache Disorders, 3rd edition, beta (ICHD-3 beta) [7] (Table 1). The most common form, present in approximately 35-50% of female migraineurs, is menstrually related migraine (MRM). ~80% of women suffering from this form of migraine also have attacks not related to their menstruation [8-10]. A small part of around 20% of women with MRM have migraine attacks only during menstruation and this form is called pure menstrual migraine [6]. The female hormone 17β-estradiol is thought to play an important role in the increased migraine attacks during menstruation, but also progesterone is suggested to be involved in menstrual migraine [11,12]. Possible underlying mechanisms are discussed further on in this chapter. Figure 2 shows the relation between female hormone levels and migraine attacks. Especially when estradiol levels drop, just before menstruation, after delivery or perimenopausal, migraine prevalence increases [13].

Artificially administrated hormones can also influence migraine attacks. The effect of combined oral contraceptives (COC's) and hormonal intrauterine devices on headache attacks is extensively studied [14]. Approximately 20-30% of women using hormones on a regular basis for contraception or hormone replacement therapy experience worsening of their migraines or newly develop headache or migraine attacks. This form of headache is called exogenous hormone-induced headache [7]. These attacks

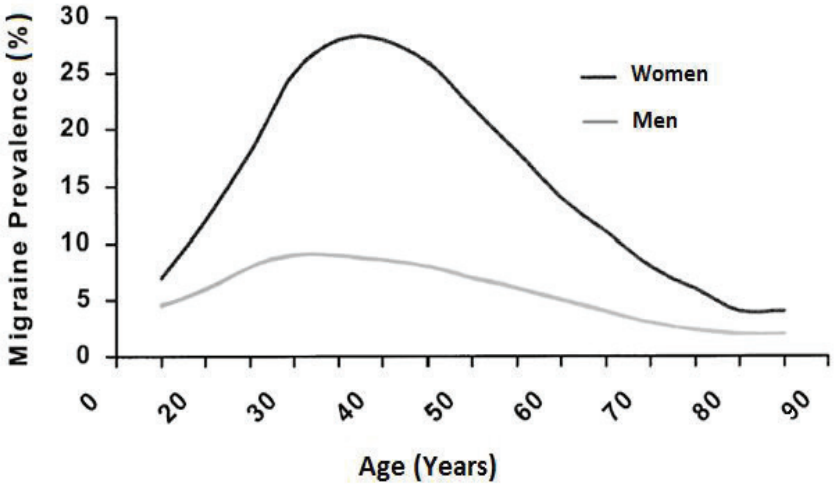

Figure 1. Migraine prevalence in women compared to men. Based on Lipton et al, 2001 [4].

Table 1. Characteristics and Prevalence of 4 subtypes of hormone related migraine in women.

	Characteristics according to ICHD-3 beta [7]	Prevalence
Pure Menstrual Migraine without aura	• Migraine attacks fulfilling ICHD-3 criteria 1.1 • Exclusively during menstruation • In at least 2 out of 3 menstruations	7% of migrainous women (~1,8% of all women) [6]
Menstrually-related migraine without aura	• Migraine attacks fulfilling ICHD-3 criteria 1.1 • In at least 2 out of 3 menstruations • Additional attacks at other times during the cycle	22% of migrainous women (~9% of all women) [6]
Headache attributed to exogenous hormones	• Headache or migraine according to ICHD-3 criteria • Headache develops or worsens significantly after hormone intake • Headache improves or resolves after reduction or ending of hormone intake	Worsening of headaches in 30% of oral contraceptive users, new onset headache in 5-13% of oral contraceptive users [16]
Estrogen withdrawal headache	• Headache or migraine according to ICHD-3 criteria • Headache or migraine develops within 5 days after interruption of daily consumption of exogenous estrogen for 3 weeks or longer (often during the pill-free interval of oral contraception or following hormone replacement therapy)	70% of oral contraceptive users [14]

often disappear after prolonged use or when the use of hormones is discontinued completely [14-16]. Improvement of migraine symptoms, especially aura, is sometimes also observed after use of hormonal contraceptives, but only in a small amount of women. Progestins might be important in this improvement, but more studies are needed to confirm this hypothesis [14,17]. Additionally, discontinuation of hormones can lead to headache or migraine attacks and is called estrogen withdrawal headache. This form of headache is most common during the pill free interval of oral contraception [18]. It is reported in up to 70% of women using oral contraception [19].

Pregnancy

During pregnancy estradiol levels are 10-100 times higher than in normal cycling women (Figure 2). 50-90% of women suffering from migraine without aura report improvement of their migraine attacks, especially during the second and third trimester of their pregnancy. In 10-20%, these attacks even disappear completely during pregnancy. Estrogen levels increase during each trimester and negatively correlate with migraine incidence. It is suggested that the absence of fluctuations in estradiol levels are responsible for the decreased migraine incidence during pregnancy [20-23]. Migraine attacks with aura can also improve during pregnancy, but more often remain the same or become worse than migraine without aura attacks [24,25]. When migraine attacks start for the first time during pregnancy, which occurs in 2-15% of pregnant women, these attacks are more often attacks of migraine with aura than without aura [24-26]. Often, no change in frequency of attacks is observed in women who already suffered from migraine with aura before pregnancy [24]. After pregnancy, migraine returns back to the pattern observed before pregnancy in most cases. 30-40% of all women suffer from headache during the first week postpartum. This is most prevalent in women who were already migraine sufferers [26]. The drop in estrogen levels caused by the delivery is thought to play an important role in the development of these headaches [27].

Lactation

Lactation can inhibit ovulation and may therefore influence female hormone levels [28]. A few studies have investigated migraine prevalence during exclusive breastfeeding and the results are contradictory. A large prospective study in Norway in women with migraine did not show any influence of breastfeeding on migraine [29] and also in a

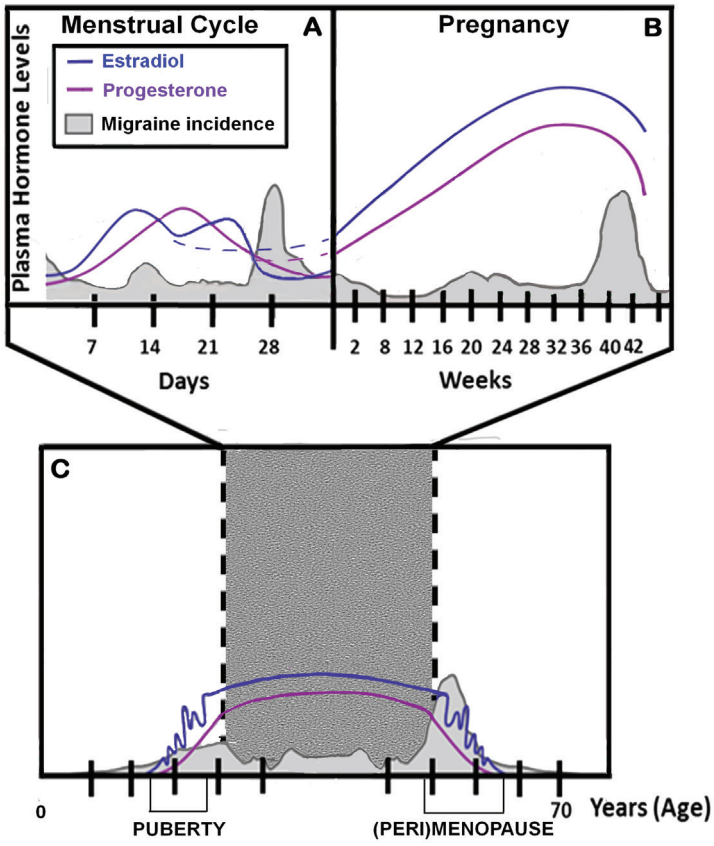

Figure 2. Migraine incidence and female hormones during the menstrual cycle, pregnancy and a woman's life. Adapted from Sacco et al, 2012 [57].

study in the USA in women with both tension type headache and migraine no effect of breastfeeding was found on headache prevalence [30]. On the other hand, studies in Japan, Brazil and Italy showed decreased recurrence of migraine in the first months after pregnancy during breastfeeding [20,26,31]. The effect of partial breastfeeding on migraine prevalence is so far never studied. So, although prevalence studies are contradictory, anovulation caused by breastfeeding is thought to lead to a decrease in menstrual migraine in breastfeeding women [26].

Menopause

The menopause is determined as the day one year after the last menstruation of a wom-

an. During the transition phase from normal ovulating women towards the menopause, worsening of migraine symptoms is often observed, probably due to changing hormone levels (Figure 2). These effects are seen on migraine attacks without aura, which are often related to the menstrual cycle, but not on migraine attacks with aura [32,33]. After the menopause the frequency of migraine attacks without aura often decreases, the attacks become less severe or they even disappear [34-36]. In a study on spontaneous postmenopausal women, a migraine prevalence of 10.5% was observed [35,37], which is considerably less than the 25% prevalence that is seen in normally ovulating women. After the menopause, estradiol levels become stable and this is thought to be the main cause of the decreased migraine incidence seen in postmenopausal women. There is also a difference in effect on migraine attacks by the type of menopause. After a surgical menopause, there is less relief of the migraine symptoms than after a natural menopause; hence it has been suggested that older ovaries may produce factors that improve migraine symptoms [38].

Pathophysiology

During the menstrual cycle, female hormone levels fluctuate (Figure 2). Estradiol levels drop abruptly just before menstruation. This drop in plasma estradiol level is thought to play an important role in the migraine attacks that are often seen at this point of the cycle. Indeed, administration of estradiol during this period can postpone a migraine attack [13,39]. Probably, prior to the drop in estradiol levels some days of sustained high estradiol levels are required to cause a migraine attack. This could explain why the increase in migraine incidence around the ovulation is only modest, while prior to ovulation also a high estradiol peak is present [40]

17β-estradiol

17β-estradiol is a small lipophilic molecule that is mainly produced by the ovaries and is capable of crossing the blood brain barrier. There are two different types of pathways via which estradiol can exert its effects, genomic and non-genomic pathways. Genomic pathways can be activated via binding to the intracellular estrogen receptors, estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ) [41]. After binding to ERα or

ERβ in the cytoplasm, the complex enters the nucleus and estradiol can bind to estrogen responsive elements on the DNA, thereby influencing gene expression [42]. Furthermore, estradiol can activate genomic mechanisms via binding to the membrane-bound G-protein coupled estrogen receptor, activating intracellular signaling pathways that influence gene expression, like the MAPK/ERK pathway [43]. Estradiol can also influence gene expression via epigenetic mechanisms, for example by changing the amount of promoter methylation of a target gene [44]. The fast non-genomic pathways can be activated through binding of estradiol to membrane-bound estrogen receptors, activating different intracellular signal transducing pathways, leading for example to vasodilatation [45] and inhibition of apoptosis [46].

Effects of estradiol on neurotransmission

Estradiol can easily cross the blood brain barrier, where it can exert its effects, but it is also thought to be locally synthesized in the brain [47,48]. Important brain structures that are involved in migraine pathophysiology, especially via transmission of nociceptive information, are the trigeminal system, cortex, brainstem and thalamus, which can all be affected by estradiol.

Cortical excitability changes during the menstrual cycle [49] and high estradiol levels can increase neuronal excitability and sensitivity of the brainstem and trigeminal nucleus caudalis (TNC) via non-genomic pathways [42]. Estradiol has shown to influence gene expression in the trigeminal ganglion of the rat [50], a structure which is involved in migraine attacks, and a decrease in estradiol levels has shown to inhibit neuropeptide Y gene expression, which is an inhibitor of synaptic CGRP release [51]. Estradiol can affect different neurotransmitter systems including the serotonergic, glutamatergic, GABA-ergic and CGRP-ergic system, which are all neurotransmitter systems involved in pain signaling in migraine pathophysiology. Serotonin synthesis and neuronal firing are influenced by estradiol levels. Monkeys treated with estradiol showed a nine-fold increase in tryptophan hydroxylase mRNA expression, a rate-limiting enzyme for serotonin synthesis, compared to controls [52,53]. Furthermore, estradiol can enhance the glutamatergic system by causing increased dendritic spine formation and it can inhibit neuronal hyperpolarization induced by GABA, an important inhibitory neurotransmitter, also enhancing neuronal responsiveness [42]. Expression of opiate receptors involved in analgesia is also influenced by estradiol, leading to increased neu-

ronal responsiveness and possible hyposensitivity to opioids during the premenstrual period [54]. Furthermore, estradiol has an effect on CGRP, which is expressed in different regions of the brain and is involved in pain pathways. In the rat dorsal root ganglion estradiol has shown to be able to increase CGRP synthesis [55].

The rise and fall of estradiol levels might thus lead to imbalanced genomic and non-genomic effects in different brain structures, leading to increased neuropeptide release, neuronal excitability and consequent migraine attacks [56].

Effects of estradiol on vasculature

Next to its central effects, estradiol affects the vasculature [57] and estradiol itself can act as a vasodilator. Estradiol induced vasodilatation is caused by the release of NO after activation of the non-genomic PI3-kinase pathway [45]. Estradiol can also affect the vasodilatory response to other stimuli. In a rat model, CGRP release caused by electrical stimulation close to the dural artery, leads to increased maximal relaxation of this artery in rats treated with estradiol compared to rats treated with placebo [58]. At the same time decreased levels of the vasoconstrictor 5-HT were observed [59]. In porcine coronary arteries the response to 5-HT decreased after physiological concentrations of estradiol [60]. However, the effects of estradiol on the vascular system do not seem to be completely straight forward. For example, in a study with 60 women, increased nitric oxide (NO) pathway activation was observed during the late luteal phase of the menstrual cycle, when estradiol levels rise, while migraine attacks occur when estradiol levels drop. This increased NO-synthase activity, via activation of PI3-kinase, can lead to an increase in NO release and thus increased vasodilation [61,62]. Further, a study was performed that compared dermal blood flow (DBF) responses after capsaicin application between women with MRM and healthy controls. DBF is a measure for the potency of the vessels of the skin to dilate. No difference in DBF were seen during the cycle in MRM patients, but in healthy controls DBF was increased at day 1-2 of the menstruation. Furthermore, estradiol levels were higher at day 19-20 of the cycle in healthy controls compared to MRM patients [63]. These studies all point to an effect of estradiol on the potency of a vessel to dilate and which is possibly increased before or at the start of menstruation, however the results seem sometimes controversial and exact mechanisms still need to be discovered.

Progesterone

Next to fluctuating estradiol levels, also progesterone levels change during the menstrual cycle, during pregnancy and perimenopausally (Figure 2). While progesterone probably is also involved in migraine pathophysiology, its effects can be synergistic as well as antagonistic compared to estradiol.

The progesterone receptor is often co-localized with the estrogen receptor and estradiol can influence its expression in the brain [42]. On the other hand, progesterone can lower estrogen receptor expression [64]. Progesterone levels are low during the follicular phase and increase during the luteal phase of the menstrual cycle (Figure 2). Increased urinary levels of progesterone metabolites were negatively correlated with migraine during the luteal phase of the menstrual cycle [12]. The authors suggest a possible preventive effect of intermediate progesterone levels on migraine attacks. Where estradiol has an excitatory effect on neuronal excitability via increased glutamate activity, progesterone can have an inhibitory effect via GABA-mediated chloride conductance [65]. Furthermore, GABA receptor induced decreased plasma protein extravasation in the trigeminal ganglion as well as decreased cFOS expression is suggested to be involved in this protective mechanism [12,66,67]. The progestins in the progestin-only oral contraceptives might thus also be involved in the decreased migraine frequency observed in some women using progestin-only oral contraceptives [17]. On the other hand, the same authors show that high levels of progesterone are also associated with worse migraine outcome. They suggest that there may be a turning point, after which progesterone is not beneficial anymore, but becomes a trigger [12]. During pregnancy both progesterone and estradiol levels are high (Figure 2) and it has been suggested that the new onset migraine during pregnancy is more often migraine with aura, because of the combined excitatory effects estradiol and progesterone can have on neuronal excitability and cortical spreading depression (CSD) [41,68].

Genetics

Family studies show a heritability of ~40% for migraine [69,70] and a monogenetic inheritance pattern has only been identified for familial hemiplegic migraine. No specific inheritance study has been performed for menstrual migraine, but no differences in inheritance of total migraine are found between women and men [71]. In a recent study a relation between estrogen receptor 1 (ESR1, the gene coding for ERα) polymorphisms,

and migraine was found [72], but the mechanisms behind this are unknown.

Epigenetics

As estradiol is known to be an epigenetic modulator, there may be a major contribution of epigenetic mechanisms. The methylene tetrahydrofolate reductase gene (*MTHFR*), encoding an important protein of the DNA methylation cycle, has been suggested to be involved in migraine [73,74], and mutations in this gene play a role in altered estradiol synthesis by the ovaries [75]. Moreover, the migraine prophylactic valproate inhibits DNA methylation and histone modifications, although no difference in effect is found between women and men [76]. It might thus well be possible that epigenetic changes of genes involved in migraine pathophysiology lead to the increased prevalence of migraine in women. A study in rats did not find changes in DNA methylation after treatment with estradiol, but the authors indicated that this could be due to insufficient statistical power [77]. Thus, more studies are needed to investigate the possible epigenetic effects of female hormones in migraine.

Summary and conclusion

Estradiol plays an important role in the increased migraine prevalence that is seen in women compared to men, but there is possibly also a role for progesterone [68]. Especially the drop in estradiol levels before menstruation and after delivery, as well as the increased fluctuations perimenopausally are thought to trigger mechanisms leading to migraine attacks [13,22]. Migraine with and without aura seem to be affected by female hormones in a different manner. While dropping estradiol levels seem to be related to migraine without aura, migraine with aura is more often observed during high estradiol levels [6,24,25,32,33]. More research is needed to elucidate the exact mechanisms and pathways behind it. Although an effect of estradiol on epigenetic changes in genes involved in migraine pathophysiology has not yet been established, both estradiol and CGRP are involved in epigenetic mechanisms and thus estradiol might also affect migraine in an epigenetic manner.

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Chapter 4 - Methylation of Migraine-related Genes in Different Tissues of the Rat.

Based on: S. Labruijere, L. Stolk, M. Verbiest, R. De Vries, I.M. Garrelds, P.H.C. Eilers, A.H.J. Danser, A.G. Uitterlinden and A. MaassenVanDenBrink, January 2014, Plos One

Abstract

Introduction - 17ß-Estradiol, an epigenetic modulator, is involved in the increased prevalence of migraine in women. Together with the prophylactic efficacy of valproate, which influences DNA methylation and histone modification, this points to the involvement of epigenetic mechanisms. Epigenetic studies are often performed on leukocytes, but it is unclear to what extent methylation is similar in other tissues. Therefore, we investigated methylation of migraine-related genes that might be epigenetically regulated (CGRP-ergic pathway, estrogen receptors, endothelial NOS, as well as MTHFR) in different migraine-related tissues and compared this to methylation in rat as well as human leukocytes. Further, we studied whether 17ß-estradiol has a prominent role in methylation of these genes

Materials and Methods - Female rats (n=35) were ovariectomized or sham-operated and treated with 17β-estradiol or placebo. DNA was isolated and methylation was assessed through bisulphite treatment and mass spectrometry. Human methylation data were obtained using the Illumina 450k genome-wide methylation array in 395 female subjects from a population-based cohort study.

Results and Discussion - We showed that methylation of the *Crcp, Calcrl, Esr1* and *Nos3* genes is tissue-specific and that methylation in leukocytes was not correlated to that in other tissues. Interestingly, the interindividual variation in methylation differed considerably between genes and tissues. Furthermore we showed that methylation in human leukocytes was similar to that in rat leukocytes in our genes of interest, suggesting that rat may be a good model to study human DNA methylation in tissues that are difficult to obtain. In none of the genes a significant effect of estradiol treatment was observed.

Introduction

Migraine is a neurovascular disorder affecting 8-17% of the population [1,2]. Little is known about the cause of migraine and the prophylactic medication that is used to prevent migraine is only effective in half of the patients [3]. Family studies show a heritability of ~40% [4,5] and a monogenetic inheritance pattern has only been identified for familial hemiplegic migraine. Hence, there may be a major contribution of environmental (including hormonal) influences, possibly via epigenetic mechanisms. The methylene tetrahydrofolate reductase gene (*MTHFR*), encoding an important protein of the DNA methylation cycle, has been suggested to be involved in migraine [6,7], although genome-wide association studies did not confirm this. However, they pointed to other genes involved in epigenetic mechanisms [8-10]. Moreover, the migraine prophylactic valproate, inhibits histone deacetylation as well as DNA methylation [11]. Finally, 17β-estradiol, which seems to be responsible for the 2-3 times higher prevalence of migraine in women compared to men, may exert at least part of its effects via epigenetic mechanisms [12,13]. Thus, DNA methylation may be involved in migraine pathophysiology. DNA methylation occurs at cytosines of CpG dinucleotides, often localized in CpG rich regions called CpG islands [14]. CpG island methylation is changed in different types of cancer, during aging, and probably also during the menstrual cycle [15-18]. Environmental factors that might be responsible for these epigenetic mechanisms include female hormones and nutrition [19,20]. DNA methylation can differ greatly between tissues, so ideally it should be studied in the tissue of interest. For complex brain diseases like migraine, this is technically impossible in humans. Yet, blood can be derived easily, and thus if the DNA methylation pattern of leukocytes is correlated to that of other tissues, this would allow conclusions from methylation studies in leukocytes.

The aim of our study was to compare the methylation of genes that are probably involved in the generation of a migraine attack and might be epigenetically regulated [21,22], as well as to examine the role of 17ß-estradiol in the methylation of our genes of interest. We focussed on the CGRP-ergic system because of its prominent role in migraine [23] We investigated DNA methylation in the Calcitonin related peptide alpha *(Calca),* receptor activity-modifying protein 1 *(Ramp1),* calcitonin receptor component protein *(Crcp),* calcitonin receptor-like receptor *(Calcrl),* upstream stimulating factor

2 *(Usf2)*, Estrogen receptor 1 *(Esr1),* G-protein coupled estrogen receptor 1 *(Gper*), nitric oxide synthase 3 *(Nos3)*) and *Mthfr* genes in several tissues relevant to the pathophysiology of migraine (dura mater, trigeminal ganglion and trigeminal caudal nucleus). We investigated methylation in leukocytes and aorta as a peripheral control. To investigate whether DNA methylation in rats may be representative of that in humans, we also studied DNA methylation in leukocytes obtained from healthy human females.

Materials and Methods

Ethics Statement

The animal experiments were performed in our laboratory with permission of the ethics committee of the Erasmus Medical Center in Rotterdam, The Netherlands (Permit Number: EMC2345(127-11-02)). All surgeries were performed under sodium pentobarbital anesthesia and all effort was made to minimize suffering.
The human blood samples were obtained from the Rotterdam Study [24], which has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of The Netherlands Ministry of Health, Welfare and Sports. All subjects provided written informed consent.

Animals

Female Sprague Dawley rats (Harlan Netherlands, Horst, The Netherlands) (N=11-12 per group, weight at the start of the study ~255 g) (Table 1) were kept at room temperature (22ºC) at a 12/12 hours dark/light cycle with unlimited access to food and water in their home cages. The animals were anesthetized (50 mg/kg) and ovariectomized or sham-treated on day 1 of the study. After 7 days, a pellet releasing placebo or 17β-estradiol (21-day release pellet, 12 μg/day, Innovative Research, USA) was implanted subcutaneously in the neck. On day 21, the animals were sacrificed via an overdose of sodium pentobarbital (200 mg/kg). A leukocyte differentiation count was performed to verify whether the proportion of different types of leukocytes was the same for all animals. 0.5 ml whole blood, dura mater, trigeminal ganglia, caudal nuclei and a 5-mm segment of thoracic aorta were snap frozen in liquid nitrogen and stored at -80 ºC for DNA isolation. Because an epigenetic study as in the current experiments has not been performed previously, we based the number of animals on our previous results, showing increased vascular endogenous CGRP responses after treatment with

17β-estradiol [25] and explored whether differences in DNA methylation caused by 17β-estradiol can be demonstrated in this model.

Body weight, vaginal smears and estradiol, CGRP and progesterone concentrations

To establish that the ovariectomy (OVX) operation and pellet implantation were successful, estradiol concentrations were measured in blood plasma at day 1, day 7 (at the end of the implantation procedure) and day 21. At day 7 and 21 an increased estradiol concentration is seen in ovariectomized animals treated with estradiol pellets (Table 2). A vaginal smear was taken at all three time points to establish the phase of the estrous cycle according to proportions of epithelial cells, cornified cells and leukocytes present in the smear. Cyclic activity was reduced after OVX and increased after consequent treatment with estradiol pellets, compared to the sham-operated animals, which showed a normal cycle (results not shown). Body weights of the animals in the three different treatment groups are shown in Table 1. No differences in bodyweight were seen at day 1 and day 7. The increase in bodyweight at day 21 in the ovariectomized animals treated with placebo pellet was significantly larger than that in the ovariectomized animals that were treated with an estradiol pellet. Concentrations of CGRP, at day 1, 7 and 21, and progesterone, at day 21, were also measured and no significant difference was found for the different treatment groups (Table 2).

Table 1. Body weight (g) and bodyweight changes (Δ, g) in rats after different treatments (n=11-14). * $p < 0.05$ compared to OVX placebo. Differences were calculated using one-way ANOVA.

	Sham-operated rats	Ovariectomized rats treated with:	
		Placebo	17β-estradiol
Day 1	254±3	256±2	255±4
Day 7	253±4	259±2	255±5
Day 21	266±3	280±6	254±3
Δ Body Weight at day 21	11±3	25±7	-1±4*

Table 2. Estradiol and CGRP concentrations measured at day 1, day 7 and day 21 and progesterone concentrations measured at day 21. * $p < 0.05$ compared to both sham and OVX animals treated with placebo pellet. Differences were calculated using one-way ANOVA.

		Sham-operated rats	Ovariectomized rats treated with:	
			Placebo	17β-estradiol
Estradiol concentration (pg/ml)	Day 1	12±1	25±8	20±5
	Day 7	19±11	9±1	130±39*
	Day 21	16±5	12±3	133±14*
CGRP concentration (pg/ml)	Day 1	69±4	74±4	73±4
	Day 7	77±4	61±7	74±4
	Day 21	67±4	68±3	69±4
Progesterone concentration (pg/ml)	Day 21 #	12±3	10±2	16±5

#) Progesterone was not measured at day 1 and 7 because of the limited availability of blood.

DNA methylation measurements

DNA of leukocytes, thoracic aorta, dura mater, trigeminal ganglia and caudal nuclei from all animals was isolated (DNeasy, Qiagen, Germantown, MD, USA) and quantified (Nanodrop, Thermo Scientific, Wilmington, DE, USA). CpG islands of the genes of interest were determined using UCSC (http://genome.ucsc.edu/) and Ensemble genome browsers (http://www.ensembl.org). Primers were designed for each CpG island located in the promoter region of the genes of interest with the EpiDESIGNER primer design software (Sequenom, San Diego, CA, USA, Table 3) . When the CpG island could not be covered in total, multiple primer sets were designed to cover the largest possible part of the CpG Island. With the BiSearch web server [26] primer sets were checked for having only one product. 500 ng DNA of all tissue samples was treated with bisulfite (EZ-96 DNA-methylation kit (Shallow), Zymo Research, Irvine, CA, USA) for 16 hours to convert all non-methylated cytosines into uracil nucleotides. After bisulfite conversion PCR, reverse transcription and uracil specific cleavage was performed. For quantitative DNA methylation measurements the MassARRAY EpiTYPEr was used (Sequenom, San Diego, CA, USA). 2.5 μg of low

methylated and 2.5 µg high methylated genomic rat DNA (EpigenDx, Hopkinton, MA, USA) were mixed into 0%, 25%, 50%, 75% and 100% methylated DNA as a control and also treated with bisulfite. To check the accuracy of the methylation measurements, methylation of a standard curve, of which the % of methylation is known (0-100%, increasing in steps of 10%), was measured for all amplicons (Figure 1).

Table 3. Primers and conditions for PCR on CpG island located in the promoter region of genes of interest.

Amplicon	Primer Sequence	Amplicon size (bp)	Number of analyzed CpG's
Calca part 1	Forward-TTTAAATGGTGTTATTTTGTTAGATGTT Reverse-TAAACAAAAACCTCAAAACTCACCT	282	7
Calca part 2	Forward-GTAATTGTGGTTGTTGGTTTTTGTT Reverse-CACCAAATAAACCCTAAAATTCCTA	405	7
Ramp1	Forward-GGGGGTTATGGTAAGTAGAGTTT Reverse-TTACAAAACAAACCCCAAAATAACT	294	17
Crcp	Forward-TTAGTAGTGGGTTTAGGAAGAGAGTG Reverse-CTAAAAAACAAAATTCTAAATACACAAAAC	290	11
Calcrl	Forward-GGGTTTTGTTGTTTGGATTTTA Reverse-TCCAAAAACTTACCTTATAACCTATTCA	24	2
Usf2	Forward-GGTAGTAGTGTTGATTTTGGTGGG Reverse-TCACCTAACCTCCATTACTCTCTATAC	411	15
Esr1 part 1	Forward-GGTAGTAGGGTATTTGGTGGTTATG Reverse-CAACTCAAAATACCCATAAAAAAAA	321	9
Esr1 part 2	Forward-TTTTTTGATTTTTTAGAAGGGTGG Reverse-TAATCTAAAAACTTTCCCCCAACTC	293	13
Gper1 part 1	Forward-GAAGATTATTTTTAGGGTTTTTTGTTTG Reverse-ACACCTTCATATCCCTCTTCCTACAA	397	7
Gper1 part 2	Forward-TTGTTGTATATGTTGATTTGTAGGAAGA Reverse-CCCAAACCTATACTTCATCAACCTAA	152	2
Nos3	Forward-TTTTTGTAAAGAAAAATTTTGGGTG Reverse-CACAATAAAAACTACCCCTAAACCT	404	11
Mthfr	Forward-GGGGTGATAGTTATTATAAGTTTATAGGTT Reverse-TTAACTAACTTCCCAAAAAACCTCC	244	12

A 10mer (aggaagagag) sequence was added to the forward primers and a T7 sequence (cagtaatacgactcactatagggagaaggct) to the reverse primers. The primers were obtained from Invitrogen (Life Technologies Corporation, Carlsbad, CA, USA).

After the validation steps, a PCR was performed in triplo for all amplicons on the bisulfite-treated samples. Percentage methylation for each CpG in all genes and tissues was calculated with Sequenom EpiTYPER software (MassARRAY EpiTYPER Analyzer software v1.0, build1.0.6.88 Sequenom, Inc, San Diego, USA). Samples were excluded when the standard deviation of triplos was larger than 10%.

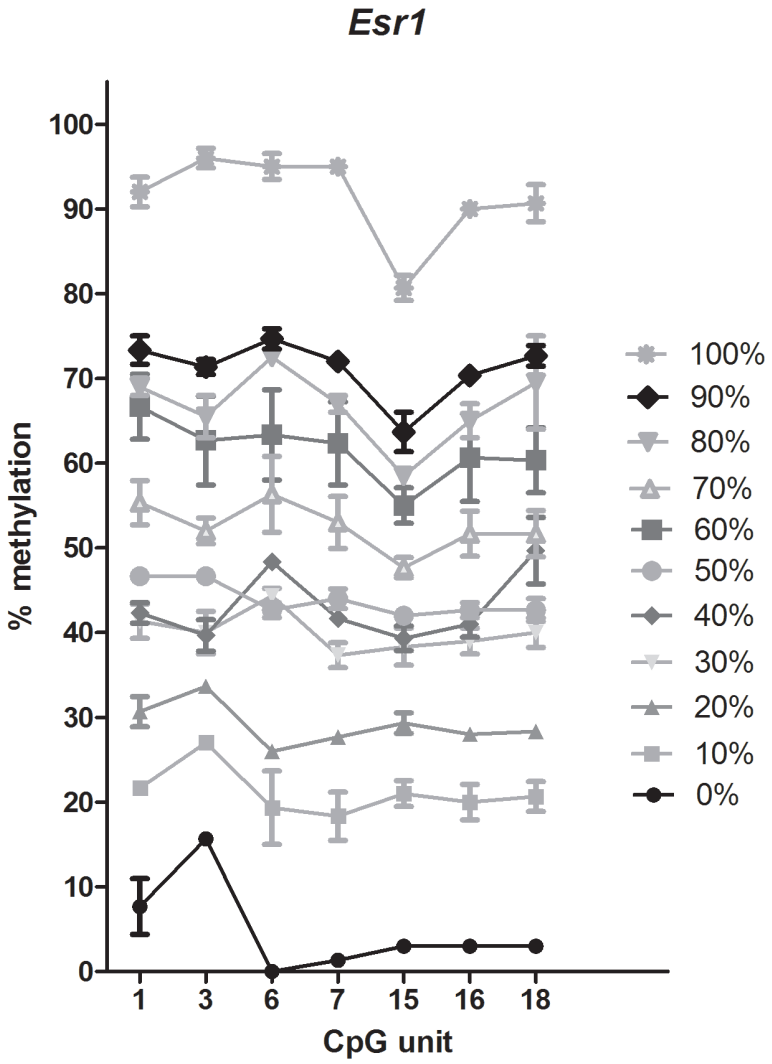

Figure 1. Example of standard curve, showing the distribution of methylation of the known samples for the Esr1 gene.

Genome-wide methylation study in humans

DNA methylation profiles from whole blood were assessed in a subset of 395 healthy women (age ≥ 45) from the Rotterdam Study-III, a population-based cohort study in the Netherlands. The design and rationale of the study has been published previously [24]. Samples were excluded when showing a low detection rate (< 99%), incomplete bisulfite conversion, or gender swaps. Probes with a detection p-value > 0.01 in > 1% samples, were filtered out. β-values for the two assay-types on the array were corrected with SWAN (Subset-Within-Array-Normalization). Mean and standard deviation for the probes in the equivalent human genomic regions were calculated for 395 samples that passed quality control filters.

Data analysis

Calculations of differences in methylation and correlation between tissues were performed using SPSS software. The linear mixed model was used to estimate the size and standard errors of the effects of tissue and treatment. The model contains random effects for the individual rats, to correct for the strong correlations between CpGs within a gene. Typically, when one CpG shows a low (or high) level of methylation for a chosen rat, all the other CpGs show a low (or high) level as well. A strong example is shown in Figure 2, for the *Crcp* gene in the trigeminal ganglion. The profiles for different rats run more or less in parallel, but at quite different levels. Subtracting the mean of all CpGs per rat removes the differences almost completely. The computations were done with the R system [27], version 2.15.2, using the function lmer in the library lme4.

Results

DNA methylation measurements

Because all CpGs per CpG island that we studied were strongly correlated, we calculated the mean methylation of a CpG island and used these values for further analysis. DNA methylation below 5% was observed for the *Calca, Ramp1, Usf2 and Mthfr* genes in all tissues (Table 4). DNA methylation higher than 90%, was observed for the *Gper* gene in all tissues (Table 4). DNA methylation differed substantially between tissues for the *Nos3, Esr1, Crcp* and *Calcrl* genes. *Nos3* showed a two times significantly higher DNA methylation in leukocytes than in the other tissues. Methylation of the

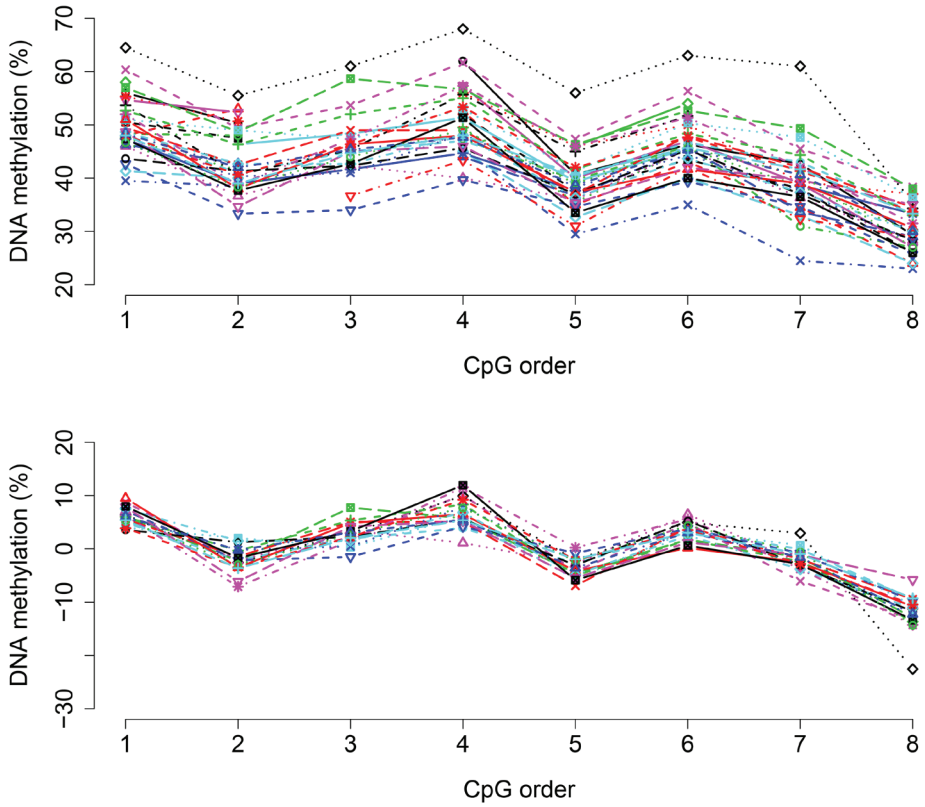

Figure 2. Methylation of the Crcp gene in the trigeminal ganglion. Methylation of each studied CpG of the promoter region of the Crcp gene is shown without correction for rat levels (upper panel) and with correction for rat levels (lower panel).

Crcp gene was high in aorta, leukocytes and trigeminal caudal nucleus, and significantly lower in dura mater and trigeminal ganglion. The *Calcrl* gene showed low methylation in leukocytes, intermediate methylation in dura mater and trigeminal caudal nucleus and high methylation in aorta and trigeminal ganglion. DNA methylation of *Esr1* was significantly higher in the aorta than in other tissues (Figure 3 and Table 4). Remarkable is that the variation in DNA methylation is small in some tissues and large in others. This is not a tissue-specific pattern, but it varies between tissues for the *Nos3, Esr1, Crcp* and *Calcrl* genes, as illustrated by the differences in standard deviation (Table 4).

Estradiol treatment did not induce any statistically significant differences in DNA methylation in any of the examined genes in any investigated tissue (Figure 3).

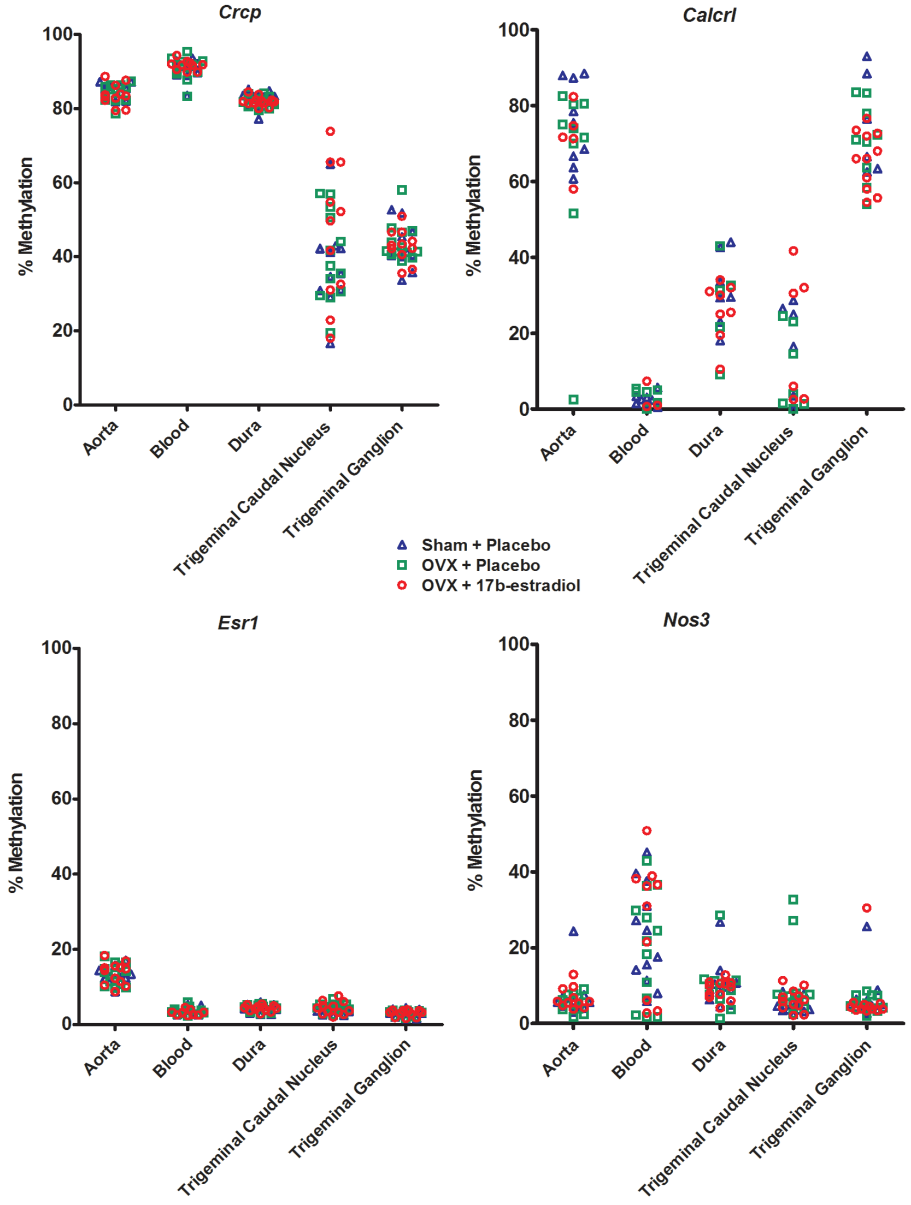

Figure 3. DNA methylation of Crcp, Calcrl, Esr1 and Nos3 in different tissues.
Mean methylation of the promoter region of the Crcp (upper left panel), Calcrl (upper right panel), Esr1 (lower left panel) and Nos3 (lower right panel) genes is shown for the different tissues studied. Each symbol represents one animal and the different colors and symbols represent the treatment of the animals. Red circle: OVX + 17β-estradiol; Green square: OVX + placebo; Blue triangle: sham + placebo.

Table 4. Mean methylation of CpG islands of candidate genes in aorta, leukocytes, dura mater, trigeminal caudal nucleus and trigeminal ganglion.

	Aorta	Leukocytes	Dura	Trigeminal Caudal Nucleus	Trigeminal Ganglion
	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)	Mean ± SD (%)
*Calca**	2.3 ± 0.6	4.2 ± 1.6	2.4 ± 0.7	2.8 ± 1.0	2.5 ± 1.1
*Ramp1**	2.3 ± 0.5	2.8 ± 0.8	2.3 ± 0.5	3.2 ± 0.7	2.5 ± 0.6
*Crcp**	84.3 ± 2.4	91.0 ± 2.5	82.1 ± 1.6	41.1 ± 14.1	43.2 ± 5.1
*Calcrl**	70.6 ± 17.6	2.9 ± 2.2	28.2 ± 9.6	15.0 ± 13.5	69.6 ± 10.3
Usf2	1.5 ± 0.3	1.7 ± 0.6	1.5 ± 0.3	1.5 ± 0.3	1.6 ± 0.3
*Esr1**	13.4 ± 2.6	3.4 ± 0.9	4.3 ± 0.9	4.1 ± 1.3	3.0 ± 0.8
*Gper**	93.7 ± 0.8	93.4 ± 1.7	92.6 ± 0.7	90.2 ± 2.2	92.9 ± 0.8
*Nos3**	6.4 ± 3.9	23.0 ± 14.6	9.7 ± 5.1	7.1 ± 6.0	6.1 ± 5.6
Mthfr	2.0 ± 0.4	2.0 ± 0.6	1.9 ± 0.5	2.2 ± 0.6	1.9 ± 0.4

*) Significant differences between tissue means are present within the respective gene. P-value < 0.0001.

Therefore, the data of the different treatment groups were pooled for further statistical analyses, where treatment effect was not included.

The DNA methylation of leukocytes and that of the other tissues were compared to each other for all genes. No correlation was seen between DNA methylation in the leukocyte samples and samples from other tissues (Table 5). A leukocyte differentiation analysis was performed, but no influence was found of leukocyte composition on methylation of blood samples for any of the genes (data not shown).

Concordance of DNA methylation in human and rat leukocytes

Human female DNA methylation data of our genes of interest were obtained from a genome wide methylation array. Human DNA methylation data of exactly the same genomic region of rat DNA was available for the *CALCA, CALCRL, USF2, ESR1, GPER* and *MTHFR* genes. For the other genes, *RAMP1, CRCP and NOS3,* the region studied in rat was very close (<100 bp) to the regions studied in human. We observed comparable values of DNA methylation in our genes of interest in human leukocytes

Table 5. Relationship of methylation of leukocytes with other tissues.

	Methylation of leukocytes compared to other tissues (Pearsons r[1])			
	Aorta	Dura	Trigeminal Caudal Nucleus	Trigeminal Ganglion
Calca	-0.23	-0.33	-0.38	-0.01
Ramp1	0.26	0.64	0.40	0.40
Crcp	0.04	0.33	0.09	0.17
Calcrl	0.08	0.29	0.52	-0.37
Usf2	0.07	0.50	0.07	-0.06
Esr1	0.43	0.53	0.18	0.58
Gper	0.01	0.02	0.17	0.07
Nos3	0.02	0.18	-0.36	-0.38
Mthfr	0.33	-0.09	-0.02	0.13

[1]) The relevance of the correlations can best be appreciated from a prediction perspective. Assume that we are interested in estimating the mean amount of methylation of some gene in some tissue. If we have no specific information, the best we can do is take the observed mean in our sample. The uncertainty is quantified by the observed standard deviation (SD). If a linear relationship with the mean amount of methylation in blood exists, a prediction model could be derived. Using basic statistical theory, one can show that the uncertainty is reduced to c * SD, where c follows form the formula c2 = 1 – r2, if r is the correlation coefficient. To get c = 0.5, thus halving the uncertainty, r has to be as high as 0.87. Conversely, when r = 0.5, c = 0.87 and thus only a 13% reduction is obtained. For reliable prediction really high correlations (e.g. r = 0.97 for c = 0.25) are needed.

as in rat leukocytes; the two genes with a high percentage of DNA methylation in the rat had also a high percentage of DNA methylation in the human samples, while the seven genes with a low percentage of DNA methylation were also concordant between the rat and human samples (Table 6, Figure 4).

Table 6. Methylation of amplicons of rat genes compared to homologues regions of the human genome.

		% DNA methylation Rat	% DNA methylation human	Location of rat amplicons in human genome (Build 37)	Location Illumina probes (Build 37)	Accession Number Rat	Accession Number Human
High DNA Methylations	*Crcp*	91.0 ± 2.5	86.0 ± 5.8	7:65587384-7:65587475	7:65583242-7:65604635	NM_053670.3	NM_014478.4
	Gper1	93.4 ± 1.7	98.3 ± 0.8	7:1131643-7:1132074	7:1132036	NM_133573.1	NM_001505.2
Low DNA methylation	*Calca*	4.2 ± 1.6	30.0 ± 3.8	11:14993934-11:14994000	11:14993929-11:14293977	NM_017338.2	NM_001505.2
	Calcrl	2.9 ± 2.2	20.8 ± 5.7	2:188312541	2:188312833	NM_012717.1	NM_005795.5
	Esr1	3.4 ± 0.9	6.1 ± 2.3	6:152129135-6:152129504	6:152129388-6:152129400	NM_012689.1	NM_000125.3
	Mthfr	2.0 ± 0.6	1.8 ± 1.0	1:11866155-1:11866365	1:11866155-1:11866236	NM_005957.4	XM_001074061.4
	Nos3	23 ± 14.6	19.7 ± 4.3	7:150710828-7:10571127	7:150710730-7:150711138	NM_021838.2	NM_000603.4
	Ramp1	2.8 ± 0.8	6.1 ± 2.3	2:238768163-2:238768240	2:238768104-2:238828641	NM_031645.1	NM_005855.2
	Usf2	1.7 ± 0.6	6.8 ± 2.3	19:345760455-19:35760666	19:35760554	NM_031139.1	NM_003367.2

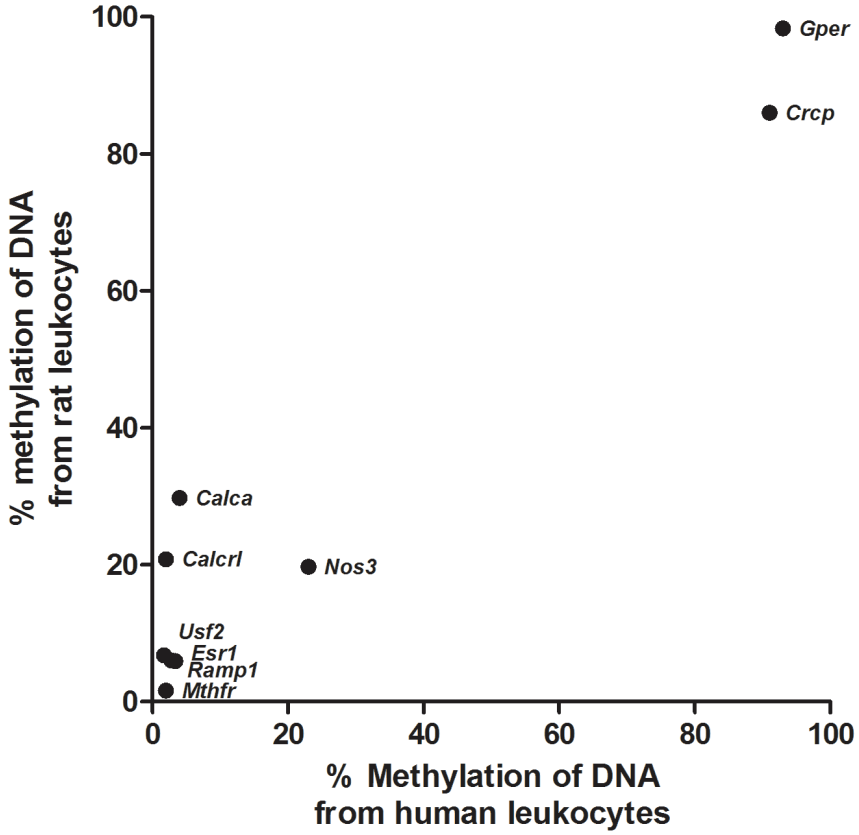

Figure 4. Rat leukocyte DNA methylation compared to human leukocyte DNA methylation. The methylation of DNA from rat leukocytes is compared to methylation of DNA from human leukocytes for different migraine-related genes. Our genes of interest that are high methylated in rat leukocytes, are also high methylated in human leukocytes and the genes that are low methylated in rat leukocytes are also low methylated in human leukocytes.

Discussion

DNA methylation and gene expression across tissues

DNA methylation and histone modifications, making the DNA accessible or locked for transcriptional regulation, are responsible for physiological processes like stem cell differentiation. Therefore, CpG island methylation may be similar between tissues but can also differ substantially. DNA methylation of the *Calca, Ramp1, Usf2* and *Mthfr* genes

was low in all tissues. Not taking into account other regulatory mechanisms, expression of these proteins would thus be expected in all investigated tissues. Notwithstanding the fact that tissue-specific information is not always available, all four genes are indeed expressed in several brain and cardiovascular tissues [28-33]. The expression in human leukocytes [34-37] suggests the expression in rat leukocytes as well.

In contrast, DNA methylation of the CpG island of the *Gper* gene was 90-94% in all investigated tissues, suggesting that expression of this gene might be low or absent. However, *Gper* mRNA is present in cells of the aorta [38] and in the central and peripheral nervous system [39] But the quantitative extent of this expression is unknown. Further, regulation of protein expression might even occur at high percentages of DNA methylation. Obviously, other regulatory mechanisms, such as histone modifications or non-epigenetic mechanisms like transcription factor binding to specific regions on the DNA, may also have an additional effect on protein expression. Hence, DNA methylation is not necessarily related to protein expression for every gene [40,41].

Our results show that the variation in methylation differs between tissues in the *Crcp*, *Calcrl, Esr1* and *Nos3* genes, which are expressed in several brain and vascular tissues [32,42]. *ESR1* and *NOS3* are also expressed in human leukocytes [43,44] Large variations could result from contamination with other tissues, but variations would then be expected to be small in tissues that are a distinct anatomical entity, such as aorta and trigeminal ganglion. Furthermore, outliers potentially caused by contaminated tissue, should then be the same in all examined genes, which was not the case. It would be tempting to quantitatively relate differences in DNA methylation to differences in gene expression and subsequent translation into proteins in the different tissues. However, such a quantification between tissues, which is clearly beyond the scope of our study, would be hampered by the different characteristics of the tissues studied, with the consequent lack of a validated internal standard between tissues. Taken together, the substantial variations in DNA methylation between tissues for *Crcp*, *Calcrl, Esr1* and *Nos3* genes in some of the tissues (Figure 2), suggests a regulatory role for DNA methylation in the expression of these genes in the respective tissues.

DNA methylation in leukocytes versus other tissues

DNA methylation is regularly examined in leukocytes with the aim of discovering

biomarkers for several diseases [45,46] because of the difficulty of obtaining affected tissues. For example, leukocyte DNA methylation is correlated to different types of cancer [47-49]. In our study, there was no correlation between DNA methylation of the candidate genes in leukocytes and that in the other tissues. Thus, it can be concluded that leukocytes are not representative for changes in DNA methylation in aorta, dura mater, trigeminal caudal nucleus and trigeminal ganglion for the migraine-related genes we investigated (Table 2).

Is the rat a valid model for human DNA methylation?

We studied whether DNA methylation of human leukocytes in our genes of interest is similar to DNA methylation of rat leukocytes. Since DNA methylation in human and rat leukocytes was found to be concordant, we hypothesize that this might also be the case for other tissues. This finding, combined with the obvious limitations in obtaining human tissues, suggests that animal models are relevant to study DNA methylation changes in specific tissues.

DNA methylation and environmental factors

DNA methylation may be influenced by environmental factors like nutrition [50] and maternal care [51]. Hormones like 17β-estradiol may also alter DNA methylation [52,53]. Interestingly, female hormones probably play an important role in migraine as migraine is much more common in females than in males especially during the fertile part of their lives, and migraine attacks in females often start at the day before menstruation [54]. It is well known that estradiol affects epigenetic mechanisms [12,13]. In addition, it potentiates the response to an important peptide in migraine pathophysiology, calcitonin gene-related peptide (CGRP) [24,25], which is widely expressed in the central and peripheral nervous system [55]. We previously demonstrated that estrogen increases vascular sensitivity to CGRP and trigeminal innervation [25]. Estrogen can also modulate CGRP expression in the spinal portion of the trigeminal caudal nucleus and the cervical spinal cord [56,57]. How estrogen causes these effects is still unknown, but a recent study showed that CGRP expression is epigenetically regulated [21]. Epigenetic mechanisms like DNA methylation may thus be involved in the differences in migraine prevalence between men and women.

When examining mean values of DNA methylation of the three different

treatment groups (Figure 2) some differences were observed between the groups, but these were not statistically significant. Because we are, to the best of our knowledge, the first to perform a study on DNA methylation of candidate genes in our field, we could not perform an adequate power calculation. We based our group sizes on our previous studies using the same animal model [25], where the same dose of and duration of treatment with 17β-estradiol as in the current study were adequate to induce changes in CGRP-ergic pathways. Furthermore, others have shown that DNA methylation patterns can change within hours or days [16,58]. A study with a similar design as our current study showed that an environmental factor, bisphenol A, can increase methylation of the promoter region of the estrogen receptor [59], indicating that the methods we applied are suitable to detect changes in DNA methylation due to environmental factors.

The large variation in methylation in our investigated tissues is likely to have reduced the statistical power of our study when investigating the effects of 17ß-estradiol treatment. We observed a maximal difference of 8% methylation with a standard deviation of 18% between animals treated with 17β-estradiol and animals treated with placebo (data not shown). Based on a post-hoc power calculation, a group size of 318 animals would be needed to obtain sufficient statistical power to investigate these hormone effects. This is practically not feasible so another study setup, possibly an in vitro study, falling beyond the scope of the current study, is needed to investigate the effect of 17β-estradiol on DNA methylation. Thus, with our experiment, we cannot categorically exclude that the methylation of the genes that we have studied may be influenced by 17ß-estradiol.

Relevance of CpG islands

Methylation of CpG islands in and close to promoter regions of genes can cause a decrease in transcription of those genes during development and in certain types of cancer, Therefore, the focus in DNA methylation studies, including our current study, is often on CpG islands [60,61]. CpG islands in or close to the promoter region of a gene are only present in 60% of the genes. Also in our case, there are genes that were of potential interest because they are involved in CGRP and estrogen signalling, as for example estrogen receptor 2 (*Esr2*) or the transient receptor potential cation channel, subfamily V, member 1 (*Trpv1*), but that are devoid of CpG islands in the rat

genome. Therefore, we did not investigate these genes in our study. Recently, it has been shown that also methylation changes in enhancer regions of the DNA [62], as well as the CpG island shores [63], may induce differences between subjects, providing another regulatory region in which DNA methylation plays a role in the regulation of transcription. The general relevance of these findings, however, needs to be confirmed in future studies.

Overall conclusion

In conclusion, our results show that DNA methylation of the rat genes we studied is variable, tissue specific and cannot be extrapolated from leukocytes to other tissues. On the other hand, we observed a high degree of concordance between human and rat DNA methylation in leukocytes, suggesting that it is possible to study effects on DNA methylation in rat tissues that are difficult to obtain from humans, for example several types of brain tissues. The large variation of DNA methylation in the *Crcp, Calcrl, Esr1* and *Nos3* genes suggests that these genes are prone to changes in DNA methylation, but challenges drawing conclusions about the effect of 17β-estradiol.

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Chapter 5 - Analysis of the vascular responses in a murine model of polycystic ovary syndrome.

Based on: Sieneke Labruijere, E. Leonie A.F. van Houten, Rene de Vries, Usha M. Musterd-Bagghoe, Ingrid M. Garrelds, Piet Kramer, A.H. Jan Danser, Carlos M. Villalon, Jenny A. Visser and Antoinette MaassenVanDenBrink, 2013, Journal of Endocrinology

Abstract

Introduction - Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of the reproductive age, but the exact pathophysiological mechanisms involved remain unclear. Cardiovascular disease risk is increased in PCOS patients and endothelial damage has been observed. We recently developed a mouse model of PCOS with reproductive and metabolic characteristics resembling those observed in women with PCOS. In this model we studied vascular function with particular emphasis on markers of vascular endothelial function.

Materials and Methods - Animals were treated for 90 days with dihydrotestosterone (DHT; 27.5 mg/day) or placebo using subcutaneous continuous-release pellets. Aortas were isolated for isometric force recordings in organ baths to investigate endothelial and vascular smooth muscle characteristics. Lungs were used to analyze endothelial nitric oxide synthase (eNOS) expression and phosphorylation. Asymmetric dimethylarginine (ADMA) levels were investigated in serum to assess endothelial damage. Expression of androgen receptor (*Ar*) mRNA was studied in aortas.

Results - DHT treatment (compared with placebo) induced i) a significant decrease in acetylcholine-induced aortic relaxations, with no change in calcitonin gene-related peptide- or sodium nitroprusside-induced relaxations, as well as 5-hydroxytryptamine-induced contractions; ii) no change in eNOS expression/phosphorylation in lungs or in plasma ADMA levels; and iii) a twofold increase in aortic *Ar* mRNA expression.

Conclusion and Discussion - Our results suggest that, in DHT-exposed mice, hyperandrogenemia specifically decreases endothelium-dependent vasorelaxation without deterioration of smooth muscle function. This study may initiate further investigations to elucidate underlying mechanism for the phenotype that is present in these animals, as well as in PCOS patients.

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age and the most common form of hyperandrogenism [1]. It has a worldwide prevalence of 5–10% [2-4], but this can be higher (13–52%) in some subpopulations [5-7]. The exact mechanisms involved in the pathophysiology of PCOS are still unclear, but high levels of androgens are considered to play a crucial role in the etiology. PCOS patients suffer from dyslipidemia, obesity and metabolic syndrome [8,9], resulting in an increased risk for type 2 diabetes mellitus and cardiovascular diseases including hypertension and atherosclerosis [10-13]. This predisposition is further aggravated by the associated endothelial dysfunction [14,15]. Studies in PCOS women showed increased circulating levels of asymmetric dimethylarginine (ADMA), a marker of endothelial dysfunction. ADMA is a competitive inhibitor of nitric oxide synthase (NOS), thus increased ADMA levels can lead to decreased vascular relaxation. ADMA levels are often increased in cardiovascular diseases [10,16]. Endothelial dysfunction in women with PCOS is often assessed by measuring flow-mediated dilation (FMD) of the brachial artery and intima media thickness of the carotid artery (CIMT). A decreased FMD and an increased CIMT have been observed in women with PCOS compared with normo-ovulatory woman [17,18]. Also women with PCOS often display increased insulin resistance. Insulin resistance is thought to be correlated with endothelial dysfunction and thus might play an important role in the development of endothelial dysfunction [19,20]. In addition, serum paraoxonase 1 (PON1) activity is decreased in women with PCOS [16,21]. PON1 is a HDL-associated enzyme that prevents LDL oxidation and thereby prevents the negative effects of LDL. Furthermore, hypertensive, but not normotensive, PCOS women displayed increased arterial stiffness [22]. Taken together, these studies suggest that women with PCOS have impaired vascular function, which may be assigned at least partly to impaired endothelial function. However, the relationship between hyperandrogenemia and endothelial dysfunction remains unclear. The results of studies on the effects of androgens on vascular reactivity in women are controversial and probably depend on the exposure period and endogenous estradiol levels in women [23]. In females taking high doses of androgens decreased vascular function was found, which was assigned to a negative effect of testosterone on endothelial NOS (eNOS)-mediated responses [24]. It is unknown whether the

effects of testosterone on endothelial function are direct effects mediated through the androgen receptor (AR) or indirect effects mediated via the conversion of testosterone into estrogens. The direct effect of androgens can be experimentally investigated by studying the effects of the nonaromatizable androgen dihydrotestosterone (DHT) on endothelial function. Several experimental animal models for PCOS have been developed through prenatal or postnatal exposure to androgens, but these animal models were mainly used to investigate ovarian and metabolic function and the vascular phenotype was not studied [25,26]. In pregnant rats treated with testosterone, increased blood pressure and decreased endothelial function were observed [27].

Recently, we have developed a mouse model of PCOS, facilitating future use of transgenic animals, in which prepubertal female mice were exposed to DHT for 90 days. These DHT-exposed mice display both reproductive and metabolic characteristics resembling those observed in women with PCOS, such as acyclicity, cyst-like follicles, increased adiposity, increased leptin and decreased adiponectin levels, and impaired glucose tolerance [28]. We used this mouse PCOS model to study the effect of hyperandrogenemia on vascular function. The effect of DHT on in vitro vascular responses to several vasoactive agents was analyzed, with particular emphasis on markers of vascular endothelial function (i.e. lung expression/phosphorylation of eNOS and serum levels of ADMA).

Materials and methods

Animals

C57BL/6J mice at postnatal day 19 were s.c. implanted with a 90-day continuous-release pellet containing either DHT (2.5 mg, 27.5 mg/day; nZ14) or placebo (n=16; Innovative Research of America, Sarasota, FL, USA) as described previously [28]. A separate group of mice was implanted with a 60-day release DHT pellet (1.5 mg, 25 mg/day; n=9) or placebo (n=9; Innovative Research of America). Mice were killed at the end of the treatment period (60 or 90 days). Blood samples were collected by orbital puncture after the mice were anesthetized with isoflurane. Mice were killed by decapitation and tissues were isolated. The same animals as reported in the study by van Houten et al. (2012)[28] were used in this study. Mice were kept under standard animal housing conditions in accordance with the National Institutes of Health Guidelines

for the Care and Use of Experimental Animals. The experiments were performed with permission of the Local Ethics Committee.

Tissues

Thoracic aortas from the 60- (n=18) and 90-day-treated animals (n=18) were placed in cold Krebs buffer (4°C), aerated with 5% CO_2 and 95% O_2 and stored overnight for organ bath experiments (composition of Krebs buffer in mmol/l: 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). In addition, aorta segments of the 90-day-treated mice (n=12) were directly snap frozen in liquid nitrogen and stored at -80 °C until RNA isolation. Furthermore, from the 90-day-treated animals, the lungs were collected (n=6 for both DHT- and placebo-treated animals) and cut into pieces. Pieces were divided into two groups and incubated for 1 min with either vehicle or 100 mmol/l acetylcholine in Krebs buffer. Samples were snap frozen in liquid nitrogen and stored at -80 °C for protein isolation. Lungs were used for this experiment, because lungs are very densely vascularized with small resistance vessels. A small amount of blood serum was available from all the treatment groups and was used for the measurement of ADMA as a measure of endothelial damage.

Functional experiments

Two-millimeter aortic rings (inner diameter 1–2 mm) were mounted in organ baths (Danish Myo Technology, Aarhus, Denmark) between two aluminum wires (wire diameter 40 mm), which were attached to a force displacement transducer and a computer on one side and a displacement device on the other side. The temperature-controlled organ baths (37 °C) were filled with Krebs buffer and aerated with 5% CO_2 and 95% O_2. The tension of the aortic rings was normalized to 90% of the estimated diameter at 100 mmHg pressure and stabilized for 30 minutes [29]. The rings were exposed to 30 mmol/l KCl and after washout to 100 mmol/l KCl to compare reactivity and maximal contractile response of the different rings. For vasodilator studies, rings were precontracted with 10–100 nmol/l U46619, a thromboxane A_2 analog, until a contraction of 50% of the contraction induced by 100 mmol/l KCl was reached. Smooth muscle cell-dependent vasorelaxation was determined in response to increasing concentrations of the vasodilating compounds calcitonin gene-related peptide (CGRP; rat α-CGRP, NeoMPS, Polypeptide Group; Strasbourg, France) and sodium nitroprusside (SNP;

Sigma Chemical Co.). CGRP activates the CGRP receptor present in smooth muscle cells [30]. SNP stimulates the direct release of NO, which can enter the smooth muscle cells [31]. Endothelium-dependent responses were examined using acetylcholine (Sigma Chemical Co.). To measure the maximal relaxation, 100 mmol/l SNP were added following the relaxation experiments to CGRP and acetylcholine. Contractile responses were investigated with increasing concentrations of the vasoconstrictor 5-hydroxtryptamine (5-HT; Sigma Chemical Co.). Half of the rings of the 90-day DHT treatment group were first incubated for 30 min with 100 mmol/l (6R)-5,6,7,8-tetrahydro-L-biopterin.2HCl (BH4) (Calbiochem, Merck KGaA, Darmstadt, Germany) (an inhibitor of both reactive oxygen species (ROS) and eNOS uncoupling), 30 mmol/l N-acetyl-cysteine (NAC; Sigma Chemical Co.) or 100 mmol/l tempol (Sigma Chemical Co.; both ROS inhibitors) prior to precontraction with U46619. These concentrations have been determined previously [32]. SNP, acetylcholine and 5-HT were dissolved in bidistilled water; NAC and tempol were both dissolved in DMSO.

Quantitative real-time RT-PCR

RNA of thoracic aortas was isolated using an RNA isolation kit for fibrous tissue (RNEasy fibrous tissue; Qiagen). RNA was reverse transcribed using the QuantiTect RT kit. A quantitative real-time RT-PCR was performed using TaqMan probes for the AR and b-actin as a housekeeping gene (Applied Biosystems). The expression of the target gene was normalized to the expression levels of b-actin using the 2-ΔΔCt method.

Quantification of eNOS phosphorylation using western blotting

Lung tissue was isolated, cut into small pieces and incubated with acetylcholine (100 mmol/l) or vehicle (Krebs buffer) for 1 min. Western blotting was performed with 15 mg of protein using total eNOS antibody (diluted 1:1000) or phosphorylated eNOS (Ser1177) antibody (diluted 1:1000). For visualization, a peroxidase-conjugated goat anti-rabbit antibody was used (all antibodies were obtained from Santa Cruz Biotechnology). The blot was scanned and analyzed with the use of ImageJ Software (NIH, Bethesda, MD, USA).

ADMA levels

Blood serum was collected from all treatment groups andserum levels of ADMA were measured using the ADMA ELISA kit (DLD Diagnostika GmbH, Hamburg, Germany).

Statistical analysis

The relaxant responses elicited by the vasorelaxant compounds were expressed as percentage of the maximum contraction induced by U46619 (10–100 nmol/l, 100%). The contractile responses to 5-HT were expressed as percentage of the previous response to 100 mmol/l KCl. All data are presented as mean±S.E.M. Statistical analysis of the concentration response curves was accomplished with GraphPad Prism 5 Software (La Jolla, CA, USA), using unpaired t-tests and ANOVA. Moreover, differences in % AR expression (compared with the household gene β-actin using the 2-ΔΔCt method) and the differences in eNOS expression and phosphorylation between the treatment groups were assessed using an unpaired t-test. Statistical significance was accepted at $P<0.05$ in all cases.

Results

Effect of 90 days DHT exposure on acetylcholine-induced relaxation in thoracic aortas

Acetylcholine-induced maximal relaxations were significantly smaller in aortas from 90-day DHT-treated mice than in those from placebo-treated mice ($P=0.04$; Fig. 1A). The pEC50 values did not differ between DHT- and placebo-treated animals (Table 1). Furthermore, no difference was observed in response to 100 mmol/l SNP, which was added after the concentration response curve to acetylcholine was finished (Fig. 1B).

Effect of 90 days DHT exposure on SNP- and CGRP-induced relaxations and 5-HT-induced contractions

In contrast to the relaxation response to acetylcholine described above, the maximal relaxations and pEC50 values to CGRP (Fig. 2A) and SNP (Fig. 2C) were not different between the 90-day DHT- and placebo-treated groups. Furthermore, no difference was observed in the subsequent response induced by 100 mmol/l SNP after the CGRP

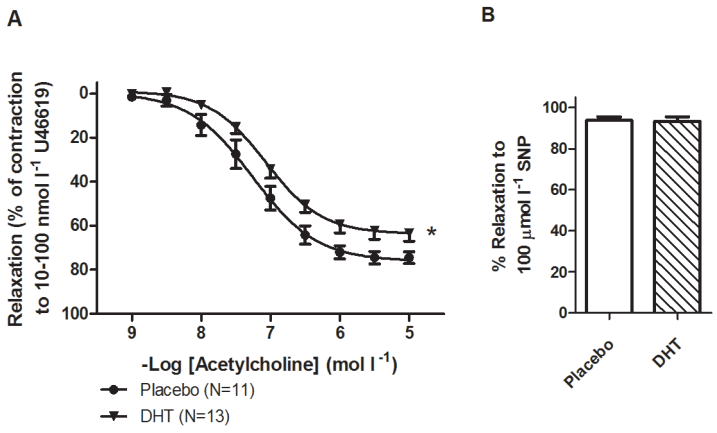

Figure 1. (A) Relaxations to acetylcholine in mice thoracic aortas after chronic treatment with DHT during 90 days. (B) Subsequently, the vasorelaxation to 100 µmol/l SNP was measured. All values are relative to precontraction with 10-100 nmol/l U46619. *Significantly different from placebo, P<0.05.

Table 1. E_{max} and pEC_{50} values for acetylcholine, CGRP, SNP and 5-HT in aortas of mice treated for 90 days with DHT of placebo.

	Placebo 90 days		DHT 90 days	
	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}
Acetylcholine	75±3	7.2±0.1	64±4*	7.1±0.1
CGRP	89±13	8.3±0.1	99±3	8.3±0.1
SNP	96±7	7.5±0.1	91±8	7.4±0.1
5-HT	96±7	6.9±0.1	91±8	6.8±0.1

* Signicantly different from placebo

curve (Fig. 2B). Likewise, no differences were observed in the concentration response curves to 5-HT (Fig. 2D), E_{max} and pEC_{50} values are shown in Table 1.

Effect of BH4, NAC or tempol on the relaxations to acetylcholine in 90 days DHT-exposed vessels

Since impaired endothelial function might be caused by a decreased NO bioavailability or decreased NO production by eNOS, the relaxations to acetylcholine were also measured

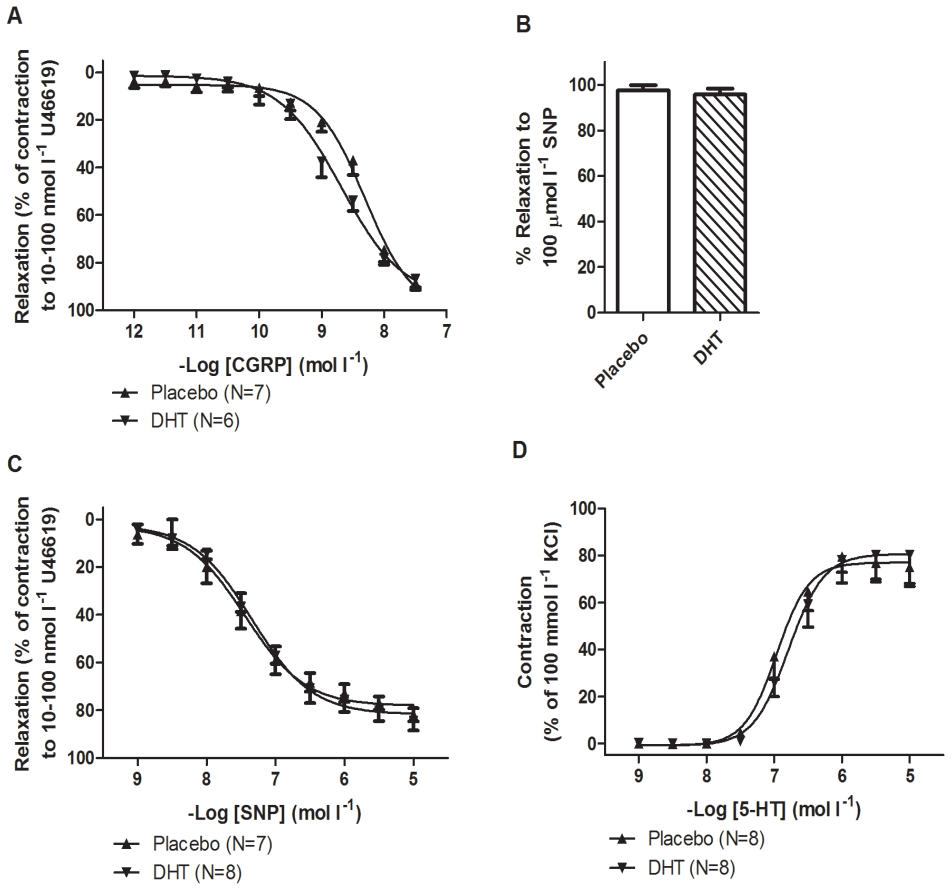

Figure 2. Relaxations to CGRP (A) and relaxations to 100 µmol l-1 SNP after relaxations to CGRP (B), as well as relaxations to SNP (C) and contractions to 5-HT (D) after 90 days of treatment with DHT or placebo.

in the presence of 100 mmol/l BH4, 30 mmol/l NAC or 100 mmol/l tempol in thoracic aortas of mice treated for 90 days with placebo and DHT to investigate the role of ROS and eNOS uncoupling in the acetylcholine response. Both NAC and tempol are ROS inhibitors and BH4 is needed for eNOS functioning. Thirty minutes of incubation with NAC or tempol did not cause any difference in acetylcholine induced relaxation in DHT- or placebo-treated mice (Fig. 3A and B). Interestingly, whereas incubation with BH4 had no effect on the acetylcholine-induced maximal vasorelaxation in placebo-treated mice, it induced a significant increase in vasorelaxation in DHT-treated animals

(Emax DHT: 64±4% vs Emax DHT+BH4: 81±3%, P=0.02). The pEC_{50} values did not differ (pEC_{50} DHT: 7.1±0.1 vs pEC_{50} DHT+BH4: 6.8±0.1, P=0.4; Fig. 3B).

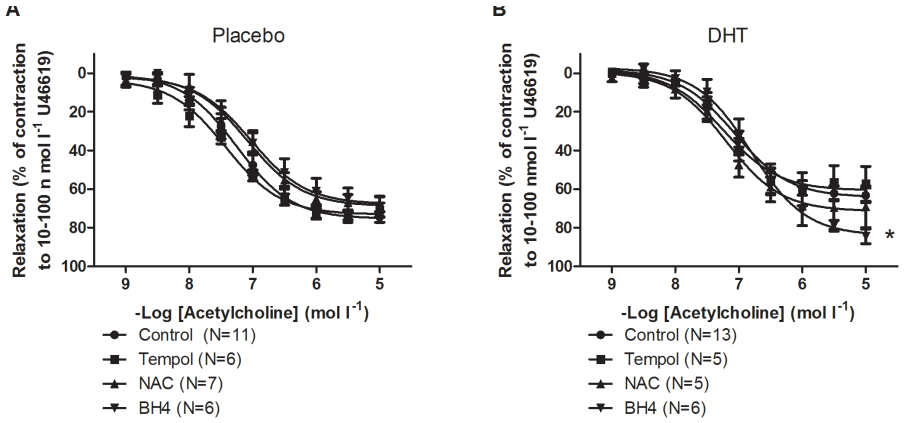

Figure 3. Acetylcholine-induced relaxations after 30 minutes incubation with BH4, NAC or tempol in Placebo- (A) and DHT- (B) treated animals.
*) Significantly different from placebo, P<0.05.

Endothelial damage as a primary or secondary phenomenon

To investigate whether the endothelial damage that we observed was a consequence of the metabolic changes in the DHT-treated mice, we studied relaxations to acetylcholine and SNP in mice that were treated with DHT for 60 days. DHT treatment for 60 days induced neither a reproductive nor a metabolic phenotype (results not shown), but also resulted in significantly lower plasma DHT levels at the end of the treatment period compared with the animals that were treated with the 90-day release pellet (0.8±0.1 vs 2.81±0.3 nmol/l, P<0.05). Comparable with the 90-day treatment group, acetylcholine-induced maximal relaxations were significantly smaller in aortas from 60-day DHT-treated mice than in those from placebo-treated mice (P=0.04; Fig. 4A). The pEC50 values did not differ between DHT- and placebo-treated animals (Table 2). Furthermore, no difference was observed in response to 100 mmol/l SNP, which was added after the concentration response curve to acetylcholine had been finished (Fig. 4B).

Table2. E_{max} and pEC_{50} values for acetylcholine in aortas of mice treated for 60 days with DHT of placebo.

	Placebo 60 days		DHT 60 days	
	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}
Acetylcholine	75±4	7.3±0.2	56±4% *	7.2±0.3

* Signicantly different from placebo

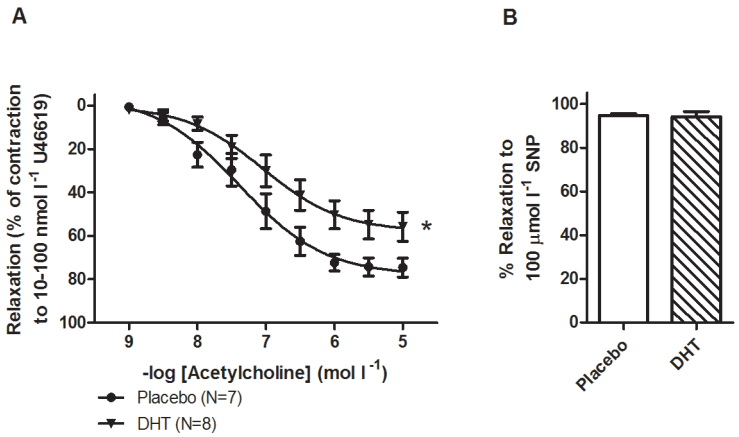

Figure 4. Relaxations to acetylcholine in mice thoracic aortas after chronic treatment with DHT during 60 days (A). Subsequently, the vasorelaxation to 100 µmol l-1 SNP was measured (B). All values are relative to precontraction with 10-100 nmol l-1 U46619. *) Significantly different from placebo, P<0.05.

Expression and phosphorylation of eNOS as well as serum levels of ADMA

To investigate whether there were differences in eNOS availability and functioning between the treatment groups, total eNOS and eNOS activation through Ser1177 phosphorylation were analyzed in lungs of 90-day placebo- and DHT-treated animals after 1 min incubation with acetylcholine or vehicle. Values were corrected for b-actin expression. No differences were seen in the total expression level of eNOS (Fig. 5A). Also DHT treatment had no effect on the eNOS Ser1177 phosphorylation level (Fig. 5B). Likewise, serum levels of ADMA, a competitive inhibitor of eNOS, did not significantly differ between 90-day DHT- and placebo-treated animals (Fig. 6).

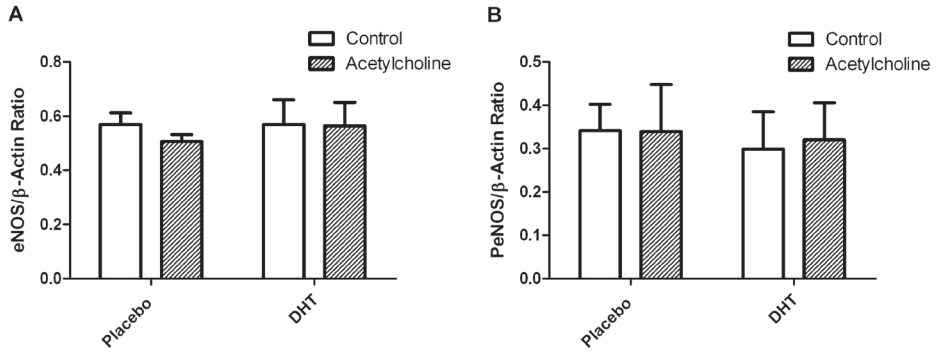

Figure 5. eNOS (A) and Ser1177 phosphorylated eNOS (B) expression in lungs of mice treated for 1 minute with acetylcholine (100 μmol l-1) (Placebo N=7, DHT N=5). No significant difference was observed between DHT- and placebo-treated groups.

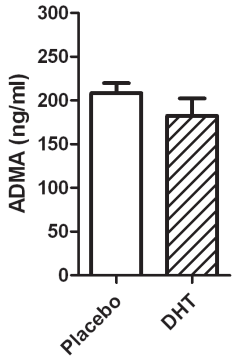

Figure 6. Serum levels of ADMA in mice chronically treated with a placebo (N=8) or DHT (N=6) pellet. No significant difference was observed between DHT- and placebo-treated group.

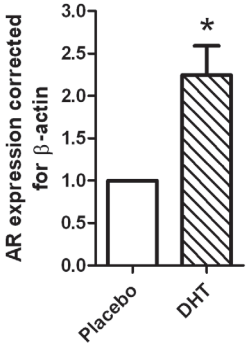

Figure 7. AR mRNA expression in thoracic aortas of mice treated for 90 days with placebo (N=5) compared to DHT-treated mice (N=5). Values are corrected for the household gene β-actin. *) Significantly different from placebo, P<0.05.

Effect of DHT exposure on *Ar* mRNA expression in thoracic aortas

To investigate if AR signaling might be involved in the difference in endothelial functioning between the DHT- and placebo-treated animals, *Ar* expression was examined. A 2.2-fold increase in *Ar* mRNA expression was observed in thoracic aortas of mice treated for 90 days with DHT when corrected for b-actin expression (P=0.02; Fig. 7).

Discussion

Our results show that in aortic vessels chronic DHT exposure i) specifically decreased the vasodilatation to acetylcholine but not that to vasodilators directly acting at the smooth muscle layer, such as SNP and ii) increased *Ar* mRNA expression. This implies a decreased endothelial function, unrelated to a deterioration of vascular smooth muscle function and/or an eNOS decreased expression/phosphorylation. These results suggest that this DHT-induced mouse model, besides having a PCOS-resembling metabolic and reproductive phenotype, also has a vascular phenotype. This mouse model for PCOS therefore may be an interesting model to investigate the cardiovascular alterations observed in PCOS patients.

Specific decrease in aortic endothelial function after DHT treatment in mice: resemblance with other PCOS experimental models

Our data show that the aortic vasodilatation to acetylcholine, which acts on the vascular endothelium to induce vascular smooth muscle relaxation [33], was significantly decreased after chronic treatment with DHT. In contrast, the vasodilatation to SNP, an NO donor acting directly on vascular smooth muscle, remained unaffected. These findings suggest that chronic DHT treatment results in a decreased aortic endothelial function. Consistent with this conclusion, the concentration response curves to the direct vasodilator CGRP and to the vasoconstrictor 5-HT remained unaffected upon chronic DHT exposure. Because CGRP and 5-HT receptors have been shown to be present on the membrane of vascular smooth muscle cells and, therefore, can produce direct vascular effects [33-35], our findings suggest that chronic DHT treatment does not cause deterioration of vascular smooth muscle function. In contrast, in a rat model of PCOS, contractions to noradrenaline, mediated via the PLC signaling pathway similarly as contractions to 5-HT in our model, were decreased [36]. Thus, although contractions to 5-HT remained unaffected after chronic DHT treatment, we cannot exclude that the effect of other constrictors might be affected in our model. On the other hand, our results are in agreement with a recent study in a DHT-induced rat PCOS model, using a comparable DHT treatment schedule as in our mouse study, where DHT treatment resulted in specific decrease in acetylcholine-induced vasodilator

responses [37]. Endothelial dysfunction was also observed in a rat PCOS model developed by daily injections of the antiprogestin mifepristone [38]. Clearly, endothelial dysfunction can be a predictor of cardiovascular disease or hypertension. This is also seen in women with PCOS [11-13]; therefore it is important to elucidate the underlying mechanisms for the development of better treatment options.

ROS inhibition and eNOS uncoupling

Binding of acetylcholine to its receptor on endothelial cells leads to the activation of eNOS. We measured expression and phosphorylation of eNOS in lung tissue, which is densely vascularized, as an alternative for the aortic tissues, because aortic tissue was not available in sufficient quantities due to the functional studies. We did not observe any effect of DHT treatment, although we obviously cannot exclude that there may be differences in eNOS expression and phosphorylation between lung and aorta tissues. eNOS signaling can be disturbed by ROS, which decrease available NO, induce eNOS uncoupling and decrease the effectiveness of eNOS [39]. Both tempol and NAC are ROS inhibitors but acting through different mechanisms [40-42]. BH4 is a cofactor of eNOS and in the presence of reduced BH4 levels eNOS uncoupling occurs, leading to superoxide production instead of NO [39,43]. Since no differences in acetylcholine-induced aortic vasodilatation occurred after the treatment with NAC or tempol it is reasonable to suggest that ROS formation is not responsible for the decreased vasodilatation to acetylcholine in DHT-treated mice. In contrast, the increase in acetylcholine-induced aortic vasodilatation after treatment with BH4 implies that eNOS uncoupling was increased, leading to a disturbed eNOS function; however, we cannot explain why we did not observe increased ROS production as a consequence.

Increase in *Ar* mRNA expression after DHT treatment

We observed a strong increase in *Ar* mRNA expression in the chronically DHT-treated animals. This is in accordance with earlier studies reporting increased AR expression in endometrium of PCOS patients [44,45], as well as in ovaries in a rat PCOS model [46]. It is known that AR signaling can be both genomic and nongenomic and may, in turn, activate other signaling pathways [47]. Thus, it is tempting to suggest that an increased AR signaling may underlie the reduced endothelial function (i.e. a decreased vasorelaxation to acetylcholine) observed in the aortas after treatment with DHT.

Admittedly, no experimental evidence is reported in the literature to support this view.

Endothelial dysfunction as a primary or secondary phenomenon

Chronic androgen exposure can increase blood pressure in women with PCOS and rats treated chronically with DHT or testosterone displayed elevated blood pressure [27,37]. Increased blood pressure can lead to decreased endothelial function. However, our PCOS mice also display a metabolic phenotype. To decide whether hyperandrogenemia directly induces endothelial dysfunction or whether this is primarily mediated through metabolic alterations, we also studied endothelial function in mice implanted with a 60-day DHT-release pellet, which resulted in lower serum DHT levels at the end of the experiment (60 days after implantation). These 60-day DHT-treated mice did not develop the metabolic or reproductive PCOS-resembling phenotype. Yet, these mice did show decreased vasorelaxation to acetylcholine to a similar extent as the 90-day DHT-treated mice. This suggests that the endothelial dysfunction observed upon DHT treatment most likely is a direct effect of androgens, not secondary, on metabolic changes. In conclusion, our results in a DHT-induced mouse model for PCOS suggest that hyperandrogenemia specifically decreases the endothelium-dependent vasorelaxation, without deterioration of smooth muscle function. This study may initiate further investigations to resolve the mechanisms behind the vascular pathologies observed in PCOS patients.

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Part 3 - **The serotonergic system**

Chapter 6 – Activation of 5-hydroxytriptamine$_{1B/1D/1F}$ receptors as a mechanisms of action of antimigraine drugs

Based on: Martha. B. Ramirez-Rosas, Sieneke Labruijere, Carlos M. Villalon and A. MaassenVanDenBrink, August 2013, Expert Opinion on Drug Discovery.

History of 5-hydroxytryptamine and migraine

Migraine is currently considered a neurovascular syndrome [1-3] that affects a significant fraction of the world population, with a higher prevalence in females (15%) than in males (6%) [4]. This syndrome is characterized by an intense and throbbing unilateral headache associated with nausea, vomiting, photophobia, phonophobia and aggravation by routine physical activity [5]. Sometimes the headache may be preceded by a focal neurological phenomenon ('aura') followed by headache (migraine with aura).

Of the many factors that have been implicated over the years in the pathophysiology of migraine, during the twentieth century none seemed to have a better claim than 5-hydroxytryptamine (5-HT; serotonin, for references see [6, 7]). Indeed, several acute (e.g., the ergot alkaloids and the triptans) and prophylactic (e.g., methysergide and lisuride) antimigraine drugs interact with 5-HT receptors and, in the case of the triptans, their development was originally based on the involvement of 5-HT in migraine [8, 9]. Indeed, multiple findings support the role of 5-HT in the pathophysiology of migraine [10, 11], namely: i) during an attack of migraine, high quantities of 5-hydroxyindole acetic acid are excreted [12]; ii) some drugs that deplete monoamines (reserpine) can provoke a migraine attack [13] and iii) a slow i.v. infusion of 5-HT can abort an attack of migraine [13,14]. The factor restricting the clinical use of 5-HT as an antimigraine agent was the prevalence of side effects [13, 14], including changes in heart rate, vasodilatation in some vascular beds (e.g., cutaneous blood vessels) and vasoconstriction in others (e.g., the external carotid bed), gastrointestinal effects, etc. [15]. The antimigraine efficacy of 5-HT, nevertheless, suggested the existence of a specific 5-HT receptor involved in the relief of migraine headache. Interestingly, before the above findings, ergot (the product of the fungus Claviceps purpurea that grows on rye) had already been introduced in 1884 by W.H. Thomson as an effective remedy for migraine [16]; physicians, however, were aware of the intoxication risk when taken frequently (ergotism or St. Antony's Fire), with descriptions dating back to the Middle Ages [17]. Ergotism is characterized by gangrene of the feet, legs, hands and arms due to a potent and long-lasting vasoconstriction. Thus, the introduction of the first pure ergot alkaloid, ergotamine, by Stoll in 1920 [18] represented a remarkable accomplishment as the beginning of an effective and rational therapy for the acute treatment of migraine. However, its side effects, contraindications and interactions

with an array of different receptors led to the speculation that a more selective cranial vasoconstrictor agent would be an effective antimigraine drug devoid of the above inconveniences associated with 5-HT and ergotamine. Hence, it was proposed [19] that a drug which could mimic the beneficial effects of 5-HT and ergotamine without its side-effect profile would provide an effective therapy for migraine. On this basis, in 1972, Humphrey and colleagues initiated a long-term project aimed at identifying novel therapeutic agents for the treatment of migraine [9]. The goal of this project was to develop selective vasoconstrictors of the cranial extra-cerebral circulation (abnormally dilated during migraine) based on the vascular theory of migraine and the lines of evidence supporting the role of 5-HT in its pathogenesis (see above). This goal was strengthened by the knowledge that 5-HT appeared to be intrinsically more active as a vasoconstrictor of cephalic blood vessels (i.e., the external carotid bed), and that the 5-HT receptors on such vessels are different to those on peripheral blood vessels [20, 21]. This research led them to identify a then unknown 5-HT receptor type (now called 5-HT_{1B}) that is largely located in cranial rather than peripheral blood vessels. They then went on to design novel agonists that specifically stimulated these receptors to produce selective vasoconstriction of cranial blood vessels, such as meningeal arteries, which can become distended and inflamed during migraine [9, 19]. Therefore, the synthesis of many tryptamine derivatives was followed by their analysis in various vascular preparations and in animals.

One of the first agents of importance identified using this strategy was 5-carboxamidotryptamine (5-CT), which potently contracted the dog isolated saphenous vein, a blood vessel believed to contain a novel 5-HT receptor that was also predominantly located in the canine carotid circulation [9]. 5-CT was also found to induce significant vasodilatation/hypotension in intact animals, presumably by concomitant activation of other 5-HT receptors (presently known as 5-HT_7 [22, 23]). Therefore, 5-CT was not developed for possible clinical applications, but it was extremely valuable as an experimental tool to characterize 5-HT receptors. Fortunately, other tryptamine derivatives (i.e., sumatriptan) displayed a more promising profile as antimigraine drugs (as will be discussed below) and helped in the further characterization of 5-HT receptors. After analysing additional tryptamine derivatives, one in particular drew special attention, namely 3-[2-(di-methylamino) ethyl-N-methyl-1H-indole-5-methane sulphonamide (sumatriptan, previously known as GR43175), which was synthesized in

1984. This compound was more selective than 5-CT for the 5-HT receptors producing vasoconstriction in the dog saphenous vein and extracranial blood vessels, and displayed much less activity in other vascular systems [19, 24]. Moreover, many studies showed that sumatriptan is an agonist at 5-$HT_{1B/1D}$ receptors (for references, see [22]), and that it produces constriction of cranial large arteries, including the canine external carotid bed [25-27]. When parenterally administered, sumatriptan is effective and well-tolerated in most patients [28, 9]. Thus, by developing such a drug that selectively activated 5-$HT_{1B/1D}$ receptors, without activity at most of the other 5-HT receptor types, the vast array of unwanted effects seen with exogenous 5-HT was avoided (e.g., platelet aggregation, bronchoconstriction, generalized vasoconstriction and various gastrointestinal effects). During this period, however, the presence of some 5-HT_{1B} receptors on coronary arteries was identified, and extensive studies were carried out early on during sumatriptan's clinical evaluation to evaluate any cardiovascular risk potential [29]. Although sumatriptan is contraindicated in patients with cardiovascular disease, it has proven to be an effective and well-tolerated medication when used properly; and as a testament to its safety profile over many years, sumatriptan (50 mg oral dose) is now in some countries available without prescription.

First generation of 5-HT receptor agonists

As described in the previous paragraph, the first generation of 5-HT receptor agonists used for the treatment of migraine is represented by the ergot alkaloids. 'Ergot alkaloids' is the collective term for ergotamine, dihydroergotamine (DHE) and methysergide. Ergotamine and DHE are used for the acute treatment of migraine since several decades ago [30, 31]. Methysergide is used as a prophylactic drug to prevent migraine attacks [32, 33]. Furthermore, ergotamine, DHE and methysergide are used as short-term prophylaxis in cluster headache [34, 35].

All ergot alkaloids are non-specific 5-HT_1 receptor agonists [36]. Methysergide also acts as an antagonist for the 5-HT_2 receptor. Apart from binding to different types of the 5-HT receptor, they also act as agonists at α-adrenoceptors and dopamine D2 receptors (Table 1, [31, 36]). Since dopamine and noradrenaline are also thought to be involved the pathophysiology of migraine [37], their activity at adrenergic and dopaminergic receptors may well contribute to their therapeutic action. Their most

Table 1. Binding affinity constants (pK_D, pKi, pA_2, pK_p, pEC_{50}, pIC_{50}) of dihydroergotamine (DHE), ergotamine, sumatriptan and methysregide for cloned human receptors (unless otherwise stated).

Receptor	Ergotamine	DHE	Methysergide	Sumatriptan
5-HT_{1A}	(p)8.4 [122],*	9.3 [121]	7.63 [122],*	6.4 [121]
5-HT_{1B}	(r)8.7 [122]	(r)7.8 [121]	(r)5.82 [122],*	7.3 [123]
5-HT_{1B}	(p)8.4 [122],*	8.6 [121]	N.D.	8.5 [121]
5-ht_{1e}	6.2 [125]	5.6 [124]	6.64 [125]	5.8 [121]
5-HT_{1F}	6.8 [125]	6.96 [121]	7.47 [125]	7.9 [121]
5-HT_{2A}	(p)7.7 [122],*	8.54 [121]	8.57 [122]	<5 [71]
5-HT_{2B}	(p)8.2 [126],&	7.70 [126],&	UA [127]	6.9 [71]
5-HT_{2C}	(p)7.3 [122],&	7.48 [122],* / 7.43 [121]	8.90 [22],*	<5 [71]
5-HT_3	N.D.	N.D.	N.D.	<5 [71]
5-HT_4	N.D.	N.D.	N.D.	<5 [71]
α_1	(r)8.0 [128]	8.0 [121]	5.64 [130]	< 4 [129]
α_{1A}	(r)8.0 [131],#	8.66 [131],#	< 6 [36]	N.D.
α_{1B}	(gp)7.6 [131],+	8.02 [131],#	N.D.	N.D.
α_{1D}	(r)7.5 [131],+	7.87 [131],#	N.D.	N.D.
α_2	(r)8.2 [128]	8.0 [121]	5.59 [130]	< 4 [129]
α_{2A}	N.D.	8.7 [121]	< 6 [36]	N.D.
α_{2B}	N.D.	8.0 [121]	N.D.	N.D.
α_{2C}	N.D.	9.0 [121]	N.D.	N.D.
D_2	8.3 [31],^	8.21 [121]	6.70 [130]	< 4 [129]
D_3	8.4 [31],^	8.2 [121]	N.D.	N.D.
D_4	7.9 [31],^	8.1 [121]	N.D.	N.D.

Data taken from: [121], Leysen et al. (1996); [122], Hoyer (1988); [123], Beer et al. (1998); [124], Parker et al. (1996); [125], Adham et al. (1993); [71], Tfelt-hansen (2000); [126], Glusa and Roos (1996); [127], Baxter et al. (1994); [22], Hoyer (1994); [128], Leysen and Gommeren (1984); [129], Peroutka et al. (1989); [130], Leysen (1985); [131], Görnemann et al. (2008); [36], Dahlof and MaassenVanDenBrink (2012); [31], Silberstein and McCrory (2003). All values have been presented as pKi, except for: *, pKD; #, pA2; ^, pIC50, &, pEC50; +, pKP. Abbreviations: UA (unsurmountable antagonist). ND (not determined). (p) corresponds to porcine receptors, (gp) guinea pig receptors and rodent (r) receptors.

obvious pharmacological effect is arterial vasoconstriction through binding of the 5-HT_{1B} receptors and α-adrenoceptors, but the neuronal properties of these compounds most probably also contribute to their clinical effects. These effects are likely mediated through the agonist activity at 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors on trigeminal

nerve terminals resulting in the inhibition of neuronal release of vasoactive peptides and preventing vasodilatation in migraine [36]. Finally, central 5-$HT_{1B/1D}$ and 5-HT_{1F} receptor signaling in the trigeminal caudal nucleus and ventroposteromedial thalamus are thought also to play a role in the action of ergot alkaloids [36].

Ergotamine

Ergotamine was first isolated and used for the treatment of migraine in the beginning of the twentieth century [17, 38, 39]. Besides the relief of migraine attacks, ergotamine also causes side-effects as blood pressure changes and uterine contractions. These effects are caused by vasoconstriction through binding to dopamine, noradrenaline and 5-HT receptors, and have led to the vascular hypothesis of migraine in which dilation of meningeal and cranial arteries causes headache [39]. Later, central mechanisms were also found to be involved in migraine pathophysiology, leading to the neurovascular theory in which both the vasculature and the central nervous system are involved. Apart from constriction of cranial blood vessels, ergotamine also induces constriction of other peripheral vascular beds, including the pulmonary and coronary arteries, which can lead to decreased blood flow to the brain, heart and extremities and, in rare cases, lead to gangrene [40]. Other side effects are nausea and vomiting, probably due to binding to dopamine and noradrenaline receptors. Ergotamine has a very slow diffusion from its receptor, leading to long-lasting effects and side effects (Figure 1) [29]. Thus, ergotamine should not be used in patients with cardiovascular disease and peripheral vascular disease or poor circulation. Ergotamine is still used for the treatment of migraine attacks, especially when other drugs are not effective. Intravenous and intramuscular injection of ergotamine leads to a bioavailability of 100% whereas oral availability is only around 2% and rectal availability around 6% due to low gastrointestinal absorption and high first-pass metabolism. Ergotamine is mainly available in a tablet form, combined with caffeine, which increases its absorption.

Dihydroergotamine

DHE was synthesized in the middle of the twentieth century from ergotamine by reducing an unsaturated bond [30, 31]. As ergotamine, DHE displays agonist activity at 5-HT, a-adrenergic and dopamine receptors, leading to vasoconstriction of the cranial vascular bed. Positively, it is less potent on peripheral arteries than ergotamine,

leading to a more favorable side-effect profile [36]. Due to its still significant peripheral vasoconstrictor properties (although less than ergotamine), it is contraindicated in all patients with known cardiovascular disease. DHE is available as intramuscular, intravenous and intranasal formulation and currently clinical trials are performed with an orally inhaled formulation [41, 42]. Its intramuscular bioavailability is 100% and intranasal bioavailability is as high as 40% because it avoids the first hepatic metabolism that takes place when administered orally. Its oral availability is, therefore, very low (1%). As ergotamine, DHE also diffuses very slowly from its receptor. This causes the effects to last longer than expected from the plasma concentrations and, therefore, plasma concentration data of both ergotamine and DHE cannot be used to predict a biological response [36].

Methysergide

Ten years after the advent of DHE, methysergide was developed [32, 33]. It is used as a prophylactic drug to prevent migraine attacks, and its precise mode of action is still unknown. Methysergide has, besides its agonistic effects on 5-HT_1 receptors, antagonistic effects on 5-HT_2 and 5-HT_7 receptors. It was thought that its prophylactic effect was caused by 5-HT_2 receptor blockade, but since specific 5-HT_2 receptor antagonists such as ketanserin do not have any effect in preventing migraine attacks [43], this hypothesis is unlikely. Methysergide has a pharmacologically active metabolite, the vasoconstrictor methylergometrine, which may contribute to its therapeutic action. In view of its propensity to cause retroperitoneal fibrosis, pleuropulmonary fibrosis or fibrotic thickening of cardiac valves [32], methysergide is only used in severe cases where other drugs are not effective. Methysergide is only available as a 1-mg tablet [32] and was recently withdrawn from the market in the USA and Canada because of its side effects.

Triptans

The introduction of the triptans was a great step forward in the treatment of migraine [44], because of the increased pharmacological selectivity compared to the ergot alkaloids. Triptans are devoid of many of the side effects observed with the ergot alkaloids [45]. The triptans are 5-$HT_{1B/1D}$ receptor agonists, and most of them

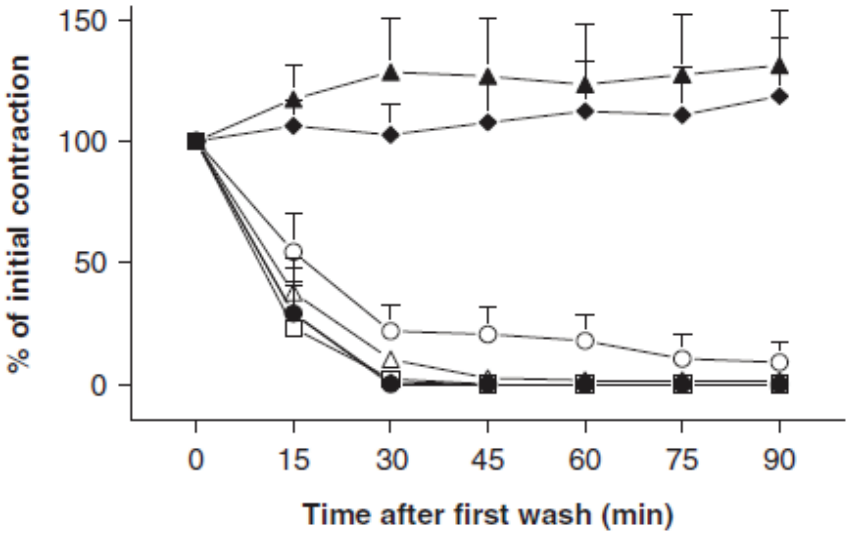

Figure 1. Effect of repeated washings (three times every 15 min) on contractions of human coronary arteries (n = 5) elicited by acutely acting antimigraine drugs (ergotamine, solid triangle; dihydroergotamine, solid diamond; sumatriptan, solid circle; zolmitriptan, open square; rizatriptan, open diamond; naratriptan, open triangle; and avitriptan, open circle). All drugs were administered once at a concentration two times their EC50.

also display a moderate affinity for 5-HT_{1F} receptors (Table 2) [22]. The triptans induce a selective vasoconstriction of cranial blood vessels by activation of 5-HT_{1B} receptors [46], which are largely located in cranial rather than peripheral blood vessels, and their expression has been shown in the medial smooth muscle cell layer of the human middle meningeal [47] and cerebral arteries [48]. In addition, sumatriptan stimulates 5-HT_{1D} receptors located in trigeminal fiber endings, which inhibits the release of proinflammatory neuropeptides [49].

As mentioned above, some triptans, apart from their affinity to 5-$HT_{1B/1D}$ receptors, also have modest to significant agonist activity of 5-HT_{1F} receptors [50]. The 5-HT_{1F} receptor, which is present in both the trigeminal nucleus and trigeminal ganglion [51], may be involved in some actions induced by triptans such as: i) inhibition of dural neurogenic inflammation mediated by presynaptic receptors and ii) inhibition of nociceptive neurotransmission within the trigeminocervical complex in the brainstem and upper spinal cord [38, 52]. The 5-HT_{1D} and 5-HT_{1F} receptors are localized prejunctionally on the peripheral and central ends of sensory trigeminal neurons and do

not seem to mediate any vasoconstrictor action [53]. Thus, the triptans, apart from their well described vasoconstrictor actions on blood vessels may also modulate the activity of trigeminovascular nociceptive neurons [45]. The presynaptic action of the triptans, inhibiting the release of neuropeptides, may largely be mediated through inhibition of release of calcitonin gene-related peptide (CGRP) and substance P [54].

After the development of the triptans, CGRP was proposed to be a key molecule in the pathophysiology of migraine [38]. Several studies have demonstrated increased levels of CGRP in the cranial circulation [55] as well as in saliva [56] during migraine attacks. Accordingly, clinical studies have shown that after treatment of a migraine attack with sumatriptan, CGRP levels were normalized [57]. Remarkably, Shields and Goadsby reported that triptans can locally inhibit ventroposteromedial neurons by a 5-HT_{1B} and/or a 5-HT_{1D} receptor mechanism, which are involved in conveying nociceptive information from the thalamus to the primary somatosensory cortex. Hence, the thalamus might be an additional site of triptan action [58]. As mentioned above, the 5-HT_{1B} receptor is not exclusive for cranial blood vessels, but it is also expressed, albeit at much lower densities, in peripheral blood vessels, including the coronary vasculature [48]. Accordingly, in vivo experiments in humans showed that triptans induced vasoconstriction, increased blood pressure and decreased the buffering capacity of conduit arteries after the intake of equipotent therapeutic dosages [59, 60]. Both in vivo [61] and in vitro [29] constriction of coronary arteries was confirmed. Consequently, the triptans are contraindicated in patients with cardiac and cerebrovascular disease [62]. Nevertheless, it is important to highlight that the main 5-HT receptor mediating coronary vasoconstriction is the 5-HT_{2A} receptor, for which the triptans do not display affinity, in accordance with their beneficial safety [47, 63]. Indeed, triptans are recommended as first-line treatment of severe migraine [64], and they are effective, safe and well tolerated [65].

As previously pointed out, sumatriptan was the first available triptan, but notwithstanding its efficacy, it has a low oral bioavailability (14%), and also a short half-life of about 2 h [66]. Whereas the initial efficacy of oral sumatriptan is about 60% in clinical trials, only about 20% of patients remain pain free for 24 h [67]. These limitations, over time, prompted the development of second generation triptans with chemical structures similar to sumatriptan, but with improved pharmacokinetic properties [68, 7]. The pharmacodynamics profile of these so-called 'second generation' triptans (in

Table 2. Affinities of triptans and other drugs for the 5-HT_1 and 5-HT_7 receptors.

Triptans and other drugs	5-HT_{1A}	5-HT_{1B}	5-HT_{1D}	5-HT_{1E}	5-HT_{1F}	5-HT_7
Almotriptan	N.D.	7.2[49]	7.8[49]	N.D.	N.D.	N.D.
Eletriptan	7.4[132]	8.0[133]	8.9[133]	7.2[133]	8.0[133]	N.D.
Frovatriptan	7.3[49]	8.6[49]	8.4[49]	<6[49]	7.0[49]	N.D.
Naratriptan	7.1-7.6[132]	8.1[133]	8.4-9.0[133]	7.7[133]	8.2[134]	N.D.
Rizatriptan	6.4[133]	6.9[133]	7.9[133]	6.8[133]	6.4-6.8[134]	N.D.
Sumatriptan	6.4[121]	6.5-8.1[121]	8.0-8.7[121]	5.6-5.8[121]	7.2-7.9[121]	(r)6.6[135]
Zolmitriptan	6.6[133]	7.7[133]	8.9[133]	7.7[133]	7.4-7.5[133]	N.D.
AS19	89.7[116]*	N.D.	N.D.	N.D.	N.D.	(r)0.6[116]*
Lasmiditan	N.D.	N.D.	N.D.	N.D.	8.7[54]	N.D.
LY-334370	7.8[134]	N.D.	N.D.	N.D.	8.7[134]	N.D.
PNU-142633	N.D.	(g)4.8[136]	(g)8.3[136]	N.D.	N.D.	N.D.
SB-269970	<5[137]	N.D.	N.D.	N.D.	N.D.	8.9[137]

Data taken from: Leysen et al. [121]; Deleu and Hanssens [49], Newman-Tandcredi et al. [132]; Napier et al. [133]; Napier et al. [133]; Wainscott et al. [134]; Rust et al. [135];Brenchat et al [116]; Nelson et al [54]; Pregenzer et al [136]; Lovell et al. [137]. All values are given as pK_i at human receptors, exceptr when stated otherwise: gorilla (g) and rodent (r) receptors. *K_i (nM). N.D.: Not determined.

alphabetical order: almotriptan, eletriptan, frovatriptan, naratriptan, rizatriptan and zolmitriptan) resembles that of sumatriptan. In contrast, they have pharmacokinetic properties that differ from that of sumatriptan, such as greater bioavailability, longer plasma half-life, active metabolites and higher lipophilicity [69], which may lead to advantages including earlier onset of action [70]. Moreover, triptan responses are time-dependent and there is evidence that triptans are more effective if they are taken early when the pain is mild [71]. However, the inter-individual variability, among other

factors, plays an important role in the determination of the efficacy/tolerability ratio in individual patients [49], and until now no factors have been identified to predict which triptan will be effective in which patient [64]. Finally, recent advances in migraine pathophysiology have underscored the need for more efficacious and specific prophylactic migraine medications [72].

Interaction of the serotonergic system with glutamate and CGRP

Glutamate is the principal excitatory neurotransmitter in the central nervous system [73]. This neurotransmitter acts on its receptors that are classified into metabotropic glutamate receptors (mGluRs, G-protein coupled) and ionotropic glutamate receptors (ligand-gated ion channels); the latter include N-methyl-D-aspartic acid (NMDA), a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), as well as kainate receptors [74]. Several lines of evidence suggest an important role of glutamate and its receptors in migraine pathophysiology, for example: i) increased levels of glutamate may elicit and support cortical spreading depression, a phenomenon that may constitute the underlying cause of migraine attacks in experimental animals ; ii) glutamate positive neurons are present in migraine pain-relay structures such as trigeminal ganglion, trigeminal nucleus caudalis (TNC), trigeminocervical complex and thalamus [75, 76]; iii) NMDA, kainate, AMPA and mGluRs as well as their messenger RNA's are expressed in trigeminal ganglion neurons [77] and the superficial lamina of the TNC in the rat [76]; iv) glutamate seems to be elevated in the spinal fluid, plasma and/or platelets of migraineurs [78]; v) all three known genes for familial hemiplegic migraine (FHM), a rare and severe variety of migraine in which a Mendelian type of inheritance has been clearly established, seem to increase the levels of K^+ and glutamate in the synaptic cleft [38] and vi) genetic studies in the more common forms of migraine are still in their early phases and need replication, but their results so far also suggest a role for glutamatergic signaling in migraine [38].

As previously stated in the paragraph on the triptans, CGRP, one of the most potent vasodilators [79], has also been suggested to play a major role in the pathophysiology of migraine [1]. Among other factors, this is based on the facts that: i) during migraine attacks the plasma levels of CGRP are increased due to activation

of the trigeminovascular system [55]; ii) this increase is normalized after abortion of the migraine attack by sumatriptan [57]; iii) an infusion of CGRP induces a migraine-like attack in migraine patients [80]; iv) CGRP receptor antagonists are effective in aborting migraine attacks [81, 82]; v) CGRP acts at second-order neurons in the TNC and at C1-C2 levels, to transmit pain signals to the thalamus and higher cortical pain regions [83] and vi) CGRP is expressed in trigeminal ganglion [84], and neurons of the trigeminal ganglion have further connections with neurons in the TNC in the brainstem [85]. Accordingly, the presence of CGRP-positive fibers has been reported in the TNC and spinal cord of different species [86, 87]. In general, the expression of CGRP and its receptor have been found both in the periphery and in the central nervous system, suggesting both vascular and neuronal actions [84].

Interestingly, there seems to be a close interaction between the serotonergic, glutamatergic and the CGRP-ergic systems. Changes in glutamatergic signaling may interact with levels of CGRP [88] and 5-HT [89]. Furthermore, it has been reported that: i) CGRP may facilitate glutamatergic neurotransmission in the dorsal horn of the spinal cord [90] and ii) mutations leading to FHM which, as mentioned above, are likely to increase glutamate levels, also may increase CGRP release in cultured murine trigeminal ganglia (TG) cells [91]. Accordingly, it has been demonstrated that the majority of glutamatergic neurons in the TG carry 5-$HT_{1B/1D/1F}$ receptors [92], and activation of presynaptic 5-HT_{1B} receptors has been demonstrated to decrease glutamate release from trigeminal primary afferents [93]. This effect has also been observed in the bed nucleus of the stria terminalis where 5-HT1B receptors decrease glutamate release from presynaptic terminals [94]. Thus, 5-HT may be relevant in the pathophysiology of migraine not only by acting as a vasoactive substance, but also as a neurotransmitter, as well as a neuromodulator [95].

Beyond the triptans

As mentioned above, most triptans, apart from their affinity for 5-HT_{1B} and 5-HT_{1D} receptors, also display affinity for 5-HT_{1F} receptors. The triptans are likely to exert their vascular effects through the 5-HT_{1B} receptor [46], while inhibition of neuropeptides release may be mediated through the 5-HT_{1D} receptor [49]. Indeed, the 5-HT_{1D} receptor agonist, PNU-142633, inhibits the trigeminovascular system [96]. In

addition, the trigeminocervical complex displays a considerable amount of 5-HT_{1F} receptor binding sites [51, 97]; consequently, the 5-HT_{1F} receptor agonists LY334370 [98] and lasmiditan [53] are effective in preclinical neuronal migraine models, e.g., inhibition of immediate early gene c-Fos in the rat TNC after trigeminal ganglion stimulation [53]. This led to the exploration of the effects of selective 5-HT_{1D} and 5-HT_{1F} receptor agonists as antimigraine drugs that would partly act like the triptans, but without their vascular effects. Despite the above trigeminal inhibition, PNU-142633 proved to be ineffective in the acute treatment of migraine [99]. On the other hand, there remain some questions about the effectiveness of this compound because, for patent reasons, it was designed using a gorilla 5-HT_{1D} receptor clone, which is not exactly the same as the human 5-HT_{1D} receptor. Furthermore, it was not tested whether the plasma levels obtained during the clinical trial were high enough to stimulate the target receptors in humans. Thus, the hypothesis that 5-HT_{1D} receptors are involved in antimigraine mechanisms may not yet be completely discarded [100]. The therapeutic potential of the 5-HT_{1F} site has been explored using the specific agonists LY334370 [101] and, more recently, lasmiditan (COL-144) [53]. These compounds are non-triptans and have a higher selectivity for 5-HT_{1F} receptors than for the 5-HT_{1B} and 5-HT_{1D} receptors. Both compounds are clinically effective in the acute treatment of migraine. Whereas the development of LY334370 was terminated because of toxicity problems of the compound, lasmiditan was recently investigated in several clinical trials [102, 103]. The efficacy of 5-HT_{1F} receptor agonists in migraine is interesting from both a clinical and pathophysiological point of view. Whereas the currently available acutely acting antimigraine drugs act, at least partly, through a vascular mechanism of action, the therapeutic efficacy of 5-HT_{1F} receptor agonists seems to be mediated through non-vascular mechanisms; these compounds did not induce vasoconstriction in animal models. Although it remains to be confirmed that the 5-HT_{1F} receptor agonists are devoid of 5-HT_{1B} receptor activity at clinical doses in human blood vessels, from a therapeutic point of view the lack of vasoconstrictor properties offers advantages for patients with cardiovascular disease where the triptans are contraindicated. In line with the expected central site of action, lasmiditan showed a high rate of central nervous system side effects in clinical trials [102, 103]. Apart from the selective 5-HT_{1F} receptor agonists, a recent progress is the development of compounds that combine 5-$HT_{1B/1D}$ receptor agonism with other properties. One such compound is NXN-188, a dual-

action compound combining 5-$HT_{1B/1D}$ receptor agonism with neuronal nitric oxide synthase (nNOS) inhibition [104]. NXN-188 can be administered orally, appears to be well tolerated [104]and inhibits CGRP release in preclinical migraine models [105]. Whether NXN-188 behaves differently from the triptans in such models and whether the inhibtion of nNOS contributes to the therapeutic potential of the compound, without increasing the side-effect burden, remains to be established. Another therapeutic approach based on the putative role of 5-HT in migraine is aimed at antagonizing 5-HT_7 receptors. Indeed, activation of 5-HT_7 receptors induces: i) direct vasodilatation of cranial blood vessels [106]; ii) excitation in neuronal systems [107]; iii) hyperalgesic pain and neurogenic inflammation [108, 109]; iv) neuroinflammatory processes [110] and v) central sensitization and activation of pain pathways [111]. All of these processes have been demonstrated to participate in migraine pathophysiology and would suggest that a selective 5-HT_7 receptor antagonist might be a potential antimigraine drug. Moreover, it has been shown in anesthetized rats that the selective 5-HT_7 receptor antagonist SB269970 caused a significant decrease in serum CGRP concentrations following electrical stimulation of the trigeminal ganglion, which was reversed by the putative 5-HT_7 receptor agonist AS19 [112]. These indirect vascular effects through CGRP release [113], as well as the direct role of the 5-HT_7 receptor in vasodilatation [114] suggest that the safety of 5-HT_7 receptor antagonists should be well considered.

Conclusion

The triptans were a major breakthrough in the specific treatment of migraine attacks. Their big advantage compared to the ergot alkaloids was their favorable side effect profile due to specific 5-$HT_{1B/1D}$ receptor binding. Although smaller than in cranial arteries, some peripheral vasoconstrictor effects remained, leading to the contraindication of triptans in patients with cardiovascular disease. Therefore, novel antimigraine drugs without peripheral vasoconstrictor properties are still needed, and compounds binding specifically to 5-HT_{1D}, 5-HT_{1F} and 5-HT_7 receptors have been or are currently being investigated. Of these approaches, 5-HT_{1F} receptor agonism seems to have the most promising prospect.

Expert opinion

As mentioned earlier in this review, the introduction of the triptans was a major improvement in the treatment of migraine. Nevertheless, there remains a place for older drugs, for example in patients who do not respond well to the triptans. One such example is DHE, which is now being investigated in novel formulations that may overcome pharmacokinetic limitations of more traditional routes of administration. Furthermore, the prophylactic drug methysergide is of particular importance for those patients who do not respond well to other prophylactic treatments. The fact that methysergide has now been withdrawn from the market in some countries is, therefore, highly undesirable, especially when considering that careful monitoring of the potential side effects is nowadays possible with imaging techniques. Notwithstanding the vast therapeutic potential of the triptans, their shortcomings have stimulated the search for novel drugs. Other avenues, such as CGRP receptor antagonism, are currently being explored. Despite their different mode of action, the clinical efficacy of the CGRP receptor antagonists generally seems to be similar to that of the triptans. Undoubtedly, CGRP receptor antagonists represent a promising therapeutic strategy that may contribute to a better treatment in future, especially in those patients where the triptans are not effective, or in patients who cannot use triptans due to their contraindications. Although the lack of direct vasoconstrictor properties of CGRP receptor antagonists seems to be an advantage over the triptans, cardiovascular adverse events induced by CGRP receptor antagonists (especially when used chronically) cannot categorically be excluded, since antagonism of CGRP receptors will inhibit the vasodilator response to endogenous CGRP. An alternative approach is the exploration of 5-HT_1 receptor subtypes as a pharmacological target. As described in this review, no definite conclusions can be drawn from the lack of clinical efficacy of the 5-HT_{1D} receptor agonist PNU-142633 since some questions remain unanswered. Unfortunately, it seems unlikely that a pharmaceutical company will invest in any further trials on selective 5-HT_{1D} receptor agonists in the coming years. The demonstrated clinical efficacy of selective 5-HT_{1F} receptor agonists is of great interest. It remains to be demonstrated whether these 5-HT_{1F} receptor agonists, at clinical concentrations, indeed do not induce contractions of human blood vessels. If so, the 5-HT_{1F} receptor agonists may be of particular interest for patients with compromised cardiovascular health. An additional interesting

question in this respect is whether the more selective ligands will have fewer propensities to induce medication overuse headache associated with most current acute antimigraine treatments [115]. A tendency quite opposite to that of the development of increasingly selective agonists is that of the above-mentioned compound NXN-188, combining the action of the triptans with nNOS inhibition. Following a similar line of reasoning, compounds that simultaneously could affect the serotonergic, CGRP-ergic and/or glutamatergic systems could be of therapeutic relevance in the treatment of migraine in view of the tight connection between these transmitter systems. However, it should obviously be born in mind that intervening with multiple systems simultaneously may also be associated with a greater side-effect potential. Thus, studies on novel compounds, based on the triptans that are now in use for over two decades, are awaited with interest.

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Chapter 7 - Dihydroergotamine and Sumatriptan in Isolated Human Coronary Arteries, Middle Meningeal arteries and Saphenous Vein

Based on: Sieneke Labruijere, Kayi Y. Chan, René de Vries, Antoon J. van den Bogaerdt, PhD, Prof Clemens M. Dirven, Prof A. H. Jan Danser, Shashidhar H. Kori, Antoinette MaassenVanDenBrink. Provisionally accepted may 2014. Cephalalgia.

Abstract

Background - Dihydroergotamine (DHE) and sumatriptan are contra-indicated in patients with cardiovascular disease because of their vasoconstricting properties, which have originally been explored in proximal coronary arteries. Our aim was to investigate DHE and sumatriptan in proximal and distal coronary artery, middle meningeal artery and saphenous vein.

Methods - Blood vessel segments were mounted in organ baths and concentration response curves for DHE and sumatriptan were constructed.

Results - In proximal coronary artery and saphenous vein, maximal contractions to DHE (proximal:8±4%; saphenous:52±11%) and sumatriptan (proximal:17±7%; saphenous:37±8%) were not significantly different. In distal coronary and meningeal arteries, contractions to DHE (distal coronary:5±2%; meningeal:26±5%) were significantly smaller than those to sumatriptan (distal coronary:17±9%; meningeal:62±14%). At clinically relevant concentrations mean contractions to DHE and sumatriptan were below 3% in proximal coronary arteries and below 6% in distal coronary arteries. Contractions in meningeal artery and saphenous vein were higher (7-40%).

Conclusions - Contractions to DHE in distal coronary arteries are smaller than those to sumatriptan, while at clinical concentrations they both induce only slight contractions. In meningeal arteries contractions to DHE and sumatriptan are significantly larger, showing their cranioselectivity. Contractions to DHE in saphenous vein are higher than those in the arteries, confirming its venous contractile properties.

Introduction

Dihydroergotamine (DHE) and sumatriptan are both 5-HT receptor agonists, and two of the most widely used drugs for the acute treatment of migraine [1-3]. DHE is part of the 'ergot alkaloid family', together with ergotamine and methysergide. Ergot alkaloids have been used for many decades for the treatment of migraine attacks. Ergotamine is derived from the fungus *Claviceps purpurea* that grows on grain, which was infamous in former centuries for causing ergotism and miscarriages when ingested through infected bread [2]. DHE is derived from ergotamine by reducing an unsaturated bond. DHE and ergotamine are both constrictors of cranial arteries. DHE is less potent in constricting peripheral arteries than ergotamine, but is more potent in constricting peripheral veins [4]. Dihydroergotamine is preferred over ergotamine because it has fewer side effects [4]. Ergotamine, and from 1945 also DHE were for decades the classical drugs for the acute treatment of a migraine attack, until the discovery of the triptans in the 90's. Sumatriptan, which is used in this study, was the first available triptan and is a specific agonist at 5-$HT_{1B/1D/1F}$ receptors, whereas DHE is a non-specific 5-HT receptor agonist and has also affinity for dopamine and noradrenegic receptors [4-6]. Next to its less specific receptor binding, the diffusion of DHE from its receptor is very slow. This may lead to longer lasting effects and side-effects than with the triptans [7]. Because triptans are not effective in all patients, ergot alkaloids are still often prescribed. Recently, a phase 3 clinical trial with a new orally inhaled formulation of DHE, MAP0004, was performed in 903 adult migraineurs and demonstrated MAP0004 to be well tolerated and effective in treating migraine compared to placebo [8]. Oral inhalation of DHE causes lower peak plasma concentrations, thus reducing side effects [3,9]. Because of their vasoconstrictive properties, both DHE and sumatriptan are contraindicated in patients with cardiovascular disease. This contraindication is based on studies in which small contractions to these drugs were observed in human proximal coronary arteries [7,10]. Recently, we showed that the effect of sumatriptan is larger in distal coronary arteries than in proximal coronary arteries [11]. This is of clinical relevance because smaller coronary arteries might also account for angina-like symptoms, especially in women [12-14]. The effect of DHE in distal coronary arteries, however, has never been reported and thus it is relevant to compare the contractile properties of those two drugs in distal coronary arteries. Furthermore, the effect of DHE has never

been studied in human middle meningeal arteries, where its main therapeutic action in migraine is most probably located. Because DHE is more potent in peripheral veins than in arteries, it is interesting to study the effects of DHE also in a venous vessel. Therefore, the aim of this study was to investigate the contractile effects of DHE and sumatriptan in 1) distal human coronary arteries next to proximal coronary arteries, 2) in human middle meningeal arteries, 3) in human saphenous vein, and 4) to relate our findings to the plasma concentrations observed in clinical practice.

Materials and methods

Tissues

Human proximal (inner diameter 2-3 mm) and distal (inner diameter 0.5-1.0 mm) coronary arteries were removed from healthy human hearts (donors died from non-cardiac disorders, 5 female, 5 male, 16-65 yrs) that were brought to the laboratory within 24 hours after circulation stop. The hearts were provided by the Heart Valve Bank Rotterdam from Dutch postmortem donors, following removal of the aortic and pulmonary valves for homograft valve transplantation. All donors gave permission for research. The hearts were stored in a sterile medium at 4°C until arrival at the laboratory. The coronary arteries were isolated from the heart, placed in cold (4ºC) Krebs buffer solution (composition: NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM and glucose 8.3 mM; pH 7.4) aerated with carbogen (5% CO_2 and 95% O_2) and stored overnight for organ bath experiments. Human middle meningeal arteries (inner diameter 0.5-1.0 mm) were obtained from pieces of the dura mater of patients that underwent a neurosurgical operation (2 male, 4 female, 44-75 yrs) and stored in a cold sterile medium M199 (Gibco, Invitrogen, Carlsbad, CA, USA) at 4ºC until arrival at the laboratory. The arteries were cleaned and placed in cold (4ºC) Krebs buffer solution 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; pH 7.4) aerated with carbogen and stored overnight for organ bath experiments in the same way as the coronary arteries. Saphenous veins (inner Ø 0.5-3 mm), obtained from patients that underwent surgery for limb ischemia or varicose vein (4 male, 2 female, 28-67 yrs) were collected and placed in physiological saline solution, transported to the laboratory and stored overnight in cold (4ºC) Krebs buffer solution (composition in mM: NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM

and glucose 11.1 mM; pH 7.4) aerated with carbogen for organ bath experiments.

Organ baths and construction of concentration response curves

Three-mm rings of the proximal coronary arteries and some saphenous vein segments too large to mount in wire myographs, were mounted in 15-ml organ baths and vessel tension was set to 15 mN as described previously [15]. Two-mm rings of the distal coronary arteries, middle meningeal arteries and the remaining part of saphenous vein segments were mounted in wire myographs (Danish Myo Technology, Aarhus, Denmark) between two small aluminium wires (Ø 40 μm). The tension was normalized to 90% of the estimated diameter at 100 mm Hg pressure [16]. All the baths were filled with warm Krebs buffer (37ºC), composed as mentioned above for the respective tissues, and aerated with carbogen. The rings were washed and stabilized for 15 minutes and 30 mM KCl was added. After washout, 100 mM (coronary and meningeal artery) or 65 mM (saphenous vein) KCl was added to compare reactivity and maximal vascular contraction. After 30 minutes, KCl was washed out. Concentration response curves were constructed to increasing concentrations of DHE (1 nM-10 μM) or sumatriptan (1 nM-10 μM) and expressed as a percentage of the 100 mM KCl-induced contractions for coronary and meningeal arteries. Endothelial function was evaluated through addition of U46619 (10-100 nM), a thromboxane A_2 analogue, and subsequent addition of substance P (1-10 nM) for coronary and middle meningeal arteries. Saphenous veins were pre-contracted using sumatriptan (1 μM) and upon plateau, endothelium-dependent relaxation was assessed using bradykinin (BK) (10 μM), because substance P is nearly inactive in saphenous veins [17]. Contractions were expressed as a percentage of 65 mM KCl-induced contractions.

Compounds

The compounds used in this study were: DHE (dissolved in DMSO as 10 mM stock solution and further diluted in H_2O, Tocris Bioscience, Bristol, UK), sumatriptan (dissolved in DMSO as 10 mM stock solution and further diluted in H_2O, Sigma Chemical Co. St. Louis, MO, U.S.A), BK (dissolved in 0.1 M acetic acid, Sigma Chemical Co. St. Louis, MO, U.S.A), U46619, Substance P and KCl (dissolved in H_2O, Sigma Chemical Co. St. Louis, MO, U.S.A).

Statistics

The contractile responses to DHE and sumatriptan were expressed as percentage of the previous response to 65 or 100 mM KCl in the same segment. All data are presented as mean ± S.E.M.. Curves that cover the full sigmoidal range were analyzed by means of a computerized curve fitting technique to obtain pEC_{50} and E_{max} values. Statistical analysis of the concentration response curves was accomplished with Graphpad Prism 5 software, using unpaired T-tests. Statistical significance was accepted at $P<0.05$ in all cases.

Results

Vascular responses to DHE and sumatriptan in human proximal and distal coronary arteries

In proximal coronary artery segments the endothelium-dependent relaxant response to 10 nM substance P was 69 ± 7% of the precontraction induced by 10 nM U46619. Maximal contractions and pEC_{50} values to DHE (E_{max}: 8 ± 4%; pEC_{50}: 6.7 ± 0.3) and sumatriptan (E_{max}: 17 ± 7%; pEC_{50}: 6.0 ± 0.2) were not significantly different, thus confirming the data in a previous study in proximal coronary arteries [7] (Figure 1).

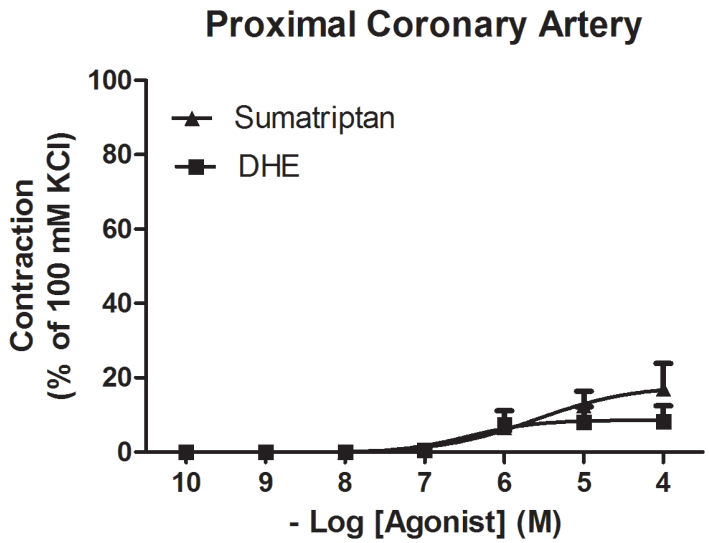

Figure 1. Contractions to DHE and sumatriptan in human proximal coronary arteries. Mean ± S.E.M., n=9.

In distal coronary arteries the endothelium-dependent relaxant response to 10 nM substance P was 91 ± 4% of the precontraction induced by 10 nM U46619 (n=8). The maximal contractile responses to DHE (5 ± 2%) were significantly smaller than those to sumatriptan (17 ± 9%) (P-value = 0.004) (Figure 2). Furthermore, the pEC_{50} values were significantly larger for DHE (7.7 ± 0.4) than for sumatriptan (6.4 ± 0.1; P-value = 0.002).

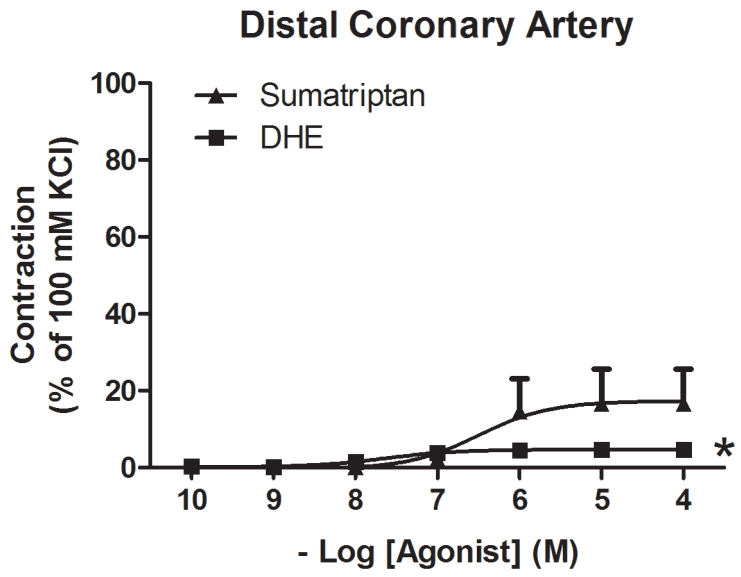

Figure 2. Contractions to DHE and sumatriptan in human distal coronary arteries. Mean ± S.E.M., n=10.

Vascular responses to DHE and sumatriptan in human middle meningeal arteries and human saphenous vein

In middle meningeal artery, the endothelium-dependent relaxant response to 10 nM substance P was 82 ± 6% of the precontraction induced by 10 nM U46619 (n=5). Maximal contractions of middle meningeal arteries to DHE (E_{max}: 26 ± 5%) were significantly smaller than maximal contractions to sumatriptan (E_{max}: 62 ± 14%, P-value = 0.04) (Figure 3). The pEC_{50} of DHE (7.9 ± 0.5) was not different from that of sumatriptan (7.0 ± 0.4). In saphenous vein, the endothelium-dependent relaxation to

10 μM bradykinin was 60 ± 10% of the precontraction induced by 1 μM sumatriptan. No differences in either E_{max} or pEC_{50} values were observed between contractions to DHE (E_{max}: 52 ± 11%; pEC_{50}: 7.4 ± 0.4) and sumatriptan (E_{max}: 37 ± 8%; pEC_{50}: 6.7 ± 0.2) (Figure 4).

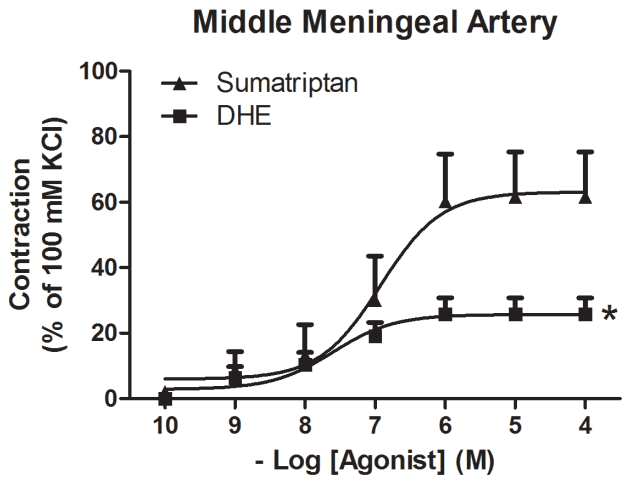

Figure 3. Contractions to DHE and sumatriptan in human middle meningeal arteries. Mean ± S.E.M., n=6.

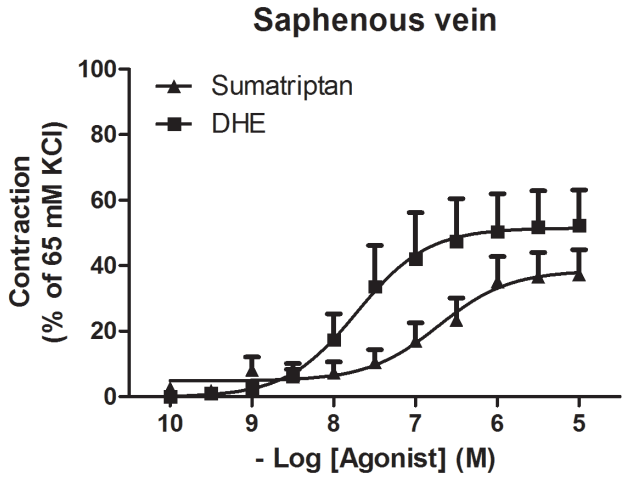

Figure 4. Contractions to DHE and sumatriptan in human saphenous veins. Mean ± S.E.M. n=6.

Comparison of response to sumatriptan and DHE between the studied vessels

The E_{max} of both DHE (26 ± 5%) and sumatriptan (62 ± 14%) in middle meningeal arteries was significantly larger than the E_{max} in proximal (sumatriptan: 17 ± 7%; DHE: 8 ± 4%) and distal (sumatriptan: 17 ± 9%; DHE: 5 ± 2%) coronary arteries (P-values < 0.02). Furthermore, the E_{max} of DHE in saphenous vein (52 ± 11%) was significantly larger than the E_{max} of DHE in proximal coronary (8 ± 4%) (P-value = 0.001), distal coronary (5 ± 2%) (P-value < 0.0001) and meningeal artery (26 ± 5%) (P-value = 0.04). The E_{max} of sumatriptan did not differ between the saphenous vein and the other vessels. Sumatriptan pEC_{50} values of both proximal (6.0 ± 0.2) and distal (6.4 ± 0.1) coronary arteries were smaller than pEC_{50} values of saphenous veins (6.7 ± 0.2) (P-value = 0.03 and 0.04 respectively). All other pEC_{50} values of both sumatriptan and DHE did not differ between the different blood vessels studied.

Contractions at clinically relevant plasma concentrations

Both DHE and sumatriptan have different formulations. Because the amount and route of administration of the drugs differ for different formulations, this leads to different plasma concentrations (Table 1). The expected contractions of the investigated blood vessels for these different plasma concentrations can be predicted by interpolating the data of the concentration response curves for DHE and sumatriptan that we constructed. At clinically relevant concentrations, mean contractions to both DHE and sumatriptan in proximal as well as distal coronary arteries are below 7% of maximal contraction obtained with 100 mM KCl (Table 1). In human middle meningeal arteries mean contractions were between 7% and 40% of maximal contraction obtained with 100 mM KCl. The contractions of the saphenous vein at clinically relevant concentrations of both DHE and sumatriptan were between 10% and 38% of maximal contraction obtained with 65 mM KCl (Table 1). There is a large variation in contractions to sumatriptan between the patients, illustrated by the large error bars in the graphs (Figure 1, 2, 3 and 4). In most of the patients, the maximal contractions of the coronary arteries at clinically relevant concentrations remain relatively small (Table 1).

Table 1. Therapeutic doses and C_{max} in patients

	Dose and administration	C_{Max} nM	Predicted Contraction Proximal	Predicted Contraction Distal	Predicted Contraction Meningeal	Predicted Contraction Saphenous
			Mean % 65-100 mM KCl maximum contraction (min-max)			
Dihydro-ergotamine	1 mg, SC [28]	3	0	0.7 (0 – 2.3)	7.2 (0.9 – 23.7)	7.7 (0 – 25.8)
	0.5 mg, OI [28]	4	0 (0 – 0.1)	0.9 (0 – 2.7)	7.8 (1.1 – 24.0)	10.1 (0 – 32.5)
	1 mg, IV [28, 29]	65	1.2 (0 – 4.1)	3.2 (0 – 12.5)	18.4 (5.1 – 37.7)	38.3 (2.4 – 92.9)
Sumatriptaan	100 mg, PO [3, 7]	143	1.4 (0 – 3.3)	5.2 (0.1 – 25.9)	36.3 (2.1 – 95.4)	19.9 (1.4 – 35.9)
	6 mg, SC [3, 7]	211	1.8 (0 – 4.3)	2.7 (0.2 – 34.6)	40 (2.7 – 101.2)	22.9 (2.1 – 40.3)

Therapeutic doses and the subsequent acquired Cmax (corrected for plasma protein binding) in patients are compared to the graphs in Figures 1- 4 to predict contraction in both proximal and distal coronary arteries as well as middle meningeal arteries and saphenous veins. Abbreviations: PO, Oral; SC; Subcutaneous; IV, Intravenous; OI, Oral Inhalation.

Conclusion and discussion

As expected, contractions to both DHE and sumatriptan in middle meningeal artery were larger than those in coronary artery, indicating the cranioselectivity of both DHE and sumatriptan. Across the various blood vessels studied, the potency of DHE was somewhat higher than that of sumatriptan (see Figure 1-4), which is in accordance with the higher affinity of DHE than sumatriptan for 5-HT_{1B} receptors, the serotonergic receptor mediating vasoconstriction to these compounds [11] (pK_i DHE: 9.2-9.5; sumatriptan: 7.4-7.8) [18-20]. As known from previous studies, both DHE and sumatriptan induced a small contraction of the human proximal coronary artery [7] and no significant differences in E_{max} were found between the two compounds. In contrast, contractions to sumatriptan in the smaller distal coronary artery were significantly higher than contractions to DHE. The smaller contractions to DHE in distal coronary artery deserve further investigation, because, as already mentioned in the introduction, in women smaller vessels are more often involved in coronary artery disease than in men [13,14]. Smaller contractions to DHE in distal coronary artery compared to that of sumatriptan may seem an advantage in view of cardiovascular safety, however, it should be kept in mind that DHE has a much longer lasting effect than sumatriptan [7], which adds to its side-effect potential.

Like in the distal coronary artery, contractions to sumatriptan were significantly larger in the middle meningeal artery than contractions to DHE. Furthermore, the different formulations of DHE lead to a lower C_{max}, than the formulations of sumatriptan (Table 1), further increasing the difference in contraction between DHE and sumatriptan in middle meningeal arteries at clinically relevant concentrations. Similar results were observed in a study of cerebral arteries by Nilsson et al [21]. Based on previous assumptions that contractions of the middle meningeal artery are responsible for the clinical efficacy, this might suggest that the anti-migraine efficacy is lower for DHE than for sumatriptan. However, in a comparison study, headache relief after subcutaneous sumatriptan was comparable to headache relief after dihydroergotamine, both around 80% [22]. This finding supports the more recent hypothesis that these anti-migraine drugs may also work through multiple other mechanisms (blocking nociception, blocking release of CGRP, etc.) in addition to vasoconstriction. The prolonged action of DHE, caused by slow diffusion from its receptors, may via a vascular mechanism

also contribute to its therapeutic action. In two previous studies we also measured contractions to sumatriptan in meningeal arteries, and the E_{max} values (107 ± 13% and 103 ± 13%) were larger than the E_{max} values in this study (62 ± 14%) [11,17]. As we described earlier for the human coronary artery [23], responses to sumatriptan in human arteries are highly variable, underlining the importance of a paired parallel setup when comparing responses of a blood vessel to sumatriptan to responses to another compound, as applied in the current study. As expected, contractions to DHE were largest in saphenous vein and in the same range as we observed before [24]. This is probably due to the fact that DHE binds to both adrenoceptors and 5-HT_1 receptors, which are present in veins. Sumatriptan has no affinity for adrenoceptors, explaining its smaller effects in saphenous vein.

There are different formulations on the market for both DHE and sumatriptan [9,25,26]. Patients prefer oral formulation over injections, but the bioavailability of DHE after oral administration is only 1%, compared to 100% bioavailability of intramuscular injections. A new orally inhaled aerosol formulation, providing a high bioavailability, recently was demonstrated to induce fewer side effects and to have a higher patient preference than the existing DHE formulations [26]. We calculated the expected contractions in both proximal and distal coronary arteries obtained in the clinical situation after using different formulations of sumatriptan and DHE that are currently available, as well as the investigational orally inhaled formulation of DHE (Table 1). For all different formulations, mean calculated contractions in proximal coronary arteries were below 3% and in distal coronary arteries below 6%. Outliers were maximal 5% in proximal coronary arteries and maximal 35% in distal coronary arteries. All contractions are percentages of their maximal contraction obtained with 100 mM KCl. As coronary blood flow remains intact until 80% constriction of original lumen diameter in healthy subjects, both sumatriptan and DHE should be safe to use in patients that do not suffer from cardiovascular diseases [27]. In contrast, in patients who do already have cardiovascular problems, even a small contraction of a vessel can lead to a reduced blood supply or even a complete occlusion and these drugs should thus not be used in these patients.

Overall, from this study we can thus conclude that 1) coronary artery contractions to DHE in distal coronary arteries are smaller than those to sumatriptan and 2) in the clinical situation both drugs are likely to induce only a small contraction in both

proximal and distal coronary arteries. 3) Contractions to DHE in middle meningeal arteries are smaller than contractions to sumatriptan, although variation of contractions to sumatriptan are large; and 4) contractions to both DHE and sumatriptan are larger in middle meningeal arteries than in coronary arteries, demonstrating the cranioselectivity of these drugs. Although contractions of both sumatriptan and DHE in coronary arteries are mostly small, higher outliers can occur and thus the use of these drugs in patients with coronary artery disease should remain contra-indicated.

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Chapter 8 – Cranioselectivity of Sumatriptan Revisited. Pronounced Contractions to Sumatriptan in Small Human Isolated Coronary Artery.

Based on: Kayi Y. Chan, Sieneke Labruijere, Martha B. Ramírez Rosas, René de Vries, Ingrid M. Garrelds, Alexander H.J. Danser, Carlos M. Villalón, Antoon van den Bogaerdt, Clemens Dirven and Antoinette MaassenVanDenBrink, January 2014, CNS Drugs.

Abstract

Background - Initial concerns about the coronary side-effect potential of the anti-migraine drug sumatriptan and second-generation triptans initiated cranioselectivity studies using proximal human coronary arteries. However, myocardial ischemia may originate from both large and small human coronary arteries.

Methods - We investigated the contractions to sumatriptan in proximal (internal diameter 2-3 mm), distal (internal diameter 1000-1500 μm) and small (internal diameter 500-1000 μm) human epicardial coronary arteries and compared these to contractions in the human middle meningeal artery. Concentration response curves to sumatriptan in human coronary arteries were constructed in the absence or presence of the 5-HT_{1B} receptor antagonist SB224289 and the 5-HT_{1D} receptor antagonist BRL15572. The effect of sumatriptan on increased cAMP levels induced by forskolin in proximal and distal coronary artery segments was investigated using a biochemical assay. Western blotting was used to analyse the 5-HT_{1B} receptor density in the human arteries.

Results - Contractions in proximal human coronary artery were significantly smaller than those in human meningeal artery, as we showed previously. In contrast, contractions to sumatriptan in distal and small human coronary arteries were not different from those in human meningeal artery. The 5-HT_{1B} receptor antagonist SB224289, but not the 5-HT_{1D} receptor antagonist, BRL15572, inhibited the contraction induced by sumatriptan in the coronary arteries. Moreover, in distal, but not in proximal coronary arteries, sumatriptan inhibited the increase in cAMP levels induced by forskolin. Contrary to our expectations, the 5-HT_{1B} receptor expression was more pronounced in the proximal human coronary artery than in the distal and small human coronary artery.

Conclusions - Based on functional experiments in distal and small human coronary arteries, contractions to sumatriptan are not as cranioselective as previously assumed. However, the vast clinical experience with sumatriptan and other triptans has proven that these drugs are cardiovascularly safe when contraindications are taken into account.

Introduction

Sumatriptan is effective and well tolerated in most migraine patients [1]. In preclinical studies, this drug has been shown to induce contraction of cranial arteries such as the middle meningeal artery via the 5-Hydroxytryptamine$_{1B}$ (5-HT$_{1B}$) receptor [2], where its clinical effect is most likely -at least partly- mediated [3]. However, the presence of 5-HT$_{1B}$ receptors on coronary arteries raised concerns about the potential coronary side effects of sumatriptan since up to 10% of patients report chest symptoms, including pressure, tightness and pain [4]. Several preclinical studies using sumatriptan and other triptans were carried out to corroborate their cranioselectivity and cardiovascular risk potential [2,5]. The results showed that, compared to the human meningeal artery, contractions induced by the triptans in the coronary artery at therapeutic concentrations are rather limited. However, these studies were exclusively performed on proximal segments of the human coronary artery, while currently techniques are available to study also more distal sections of the human coronary artery. Since myocardial ischemia may originate from large as well as small coronary arteries [6] and chest pain might be caused by microvascular spasm [7], parallel studies using proximal and distal coronary arteries are essential to properly assess the cranioselectivity and cardiovascular risk potential of the triptans. Considering the above findings, the present study was designed to characterize the differences in contraction to sumatriptan in proximal and distal parts of the human coronary artery. Subsequently, the effects of sumatriptan at therapeutic concentrations in the human coronary artery and the human meningeal artery were compared to analyse the cranioselectivity and cardiovascular risk potential of sumatriptan.

Methods

Functional experiments

Human coronary artery

The right epicardial coronary artery was obtained from 6 heart-beating organ donors who died of non-cardiac disorders less than 24 hour before the tissue was taken to the laboratory (3 male, 3 female; age 11-62 years). The hearts were provided by the Rotterdam Heart Valve Bank after donor mediation by Bio Implant Services Foundation / Eurotransplant Foundation (Leiden, The Netherlands). Dissected proximal (internal

diameter 2-3 mm), distal (internal diameter 1000-1500 μm) and small (internal diameter 500-1000 μm) right human coronary arteries were mounted in 15-ml organ baths (proximal) or small vessel myographs (distal and small) containing oxygenated (95% O_2 and 5% CO_2) Krebs bicarbonate solution at 37°C [8]. The ethics committee dealing with human experimentations at the Erasmus Medical Center Rotterdam approved the study protocol. Concentration response curves (CRCs) to 5-hydroxytryptamine (5-HT) and sumatriptan were constructed [paired parallel design, 5]. CRCs to sumatriptan were constructed in the absence or presence of the 5-HT_{1B} receptor antagonist SB224289 and the 5-HT_{1D} receptor antagonist BRL15572 (all compounds: Sigma Chemical Co., St. Louis, MO, U.S.A.).

Middle meningeal artery

In this study, coronary artery data are compared with historical data from our previous cranioselectivity study [2]. To exclude bias due to the use of historic data, three novel experiments were performed with sumatriptan in the human middle meningeal artery. Human meningeal arteries (1 male, 2 female, age 51-75, internal diameter 500-750 μm) were obtained perioperatively from patients undergoing neurosurgical procedures at Erasmus Medical Center, Rotterdam, The Netherlands. Vessels were transported to the laboratory immediately and were mounted in small vessel myographs [9]. CRCs to sumatriptan were constructed. The ethics committee dealing with human experimentations at the Erasmus Medical Center Rotterdam approved the study protocol.

Human arteries for the biochemical studies

The right epicardial coronary artery was obtained from 15 heart-beating organ donors who died of non-cardiac disorders less than 24 hour before the tissue was taken to the laboratory (9 male, 6 female; age 41-62 years). The hearts were provided by the Rotterdam Heart Valve Bank after donor mediation by Bio Implant Services Foundation / Eurotransplant Foundation (Leiden, The Netherlands). Dissected human coronary arteries were directly used for cyclic adenosine monophosphaat (cAMP) experiments or snap frozen with liquid nitrogen, and stored at –80°C until use for Western blotting. One human meningeal artery (1 female, age 40 years) obtained perioperatively from a patient undergoing a neurosurgical procedure at Erasmus Medical Center, Rotterdam, The Netherlands was also snap frozen for Western blotting.

cAMP measurements

Vasoconstriction to sumatriptan is mediated via a reduction in intracellular cAMP levels. This reduction was studied in proximal and distal segments by incubation with sumatriptan in a medium containing isobutylmethylxanthine (0.5 mM). After 30 minutes, segments were exposed to forskolin (cAMP inducer, 1 μM) for 5 min and snap frozen. Samples were analyzed with a cAMP ELISA kit (R&D Systems Europe Ltd., Abingdom, U.K.). Small coronary arteries were not used due to the limited volume of these tissues.

Western blotting

To lyse the tissues, 1 ml RIPA buffer (containing 150 mM NaCl, 1% Igepal 630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 8.0) supplemented with complete protease inhibitors (Roche, Woerden, The Netherlands) and phosphotase inhibitors (Sigma-Aldrich) were added. Tissues were homogenized by repeated 10-s sanitation for 1 min on ice. The resulting homogenates were centrifuged at 14,000 rcf for 15 min at 4°C, and protein concentrations in supernatants were measured with a BCA assay (Pierce, Etten-Leur, The Netherlands).

Equal amount of protein (15 μg) was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore) for immunoblot analysis. Blots were probed with antibodies against 5-HT_{1B} receptor (1:1,000, Abcam) and β-actin (1:50,000, Millipore). Bound antibody was detected by enhanced chemiluminescence (Pierce) using horseradish peroxidaseconjugated secondary antibodies (1:3,000, Bio-Rad, Veenendaal, The Netherlands).

Analysis of data

Curves that cover the full sigmoidal range were analyzed by means of a computerized curve fitting technique to obtain pEC_{50} and E_{max} values. E_{max} values of arteries are expressed as percentage of contraction to 100 mM KCl in the respective arterial segment. All data are presented as mean±s.e.m.. The blocking potency of the antagonists was estimated by calculating the apparent pK_B [8]. Statistical testing was performed using paired Student's t-test and ANOVA followed by Dunnett's post hoc comparisons test. Kruskal-Wallis test was used to test the predicted contraction at therapeutic plasma concentrations. $P<0.05$ was assumed to denote significant changes.

Results

Contractile responses to 5-HT and sumatriptan

No significant differences were found between the maximal contractions induced by 5-HT and sumatriptan (proximal: E_{max}: 55±11% vs. 29±17%, distal: E_{max}: 96±26% vs. 70±22%, small: E_{max}: 86±22% vs. 57±16%, respectively) in all tested coronary arteries (figure 1). The E_{max} for 5-HT and sumatriptan did not differ between the three types of vessels. The potency of 5-HT was significantly higher than that of sumatriptan in the proximal (pEC_{50}: 7.13±0.48 vs. 6.07±0.24), but not in the distal (pEC_{50}: 7.26±0.27 vs. 6.39±0.24) and small (pEC_{50}: 7.28±0.29 vs. 6.73±0.16) coronary arteries.

Contractions to sumatriptan were significantly inhibited by the 5-HT_{1B} receptor antagonist, SB224289, in proximal, distal and small coronary arteries with a pK_B of 8.0±0.6, 8.7±0.5 and 9.2±0.6, respectively (figure 1). pK_B values were not significantly different. The 5-HT_{1D} receptor antagonist, BRL15572, did not affect the contractions induced by sumatriptan

Contractile responses in meningeal vs. coronary arteries

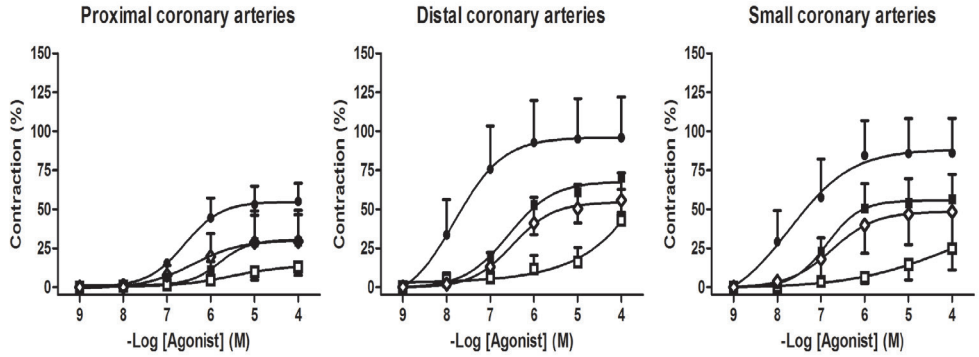

Figure 1. Contractile responses (%) to 5-Hydroxytryptamine (5-HT) (●), sumatriptan (■), sumatriptan + SB224289 (□), and sumatriptan + BRL15572 (◊) in the human isolated proximal (left), distal (middle) and small (right) coronary arteries. Data are expressed in mean±s.e.m., n=6.

Meningeal artery contractions to sumatriptan in the current series of experiments (E_{max}: 107±13%, pEC_{50}: 7.11±0.36, n=3) were not significant different from our previously published experiments (E_{max}: 103±13%, pEC_{50}: 7.70±0.28, n=5 [2]). Therefore, the his-

toric data and new data were pooled for further analysis. Maximal coronary artery contractions to sumatriptan in the present study were compared to those in human meningeal artery. Compared to the human meningeal artery (pooled data E_{max}: 105±9%, n=8, figure 2), contractile responses to sumatriptan were significantly lower in the proximal, but not in the distal and small human coronary artery. The potency of sumatriptan in the human proximal, distal, but not the small coronary arteries was significantly lower than that in the human meningeal artery (pEC_{50}: 7.48±0.23, n=8, figure 2).

Predicted contraction at therapeutic plasma concentrations

For coronary arteries from each calibre, the contractions that would occur at clinically relevant concentrations (free, i.e., unbound to plasma proteins C_{max}: 112-157 nM after the 100 mg oral and 193-224 nM after the 6 mg subcutaneous dose [5]) was calculated

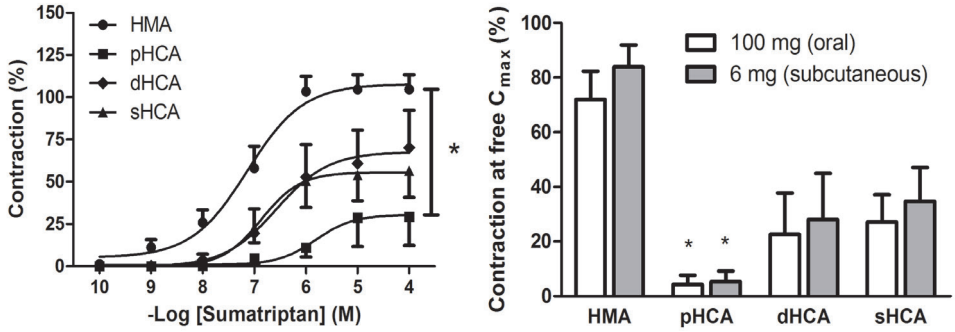

Figure 2. Contractile responses (left) to sumatriptan and the predicted contraction at free Cmax of sumatriptan (right) in human proximal (pHCA), distal (dHCA) and small (sHCA) coronary arteries as well as in human meningeal arteries (HMA). Data are expressed as % in mean±s.e.m., n=5-8. * $p<0.05$, HMA vs. pHCA.

by interpolation of the concentration response curves. The predicted contraction at free C_{max} after the 100 mg oral and 6 mg subcutaneous dose in human meningeal artery (72±10% and 84±8%, respectively) was significantly higher than that in proximal (4±3% and 5±4%), but not in distal (23±15% and 28±17%) or small (27±10% and 35±12%) coronary arteries.

Effect of sumatriptan on cAMP levels induced by forskolin

Sumatriptan inhibited the increase in cAMP levels induced by forskolin only in the distal (33±9 vs. 12±2 pmol/mg protein), but not in the proximal (34±7 vs. 27±6 pmol/mg protein, n=11) coronary artery (figure 3).

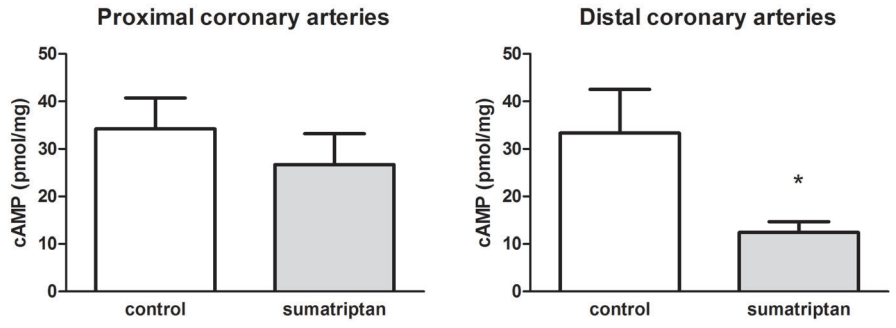

Figure 3. Effect of sumatriptan (1 μM) on the increase in cyclic Adenosine monophosphate (cAMP) levels induced by forskolin (10 μM) in human proximal (left panel) and distal (right panel) coronary arteries. Data are expressed in mean±s.e.m., n=11.

5-HT$_{1B}$ receptor density in human meningeal and human coronary arteries

The 5-HT$_{1B}$ receptor density (40 kDa) was highest in proximal sections of the coronary artery, while the levels were lower in distal and small coronary artery. The 5-HT$_{1B}$ receptor density in the human meningeal artery was higher than the receptor density in most proximal coronary arteries (figure 4). At 25 kDa an aspecific band was observed, that may potentially represent a degradation product of the 5-HT$_{1B}$ receptor protein [10].

Discussion

The present study investigated whether the cranioselectivity of sumatriptan, which was described earlier based on experiments in the proximal coronary artery, is also present for distal and small parts of the human coronary artery. In addition, we studied whether the contraction to sumatriptan is also mediated via the 5-HT$_{1B}$ receptor in distal and small coronary arteries. The results confirm our earlier results in proximal coronary artery, but also indicate that, in contrast to the proximal coronary

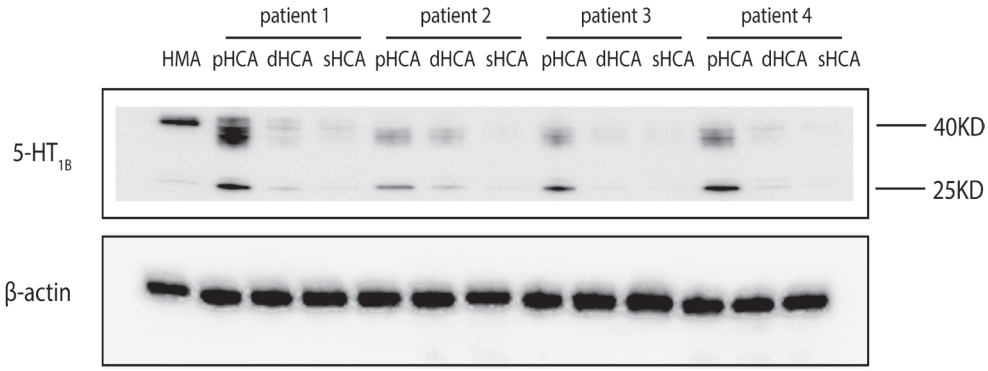

Figure 4. Representative Western blots of lysates from human proximal (pHCA), distal (dHCA) and small (sHCA) coronary arteries of four donors as well as from human meningeal artery (HMA). Blots were stained for 5-HT1B receptor (40 kDa) and β-actin.

artery, contractions to sumatriptan in distal and small parts of the coronary artery do not differ significantly from that in meningeal artery. As expected, the contractions in all parts of the coronary artery were mediated via the 5-HT_{1B} receptor. In accordance with our functional data, biochemical measurements indicated a stronger reduction of cAMP levels in distal than in proximal coronary artery. Longmore at al [11] showed higher 5-HT_{1B} receptor expression with autoradiography in human meningeal artery than in human proximal coronary artery, which is in accordance with our findings in the proximal coronary and the meningeal artery. Based on our functional results, the 5-HT_{1B} receptor density in distal coronary artery would be expected to be comparable with that in the human meningeal artery. However, contrary to our expectations, in our Western blot study, the 5-HT_{1B} receptor density was highest in proximal sections of the coronary artery, while the levels were lower in distal and small coronary artery. This intricate relationship between the functional responses in our study and the receptor density may be assigned to several factors. Firstly, it could be that receptor signaling is more efficient in distal than in proximal coronary artery, thus leading to the highest functional response in the distal and small coronary artery segments, notwithstanding their lower 5-HT_{1B} receptor expression. Alternatively, however, there might be several confounding factors such as more atherosclerosis in proximal coronary artery segments, which may introduce a bias. Moreover, it should be kept in mind that the functional experiments

and the Western blots were performed on coronary arteries obtained from different donors. Thus, from the current Western blot experiment, we can only conclude that the magnitude of the functional response is not necessarily directly related to the receptor density. This issue should be investigated in detail in future studies, making a paired comparison between functional experiments and Western blot studies, in both healthy and diseased coronary arteries of different calibre.

Our functional findings suggest that, at therapeutic concentrations, sumatriptan does not only induce considerable contractions in human meningeal artery, but also in human distal and small coronary arteries. Therefore, based on these data, sumatriptan and probably also other triptans would be considered to have limited cranioselectivity. This may well be a class effect of the triptans, independent from their differences in pharmacological selectivity [12], since the current findings are in accordance with previous observations from our group, where the contractions to zolmitriptan were also more pronounced in distal than in proximal coronary arteries [8].

However, the two decades of clinical experience with the triptans have demonstrated that the use of triptans, taking the contraindications into account, is safe [4]. Indeed, in cardiovascularly healthy subjects, coronary artery flow is not reduced up to a decrease in diameter of about 80% [13]. As a testament to its safety profile over many years, sumatriptan is now available without prescription in some countries [1,14]. It has even been reported that the number of adverse events was not increased when patients were treated with cardiovascularly active drugs and triptans [15], suggesting that the triptans are also safe in patients with increased cardiovascular risk. However, our current findings in the distal and small human coronary artery indicate that triptans should remain contraindicated in patients with cardiovascular disease. This supports the concerns about safe use of these drugs when triptans are available without prescription [14].

Conclusion

In conclusion, based on the functional experiments in distal and small human coronary arteries, contractions to sumatriptan are not as cranioselective as previously assumed. However, the vast clinical experience with sumatriptan and other triptans has proven that these drugs are cardiovascularly safe when contraindications are taken into account.

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Part 4 - The CGRP-ergic system

Chapter 9 – Discovery Techniques for Calcitonin gene-related peptide receptor antagonists for potential anti-migraine drugs.

Based on: S. Labruijere, K. Ibrahimi, K.Y. Chan and A. MaassenVanDenBrink**,** September 2013, Expert opinion on drug discovery.

Introduction

Migraine is a disabling neurovascular disorder, characterized by a unilateral throbbing headache that lasts from 4 to 72 hours. The headache is often accompanied by nausea, vomiting, phonophobia and photophobia and worsens by physical exercise [1]. There are different types of migraine where migraine with aura (MA) and migraine without aura (MO) are the most common forms [2]. A migraine attack can have different phases, the prodromal or premonitory phase, the aura phase, the headache phase and the postdromal phase. During the premonitory phase patients have often symptoms like yawning, nausea and craving for food. The migraine aura is thought to be caused by a cortical spreading depression (CSD). During the postdromal phase, which can last hours to days after the resolution of the headache, patients are often tired [3]. Migraine has a worldwide prevalence of 10% and a prevalence of 15 – 20% in the Western Countries [4]. This shows that migraine is a major health problem, which also involves a high financial burden to society. Migraine pathophysiology is not clearly understood, but the trigeminovascular system is thought to play an important role in it [5]. Apart from the more general analgesics there are different medicines available to treat migraine. These drugs can be divided into two types: 1) drugs aimed at abolishing migraine attacks and 2) drugs aimed at preventing migraine attacks. The latter group is prescribed to patients that have frequent attacks and in which the acute drugs have no effect, in order to reduce the severity or the number of attacks [6].

The first drugs used to treat acute migraine attacks were the ergot alkaloids, which were discovered in the beginning of the 20th century. Ergot alkaloids are vasoconstrictors that act by binding of 5-hydroxytryptamine (5-HT) receptors, α-adrenoceptors and dopamineD_2 receptors. Due to their multiple receptor binding properties these drugs have many side effects [7]. In the 1990s of the former century, the triptans were introduced. These drugs have shown to be as effective as the ergot alkaloids in treating migraine attacks, but with less side-effects due to their specificity for 5-$HT_{1B/1D}$ receptors [8,9]. Triptans are not effective in all migraineurs and contra-indicated in patients with cardiovascular disease, so it is important that more drugs are developed to abolish migraine attacks [10]. In the early nineties, Edvinsson and Goadsby discovered an increase in calcitonin gene related peptide levels in plasma of the jugular vein during a migraine attack and the CGRP levels

normalized after treatment with triptans [11,12]. CGRP is a potent vasodilator and is widely present in the brain and the cardiovascular system together with its receptor, which is composed of three parts: receptor activity modifying protein 1 (RAMP1), calcitonin-like receptor (CLR) and receptor component protein (RCP) [13,14]. A migraine attack is thought to start in the brainstem due to certain triggers, which have not been exactly identified yet, leading to the release of CGRP at trigeminal nerve endings into meningeal arteries [15]. CGRP binds to its receptor that is present on the smooth muscle cell membrane, activating adenylate cyclase, causing an increase in intracellular cyclic AMP (cAMP). This leads to activation of different signalling pathways and vasodilatation, where activation of nitric oxide synthase (NOS), leading to an increase in nitric oxide (NO) [16], may also be involved. The vasodilatation thought to affect sensory neurons which, in turn, activate the trigeminal system, passing on sensory information to higher brain regions, but also leading to other neurological symptoms as phono- and photophobia [17,18]. The first potent and selective CGRP receptor antagonist was olcegepant and showed to be effective in the acute treatment of migraine attacks similar to the triptans, but without its cardiovascular side effect profile [19]. Because of its low oral bioavailability, other CGRP receptor antagonists with improved oral bioavailability were developed [20,21]. Telcagepant was the first improved, orally available CGRP receptor antagonist that was tested in multiple clinical trials, as well as other ones developed later on. These clinical trials are discussed below. It is crucial that various effects and side effects of newly developed CGRP receptor antagonists are characterized to be sure the developed drugs are both safe and effective. Furthermore, as migraine is a syndrome involving multiple physiological systems, it is very important to obtain more knowledge about the underlying mechanisms causing migraine. This may eventually lead to more specific treatment for specified migraine patient groups. Since migraine involves multiple organ systems, both vascular and neuronal, there are many different research models, which are used to study different aspects of migraine and antimigraine medication.

The aim of this review is to describe the experimental *in vivo* and *in vitro* models that can be used for the discovery and characterization of new CGRP receptor antagonists.

Clinical trials with CGRP receptor antagonists

CGRP receptor antagonists have proven to be effective in the acute treatment of migraine in multiple clinical trials (Table 1). Olcegepant (BIBN4096BS) was the first CGRP receptor antagonist to be tested in clinical trials. In phase I trials olcegepant showed good safety and tolerability [19,22] and adverse events (AE's) reported were only transient and mild paresthesias. In a Phase II trial in 126 migraine patients, olcegepant proved to be an effective drug for the acute treatment of migraine and headache relief after 2 hours was achieved in 66% of the migraineurs [23]. Due to the high molecular weight of olcegepant it can only be administered intravenously and is consequently unfeasible in a clinical setting. It was clear that a CGRP receptor antagonist with an improved pharmacokinetic profile was required. Telcagepant (MK-0974) was the first orally bioavailable CGRP receptor antagonist that was discovered. In a Phase II trial, where several doses of telcagepant were compared to rizatriptan, telcagepant proved to be effective in the acute treatment of moderate or severe migraine attacks. Headache relief after 2 h was achieved in 68% of the migraineurs when treated with the highest dose that was used in this trial (600 mg), compared to 69% when treated with rizatriptan [24]. In Phase III trials telcagepant repeatedly acted effectively as acute treatment of migraine when compared to placebo [25,26]. However, when raised levels of liver transaminases were detected in a few participants in a Phase II trial (NCT00797667) where telcagepant was assessed as a prophylactic treatment for migraine, the development of telcagepant was suspended [27]. The development of another CGRP receptor antagonist (MK-3207) with higher bioavailability than telcagepant and also effective in the acute treatment of migraine, was discontinued as well after reports of raised levels of liver transaminases [21,28]. The CGRP receptor antagonist BI 44370 TA has proven to be successful in treatment of acute migraine in a Phase II trial. Headache relief after 2 hours was reached in 27% of the participants for BI 44370 TA (400 mg), compared to in 9% of the participants for placebo and there were very few reports of AE's, with an incidence of ≤ 3 % [29]. Finally, in a recent Phase I trial, the CGRP receptor antagonist BMS-927711 showed to be safe and tolerable in 8 healthy subjects at single doses up to 1500 mg and multiple doses up to 600 mg. No serious AE's were reported [30]. Though clinical trials provide an excellent opportunity to study the pharmacokinetics and pharmacodynamics of a drug, they

Table 1. Overview of clinical trials performed with CGRP receptor antagonists
CGRP receptor antagonist

CGRP receptor antagonist	Administration route	Last trial phase completed (headache relief after 2h)	Adverse events reported (>5%)	Status
Olcegepant [23]	i.v.	II (66%)	Mild paresthesias	Discontinued due to low oral availability
Telcagepant [25]	Oral	III (24-27 %)	Dry mouth, nausea, somnolence, dizziness and fatigue	Discontinued due to increased liver enzymes
MK-3207 [28]	Oral	II (36%)	Dry mouth, nausea, vomiting, dizziness, fatigue and headache	Discontinued due to increased liver enzymes
BI 44370 TA [29]	Oral	II (27 %)	None	In development
BMS-927711 [30]	Oral	I (unknown)	Headache, constipation and nausea	In development

provide little information of the underlying mechanisms of action. Consequently, *in vitro* and *in vivo* research models are needed to allow us insight into these mechanisms.

Experimental Models

Clinical trials in healthy subjects and patients cannot be performed without prior characterization studies of the potential drug in experimental models. There is no experimental model in which a complete migraine attack can be studied. All available models focus on a specific component of the disease. Different *in vitro* and *in vivo* models are used to investigate effects and side effects of prospective antimigraine drugs (Table 2 and Figure 1).

In vitro models

Because migraine is a heterogeneous disease and pain is difficult to measure in animals, different *in vitro* models have been developed to study separate components of the disease. Since migraine is a neurovascular disease, various models have been developed that focus on the vasculature. CGRP is a very potent vasodilator [31], so newly developed CGRP receptor antagonists can be studied for their potency in different types of isolated blood vessels. Apart from its effects on the vasculature, CGRP also plays an

important role in different brain regions. CGRP has shown to be present in perivascular nerves, but also in the trigeminal system, amygdale, striatum, colliculi, hypothalamus, cerebellum and brainstem [13]. *In vitro* models focussing on brain tissue can be used to study effects of CGRP receptor antagonists on its neurotransmitter function. In general, *in vitro* models have a number of advantages: i) multiple tissue samples can be studied of the same tissue, making it possible to perform a detailed pharmacological analysis; and ii) there is no influence of varying blood pressure, circulating hormones or central and autonomous nervous system, reducing the number of confounding factors when performing pharmacological analyses. However, *in vitro* models are not sufficient to completely predict a therapeutic effect or to predict the pharmacokinetics, including brain penetration, of a compound, and should thus be used complementary to *in vivo* models, which will be described further on in this review.

Isometric tension measurements in blood vessels from experimental animal and humans

Organ baths for isometric tension measurements are experimental models in which vasoconstrictor or vasodilator properties of compounds can be studied in isolated blood vessels. There are different sizes of organ baths, made for blood vessels with different diameter size. Vessel segments with a diameter of 0.05 – 3 mm and a length of 2 – 5 mm can be mounted in wire myographs between two wires. One of the wires is connected to a force transducer and computer and the vessels are stretched to a force that is comparable to a normal blood pressure. Different organ bath studies have been performed on the characterization of the CGRP receptor antagonist olcegepant and telcagepant. As may be expected from their mechanism of action that differs from that of the triptans that are vasoconstrictors, CGRP receptor antagonists have been found to be devoid of vasoconstrictor effects per se [22,31]. However, it is important to assess their potency to antagonize vasodilator responses to CGRP in isolated blood vessels. In both human and bovine cerebral arteries it was shown that olcegepant blocked the effect of exogenously administrated CGRP [32,33]. Furthermore, it was shown in human isolated coronary as well as in cranial arteries that telcagepant, similar to olcegepant, antagonized vasodilator responses to CGRP, and moreover did not induce any contraction or relaxation of the blood vessels (Figure 2) [31,34]. A disadvantage of this model is that the compounds under investigation reach the blood vessels from both the luminal and abluminal side, which is not physiological, because neuropeptides like

Table 2. Overview of experimental models.

Experimental model	*In vivo/ in vitro*	Description	Advantages	Disadvantages
Isometric tension measurement [31]	*In vitro*	Study of vasoconstrictor or vasodilator properties of compounds on isolated blood vessel segments in organ baths	Multiple segments can be studied at once	No difference can be made between luminal and abluminal side
Pressure myography [35]	*In vitro*	Study of vasoconstrictor or vasodilator properties of compounds on islated blood vessel segments in organ bath in which compounds can be administrated luminally or abluminally	Effects can be studied on luminal and abluminal side of the blood vessels	Mounting of segements is time consuming so only a few segments can be studied at once
Brain slices [21]	*In vitro*	Binding of CGRP receptor antagonists in the brain can be studied	Binding properties can be studied in detail in the brain	No information on brain access, penetration or functional response
Vascular animal models [37]	*In vivo*	Effects of both exogenous an endogenous CGRP can be studied in living animals	Hemodynamic effects can be studied	No information on brain activity
Intravital microscopy via closed cranial window [40-42]	*In vivo*	Study of dural blood vessel properties via a thinned skull (closed cranial window)	The effect on dural vessels can be studied in living animals	No information on brain activity
Imaging models [47,50]	*In vivo*	Real time imaging of blood vessels and brain via MRI, Bold-MRI and PET	Effects in living animals or humans	Expensive and time consuming
Laser Doppler perfusion imaging [56]	*In vivo*	Measurement of skin perfusion with laser Doppler scanner	Small resistance vessels are studied, non-invasive	No measurements of dural blood vessels
Human migraine models [60,63,67,72]	*In vivo*	Infusion of migraine-inducing compounds in either migraine patients or healthy volunteers	Effect of CGRP receptor antagonists can be studied in human	Not all subjects get headache. Headache is not identical to migraine
Fos expression [77	*In vivo*	Expression of the Fos protein is related to neuronal activation and can be used to study activation of the trigeminovascular system	Central effects of CGRP receptor antagonists can be studied	Fos is not expressed in all neurons and stimulations neccesary to induce Fos expression are sometimes higher than physiological
Electrophysiological recordings [91]	*In vivo*	Measurement of action potential activity in brain of living animal via electrodes	Living animals, physiological	Small area can be measures, no information on vascular system
Transgenic mice models [127]	*In vivo*	Mice in which a gene of another species is inserted and expressed	The physiological role of the CGRP-ergic sytem can be studied	Difficult to examine migraine symptoms in animals

CGRP are released form nerve afferents on the abluminal side of the artery. In contrast, drugs are transported via the blood and need to cross the blood vessel wall to reach the smooth muscle cells, where for example CGRP receptors are located [34]. This is especially important in the brain, were the blood brain barrier is present. Therefore another model was developed, which is described below.

Pressure myography in blood vessels from experimental animals and humans

In the pressure myograph model, a segment of a blood vessel is placed over a small glass pipette tip at both sides and securely tied up by small wires. A flow is established through the vessel, leading to a certain pressure, which can be measured. The blood vessel is mounted in an organ bath, with the abluminal side of the vessel completely separated from the luminal side. This model can be used for example to investigate ability of a substance to cross the endothelial layer of a blood vessel (e.g. the blood brain barrier) [35], but also to study receptors that are present on smooth muscle cells and activated by release of proteins via nerve endings, in a more physiological way [35]. It was shown with this model that luminally applied olcegepant could not block the relaxing effect of abluminally applied CGRP in middle cerebral arteries. This indicates that olcegepant possibly cannot cross the blood- brain barrier [36]. A disadvantage of this model is that only a few pieces of vessel can be studied at a time resulting in a very time consuming method. Furthermore, because a stable intraluminal pressure is needed, the construction of concentration response curves is difficult from the luminal side of the blood vessel.

Brain slices of experimental animals

Brain slices can be used to study binding of antagonists to a receptor. A study in rhesus monkey brain slices on a new CGRP receptor antagonist, MK-3207, showed a very high density of binding in the meninges, cerebellum and brainstem and a very low binding in the cortex, providing information about the potential central sites of action of the CGRP receptor antagonist [21]. Obviously, results obtained from such methods should be complemented by investigations on the brain penetration of the CGRP receptor antagonists

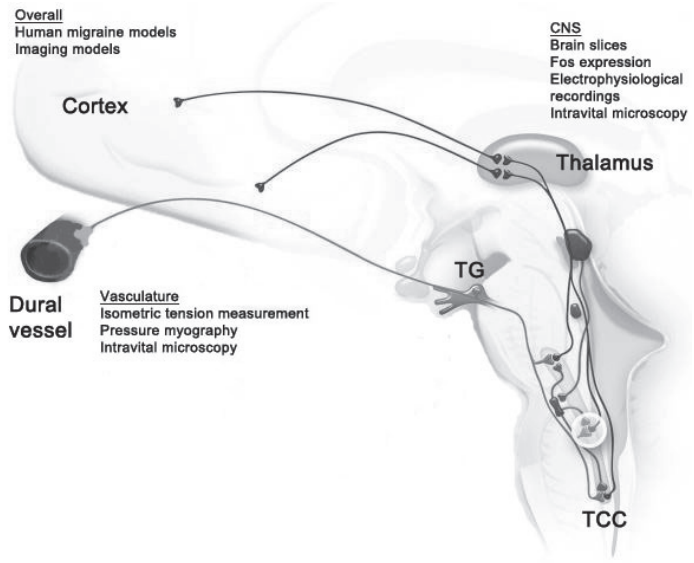

Figure 1. Experimental models in the trigeminovascular system.

In vivo models

Major limitations of the *in vitro* models that are used to study migraine are that they are not physiological and thus cannot be used without complementary *in vivo* studies to predict therapeutic effects and pharmacokinetics of investigational drugs. In the next part, *in vivo* models for migraine that can be used to characterize effects of CGRP receptor antagonists on vasculature as well as the central nervous system are discussed.

Vascular models in experimental animals

A main part of research performed for the development of the triptans was based on purely vascular models in experimental animals (for review, see Villalón and Gupta, [10]). For research on CGRP receptor antagonists, a neuronal component should be implicated as well, since otherwise only effects on exogenously administered CGRP can be studied. Administration of capsaicin induces endogenous CGRP release, which in turn leads to vasodilatation. This model has been applied in anaesthetized vagosympathectomized dogs [37]. An alternative approach to induce endogenous CGRP release is electrical spinal stimulation, for example in pithed rats [38]. These models may well be used to study the hemodynamic effects of CGRP receptor antagonists.

Closed cranial window model in experimental animals

The dilation of blood vessels in the meninges is caused by activation of the trigeminovascular system [39]. The closed cranial window model is a preclinical *in vivo* model, which is based on intravital microscopy for a direct measurement of dural blood vessel diameter through a closed cranial window in anaesthetized rats [40] or mice [41]. In this model the skull is not opened, thus avoiding alteration of vessel reactivity due to brain swelling or change in pressure. Dilation of the dural artery can be induced by intravenous injection of CGRP or capsaicin [40] as well as by electrical stimulation on the surface of the skull [42]. Due to the different stimulations to induce dural vasodilatation, effects of antimigraine drugs can be studied on three levels; postsynaptic at the blood vessels due to exogenous CGRP injection, presynaptic at the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor due to capsaicin injection and in trigeminovascular neurons due to electrical stimulation. Exogenous CGRP exerts its effect by binding to the CGRP receptor located on the vascular smooth muscle cells, capsaicin induces endogenous CGRP release by binding on the TRPV1 receptor of TRPV1 receptor containing neurons and electrical stimulation induces endogenous CGRP release by depolarization of perivascular trigeminal nerves.

In the past years, different potential antimigraine drugs like the neuronal NOS inhibitor, s-methyl-l-thiocitrulline [43], the antiepileptic drug, topiramate [44] and different P/Q-,N- and L-type calcium channel blockers [45] have shown to block electrical stimulation-induced neurogenic vasodilatation, but not exogenous CGRP-induced vasodilatation. This suggests that all these compounds inhibit CGRP release from the perivascular nerve via presynaptic blockade of trigeminovascular neurons. In contrast, Chan *et al.* showed that the glutamate receptor antagonist LY466195, which shows antimigraine effects, did not affect CGRP release or its vasodilator effects, suggesting a central mechanism not involving CGRP-ergic pathways [46]. The cranial window model can be used to study the effect of potential CGRP receptor antagonists on dural vasodilatation *in vivo*. As may be expected from their mechanism of action, CGRP receptor antagonists inhibit vasodilator responses to both exogenous and endogenous CGRP [41]. Furthermore, this model can be used to study differences between exogenous and endogenous CGRP on dural vasodilatation, providing information about (patho)physiological factors affecting either the release of CGRP, or the response mediated by its receptors.

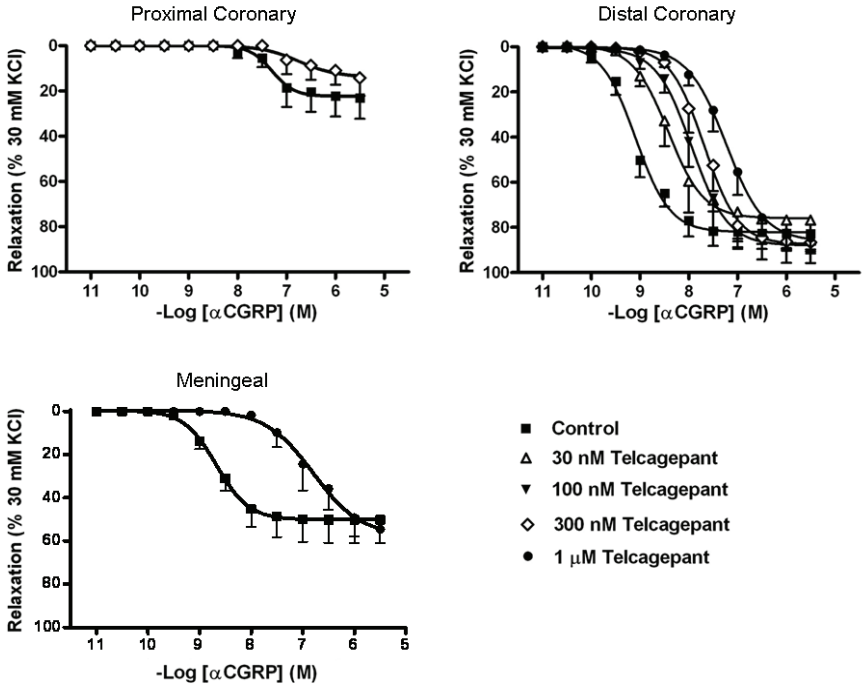

Figure 2. Relaxant effect of α-CGRP on human proximal (top left) and distal (top right) coronary arteries and human meningeal arteries. Concentration-response curves to α-CGRP were constructed in the absence or presence of 300 nM telcagepant (proximal), 30 nM - 1 μM telcagepant (distal), and 1 μM telcagepant (meningeal). Values given represent mean ± S.E.M. (n = 4–8). Adapted from: Chan et al. [31] and Edvinsson et al. [34].

Imaging models in human

In recent years, imaging of the brain and its related vasculature has become important in migraine drug research. Positron emission tomography (PET), a 3D imaging technique that can be used to image functional processes of the body, has successfully been used to study the effects of antimigraine drugs. With PET imaging, rizatriptan was shown to cause a decrease of cerebral blood flow (CBF) and blood volume of around 13% [47]. PET imaging was also used to show that sumatriptan can normalize the migraine-related increase in brain serotonin synthesis [48]. More recently, central CGRP receptor occupancy was investigated with PET imaging in healthy subjects. The investigators showed low receptor occupancy (4 - 10%) after dosing with the lowest clinically effective dose of telcagepant and moderate receptor occupancy (43 - 58%)

after dosing with a supra-therapeutic dose of telcagepant. They therefore concluded that central antagonism of CGRP receptors may not be required for a CGRP receptor antagonist to be effective in migraine treatment [49]. Due to the low temporal and spatial resolution of PET imaging, this technique is limited to investigating large areas of the brain. Consequently, magnetic resonance imaging (MRI) and especially, blood oxygenation level-dependent functional MRI (BOLD-fMRI) has become more accepted as the technique to assess altered brain activation and functional connectivity between brain regions [50]. BOLD-fMRI is favored because it is a noninvasive technique with high spatial and temporal resolution [51]. The effect of CGRP infusion and subsequent application of a subcutaneous therapeutic dose of sumatriptan on the visual cortex BOLD signal response was investigated and no changes in the visual neuronal activity after either CGRP infusion or subcutaneous sumatriptan were detected [52]. Using magnetic resonance angiography, an MRI technique to image blood vessels, Asghar *et al.* showed dilatation of the middle meningeal artery after infusion of CGRP and the reversal of this dilatation by subcutaneous sumatriptan in healthy volunteers [53]. The application of imaging techniques in the study of migraine headache, spontaneous or provoked by infusion of drugs, can provide information on the yet poorly understood underlying mechanism of migraine. In the research of CGRP receptor antagonist, imaging techniques can offer insight into the mode and location of action of the drugs.

Dermal blood flow in response to stimulation with capsaicin as well as electrical stimulation in animal models and humans.

Capsaicin, the active ingredient of chili peppers, stimulates the TRPV1 receptor and causes release of CGRP from perivascular nerve terminals [54]. Topically applied capsaicin on the human forearm skin is known to increase dermal blood flow (DBF), which can be measured with laser Doppler perfusion imaging [55]. This increase in DBF was inhibited by the CGRP receptor antagonist telcagepant [56]. Endogenous CGRP release can also be accomplished by electrical stimulation [11,57]. In humans, current-induced vasodilatation can be inhibited by local anesthesia and reduced by desensitization of C-nociceptive fibers [58]. Though not confirmed with the application of a CGRP receptor antagonist, current induced vasodilatation is thought to be mediated mostly by CGRP and substance P. Current-induced vasodilatation may be used to study the release of endogenous CGRP, without the involvement of the

TRPV1 receptor. A combination of the capsaicin application response and the current-induced vasodilatation provides a suitable model to study CGRP receptor antagonists, especially to elucidate the role of the TRPV1 receptor in migraine headaches [59]. An advantage of this model is that due to its direct translatability between species, this model can be applied in humans and animal models.

Human migraine models

Human migraine models play an important role in the research for new antimigraine drugs. There are several models in either healthy volunteers or migraine patients. In the latter group, migraine-like attacks can be triggered via infusion of drugs.

CGRP infusion - Infusion of CGRP in migraine patients, but not in healthy volunteers can induce delayed (1-12 hours after infusion) headache fulfilling the International Headache Society (IHS) criteria for 67% of MO patients and in 57% of MA patients [60-62]. The mechanism behind migraine after CGRP infusion is still not clear. Recently a study by Asghar *et al.* revealed that CGRP infusion leads to dilation of the middle meningeal artery but not to dilation of the middle cerebral artery [53]. It would be interesting to investigate the effects of CGRP receptor antagonists in the CGRP infusion model on the delayed migraine-like headache.

Glyceryl trinitrate infusion - Glyceryl trinitrate (GTN) infusion in migraine patients also triggers headache attacks, fulfilling the IHS criteria for MO, 4-5 hours after infusion in 50% of patients with MA and in 80% of patients with MO. In healthy volunteers GTN infusion does not cause a delayed migraine attack, although headache during and immediately after infusion does occur [63,64]. As with the CGRP infusion model, the exact mechanism behind the GTN migraine model is not known. GTN infusion leads to the production of NO, a potent vasodilator, in the body. NO-inhibitors are currently under research as possible new therapy target for migraine [65]. Although more studies are needed to draw categorical conclusions, the GTN migraine model may not be suitable to study the effects of CGRP receptor antagonists, since Tvedskov *et al.* showed that GTN-induced migraine was not prevented by olcegepant [66]. A possible explanation could be that CGRP exerts its effect earlier than NO in the events leading to migraine after GTN infusion.

Pituitary adenylate cyclase activating polypeptide infusion - The pituitary adenylate cyclase activating polypeptide (PACAP) migraine model is a model that also makes

use of a strong vasodilator. PACAP infusion induces migraine in 58% of patients with migraine without aura [67]. Infusion of vasoactive intestinal peptide (VIP) does not cause migraine in migraine patients [68]. VIP and PACAP share receptors, except the PACAP type 1 receptor (PAC_1), which suggests the involvement of PAC_1 in the migraine attacks seen after PACAP infusion [69]. PACAP and CGRP are hypothesized to share the cAMP signaling pathway and it would be interesting to investigate whether CGRP receptor antagonists can abort PACAP-induced migraine [70,71].

Prostaglandin infusion - Finally, infusion of prostaglandin E_2 can also induce migraine [72]. After prostaglandin infusion, 75% of patients with migraine without aura experience migraine-like attacks [73]. The migraine-like attacks occur during and immediately after infusion, which is different from the other pharmacologically induced migraine models. The immediate onset of the attacks points towards a role for prostaglandins in the late phase of migraine development. This is an interesting model to investigate the effect of CGRP receptor antagonists in this phase of migraine.

Fos expression in experimental animals

Immunoreactivity of the Fos protein, which is a nuclear protein that regulates transcription of other target genes, has been shown to be a marker of neuronal activity [74,75]. To identify related nociceptive pathways in migraine, activation of Fos expression in the trigeminovascular system has been used [76]. The expression of the Fos protein occurs *in vivo* after a certain stimulus, although obviously the measurement of the protein is performed *in vitro*. Fos expression can be activated by applying mechanical, electrical or chemical stimuli in either extracranial or intracranial tissues innervated by the trigeminal nerves. This includes electrical stimulation of the trigeminal ganglion and superior sagittal sinus (SSS) as well as chemical stimulation of the meninges (dura mater) with capsaicin or other irritant substances [75].

Stimulation of the trigeminal ganglion and meninges - Trigeminal ganglion activation is a well-established method for the activation of the trigeminovascular system. Electrical stimulation of the trigeminal ganglion induces expression of c-Fos mRNA and Fos protein in trigeminal nucleus caudalis (TNC) of pigs [77] and rats [78]. However, trigeminal ganglion stimulation may induce widespread Fos expression by discharging many other afferents than those innervating the meninges, which is a limitation of this method [75]. A more direct activation of the meninges is chemical stimulation

using capsaicin or other irritant substances [75]. Primary sensory fibers supplying the meninges are activated by injection of capsaicin or other irritant substances via a small catheter into the cisterna magna through the atlanto-occipital membrane. Fos protein immunoreactivity is detected in both sides of the TNC 2 hours after injection and capsaicin increases Fos protein immunoreactivity in a dose-dependent manner [79]. The limitation of chemical stimulation of the meninges is that no comparison can be made between the two sides of the TNC. To provide more information about the site of action in the TNC, stimulation of the SSS to induce Fos expression seems to be the most suitable model.

Stimulation of the superior sagittal sinus - Mechanical and electrical stimulation of the SSS has been used to induce Fos immunoreactivity in the TNC of rat [80], cat [81] and non-human primate [82]. Activation of brainstem structures after SSS stimulation, which is observed in different studies, reflects to the neurovascular activation of pain-sensitive structures during a migraine attack [75]. Moreover, neurons in the TNC that are activated through SSS stimulation are primary located in the deeper part of the nucleus caudalis, lamina V which correspond to Aδ-fiber input [81,83] and SSS stimulation induces the release of neuropeptides like CGRP and VIP in a pattern similar to that observed during migraine attacks [12,84].

Systemic infusion of stimuli - Systemic infusion of noxious stimuli can also be used to induce Fos protein expression. Glyceryl trinitrate (an NO donor) infusion induced Fos expression in the trigeminocervical complex (TCC, TNC and C1/2 spinal cords levels) [85], which suggests a role of NO donors in activating the trigeminovascular system. This is supported by the results that the NOS inhibitor N-Nitro-L-Arginine Methyl Ester (L-NAME) decreases capsaicin- as well as electrically-induced Fos expression [83,86]. Infusion of CGRP has also been shown to induce Fos expression in TNC [87]. The CGRP receptor antagonist, olcegepant inhibits systemically administered capsaicin-induced Fos expression in the spinal trigeminal nucleus, but not the increased phosphorylated extracellular signal-regulated kinase in the trigeminal ganglion when capsaicin is injected unilaterally in the face. This might suggest that olcegepant exerts its effect mainly in the central nervous system rather than periphery including the trigeminal ganglion, which is activated when capsaicin is injected in the face [88]. On the other hand, olcegepant was administered intravenously so it is questionable if the concentration that reached the central nervous system is high enough to exert any

effects, because of its incapability of crossing the blood brain barrier. More studies with CGRP receptor antagonists comparing central and peripheral effects need to be done to investigate this proposition.

Fos protein expression models are only good models when the stimulus drives the expression of Fos protein [75]. However, to induce a Fos expression to quantifiable levels, a strong, consistent stimulation is required that is often not physiological [89]. Moreover, since Fos is not expressed in all neurons [74], lack of Fos expression does not mean that there is no neuronal activity [90]. Despite these limitations, the Fos expression model with its different stimulations is a good model to investigate the neuronal activity of the trigeminovascular system on different levels [75,90]. The different stimulations have allowed the identification of a subpopulation of neurons that is activated in response to noxious stimuli and thus identifying related nociceptive response [90]. The Fos expression model already greatly increased our understanding of the trigeminovascular system [75,76] and the role of CGRP signaling in this system. In addition, the therapeutic mechanisms of possible CGRP receptor antagonists in the trigeminovascular system can be investigated via determination of neuronal activity.

In vivo electrophysiological recording in experimental animals

Electrophysiological recording is a method to measure action potential activity or local field potentials, which represent the net activity of a population of cells, in neurons by placing electrodes into the brain area of interest in a living animal [76]. In migraine research, electrophysiological recordings are performed in different parts of the trigeminovascular system, like the trigeminal ganglion [91] and the TCC [92,93] . Moreover, since functional imaging studies in spontaneous migraine attacks as well as human models using trigeminal nociceptive stimulation have shown a consistent activation of the thalamus [94-96] and the majority of the secondary neurons in the TCC project to the thalamus [97], the ventral posteromedial thalamic nucleus (VPM) is also an area of interest [76]. Indeed, electrophysiological studies in experimental models demonstrate that trigeminovascular nociceptive stimulation activates neurons in the VPM [12,98]. Action potentials in the TCC and VPM, measured by electrophysiological recordings, can be triggered by electrical stimulation of SSS (see Fos protein), electrical stimulation of dura mater (see cranial window) and micro-iontophoresis of $_{L}$-glutamate in either TCC [93] or VPM [99].

This model can be used to investigate the effect on neuronal activity in the second order neurons of TCC and the third order neurons of VPM for a possible site of action of antimigraine drugs [76]. Moreover, using micro-iontophoresis of $_{L}$-glutamate, effects on postsynaptic action of neuronal elements including dendrites and cell bodies distal to the synaptic cleft can be studied more specifically [99]. The great advantage of this model is the real-time resolution of the response to a stimulus or pharmacological intervention. Although the neuronal activity can only be studied in a few neurons at the same time [76], potential targets of triptans [92,98] as well as prophylactic antimigraine drugs have been discovered with this model [99,100].

CGRP release has been shown in many structures of the trigeminovascular system, including second-order neurons in the TCC [39]. Therefore, CGRP receptor antagonists might also affect the TCC and the VPM. Intravenous administration of olcegepant has been shown to reduce spinal trigeminal activity induced by electrical stimulation of the dura mater [91] and thalamic cell firing in VPM [101] as well as TCC [102] by SSS stimulation. Moreover, the CGRP receptor antagonist, $CGRP_{8-37}$, is able to inhibit cell firing induced by micro-iontophoresis of $_{L}$-glutamate and SSS stimulation in the VPM [101,102]. These results suggest that VPM might be a possible therapeutic target for CGRP receptor antagonists [102]. Because of the positive results obtained with the CGRP receptor antagonists olcegepant and $CGRP_{8-37}$, electrical recording of TCC and VPM seems an excellent experimental model to identify the effect of CGRP receptor antagonists in the second-order neurons of the TCC and the third order neurons of the VPM as a possible site of therapeutic action.

Cortical spreading depression - CSD is regarded to be a main pathogenic step in migraine with, and possibly also without aura. Although the potential role of CGRP in CSD has not yet been examined in detail, a recent study suggests that CGRP is involved in CSD and that CGRP receptor antagonism reduces CSD. This suggests, in contrast to the low CGRP receptor expression in cortex [21] and the limited CNS penetration of telcagepant [49] mentioned earlier in this review, that central CGRP receptors may be a relevant target for CGRP receptor antagonism.

Transgenic mice

Another model in which the physiological role of the CGRP-ergic system can be studied are transgenic mice. In these animals a gene of another species is inserted and

expressed. A recently developed transgenic mouse model is the *nestin/hRAMP1* mouse model. These animals over-express the RAMP1 part of the human CGRP receptor in the central and peripheral nervous system and show an aversive behaviour against light, which increases enormously after injection of intracerebroventricular CGRP [103]. It was shown that this light aversive behaviour in these animals could be blocked by olcegepant. Furthermore, some CGRP knockout mice show decreased pain responses and increased blood pressure [104,105]. These transgenic animals can thus be used to study effects of newly developed CGRP receptor antagonists.

Future developments

CGRP receptor blockade for the prophylactic treatment of migraine

While blockade of CGRP receptors has been demonstrated to be effective and well tolerated in the acute treatment of migraine, this concept has also been proposed as a mechanism of action for prophylactic antimigraine drugs, which are used by patients who suffer from highly frequent attacks, or where acutely acting drugs are contraindicated. Indeed, there is a need for effective preventive antimigraine treatments. Of the current prophylactics, none is specific for migraine (e.g., valproate was developed as an anti-epileptic drug and beta-blockers were developed to treat high blood pressure), explaining the high number of side effects due to their low specificity [106]. In addition, prophylactic drugs are generally effective in only 50% of patients.

Chronic CGRP receptor blockade can be reached via different strategies. Firstly, CGRP receptor antagonists can be administered on a (twice) daily basis. As described above in paragraph 3, this approach has been followed for telcagepant, which induced raised levels of liver transaminases and subsequent termination of the development of telcagepant. Alternatively, chronic CGRP receptor blockade may be reached by the administration of antibodies directed against the CGRP receptor. Using this latter approach, the CGRP receptor is blocked for up to 7 days after one single administration [107]. While a permanent blockade of the CGRP receptors could indeed be effective against migraine, it should be born in mind that, under such conditions, the physiological role of CGRP is constantly repressed. CGRP is one of the main contributors to the maintenance of normal vascular tone and protects the brain as well as peripheral organs, including the heart, against excessive vasoconstriction [108-112].

These important protective functions of CGRP would most likely be blocked when CGRP receptor antagonists or antibodies directed against the CGRP receptor are used as a prophylaxis, which could have potentially harmful consequences. On the other hand, in mice and rat no evidence was found that CGRP receptor antagonists have a negative effect on ischemic injury [113] and in rat and pig no effect of CGRP receptor antagonists olcegepant and $CGRP_{8-37}$ was seen on infarct size in ischemia/reperfusion studies [114,115]. Because these results are still contradictive, it is of crucial importance that extensive preclinical, as well as clinical studies are performed to elucidate whether it is safe to constantly repress the function of CGRP.

An alternative approach to blockade of the CGRP receptor is the use of an antibody directed against the CGRP protein itself [116]. Currently, the antibody LY2951742 is under investigation in a clinical trial. The potential objections mentioned above for repression of the CGRP receptor function obviously also apply for eliminating the function of the CGRP protein itself.

CGRP receptor antagonists against migraine and hot flushes during menopause?

Menopause is known to disturb the secretion of CGRP, resulting in lower levels of plasma CGRP in postmenopausal women compared to fertile women [117]. Vasomotor changes, such as hot flushes, on the other hand, are accompanied by a temporary rise in plasma CGRP levels in menopausal women. Indeed, infusion of CGRP can cause facial flushing [118]. Thus, CGRP may be an important factor in the occurrence of hot flushes [119]. As about one quarter of women visiting menopause clinics also report migraine [120], a combined treatment of both the vasomotor symptoms and the headaches would seem useful. The vasomotor changes in menopausal women are until now treated with hormone replacement therapy (HRT) [121]. However, at the onset of HRT migraine attacks may become more frequent [122]. In addition, HRT is associated with cardiovascular side effects [123]. An alternative treatment for the vasomotor changes during menopause seems to be required. The perimenopausal migraine headaches are not differently treated from any other migraine headaches [124]. CGRP receptor antagonists could be effective against both the perimenopausal migraine, as well as the vasomotor symptoms. However, considering the transient character and short duration of the hot flushes, CGRP receptor antagonists should probably be administered as prophylaxis. Obviously, the same potential drawbacks as

described above for the use of CGRP receptor antagonists as prophylactic treatment against migraine apply, and the safety should be well investigated.

Conclusion

CGRP receptor antagonists seem to be promising new compounds for the acute treatment of migraine attacks, but no compound has passed clinical trials yet. The advent of novel compounds is awaited with great interest. Before a compound is tested in clinical trials, different preclinical experimental models may be used to characterize its pharmacological properties. As migraine is a neurovascular disease and no integrative model exists encompassing all components of this disease, the models used to study new CGRP receptor antagonists include both vascular models as well as models of the central nervous system. All these models study specific properties of a compound, and their results should be integrated to get a complete picture of the effects and side-effects. Furthermore, these models can also be applied for more basic studies to obtain a better understanding of the pathophysiology of migraine.

Expert Opinion

The most widely used acute antimigraine drugs available are the triptans. Although they provided a major breakthrough in the acute treatment of migraine attacks when they were discovered, they are not effective in all patients and because of their vascular side-effect potential contraindicated in patients with cardiovascular disease. Thus, a major proportion of migraine patients cannot use, or has no profit of the use of triptans. Therefore it is highly relevant to find novel types of antimigraine drugs with fewer side effects. The discovery of the important role of CGRP in the pathophysiology of migraine prompted the search for a new type of acute antimigraine drugs, the CGRP receptor antagonists. The outcome of the first studies with the CGRP receptor antagonists, olcegepant and telcagepant, was positive. Both drugs showed little side effects and their effectivity against migraine was comparable to that of the triptans. CGRP receptor antagonists do not cause any constriction of coronary arteries [31], which may be an advantage compared to the triptans in view of cardiovascular safety. Furthermore, the patients that respond well to CGRP receptor antagonists are not necessarily the same patients that respond well to the triptans [125]. Therefore,

CGRP receptor antagonists may fulfil a complementary role in the acute treatment of migraine and may hopefully also be of benefit to non-responders to triptan therapy.

It is still a question where CGRP receptor antagonists exert their therapeutic effects in migraine. Some studies, for example the above-mentioned study showing only moderate brain receptor binding for telcagepant [49], suggest that a central action may not be essential for the effects of CGRP receptor antagonists. Other studies, however, suggest that vascular changes in migraine are absent or an irrelevant epiphenomenon [126]. While it is still unclear where the therapeutic site of action of the CGRP receptor antagonists is located, possibly the efficacy or lack of efficacy of antibodies directed against the CGRP receptor, that are unlikely to penetrate into the brain in view of their high molecular weight, may shed more light on this discussion. It is important in this light to realize that the trigeminal ganglion is located outside the blood-brain barrier, and could also serve as a target for ligands without central penetration.

As described above, it remains to be demonstrated whether continuous blockade of CGRP receptors does not increase the risk for myocardial or cerebral ischemia. Further, since overexpression of the CGRP receptor in mice leads to increased CGRP levels in cerebrospinal fluid [103], it is important to investigate whether blockade of CGRP receptors would lead to an opposite effect, that is decrease the levels of CGRP.

Since CGRP receptor antagonists act through a different mechanism of action than the triptans, it is important that their effects are assessed in adequate models. Currently available experimental migraine models only study a small part of all potentially relevant processes that are going on during a migraine attack. Integrated neurovascular animal as well as human models that measure both vascular and neuronal components simultaneously would increase the knowledge on the mechanism of action of prospective CGRP receptor antagonists. Further, more emphasis should be put on the translation of the human infusion model to animal models, where effects of infusion of migraine triggers may be studied in detail. It would be desirable to perform more studies in awake animals, where behaviour can be studied and results are not confounded by the use of anaesthetics. The use of transgenic animals in these models may be of additive value.

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Chapter 10- Long-lasting physiological antagonism of cacitonin gene-related peptide towards endothelin-1 in rat mesenteric and human coronary arteries.

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Abstract

Introduction - Endothelin-1 causes long-lasting contraction via endothelin type A receptor (ETAR) in isolated rat mesenteric arteries(RMA) that cannot be readily terminated by removing the agonist, or by adding the ETAR antagonist BQ123 or the NO donor sodium nitroprusside. It could be terminated by adding calcitonin gene-related peptide (CGRP), most likely because CGRP causes ET-1/ETAR dissociation. Here we investigated this phenomenon in human coronary microarteries (HCMA). We simultaneously verified the effects of CGRP in RMA and HCMA towards other vasoconstrictors, i.e., the α1-adrenoceptor agonist phenylephrine, the thromboxaneA2 analogue U46619 (9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin F2α) and KCl.

Methods and Results - Long-lasting contraction (remaining after washing away the agonist) was observed for ET-1 in RMA, but not HCMA. Constrictions to phenylephrine, U46619 or KCl did not last upon washing. When added on top of ET-1-initiated contraction in RMA, CGRP effectively counteracted vasoconstriction, i.e., it caused full relaxation. Inhibitory effects of CGRP were also observed when briefly exposing RMA and HCMA to CGRP 1h before the addition of ET-1. Similar inhibitory effects of transient CGRP pre-incubation were seen towards phenylephrine, U46619 or KCl in RMA and HCMA.

Conclusion and Discussion - In conclusion, our data imply that CGRP, likeET-1, causes long-lasting effects that remain apparent up to 1h after its removal from the organ bath. Thus, in addition to the reported dissociation of ET-1/ETAR complexes, CGRP causes long-lasting non-selective arterial smooth muscle relaxation that may add to the neuropeptide being a physiological antagonist of arterial effects of ET-1. Long-lasting, washout-resistant ET-1/ETAR interaction does not occur in HCMAs.

Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictor in a wide variety of blood vessels via endothelin type A (ETA) receptor stimulation [1]. When applied in vitro in an organ bath setup, its effects are long-lasting and even persist upon repeated washing of the organ bath fluid (i.e., after removing all exogenous ET-1) [2]. This implies a very tight binding of ET-1 to ETA receptors, as illustrated by the half-life of this complex of almost 3 days [3]. As a consequence, functional antagonism by vasodilators such as NO can only transiently reduce such constrictions [4]. Remarkably, this is also true for ETA receptor antagonists like BQ123 [4]. ET-1 binding to its receptor is therefore believed to involve 2 binding sites, a high-affinity site which also binds BQ123 and a low-affinity site which determines signaling [4]. In contrast to NO and BQ123, calcitonin-gene related peptide (CGRP) was recently shown to permanently terminate the vasopressor response to ET-1 in vitro and in vivo [4,5]. It was also observed that CGRP, in addition to its relaxant effects, promotes dissociation of the binding of ET-1 in the intact arterial wall via stimulation of postjunctional CGRP receptors. Results were identical with exogenously added and endogenously released CGRP. The under- lying mechanism has not yet been fully elucidated; it involves G protein βγ subunits but not cyclic nucleotides [6]. In the present study, we set out to investigate to what degree arterial CGRP effects are specific for ET-1. To this end, we evaluated the effects of CGRP towards other vasoconstrictors, the α1-adrenoceptor agonist phenylephrine, the thromboxane A2 analog U46619 (9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin F2α) and KCl. A comparison was made with BQ123 and the NO donor sodium nitroprussside (SNP). Studies were performed in the same preparation previously used to address CGRP/ET-1 responses, i.e., the rat mesenteric artery (RMA). In addition, we verified whether similar effects occur in rat iliac arteries and human coronary micro-arteries (HCMA).

Materials & methods

Vessel collection

Wistar rats (320–400 g, n = 16) and WKY rats (310–360 g, n = 6) were obtained from Charles River. All studies were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC (protocol number 118-12-03). Rats were

housed in individual cages and maintained on a 12-h light/dark cycle, having access to standard laboratory rat chow and water ad libitum. RMAs (diameter 150–200 mm) and iliac arteries (diameter 500–600 mm) were isolated and kept in ice-cold, oxygenated Krebs bicarbonate solution (in mmol/l: 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) until the start of the experiment the same day.

HCMAs were obtained from 10 organ donors (5 men, 5 women, mean age 44 ± 5 years, cause of death: 2 myocardial infarction, 2 severe head trauma, 1 brain tumor, 4 subarachnoid bleeding, 1 suffocation). Hearts were provided by the Rotterdam Heart Valve Bank from Dutch postmortem 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. The study was approved by the Ethics Committee of the Erasmus MC. The hearts were stored in ice-cold sterile organ protecting solution after circulatory arrest. After arrival at the laboratory, a tertiary branch of the left anterior descending coronary artery (diameter 250–400 mm) was removed and stored overnight at 4 1C in oxygenated Krebs bicarbonate solution.

Myograph studies

Following isolation, RMAs, rat iliac arteries, and HCMAs were cut into segments of approximately 2 mm length and mounted in a Mulvany myograph (Danish Myo Technology, Aarhus, Denmark) with separated 6-ml organ baths containing Krebs bicarbonate solution, aerated with 95% O_2 and 5% CO_2, and maintained at 37 °C. Tissue responses were measured as changes in isometric force, using Powerlab with Labchart software. Following a 30-min stabilization period, the optimal internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure as described by Mulvany and Halpern (1977). To determine the maximum contractile response, the tissue was exposed to 100 mmol/l KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min. To study CGRP- induced ET-1-ETA receptor dissociation, we used the protocol proposed by Meens et al. (2010), shown in Fig. 1. In short, first a concentration–response curve (CRC) was constructed to ET-1, phenylephrine or U46619 (CRC1). Agonists were then removed from the organ bath by rapidly washing three times. This allows the evaluation of any remaining contractile response due to tight ('irreversible') binding of an

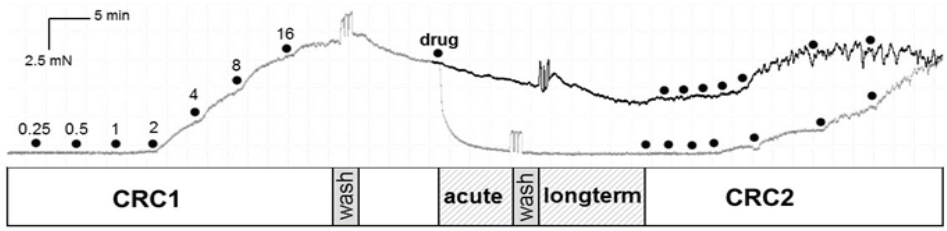

Figure 1. Experimental protocol illustrated by a representative tracing. A concentration-response curve was constructed to ET-1, phenylephrine or U46619, and this was followed by a rapid wash. After 8 min, the acute (relaxant) effect of 1 μmol/l BQ123, 1μmol/l SNP, or 100 nmol/l CGRP (denoted as drug) was evaluated (control segments received no drug), after which the vessel segment was washed again. Then the remaining (longterm) effect of these drugs was quantified, after which a second CRC to the above 3 constrictors was constructed. The tracing shows an ET-1 CRC1, followed by exposure to either CGRP (gray line) or no drug(black line), and the ET-1 CRC2. The ET-1 concentrations (nmol/l) in the organ bath are provided for the first CRC, and were identical for CRC2.

agonist to its receptors. Next, after a period of 8 min, segments were incubated for 10 min with 1 mmol/l BQ123, 1 mmol/l SNP, or 100 nmol/l CGRP to study their acute effects on top of any remaining constriction. Control segments received no drug. After 10 min the organ bath was washed again three times, and any remaining 'long-term' (i.e., remaining after washing) effect was quantified. This allows the evaluation of the possible return of the contractile response. Finally, 8 or 60 min later a second CRC (CRC2) was constructed to ET-1, phenylephrine or U46619. This provides information on the reproducibility of the ETA, α and thromboxane A2 receptor-mediated responses, and the possibility that the effect of BQ123, SNP or CGRP lasts far beyond their washout. In an additional set of experiments, segments were incubated for 30 min in the absence (control) or presence of 1 mmol/l BQ123, 1 mmol/l SNP, or 100 nmol/l CGRP. Next, CRCs were constructed to ET-1, phenylephrine or U46619, either directly, or, in the case of CGRP, 10 min after rapidly removing CGRP by washing the organ bath 3 times. Moreover, the relaxant effect of CGRP was evaluated in HCMAs that were preconstricted with 30 mmol/l KCl. Thereafter, the organ bath was washed 3 times, and, to determine any longlasting relaxant effect of CGRP (i.e., when no longer present in the organ bath fluid), the reappearance of the contractile response to KCl was verified after 5, 30 or 60 min by adding 30 or 60 mmol/l KCl to the organ bath.

During the experiments in RMAs 10 mmol/l indomethacin and 100 mmol/l L-NAME were continuously present in the organ bath fluid to suppress prostaglandin and NO production. This was not the case in the HCMA experiments because in those vessels, in contrast to RMAs, L-NAME + indomethacin increased baseline by ~ 60%. All drugs were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands), except for CGRP (rat α-CGRP), which was from neoMPS (Strasbourg, France).

Data analysis

Data are provided as mean ± S.E.M. All contractile responses are expressed as a percentage of the contraction to 100 mmol/l KCl. CRCs were analyzed as described [7] to obtain pEC_{50} ($-^{10}\log EC_{50}$) values. In experiments where no clear maximum effect (E_{max}) was reached, E_{max} was defined as the effect obtained at the highest concentration tested. pEC_{50} values were not calculated when E_{max} was < 20%, and in such cases statistical analysis was performed under the assumption that pEC_{50} equaled the highest concentration tested. Relaxant effects following CRC1 were expressed as a percentage of the maximum contractile response to ET-1, phenylephrine or U46619. Relaxant effects of CGRP following KCl preconstriction were expressed as a percentage of the contraction to KCl. Data were analyzed by Student's t-test or one-way ANOVA, followed by post-hoc evaluation according to Bonferroni. $P < 0.05$ was considered significant.

Results

Rat arteries

Washout-resistant ET-1-, phenylephrine- and U46619-induced vasoconstriction?

ET-1, phenylephrine and U46619 concentration-dependently constricted Wistar RMAs (pEC_{50} 8.5 ± 0.1, 6.2 ± 0.2 and 7.1 ± 0.1, respectively; Fig. 2). Washing the organ bath 3 times virtually abolished the contractile responses to phenylephrine and U46619 within 1 min, whereas the response to ET-1 was reduced by only 17 ± 10% after 10 min (Fig. 3A). As a consequence, adding BQ123, SNP or CGRP exerted no detectable (further) relaxant effect in the RMAs that had been exposed to phenylephrine or U46619. Yet, in the ET-1-exposed vessel segments, all 3 drugs induced relaxation (Fig. 3A), and the relaxation was almost complete with both SNP and CGRP. Subsequent washing allowed the partial return of constriction in the segments exposed to BQ123 and SNP, whereas

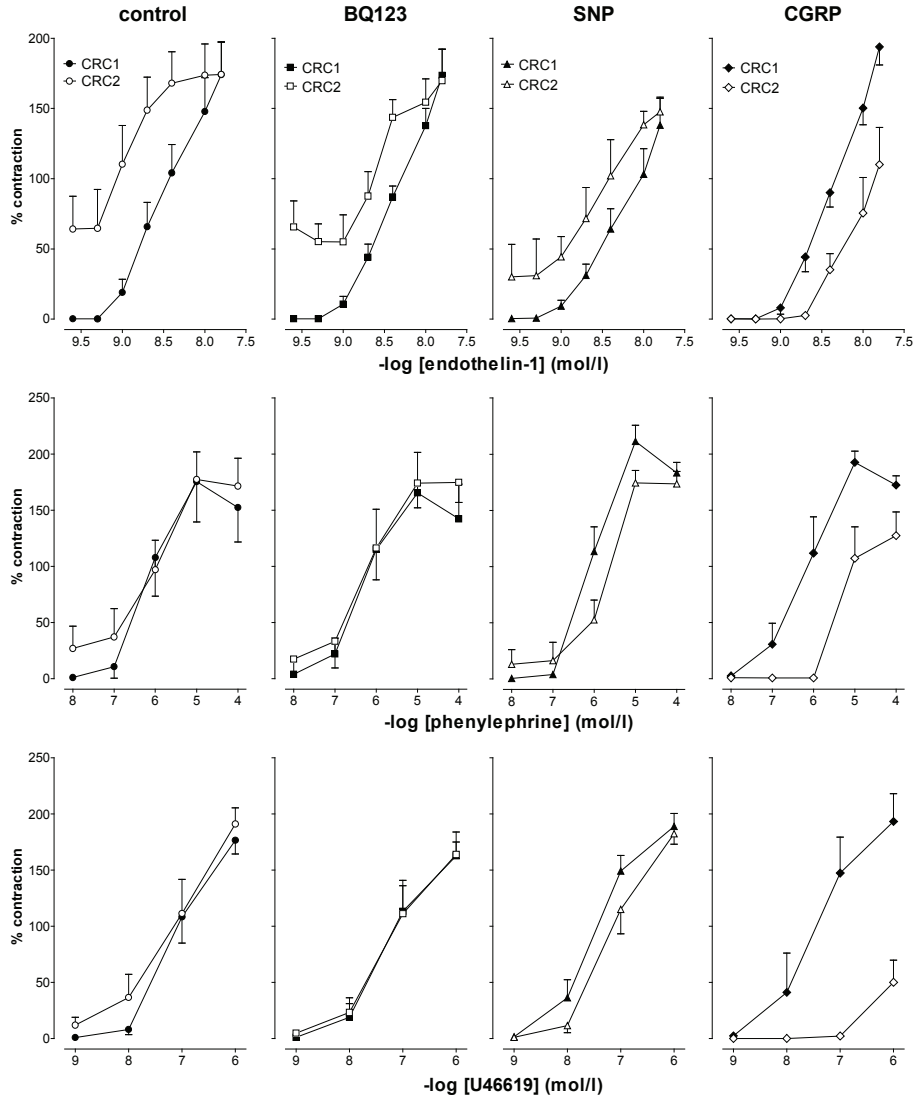

Figure 2. Endothelin-1, phenylephrine and U46619-induced concentration response curves in Wistar rat mesenteric arteries. The used protocol is explained in Fig. 1, i.e., exposing the vessel segments in between to either no treatment (control), 1 μmol/l BQ123, 10 μmol/l SNP or 100 nmol/l CGRP. Data are mean ± S.E.M. of n = 6, and have been expressed as a percentage of the contraction to 100 mmol/l KCL.

no change occurred in the segments that had been exposed to CGRP (Fig. 3A). Taken together, these data show that ET-1-induced constriction is largely washout-resistant,

and that BQ123, SNP and CGRP all relax ET-1-constricted RMAs, although the effect of BQ123 was very modest. The relaxant effect remained present upon subsequent washing.

Desensitization vs. long-lasting effects of BQ123, SNP or CGRP

Constructing a second CRC under control conditions yielded curves that were identical to the first CRC in the case of phenylephrine or U46619 (Fig. 2). In the case of ET-1, because of the remaining degree of contraction, CRC2 started at 64 ± 23% of the effect of 100 mmol/l KCl, but the pEC_{50} and E_{max} were unaltered (Fig. 2). Results for ET-1, phenylephrine and U46619 in the RMAs that had been exposed to BQ123 or SNP after CRC1 were identical to those in control RMAs not exposed to these drugs. However, in the RMAs that had been exposed to CGRP, the second CRC to all 3 agonists was shifted to the right (pEC_{50} 8.2 ± 0.1, 5.3 ± 0.2 and < 6.0, resp., $P < 0.05$ vs. CRC1 for all). This raises the possibility that the relaxant effect of CGRP remained presently long after washing. To test whether a prolonged incubation after washing would diminish this effect, CRC2 was also constructed at 1 h after the second wash. Results were identical to those when constructing the CRC after 8 min (data not shown, n = 4). Furthermore, results in WKY RMAs were identical to those in Wistar RMAs (n = 6, data not shown), and replacing our Krebs solution by the Krebs solution used by Meens et al. (containing 5.5 instead of 8.3 mmol/l glucose) [2] also did not alter the results (n = 6, data not shown). Thus, desensitization did not occur for any of the tested constrictors, and only transient exposure to CGRP shifted the CRCs of the various constrictors to the right.

Mesenteric vs. iliac arteries

Rat iliac arteries responded marginally to ET-1 and CGRP and could therefore not be used for the above protocols (n = 3).

BQ123, SNP or CGRP preincubation prior to ET-1, phenylephrine or U46619 exposure

Preincubating Wistar RMAs for 30 min with BQ123 fully abolished the CRC to ET-1, but did not affect the phenylephrine and U46619 CRCs (Fig. 4A). Preincubation with SNP or CGRP shifted the CRCs of ET-1 and phenylephrine ~ 10-fold to the right ($P < 0.01$ for both; Fig. 4A), whereas a shift of almost 2 orders of magnitude occurred in

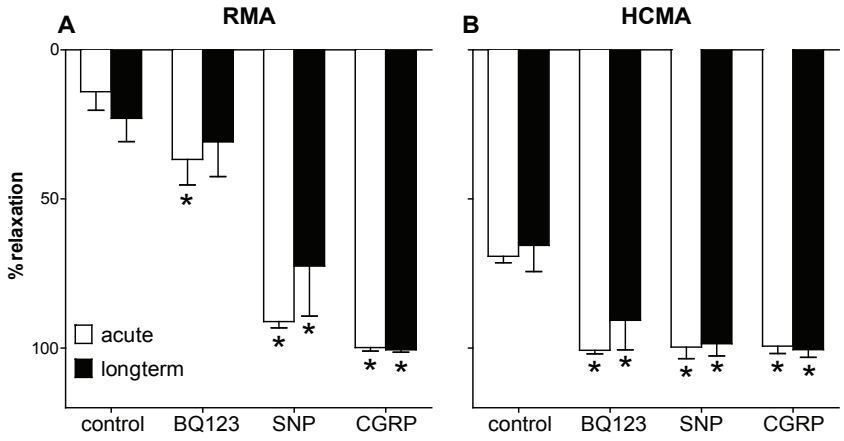

Figure 3. Acute and longterm effect of either no treatment (control), 1 µmol/l BQ123, 10 µmol/l SNP or 100 nmol/l CGRP on ET-1 induced constriction in Wistar rat mesenteric arteries (n=6, panel A) or human coronary microarteries (n = 4-5, panel B) according to the protocol shown in Fig 1. Data are mean ± S.E.M. and have been expressed asa percentage of the contractile response to each agonist before washing. *P <0.05 vs. control.

the case of U46619 (P < 0.01; Fig. 4A). The effect of CGRP preincubation was also apparent when washing away CGRP immediately prior to the construction of the ET-1, phenylephrine and U46619 CRCs (Fig. 4B). This implies that the relaxant effect of CGRP is long-lasting, applies to all tested constrictors, and does not require its continuous presence in the organ bath.

Human arteries

Washout-resistant ET-1-, phenylephrine- and U46619-induced vasoconstriction and desensitization

ET-1 concentration-dependently constricted HCMAs (pEC_{50} 8.4 ± 0.1; Fig. 5). Washing the organ bath 3 times diminished the contractile response by 69 ± 2% within 10 min (Fig. 3B). Adding BQ123, SNP or CGRP after washing induced near-complete (BQ123) or complete (SNP, CGRP) relaxation (Fig. 3B). Subsequent washing did not allow a return of the contractile response, and when constructing a second ET-1 CRC, it was identical to the first CRC, except in the segments exposed to CGRP, where CRC2 was modestly shifted to the right (P = NS) vs. CRC1 (pEC50 8.1 ± 0.1; Fig. 5). Thus, ET-1-induced constriction is HCMAs is not washout-resistant, and desensitization to ET-1 did not occur.

BQ123, SNP or CGRP preincubation prior to ET-1, phenylephrine, U46619 or KCl exposure
Preincubating HCMAs with BQ123 fully abolished the response to ET-1 (Fig. 6), whereas preincubation with SNP or CGRP shifted the ET-1 CRC ~ 10-fold to the right ($P < 0.05$ for both; Fig. 6). CGRP fully relaxed 30 mmol/l KCl-preconstricted HCMAs (pEC50 9.1 ± 0.1; Fig. 7A). When re-introducing 30 mol/l KCl in the organ bath at 5, 30 or 60 min after washing, the degree of constriction was low or absent. Doubling the KCl concentration resulted in a higher degree of constriction (Fig. 7B), although it was still below the constriction obtained with 30 mmol/l prior to the exposure to CGRP at 30 and 60 min. These data illustrate that the relaxant effects of CGRP last for at least 60 min after washing the agonist away from the organ bath fluid.

Discussion

This study is the first to show that the relaxant effects of CGRP in RMAs and HCMAs last long after its washout. As a consequence, CGRP is capable of antagonizing vasoconstrictors like ET-1 even when it has been washed away from the organ bath. The ETA receptor-mediated effects of ET-1 in RMAs are similarly long-lasting: repetitive washing of the organ bath (i.e., removal of unbound ET-1) only marginally diminished the contractile response to ET-1. This supports the very tight binding of ET-1 to ETA receptors that has been observed before in RMAs [2]. In contrast, in HCMAs, no such long-lasting effects of ET-1 occurred, and repetitive washing easily annihilated its effect. Finally, ET-1 exerted virtually no constrictor effect in rat iliac arteries. One explanation of the findings on ET-1 could be that the ETA receptor density in these 3 preparations follows the order: rat iliac artery < HCMA < RMA. Clearly, assuming the same tight (but not irreversible) binding in all preparations, the more receptors are occupied by ET-1, the larger the chance that an effect remains upon washing, particularly if only a small fraction of the receptors needs to be stimulated to obtain a maximum effect. Alternatively, the persistent ET-1 effects in certain vessels may relate to differences in second messenger coupling, mediated via $G_{q/11}$ or G_{12}, and not necessarily requiring the continuous stimulation of ETA receptors [8-11]. In fact, it has even been suggested that sustained effects on intracellular calcium (Ca^{2+}) relate directly to the level of ETA receptor expression, intracellular Ca^{2+} remaining elevated only in cells with high ETA receptor levels [8]. Adding the ETA receptor antagonist BQ123 to the

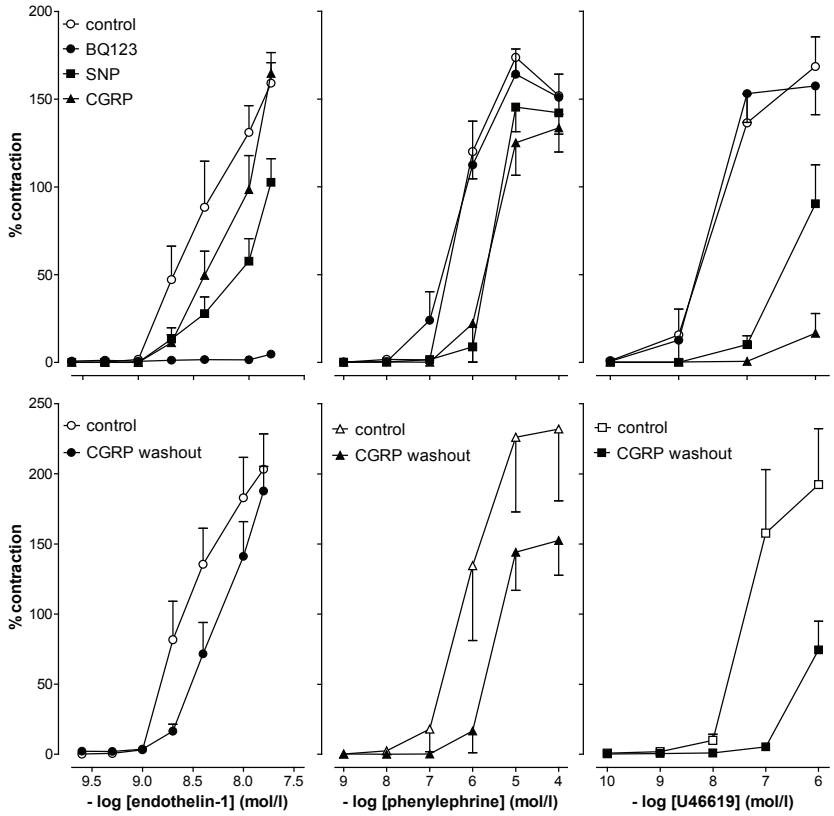

Figure 4. Endothelin-1, phenylephrine and U46619-induced concentration-response curves in Wistar rat mesenteric arteries after no treatment (control) or a 30-min preincubation of the vessel segment with 1 μmol/l BQ123, 10 μmol SNP or 100 nmol/l CGRP (Panel A). Data are mean ± S.E.M. of n = 6, and have been expressed as a percentage of the contraction to 100 mmol/l KCL. Panel B shows the same approach, but now CGRP was washed away after its 30 min preincubation period. Data are mean ± S.E.M of n = 3-4, and have been expressed as a percentage of the contraction to 100 mmol/l KCl.

organ bath after washing away ET-1 induced a modest further reduction of the ET-1 contractile response in both RMAs and HCMAs, in agreement with the data by Meens et al. (2010). In HCMAs even complete relaxation was obtained. These data suggest that ET-1, once dissociated, will rapidly rebind to unoccupied neighboring ETA receptors, thereby allowing continuation of the constriction, unless BQ123 is present. In case of the latter, at sufficiently high concentrations, the blocker will competitively prevent rebinding, thereby (further) reducing the degree of constriction. Subsequently washing

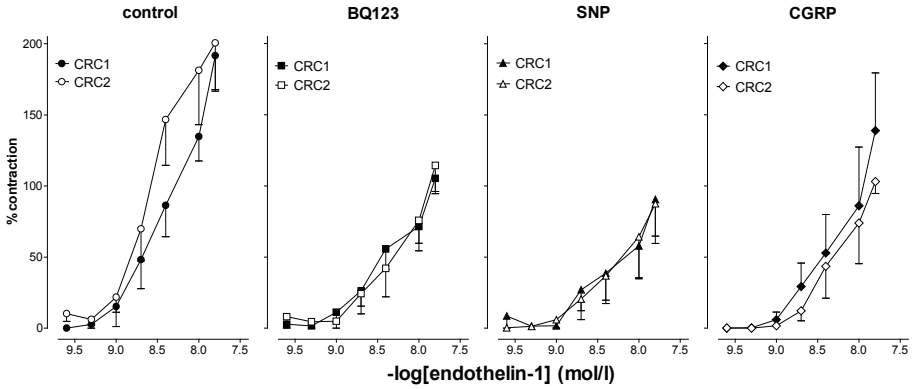

Figure 5. Endothelin-1 induced concentration-response curves (CRC1 and CRC2) in human coronary microarteries using the protocol explained in Fig. 1, i.e., exposing the vessel segments to either no treatment (control) or 1 μmol/l BQ123, 10 μmol SNP or 100 nmol/l CGRP. Data are mean ± S.E.M. of n = 4-5, and have been expressed as a percentage of the contraction to 100 mmol/l KCL.

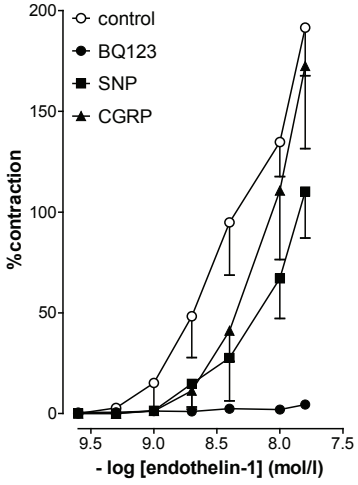

Figure 6. Endothelin-1 induced concentration-response curves in human coronary microarteries after no treatment (control) or a 30 min preincubation with 1 μmol/l BQ123, 10 μmol SNP or 100 nmol/l CGRP. Data are mean ± S.E.M. of n = 3-5, and have been expressed as a percentage of the contraction to 100 mmol/l KCL.

away BQ123 did not result in a return of the pre-BQ123 ET-1 constriction. This implies that the ET-1 that had been displaced by BQ123 was effectively removed by washing, i.e., could no longer rebind. These observations are in contrast to those of Meens et al. (2010) in WKY RMAs, who observed a return to the pre-BQ123 degree of constriction after washing away the antagonist, and on this basis proposed a two-binding-sites model for ET-1. Importantly, constructing a second ET-1 CRC after washing away BQ123 occurred in an identical manner as in the segments that had not been exposed to BQ123, i.e., without a change in pEC_{50}. Apparently therefore, washing the or-

gan bath 3 times had effectively removed all BQ123. This is in full agreement with its fast rate of dissociation [12], allowing its complete removal upon repetitive washing. When given on top of BQ123, ET-1 was unable to induce vasoconstriction, at least up to concentrations of 30 nmol/l (Figs. 4 and 6). This confirms that these effects are mediated by ETA- receptors. Previous studies have already shown that higher ET-1 concentrations are needed to competitively displace ETA receptor- bound BQ123 [13,14].

Next, we tested functional antagonism towards ET-1-initiated constriction by applying SNP and CGRP to the organ bath. Both relaxants caused complete relaxation of vessel segments that had not yet relaxed fully after washing away ET-1 from the organ bath. The relaxation remained present when washing away SNP or CGRP. Meens et al. (2010) concluded from the latter that CGRP, unlike BQ123, caused ET-1-ETA receptor dissociation, and they were able to confirm this by analysis of the binding of fluorescently-labeled ET-1 in intact arterial segments. Yet, not all rat arteries displayed this phenomenon in their hands, and in particular in rat coronary and basilar arteries it was absent [5]. In the present study, reconstructing a second ET-1 CRC after the last wash showed that, after SNP, CRC2 was identical to CRC1. Yet, after CGRP, CRC2 was shifted to the right, implying that the relaxant effect of CGRP remained present after its removal. Results were identical when performing CRC2 60 min (instead of 8 min) after removing CGRP. Moreover, when applying the same experimental setup to different constrictors (phenylephrine or U46619), the findings were similar to those observed for ET-1. In fact, the long-lasting effect of CGRP towards U46619 was even stronger, possibly because both agonists interfere, in an opposite manner, with protein kinase C [15-17]. To evaluate the latter observations in further detail, we also constructed CRCs to ET-1, phenylephrine and U46619 in the presence of SNP or CGRP and after preincubation with these agonists followed by a washout (i.e., in their absence). Under these conditions, CGRP could not yet have induced agonist–receptor dissociation. It then became clear that, when still present in the organ bath, SNP and CGRP both shifted the 3 CRCs to the right, and that even after CGRP washout, its effect remained present. Long-lasting effects of CGRP were also observed in HCMAs, given our observation that 30 mmol/l KCl was unable to induce constriction in vessels that had been washed thoroughly after they had been preconstricted with 30 mmol/l KCl and relaxed with CGRP. Normally, a second exposure to 30 mmol/l KCl matches the constriction reached after the first exposure [18,19]. At what level CGRP counteracts KCl cannot

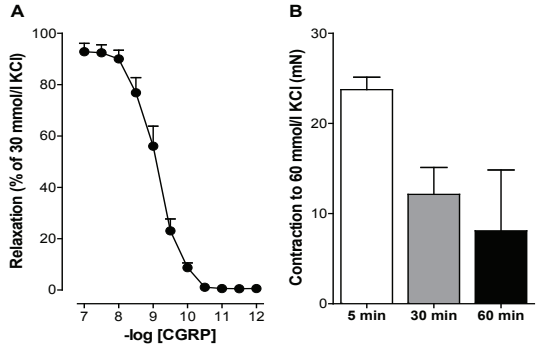

Figure 7. Panel A. CGRP-induced relaxation of 30 mmol/l KCL preconstricted human coronary microarteries. Data are mean ± S.E.M. of n = 5-10. Panel B. KCL-induced constriction at 5, 30 or 60 min after washing the organ bath following the experiment shown in panel A.

be concluded from our data, but given the promiscuity of CGRP receptors, there are ample possibilities, including the generation of NO [20].

Summarizing, our data reveal strong and long-lasting relaxant effects of CGRP after it has been removed from the organ bath. This may either involve continuous stimulation of CGRP receptors by tightly-bound CGRP (comparable to the tight binding of ET-1 to ETA receptors), or, perhaps more likely, a long-term activation of post-receptor relaxant mechanisms. Indeed, agonist-bound CGRP receptors have been observed to internalize in cells and to continue signaling [21]. The long-lasting non- selective arterial relaxing effect of CGRP observed here and the reported dissociating effect of the neuropeptide on ET-1/ETA- receptor complexes may both participate in effective physiological antagonism of ET-1-induced vasoconstriction.

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Chapter 11 - Comparison of the vasodilator responses of human and rat middle meningeal arteries to migraine related compounds.

Based on: Gustaf Grände, Sieneke Labruijere, Kristian Agmund Haanes, Antoinette MaassenVanDenBrink and Lars Edvinsson, April 2014, The Journal of Headache and Pain

Abstract

Introduction - Migraine attacks occur spontaneously in those who suffer from the condition, but migraine-like attacks can also be induced artificially by a number of substances. Previously published evidence makes the meninges a likely source of migraine related pain. This article investigates the effect of several vasodilators on meningeal arteries in order to find a connection between the effect of a substance on a meningeal vessel and its ability to artificially induce migraine.

Methods - A myograph setup was used to test the vasodilator properties of the substances acetylcholine (ACh), sodium nitroprusside (SNP), sildenafil, prostaglandin E_2 (PGE_2), pituitary adenylate cyclase activating peptide-38 (PACAP-38), calcitonin gene-related peptide (CGRP) and NaCl buffer on meningeal arteries from human and rat. An unpaired t-test was used to statistically compare the mean E_{max}(%) at the highest concentration of each substance to the E_{max}(%) of NaCl buffer.

Results - In the human experiments, all substances except PACAP-38 had an E_{max}(%) higher than the NaCl buffer, but the difference was only significant for SNP and CGRP. For the human samples, clinically tested antimigraine compounds (sumatriptan, telcagepant) were applied to the isolated arteries, and both induced a significant decrease of the effect of exogenously administrated CGRP. In experiments on rat middle meningeal arteries, pre-contracted with $PGF_{2\alpha}$, similar tendencies were seen. When the pre-contraction was switched to K^+ in a separate series of experiments, CGRP and sildenafil significantly relaxed the arteries.

Conclusions - Still no definite answer can be given as to why pain is experienced during a migraine attack. No clear correlation was found between the efficacy of a substance as a meningeal artery vasodilator in human and the ability to artificially induce migraine or the mechanism of action. Vasodilatation could be an essential trigger, but only in conjunction with other unknown factors. The vasculature of the meninges likely contributes to the propagation of the migrainal cascade of symptoms, but more research is needed before any conclusions can be drawn about the nature of this contribution.

Introduction

The dura mater and its vasculature, the middle meningeal artery (MMA) and the venous system have for decades been central to many hypotheses aimed to explain migraine pathophysiology [1,2]. Ray and Wolff showed in the 1940s that direct stimulation of the dura mater may result in headache with migraine-like qualities [3]. Further research during the last two decades has demonstrated that sensitization and local inflammation in the dura mater can be elicited by various agents and stimulation paradigms [4]. The local administration of an "inflammatory soup" or the direct stimulation at different parts of the dura mater may result not only in vasoactive responses but sometimes also in mast cell degranulation and in plasma extravasation. Histamine, a major amine released from mast cells, is known to induce a migraine-like pain upon infusion into test subjects previously diagnosed with migraine [5]. Some migraine-associated symptoms, including the characteristic pain, could therefore stem from the activation of meningeal mast cells and aseptic inflammation initiated by histamine release [6]. However, local administration of calcitonin gene-related peptide (CGRP), which is a sensory neuropeptide in the dura mater and a known migrainogenic substance [7], results only in vasodilatation [8]. Despite the fact that mast cells have CGRP receptors [9,10], no activation or sensitization of meningeal nociceptors could be detected upon topical or systemic administration of this compound in rats [8].

Apart from CGRP and histamine, other substances that upon intravenous infusion can provoke migraine-like attacks in humans previously diagnosed with migraine are prostaglandin E_2 (PGE_2), pituitary adenylate cyclase activating peptide-38 (PACAP-38), epoprostenol (prostacyclin, PGI_2), sildenafil and nitric oxide (NO) [11-15]. The only common factor among these substances is their vasodilator properties, but still there exist several other vasodilators which do not induce migraine-associated symptoms upon infusion. Vaso intestinal polypeptide (VIP) and carbachol, both associated with the parasympathetic system, are noted examples, although their ability to dilate meningeal arteries *in vivo* remains to be verified [16,17]. This shows that factors apart from the vasodilatation have to be taken into consideration. There is still controversy regarding whether vasodilatation directly contributes to any of the migraine-related symptoms or whether this is just a side-phenomenon. Several hypotheses exist that downplay the role of vasodilatation, including the observation of prodromal aura

symptoms preceding any activity in the vasculature [18], that the vasoconstrictor triptan drugs are not an effective treatment in all migraine patients and that the extravascular tissue surrounding the dural arteries could be too rigid to allow for any significant expansion of the vessel diameter [19].

We have recently investigated the vasoactive effects of several substances on cerebral arteries from human and rat *in vitro* in order to find common denominators that would allow us to understand more about the possible factors behind the migraine-associated symptoms [20]. However, cerebral arteries are different from meningeal arteries regarding several morphological aspects, including receptor expression, anatomical origin (internal carotid artery (ICA) versus external carotid artery (ECA)) and the lack of a blood-brain barrier (BBB) in the meningeal arteries [21]. Nevertheless, both vascular regions receive sensory input from the first division of the trigeminal ganglion [22]. Therefore it is possible that the two artery types contribute to migraine differently if there indeed is a vascular component.

The aim of the present study is to evaluate whether there is a connection between the ability of a substance to dilate meningeal arteries and the previously reported migrainogenic properties of each substance. This is useful to verify whether vasodilatation of the meningeal arteries indeed is the trigger for the migraine-like pain triggered by these substances. Since the meningeal arteries lack BBB properties, systemic drugs are freely diffusible to endothelial and smooth muscle receptors to elicit a vasomotor response. A secondary goal of this study is to compare the functional aspect of the middle meningeal artery (MMA) between the species because rat is often used in mechanistic studies due to the difficulty in obtaining human tissue samples.

Materials and Methods

The experimental protocol (M11104) was approved by the Animal Protocol Review committee at the University of Lund. All human artery procedures were carried out strictly according to national laws and guidelines and approved by the Ethical Committee at the University of Lund (LU-818-01) and the local Ethics Committee at the Erasmus MC, Rotterdam.

Obtaining the MMA

Fresh samples of MMA were obtained from the meninges of male Sprague-Dawley rats by the following procedure. The rats were anesthetized using CO_2 and decapitated. The cranium was opened from the top and the brain removed, which exposed the dura mater. The cranium and dura mater were divided sagitally and each half was placed in a buffer solution composed of NaCl 119 mM, $NaHCO_3$ 15 mM, KCl 4.6 mM, $MgCl_2$ 1.2 mM, NaH_2PO_4 1.2 mM, $CaCl_2$ 1.5 mM and glucose 5.5 mM. The dura mater was carefully separated from the cranium and moved to a Petri dish, where it was submerged in buffer solution, stretched and suspended by needles. The MMA was removed from the dura mater by a dissecting microscope, cut into segments 1-2 mm long that were placed in parallel tissue baths of ice-cold bicarbonate buffer solution aerated with a gas composed of 95% O_2 and 5% CO_2, with a resulting pH of 7.4 [23].

Human samples of intracranial MMA branches were acquired from patients undergoing neurosurgery at the University Hospital of Lund and the Erasmus University Medical Center (Rotterdam, the Netherlands). The vessel segments themselves were from visually healthy tissue.

The artery samples were upon removal immediately placed in cold Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Carlsbad, CA, USA) in Lund and in cold medium M199 (Gibco, Invitrogen, Carlsbad, CA, USA) in Rotterdam and immediately transported to the laboratory where they were prepared in an identical way to the rat MMA.

Myography

Each segment of MMA was mounted on a pair of thin metal wires (human: 40 μm, rat: 20/25 μm) in an arterial myograph. One wire was connected to a micrometer screw, allowing for fine adjustment of the vascular tone by varying the distance between the wires. The other wire was connected to a force displacement transducer, paired with an analogue-digital converter (ADInstruments, Oxford, UK). Data was recorded on a computer using a PowerLab unit (ADInstruments).

The aerated bicarbonate buffer was heated to +37°C and used to submerge the wires with the MMA-segment during the experiment. The segment was normalized, attaining 90% of the internal circumference that a fully relaxed vessel would have under a transmural pressure of 100 mmHg or 50 mmHg (see below). A reference value for

the contractile capacity of the segment was determined by temporarily replacing part of the NaCl in the buffer solution with 60 mM K^+.

The effects on the segment of the different vasoactive substances was tested by first recording the spontaneous resting diameter of the artery segment and define it as the maximum dilatation possible. Next, the segment was pre-contracted with 10^{-6} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to achieve a stable tension for the duration of the experiment. Finally, concentrations of the tested substances in the range of 10^{-10} - 10^{-5} M (the exact range varied between substances) were applied cumulatively and the vessel response was recorded. Because of the unstable response to $PGF_{2\alpha}$ in the first rat experiments, we changed the protocol and used 30 mM K^+ as a more stable contraction. In addition, the transmural pressure was lowered to 90% of 50 mmHg in these experiments.

Artery segments were omitted from the study if they failed to fulfill the inclusion criteria. Segments included in the final calculations were required to respond to initial testing with K^+ and to the pre-contraction with a maximum contractile capacity of at least 1 mN for human arteries and 0.1 mN for rat arteries.

Studies with clinically tested migraine compounds

The vessel segments were precontracted with 30 mM K^+. After precontraction the vessel segments were dilated with 4 nM CGRP. When a stable dilatation was achieved, sumatriptan or telcagepant was added to the arteries in concentrations corresponding to the C_{max} (160 nM and 5 μM, for the 100 mg and 300 mg oral doses, respectively) [24-26], as well as in concentrations corrected for plasma protein binding. The C_{max} for sumatriptan was corrected for 17.5% plasma protein binding [27], while the C_{max} for telcagepant was calculated using a plasma protein binding of 96.4% [28].

Calculations

The maximum dilatory response (E_{max}(%)) for each tested substance was obtained for the vessel segments as the percentage tonus relaxed by the maximum concentration of substance from the $PGF_{2\alpha}$- or K^+-induced pre-contraction towards the resting tonus sampled before the lowest concentration of substance was added. In the case of significant relaxation the negative logarithm of the concentration of substance required for dilation of the vessel segments to half of their E_{max}(%) (pEC_{50}) was obtained for the purpose of comparing the potency of the different substances, calculated using Graph-

Pad Prism software (GraphPad Software, San Diego, California, USA.).

If a test subject contributed with more than one vessel segment to the testing of a substance, all results obtained from that individual regarding that substance were pooled together into an average before further processing of data. The hypothesis was that each substance would induce vasodilatation and to determine the significance of the dilatation an unpaired t test was performed using the GraphPad Prism software (GraphPad Software, San Diego, California, USA.) comparing the average E_{max}(%) of the highest concentration of each substance to the average E_{max}(%) of NaCl buffer at the end of the session.

Figures were compiled where it is shown how the $PGF_{2\alpha}/K^+$ precontraction (100%) during a session is restored towards the original resting baseline at different concentrations of each of the substances tested and NaCl buffer (Figure 1-3,4). The contractile responses to telcagepant and sumatriptan were expressed as percentage of the previous relaxant response to 4 nM CGRP in the same segment (Figure 2). All data are presented as mean ± S.E.M.. Statistical analysis and pEC_{50} calculations were performed using Graphpad Prism 5 software. Statistical significance was accepted at $P<0.05$.

Chemicals

The vasoactive substances used in the present study were: acetylcholinechloride (ACh), sodium nitroprusside (SNP), sildenafil, PACAP-38 (all purchased from Sigma Aldrich), PGE_2 (Larodan fine Chemicals AB). The inhibitors used were the following: Telcagepant (MK-0974) (Dissolved in DMSO and further diluted in H_2O) (MSD, Whitehouse Station, NJ, USA), sumatriptan (dissolved in H_2O) (Sigma Aldrich).

Results

Functional results from the MMA

Intracranial MMA segments from 6 humans were used to test the vasoactive properties of six different compounds including NaCl buffer but excluding CGRP. The vessels were divided into several segments of 1-2 mm in length and studied in parallel tissue baths. Not every segment was exposed to all compounds. Vessels from 4 additional human patients were used for the testing of CGRP.

Human MMA (100 mmHg, pre-contracted with $PGF_{2\alpha}$)

Of the compounds tested, SNP and CGRP on human artery showed significant dilatation compared to NaCl, with CGRP being the strongest vasodilator (Figure 1, Table 1). ACh showed a non-significant tendency of relaxation. PGE_2, PACAP-38 and sildenafil showed no vasomotor activity distinct from the gradual loss of pre-contraction seen when using only NaCl buffer (Figure 1, Table 1). The very high concentration of sildenafil (10 μM) was not tested on human vessels.

Table 1. Human MMA, 100 mmHg, pre-contracted with $PGF_{2\alpha}$

Compound	E_{max}	pEC_{50}	p-value
Ach (n = 6)	17 ± 6	N.A.	0.44
SNP (n = 6)	32 ± 6	~3.5	0.034
Sildenafil (n = 6)	11 ± 2	N.A.	0.91
PGE_2 (n = 6)	8 ± 1	N.A.	0.62
PACAP-38 (n = 5)	9 ± 3	N.A.	0.89
CGRP (n = 4)	62 ± 8	8.1 ± 0.19	0.0015
NaCl (n = 4)	10 ± 4	N.A.	/

p-value for comparing agonist versus the NaCl effect. N.A. = Not Applicable. Values are ± SEM, n given in parenthesis.

Human MMA and CGRP (100 mmHg, pre-contracted with 30mM K^+)

For human samples the clinically tested migraine compounds were applied to the isolated arteries that that had been precontracted with 30 mM K^+. CGRP induced a significant vasodilatation of meningeal arteries (84 ± 10% of precontraction with 30 mM K^+). The concentrations corresponding to the Cmax obtained after oral administration of 100 mg sumatriptan (160 nM) or 300 mg telcagepant (5 μM) both lead to a significant decrease of the effect of the exogenous administrated CGRP (-8 ± 18% and 8 ± 8% respectively) (Figure 2). Also the concentrations of both sumatriptan and telcagepant corrected for plasma protein binding abolished the effect of exogenous CGRP

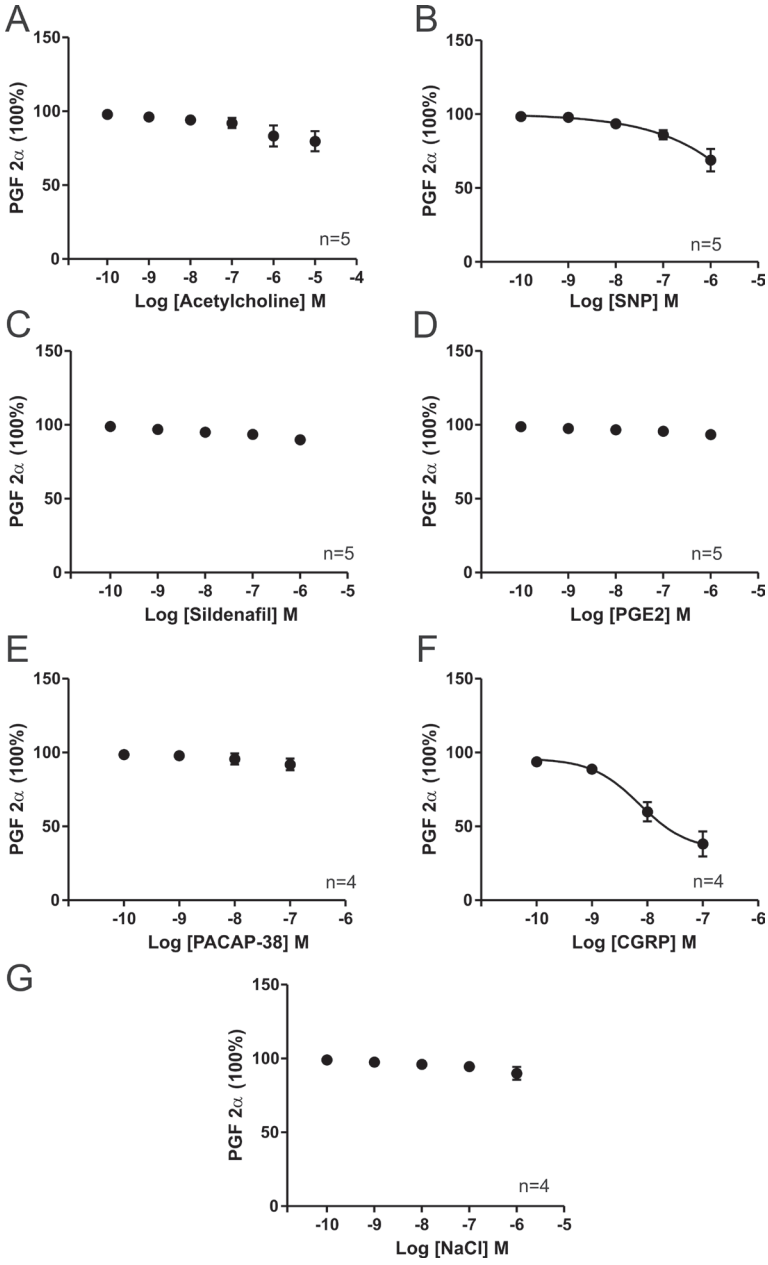

Figure 1. The effect of substances on human middle meningeal arteries precontracted with PGF2α. The relaxation relative to $PGF_{2\alpha}$ induced tone in human meningeal arteries by increasing concentrations of each of the tested substances (A-F) and NaCl buffer (G). Values are given as mean ± SEM (n = 4 – 6), where $PGF_{2\alpha}$ precontraction is set to be 100 %.

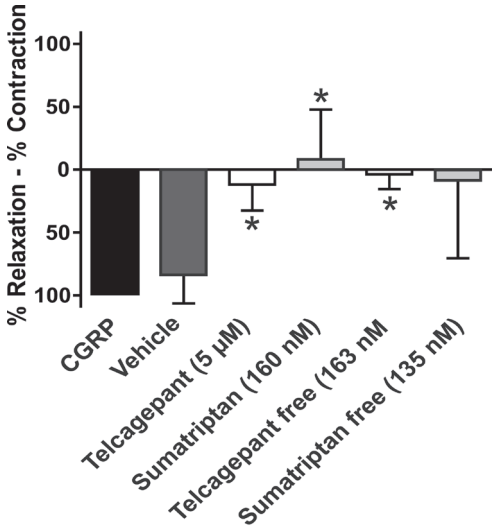

Figure 2. Effect of telcagepant and sumatriptan on CGRP induced relaxation of the middle meningeal artery. Arteries were precontracted with 30 mM K+, dilated with 4 nM CGRP and sumatriptan or telcagepant was subsequently added (* =p<0.05). Values are given as % of the CGRP relaxation (mean ± SEM, n = 5)

(8±36% for sumatriptan and 4±7% for telcagepant, respectively) (Figure 2).

Table 2. Rat MMA, 100 mmHg, pre-contracted with $PGF_{2\alpha}$

Compound	E_{max}	pEC_{50}	p-value
Ach (n = 3)	33 ± 22	N.A.	0.68
SNP (n = 3)	70 ± 18	8.3 ± 0.7	0.066
Sildenafil (n = 3)	52 ± 20	~7	0.23
PGE_2 (n = 3)	23 ± 12	N.A.	0.82
PACAP-38 (n = 4)	22 ± 14	N.A.	0.93
NaCl (n = 3)	23 ± 4	N.A.	/

p-value for comparing agonist versus the NaCl effect. N.A. = Not Applicable. Values are ± SEM, n given in parenthesis.

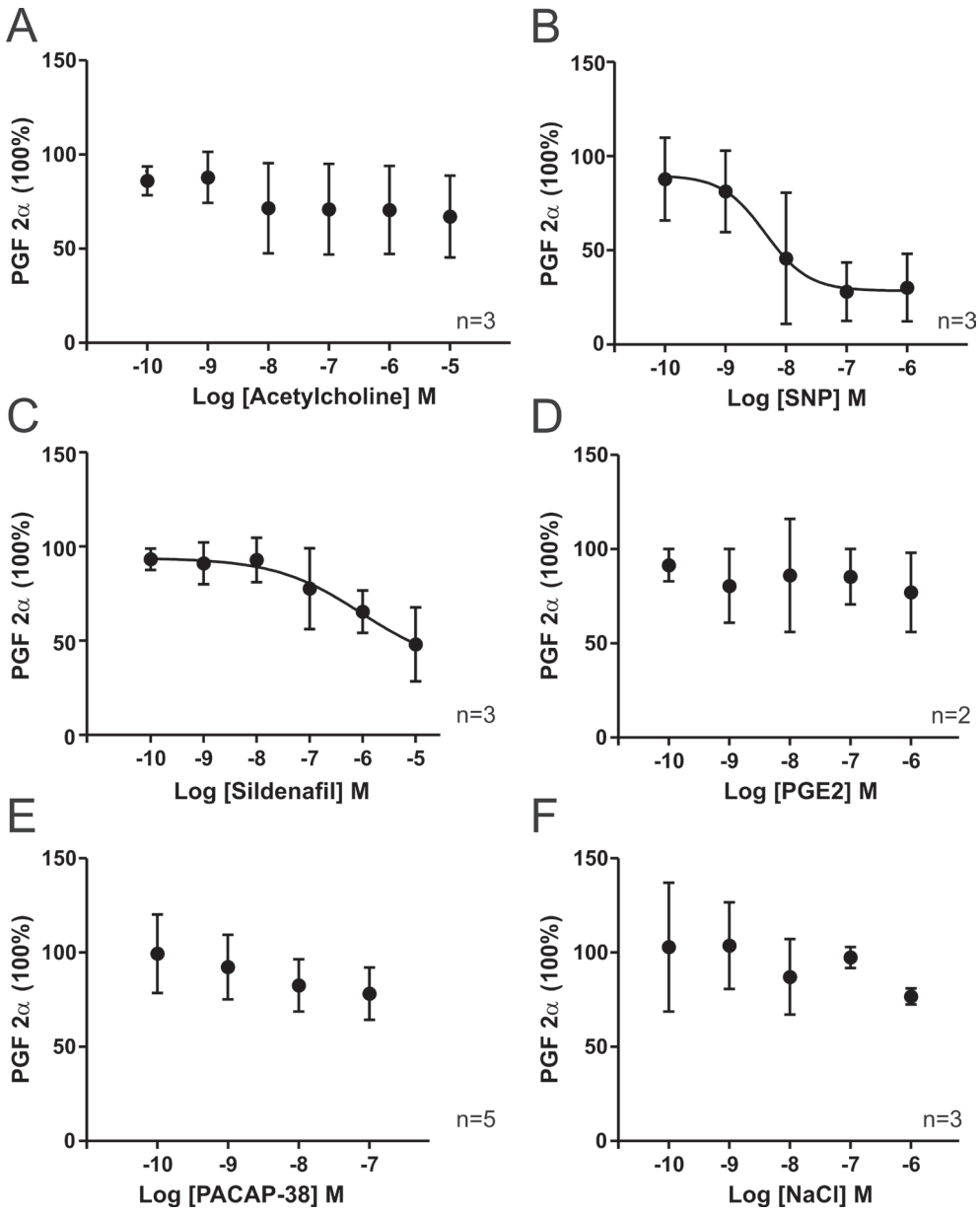

Figure 3. The effect of substances on rat middle meningeal arteries precontracted with $PGF_{2\alpha}$. The relaxation induced in rat meningeal arteries pre-contracted with $PGF_{2\alpha}$ at increasing concentrations of each of the tested substances (A-E) and NaCl buffer (F). Values are given as mean ± SEM (n = 2 – 4), where $PGF_{2\alpha}$ precontraction is set to be 100 %.

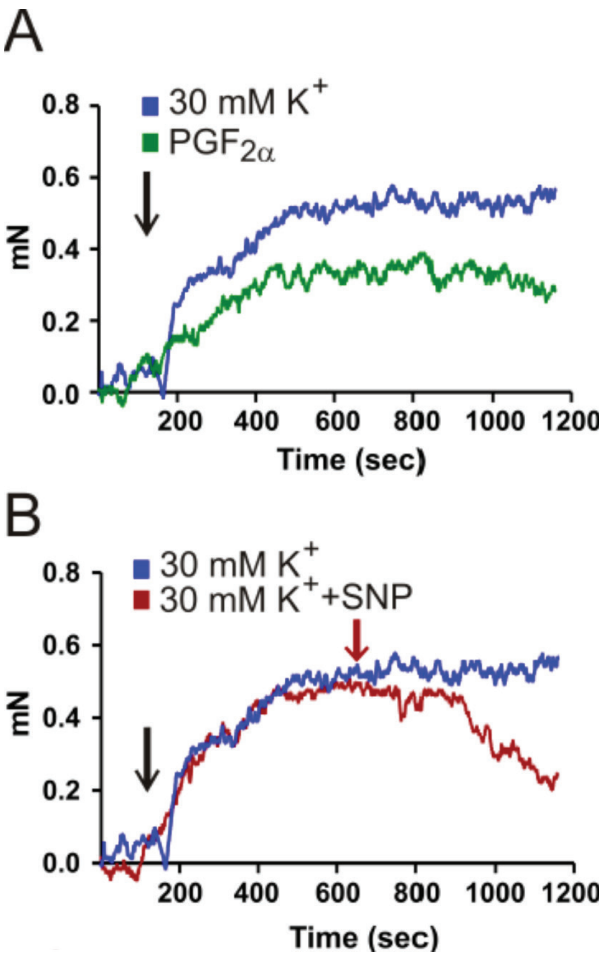

Figure 4. Comparison of 30 mM K+ and PGF2α on rat middle meningeal arteries, and sample relaxation. A) Sample contraction induced by of 30 mM K+ or PGF2α in rat meningeal artery. One can observe the slight positive drift for of 30 mM K+ and slight the negative drift for PGF2α. B) Sample relaxation in an artery precontracted with 30 mM K+ and the cumulative relaxation of added sodium nitroprusside (SNP). Small plateaus were allowed before next concentration was added.

Rat MMA, (100 mmHg, pre-contracted with $PGF_{2\alpha}$)

SNP and sildenafil showed a tendency to relax the arteries but the observed dilatation was not statistically significant (Figure 3, Table 2). ACh, PGE_2 and PACAP-38 did not display any discernible vasodilatation compared to NaCl (Figure 3, Table 2). The results from these rat experiments showed a larger spread compared to the experiments

using human artery segments or a different protocol, and thus had a higher ratio of not passing the inclusion criteria. For the first experiments above, the same protocol as for the human vessels (100 mmHg and pre-contracted with $PGF_{2\alpha}$) was used to test all substances including NaCl on MMA segments from 5 rats. These vessels showed a large variation and had a high ratio of not passing the inclusion criteria of 0.1 mN. Therefore, the protocol was changed by lowering the transmural pressure to 50 mmHg and the experiments were repeated using an additional 5 rats. This markedly improved the success rate. In addition, the MMAs pre-contracted with 30 mM K^+, did not have a negative drift over time (Figure 4A). A sample relaxation to a cumulative dose of SNP is also shown (Figure 4B).

Rat MMA, (50 mmHg, pre-contracted with 30 mM K^+)

The results showed significant relaxation upon exposure to SNP, CGRP and sildenafil compared to the NaCl control group, SNP being the strongest vasodilatator (Figure 5, Table 3). PGE_2, ACh and PACAP-38 showed no significant response compared to the control group (Figure 5, Table 3). These results correlate with the tendency seen for the above MMA.

Table 3. Rat MMA, 50 mmHg, pre-contracted with 30 mM K^+

Compound	E_{max}	pEC_{50}	p-value
Ach (n = 5)	-26 ± 13	N.A.	0.85
SNP (n = 5)	67 ± 22	6.1 ± 1.3	0.015
Sildenafil (n = 5)	40 ± 17	5.7 ± 3.8	0.0069
PGE_2 (n = 5)	-31 ± 5	N.A.	0.311
PACAP-38 (n = 5)	27 ± 8	N.A.	0.94
CGRP (n = 5)	-1 ± 6	~ 8	0.0015
NaCl (n = 5)	-24 ± 5	N.A.	/

p-value for comparing agonist versus the NaCl effect. N.A. = Not Applicable. Values are ± SEM, n given in parenthesis.

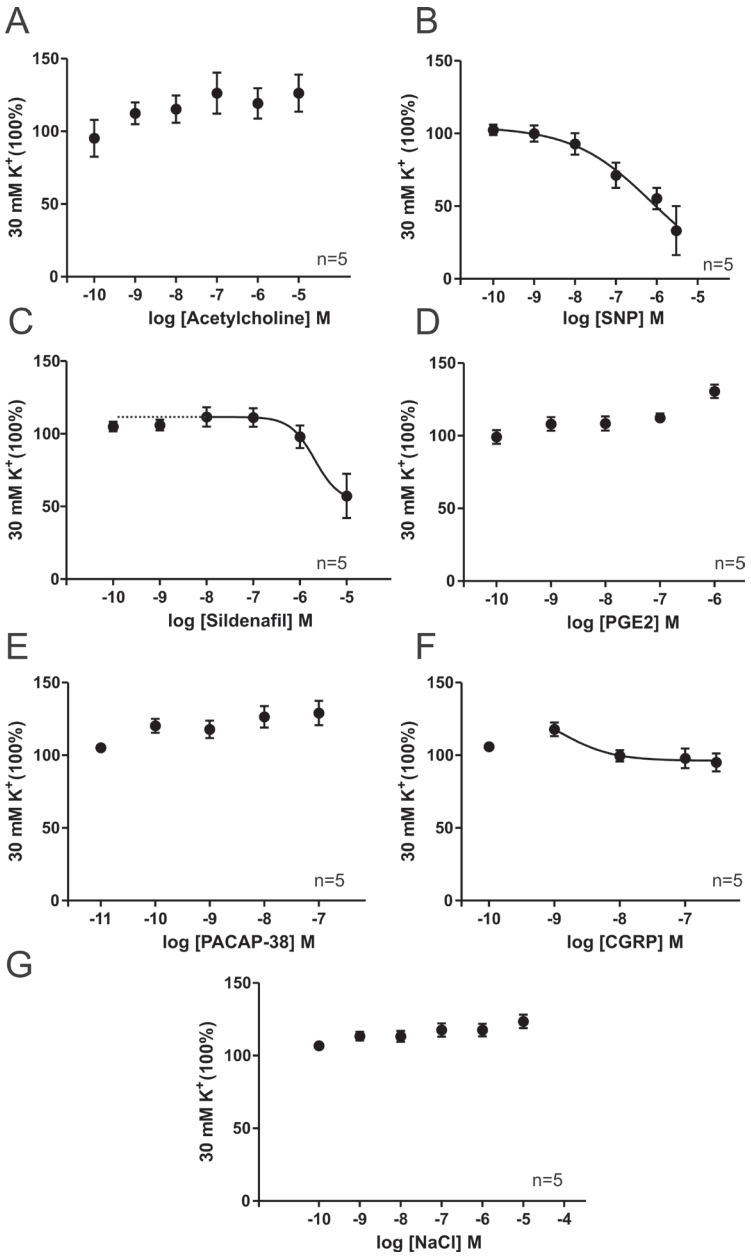

Figure 5. The effect of substances on rat middle meningeal arteries precontracted with K+. The relaxation induced in rat meningeal arteries pre-contracted with K+ at increasing concentrations of each of the tested substances (A-F) and NaCl buffer (G). Values are given as mean ± SEM (n = 5), where 30 mM K+ precontraction is set to be 100%).

Discussion

The addition of substances on human vessels were studied at a transmural pressure of 100 mmHg and pre-contracted with $PGF_{2\alpha}$. Two different approaches for the rat MMAs were used, and in this article the results from both methods are presented. Lowering of the transmural pressure from 100 mmHg to 50 mmHg greatly improved the success rate, and the more constant 30 mM K^+ pre-contraction improved reproducibility. The gradual failing of the $PGF_{2\alpha}$ pre-contraction and increase of the K^+-induced pre-contraction was mitigated by comparing the vasomotor activity of the substances with the spontaneous activity observed when subjecting the vessel segments to only NaCl buffer. It is important to inform the reader, that the isolation and insertion of wires into the rat MMA, destroys the endothelial cell layer because of the small diameter of the rat MMA (around 100 μm in o.d.). The lack of endothelium can be seen in figure 4A because ACh induces a contraction, most likely acting on ACh receptors on the smooth muscle cells. The endothelial function in the human vessels is not substantial either, as no significant relaxation to ACh is observed. The lack of full endothelial function and the lack of (para)sympathetic tone in the vessels in this study could alter some of the effects of the substances added. For example, sildenafil has both endothelial-dependent and -independent effects [29], which could be different in human and rat.

The results from both types of experiments on rat tissue were consistent and showed that SNP, CGRP and sildenafil induced vasodilatation, while ACh, PGE_2 and PACAP-38 had little effect. When used on human vessels, the same myography method yielded significant results for SNP and CGRP, where a strong dilatation was observed. An alternative method that has been used in rodents with some success is the cranial window method where substances can be applied topically through a thinned calvarium [30], but with this method quantification is difficult, as the concentration of active substance that reaches the vessel is difficult to control, hence pEC_{50} and E_{max} cannot reliably be calculated. Further, there are more factors (e.g. blood pressure) that can influence the results than when using isolated vessels *in vitro*.

For the experiments on human vessels, the greatest difference in response between MMA and the previously published data for MCA [20] was seen when subjecting the vessels to sildenafil and PACAP-38, which in both cases constituted a stronger dila-

tation in the MCA. This was surprising in the case of sildenafil since there are previous reports of rat MMA containing higher levels of phosphodieterase type 5 (PDE5) than reported for rat MCA [31]. If the same conditions were valid for human vessels, the higher concentration of the target enzyme in the MMA means that sildenafil should have had more effect there. However, the effect is also dependent on basal NO and cGMP levels, and we cannot rule out that there could be an influence of the age of the human compared to the rat vessels. In addition, the parasympathetic tone *in vivo* could be counteracted by increased NO production, which would not occur in our setup. Therefore, sildenafil, could be effective *in vivo*, but not in the myographs. It is hard to draw conclusions about the difference in E_{max}(%) of PACAP-38 due to previous conflicting observations of the activity of PACAP-38 in rat. Previously published *in vitro* experiments where human intracranial MMA was subjected to the substance suggests that PACAP-38 could indeed dilate human MMA (E_{max}(%) = 34 ± 12; pEC_{50} = 6.9 ± 0.1) [32] and that the absence of vasodilatation observed in the rat experiments in the present study is either due to lack of receptors or technical issues. However, there is no data suggesting an endothelial response to PACAP-38. This suggests that the rat is not a good model for studies of the PACAP-38 mechanisms.

Previous *in vivo* studies exist where magnetic resonance angiography (MRA) was used to measure the diameter of the extracranial part of the MMA of live human test subjects before, during and after spontaneous and artificially induced episodes of migraine. Intravenous infusion of CGRP [33], PACAP-38 [34] and nitroglycerine [35] in healthy non-migraineurs were shown to increase the diameter of the extracranial MMA. These substances are known to induce a headache lacking the migraine-related characteristics in non-migraineurs and this type of pain coincided with the observed vascular dilatation. When CGRP was infused to migraineurs, the results were reproduced and an additional, migraine-like headache with delayed onset occurred [36]. On the other hand, spontaneous migraine attacks reported in patients were associated with a modest increase in diameter (a magnitude of 11.4-13.0%) of the MCA and the intracranial parts (cavernous and cerebral) of the ICA [37]. The intracranial basilar artery (BA) and the extracranial vessels of ECA, superficial temporal artery (STA), the cervical part of the ICA and the extracranial part of the MMA were not significantly dilated.

Migraine-aborting triptan drugs had a constricting effect on arteries outside of the BBB, like the MMA and the extracranial part of the ICA, but not on cerebral

arteries including the intracranial part of the ICA. Regardless of whether an episode of migraine was triggered naturally or by an infused substance, the pain was mitigated and the extracranial MMA contracted from the use of triptan drugs. However, it should be noted that these studies did not include the intracranial branches of the MMA, which is the source of the vessel segments used presently in the *in vitro* studies, and it is still unknown how this part of the artery would be affected *in vivo* by a spontaneous episode of migraine or a therapeutic dose of sumatriptan. The question is relevant, because all arteries shown to significantly dilate during an episode of migraine are all intracranial. Intracranial meningeal arteries would be unique in that they are both intracranial and without BBB properties. They are thereby a type of artery that could possibly be both dilated during an episode of migraine and contracted by drugs. In the *in vitro* study presented here, the effect of two well-known clinically tested migraine compounds were applied to the isolated human MMAs. Both sumatriptan (5-HT receptor agonist) and telcagepant (a CGRP receptor antagonist) significantly reduced the CGRP induced vasodilation. This shows that the vasoactive clinically tested compounds can exert their effect directly on the MMA.

In vivo experiments in rat, using the cranial window method to directly view and apply substances to the intracranial portion of the MMA, have yielded similar results to the *in vivo* observations in human regarding the extracranial part of the vessel. The MMA of rats was significantly dilated by α- and βCGRP [38,39], sildenafil [31], VIP, PACAP-38, PACAP-27 [40], histamine [41] and ACh [42]. VIP is interesting in that it is not a migrainogenic substance [16]. It has not been established what effect VIP could have on human MMA *in vivo*, but since VIP is known to dilate human MMA branches *in vitro* [32] one can assume that the *in vivo* effect would mirror that found in the rat. Vasodilatation without migraine-related pain speaks against the dilation of the MMA as a direct cause of the pain. As with the effect of other substances on meningeal arteries, there are conflicting reports regarding VIP. No significant dilatation was observed during *in vitro* experiments using the myography method [43] or the pressurized arteriography method [44,45].

When the vasomotor responses from our experiments are compared with the migrainogenic properties of the tested substances, there seems to be little correlation between the ability of a substance to induce migraine-like attacks and their vasoactive properties in human meningeal arteries. Sildenafil, PGE_2 and PACAP-38 showed no

tendency of dilatation of the arteries in the current study, despite having known migrainogenic properties. One may wonder why and how the migrainogenic substances induce headache if vasodilatation of meningeal or cerebral arteries is not the mechanism. Endothelial nitric oxide (NO) is not the key molecule since the intracellular pathways activated by the migrainogenic substances CGRP and PACAP-38 are not dependent of NO in the cranial vessels [20]. The remaining factors are the perivascular and dural nerve fibers. It has been shown before that the dura mater contains a rich supply of sensory substance P and CGRP fibers, and of sympathetic noradrenaline and neuropeptide Y, but only a minor amount of parasympathetic VIP and PACAP containing fibers [46]. It is tentative to suggest that local release or systemic administration of the various substances may modify the activity of the dural nerve fibers via specific receptors or mechanisms. This clearly deserves future attention.

Conclusion

The lack of correlation between the vasoactive and migrainogenic properties of the tested substances leads us to the conclusion that direct vasodilatation of intracranial meningeal arteries is most likely not the sole trigger of the artificial migraine-like pain experienced by migraine sufferers upon infusion of these substances into the blood stream. This opinion is based on the fact that several migrainogenic substances induced no significant vasodilatation when applied *in vitro* to human meningeal arteries. We can however not rule out that the cranial pain and migrainogenic properties associated with the infusion of these substances could have a relation to cranial vasodilation *in vivo*. The *in vivo* vasodilatory responses to the substances could be affected by the vascular tone, endothelial interplay and the activation of perivascular nerve fibers around the dural arteries or in the trigeminal ganglion.

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Part 5 - **Discussion and Summary**

Chapter 12 - Summarizing Discussion and Future Perspectives.

Summarizing discussion

Migraine is a very common disorder, affecting around 10% of males and 25% of females worldwide [1]. The exact mechanisms behind a migraine attack are still not clear, but the trigeminal system of the brain as well as the meningeal vasculature that is innervated by the trigeminal nerve are found to be involved. Furthermore, the neurotransmitter and potent vasodilator CGRP is thought to play a key role in migraine [2]. The different forms of migraine, all having different characteristics, suggest that there are multiple mechanisms involved in migraine pathophysiology.

Migraine is much more common in females than in males and during major hormonal changes such as during menstruation, pregnancy and menopause, migraine prevalence in females changes dramatically [3-5]. This is suggesting an important role for female hormones in migraine pathophysiology and, already 40 years ago, Sommerville et al. demonstrated the role of estradiol in migraine [6,7]. In **chapter 3** the prevalence, characteristics and pathophysiology of hormone-related migraines are described. Although a lot of research has been performed on female hormones and migraine since the first studies of Sommerville, the underlying mechanisms still remain unclear. Female hormones are mostly involved in migraine without aura and these attacks occur when estradiol levels drop [7]. Sometimes an effect of female hormones is also seen on migraine with aura, but at different hormonal levels, for example when estradiol and progesterone levels are high [8,9]. The evidence that female hormones differently affect migraine with and without aura, suggests that different mechanism are involved in these forms of migraine.

Environmental and endocrinological factors, such as fluctuating female hormones, are known to be able to change gene and protein expression via epigenetic mechanisms [10]. As described in **chapter 4,** epigenetic mechanisms might be involved in migraine. Valproate is used as a prophylactic antimigraine drug, and is a DNA methylation and histone modification inhibitor. Furthermore, it was demonstrated that CGRP is involved in epigenetic mechanisms [11]. In our study we showed that DNA methylation is tissue-specific and DNA methylation data of one tissue cannot categorically be extrapolated to another tissue. However, we discovered that DNA methylation of the genes we studied is comparable between rat and human, making rat possibly a good alternative to study DNA methylation in tissues that are difficult to obtain in hu-

man. Unfortunately, we could not demonstrate an effect of either high or low estradiol levels on DNA methylation of the promoter region of nine genes involved in migraine pathophysiology. There are, nevertheless, some reasons why an effect of estradiol also cannot be excluded based on this study. First of all, the variation of the DNA methylation was very large for some genes. These big differences suggest that DNA methylation of some genes is not very stable and thus possibly can be regulated by environmental factors. This big variation decreased the power of the study to such an extent that no conclusion could be drawn about an estradiol-related effect. Secondly, the approach of the study was candidate gene-based and the promoter regions of only nine genes were studied. There are of course more proteins involved in migraine pathophysiology and also other regions than the promotor region can be involved in regulation of protein expression via DNA methylation. Therefore, it is very well possible that we might have missed the right region or gene and much more research is needed to establish the role of female hormones on DNA methylation of genes involved in migraine pathophysiology.

Because of the neurovascular character of migraine, we were also interested in the effect of hormones on vasculature. Female hormone studies done by our department showed that estradiol can enhance neurogenic vasodilatation and potentiates vascular relaxations to CGRP [12,13]. These effects were observed at high estradiol levels, while patient studies indicate that most hormone-related migraine attacks occur when estradiol levels are dropping. Sommerville, though, 40 years ago showed that a drop in estradiol levels only induced a migraine attack after a certain period of sustained high estradiol levels, such as occurring just before the menstruation. The effect of the drop in estradiol levels on vascular function has not been studied yet. In women suffering from PCOS, estradiol levels do not fluctuate due to high testosterone levels. Therefore, one would expect less migraine without aura those women. In **chapter 5**, the vascular function of a PCOS mouse model is characterized and we observed decreased endothelial function in these animals. Besides making this model a good model to study cardiovascular alterations seen in women with PCOS, this decreased dilatory function of the vessels is also interesting when focussing on migraine, as the migraine-related neuropeptide CGRP is a potent vasodilator and both triptans and ergot alkaloids are potent vasoconstrictors. Only one study has been performed on the prevalence of migraine in patients with PCOS [14] and no correlation was found between migraine and

PCOS. Unfortunately, no distinction between different forms of migraine was made. Thus, we are planning to investigate this relation in the near future.

As already mentioned, most specific antimigraine drugs have a significant effect on the vasculature. The oldest antimigraine drugs are the ergot alkaloids, but they are also the least pharmacologically specific ones. Their antimigraine effect is thought to be mediated via binding to 5-HT receptors, but they can also bind to dopamine$_{D2}$ and noradrenergic receptors [15]. Although they can be very effective in aborting a migraine attack, they have many side effects, especially on the cardiovascular system. In the 90's the triptans were developed, which are more specific than the ergot alkaloids, binding only to the 5-HT$_{1B/1D}$ receptors [16]. The antimigraine mechanisms behind binding to 5-HT$_1$ receptors are discussed in **chapter 6.** Vascular effects and side effects of both sumatriptan and dihydroergotamine are investigated in **chapters 7 and 8.** We showed that sumatriptan is more potent in contracting distal coronary arteries than proximal, making the contraindications in patients with cardiovascular disease even more important. Because of the cardiovascular side effects of the triptans and ergot alkaloids there is still a great need for a new antimigraine drugs that do not affect the vasculature. CGRP plays a key role in migraine pathophysiology and the recently developed CGRP receptor antagonists, which do not have direct vascular effects, seemed to become these new antimigraine drugs [17,18]. Unfortunately, due to bad oral availability [17] or increased liver transaminases observed in some patients in a phase 3 clinical trial when a CGRP receptor antagonist was administered twice daily for a longer period [19], the development of several CGRP receptor antagonists was discontinued. In **chapter 9,** different techniques that can be used to investigate effects and side effects of potential CGRP receptor antagonists are described. It is shown that CGRP is a potent vasodilator via binding to the CGRP receptor, but we showed in **chapter 10** that it can also inhibit binding of endothelin-1, a potent vasoconstrictor, to its receptor. Because of the involvement of CGRP in multiple physiological mechanisms it is thus very important to study side effects of drugs that act on the CGRP-ergic system. In **chapter 11**, we studied the effect of CGRP and other substances that may artificially induce migraine-like attacks in human and rat middle meningeal arteries. We did not find a direct relation between the migraine-inducing effect of these compounds and dilatation of the middle meningeal artery *in vitro*. Thus, more research is needed to unravel the relation between meningeal vasodilation and the generation of migraine headache.

Future perspectives

Epigenetics and genome-wide methylation arrays

There is still little known about the factor(s) initiating a migraine attack. It is thought that an attack starts in the brainstem, but how this system becomes activated is not known. Furthermore, it is unknown why some people develop migraine attacks from certain triggers, while others who are exposed to the same triggers do not. Although genetics seems to be involved, a monogenetic inheritance pattern is only seen for FHM and heritability of the common migraines is only 50% [20-22]. Multiple studies point to different kinds of environmental triggers, such as diet, sleeping pattern, menstrual cycle, stress [23-26] and other triggers [27] that can start a migraine attack. For example, most women with menstrual migraine, in which dropping estradiol levels possibly trigger an attack, also have attacks that are not related to their cycle [28,29], suggesting that there are also other triggers that can initiate a migraine attack in these women. Admittedly, some of these factors also have a genetic component.

Alterations in gene expression via the influence of environmental factors can occur via epigenetic modifications of the genes as discussed in Chapter 3. Unfortunately, we could not discover alterations in DNA methylation caused by estradiol in the migraine-related genes that we studied. Recently, a more effective method has been developed to study DNA methylation, the genome wide methylation array (GWMA). One array contains 450 thousand probes, covering all known genes at different important regions as the promoter region and possible regulatory regions [30]. This process can be automated, so many samples can be measured and big groups of patients can be compared to each other. Furthermore, because DNA methylation is measured genome widely, a large amount of data is created that can be used to answer multiple research questions of different groups, making it possible to share the costs of this expensive method. Therefore, it would be very interesting to perform a GWMA specific for migraine with and migraine without aura and healthy controls. The statistical power of such a study could be increased by focusing on genes involved in pathways that have been indicated by studies on FHM and GWAS on the more common forms of migraine [31-37]. Such pathways include (glutamatergic) neurotransmission, neuron and synapse development, brain vasculature function and pain sensing systems [38]. However, there is one big problem that needs to be solved first. As we showed in Chapter 3, DNA

methylation is tissue-specific and DNA methylation of blood cannot automatically be extrapolated to DNA methylation of other tissues. To study the effect of environmental factors on the activation of the trigeminal system, a sample of this tissue should thus be obtained from each patient and healthy control. As this is almost impossible, another approach should be developed. As we showed in our study, DNA methylation of rat leukocytes is concordant to DNA methylation of human leukocytes. This might thus also be the case for other tissues, but of course this needs to be validated first. When the concordance would appear to be (relatively) generalized, the rat can be a very good model to study DNA methylation after different environmental circumstances such as for example stress, hormones or alterations in sleeping pattern. We could not demonstrate differences in DNA methylation values after estradiol treatment as described in our study in Chapter 3. This was probably because of the large variations between the animals, decreasing the statistical power. When performing GWMAs, it is even more difficult to find statistically significant differences between genes. Because of the high number of genes that are studied, they should be corrected for multiple comparisons, which also decreases the power. Therefore, a study needs to be performed to investigate whether differences in DNA methylation can be directly measured in tissues obtained from animals or humans. For example treatment of rats with hypomethylating agents such as 5-aza-2'-deoxycytidine would be a possible positive control.

If these direct studies appear to be difficult, another approach can be to treat tissues in organ baths with hormones and measure DNA methylation of genes of interest, as others have shown that methylation profiles can change in hours or days [39,40]. Possibly, this would give smaller variations, because the environment can be controlled much better in an organ bath. Furthermore, treatment of animals and or tissues with valproate, the antimigraine prophylactic and inhibitor of histone modifications and DNA methylation, would be a very interesting approach. However, first an effect of an environmental factor on DNA methylation of genes involved in migraine would be needed, to know where to find a treatment effect.

Unfortunately, there is no migraine rat model and we cannot measure migraine headache in rats while it might be possible that environmental factors only influence epigenetic mechanisms in susceptible animals and not in healthy ones, because also not all people get migraine from environmental factors, so it seems that some people are more susceptible than others. There are however, some rare inherited diseases

characterized by different forms of migraine; FHM (Familial Hemiplegic Migraine), CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) and RVCL (Retinal Vasculopathy with Cerebral Leukodystrophy) [41,42]. Tissues from genetically modified mouse models for those diseases could be very well used for these GWMA's. There are many different types of GWMA developed, which can be used for different species [43]. Because they differ in characteristics, specificity and costs, a well-considered decision should be made about which is array is most useful. A prerequisite is that DNA methylation of mice is concordant to that of humans, so this should be investigated first.

Human prevalence studies on migraine and sex hormones

Migraine prevalence is higher in women than in men [44]. When hormone levels change, for example during pregnancy, the prevalence of migraine also changes. Apart from migraine, there are some other diseases and conditions where female hormone levels are completely different from those in normally cycling women. This is for example the case in women suffering from PCOS, but also in women or men undergoing a gender transformation. One study investigated the prevalence of migraine in women with PCOS, but unfortunately no difference was made between MA an MO, while MO seems to be more related to female hormones than MA [14]. The (changing) prevalence of migraine in women or men undergoing a sex changing operation has only once been studied so far [45]. In this retrospective study, migraine prevalence was shown to increase in men undergoing a female gender transformation via treatment with estrogens and removal of testosterone. The change from female to male has never been studied. It would be very interesting to investigate migraine prevalence and mechanisms in people undergoing a transgender operation and discover more about the role of sex hormones on migraine prevalence.

Promising anti-migraine compounds

As described in chapter 6, 7 and 8, the most used specific anti-migraine drugs, the ergot alkaloids and the triptans, are all 5-HT receptor agonists. Although they can be very efficient in aborting a migraine attack, their cardiovascular side-effect profile has led to their contraindication in patients with cardiovascular diseases. Furthermore, the triptans are not effective in all migraine patients. Therefore, there is a need for other

anti-migraine drugs without vasoconstrictive properties.

5-HT1F receptor agonists

Next to binding to the 5-HT1B/1D receptors, some of the triptans can also bind to 5-HT1F receptors and it has been shown that these receptors are located in the trigeminal system. Activation of the 5-HT1F receptor does not induce vasoconstriction, and LY334370 and lasmiditan, 5-HT1F receptor agonists, have been shown to be effective in the treatment of migraine [46,47]. Unfortunately, LY334370 had multiple adverse effects, and due to safety concerns apparent from animal studies its development was discontinued [47]. Lasmiditan was tested in two phase II trials and efficacy was shown. Adverse events were reported in 65-87 % of subjects in the lasmiditan-treated group compared to 28-42% in the placebo-treated group [47,48]. However, the effect and side effects need to be compared to other antimigraine drugs, for example the triptans, to find out whether lasmiditan is an improvement in the treatment of migraine attacks and to consider its potential use in patients suffering from cardiovascular disease.

NXN-188, combination of triptan and nNOS inhibitor

Another prospective compound is NXN-188, which is a combination of a triptan and a neuronal NO synthase (nNOS) inhibitor. NO is an important molecule in vasorelaxation as well as modulation of neuronal signalling of the trigeminal system [49,50]. Furthermore, intravenous administration of glyceryl trinitrate (GTN, an NO donor) can induce migraine attacks in migraineurs. The first NOS inhibitor tested was L-NNMA, a non-specific NOS inhibitor. Although the results seemed promising, increased blood pressure, probably caused by eNOS inhibition, and poor oral absorption prohibited clinical use of this compound [51,52]. The more specific iNOS inhibitor, GW274150, that was developed afterwards was not effective in treatment of migraine [53]. Recently NXN-188 was developed, which is a specific nNOS inhibitor, combined with a triptan. In experimental models NXN-188 has shown to be effective in inhibiting CGRP release [54], and it has shown to be well tolerated in healthy volunteers [55]. However, more studies are needed to investigate the safety en efficacy of this compound in migraine patients. Primarily, a study comparing NXN-188 with different triptans is needed, to investigate its advantage compared to the triptans. This advantage compared to the triptans should be due to the inhibition of nNOS. nNOS is thought to be involved

in pain signalling mechanism, as NOS inhibition can lower the activity of neurons with meningeal input in the rat spinal trigeminal nucleus [56] and NOS inhibitors can antagonize neurogenic and CGRP induced dilation of dural meningeal vessels[57]. No specific nNOS inhibitor is tested in migraine patients yet, however, in 2012 a specific, orally tolerable nNOS inhibitor was discovered that might be interesting to study further [58], especially because no effect on the vasculature is expected for a nNOS inhibitor.

CGRP antibodies

The recently developed CGRP receptor antagonists were devoid of the cardiovascular side effects of the triptans and ergot alkaloids, while their efficacy in migraine was comparable. Unfortunately the development was stopped when increased liver transaminases were found in a phase 3 clinical trial, so there is a need for a replacement. CGRP and CGRP receptor antibodies seem to be a good candidate. These antibodies will activate the immune system, thereby inducing breakdown of CGRP or its receptor, thus preventing CGRP receptor activation. Because these antibodies are less foreign than the antagonists there is a much smaller chance that they have side effects on the liver. The first animal studies with a CGRP antibody demonstrated no effect on heart rate and blood pressure [59]. AA95, an antibody directed against the CGRP receptor, was effective in inhibiting the capsaicin-induced increase in dermal blood flow in monkeys [60]. Furthermore, LBR-101, an antibody directed against CGRP, was demonstrated to be safe in healthy volunteers in a phase I study [61]. Although these results are promising, of course extensive studies are needed to investigate the safety, tolerability and effectiveness of the CGRP and CGRP receptor antibodies in both healthy controls as well as migraine patients. Their effects are thought to be much longer lasting than those of CGRP receptor antagonists, because of their much longer half-life, which can be up to 21 days for IgG antibodies [59,62]. This would be a perfect opportunity for their use as a prophylactic, however it can also be the pitfall, as the putative side effects are therefore also much longer lasting. Furthermore, the physiological functions of CGRP and its receptor would be suppressed for a longer period of time. As it is thought that CGRP is involved in ischemia and reperfusion, this needs serious investigation before using them as a prophylactic drug.

Although we do have some very useful anti-migraine drugs at the moment, there is still a great need for more specific anti-migraine drugs, especially without car-

diovascular side effects. Fortunately, some promising compounds were discovered recently. Animal studies and clinical trials performed at the moment and in the near future should reveal their usefulness for anti-migraine treatment. Furthermore, the search for the pathophysiological mechanisms behind migraine should continue, to reveal the differences behind the different types of migraine and to discover more targets for specific anti-migraine treatment and decreasing the amount and severity of the side-effects of anti-migraine drugs.

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Chapter 13 - Nederlandse Samenvatting

Nederlandse Samenvatting

Migraine is een aandoening die wereldwijd bij ongeveer 10% van de mannen en 25% van de vrouwen voorkomt. De exacte mechanismen achter een migraineaanval zijn nog niet duidelijk, maar waarschijnlijk speelt het trigemino-vasculaire systeem, betrokken bij signalering van pijn in de hersenen, een belangrijke rol hierin. Ook de neurotransmitter en potente vaatverwijder CGRP speelt mogelijk een cruciale rol bij migraine. Er bestaan verschillende vormen van migraine met allemaal verschillende kenmerken. Dit suggereert dat meerdere mechanismen betrokken zijn bij de pathofysiologie van migraine.

Migraine komt vaker voor bij vrouwen dan bij mannen. Vooral bij grote hormonale veranderingen zoals tijdens de menstruatie, zwangerschap en menopauze kan de hoeveelheid en intensiteit van migraine aanvallen sterk veranderen. Dit suggereert een belangrijke rol voor vrouwelijke hormonen in de pathofysiologie van migraine, en inderdaad toonde Sommerville reeds 40 jaar geleden de belangrijke rol van oestradiol in migraine bij vrouwen al aan. In **hoofdstuk 3** worden de prevalentie, kenmerken en pathofysiologie van hormoon-gerelateerde migraines beschreven. Hoewel veel onderzoek is uitgevoerd naar het verband tussen vrouwelijke hormonen en migraine zijn de onderliggende mechanismen nog steeds onduidelijk. Vrouwelijke hormonen zijn meestal betrokken bij migraine zonder aura en deze aanvallen treden op wanneer het oestradiol niveau in het bloed daalt. Soms wordt echter ook een effect van vrouwelijke hormonen gezien op migraine met aura, maar dan wel bij andere hormonale niveaus in het bloed, bijvoorbeeld tijdens de zwangerschap, wanneer de spiegels van oestradiol en progesteron hoog zijn. Het feit dat vrouwelijke hormonen migraine met en zonder aura anders beïnvloeden, suggereert dat bij deze vormen van migraine verschillende achterliggende mechanismen betrokken zijn.

Omgevings- en endocrinologische factoren, zoals vrouwelijke hormonen, kunnen gen- en eiwitexpressie beïnvloeden via epigenetische mechanismen. De twee meest bekende zijn DNA methylering en histon modificaties. Deze mechanismen kunnen de vouwing van het DNA beinvloeden waardoor het meer of minder toegankelijk is voor transcriptie. In **hoofdstuk 4** is beschreven dat epigenetische mechanismen betrokken kunnen zijn bij migraine. Valproaat wordt bijvoorbeeld gebruikt als een profylactisch middel tegen migraine en is ook een remmer van DNA methylering en histon modifi-

caties. Verder is aangetoond dat CGRP betrokken is bij epigenetische mechanismen. In ons onderzoek hebben we aangetoond dat DNA methylering specifiek is voor weefsels en dat de mate van DNA methylering van het ene weefsel niet automatisch kan worden geëxtrapoleerd naar een ander weefsel. Ook ontdekten we dat DNA methylering van de genen die we onderzocht hebben vergelijkbaar is tussen bloed van de rat en de mens. Als dit ook voor andere weefsels het geval is, maakt dit de rat mogelijk een goed alternatief voor het bestuderen DNA methylering in weefsels die moeilijk te verkrijgen zijn in de mens. Helaas hebben we geen effect van hoge of lage oestradiol concentraties kunnen aantonen op DNA methylering van de promotor regio van de negen door ons onderzochte genen die betrokken zijn bij migraine. Er is echter een aantal redenen waarom een effect van oestradiol niet kan worden uitgesloten op basis van deze studie. Allereerst is de variatie van de DNA methylering erg groot voor sommige genen. Deze grote verschillen suggereren dat DNA methylering van bepaalde genen niet erg stabiel is en dus eventueel door omgevingsfactoren gereguleerd kan worden. Deze grote variatie verminderde de statistische power van de studie echter zodanig dat geen conclusies konden worden getrokken over een oestradiol gerelateerd effect. Ten tweede was de studie gebaseerd op kandidaat genen en zijn de promotor regio's van slechts negen genen onderzocht. Er zijn natuurlijk meer eiwitten die betrokken zijn bij de pathofysiologie van migraine en ook andere regio's dan de promotor regio kunnen betrokken zijn bij de regulatie van eiwitexpressie via DNA methylering. Daarom is het goed mogelijk dat we het juiste gebied of gen gemist hebben. Er is dus nog veel meer onderzoek nodig om de rol van vrouwelijke hormonen in DNA methylering van genen betrokken bij de pathofysiologie van migraine vast te stellen.

Vanwege het neurovasculaire karakter van migraine waren we ook geïnteresseerd in het effect van hormonen op de bloedvaten. Op onze afdeling zijn eerder studies gedaan naar het effect van vrouwelijke hormonen op migraine en hieruit bleek dat oestradiol de potentie van een bloedvat om te verwijden kan vergroten. Verder is aangetoond dat oestradiol de afgifte van CGRP in de bloedvatwand kan vergroten. Deze effecten werden waargenomen bij hoge oestradiol niveaus in het bloed, terwijl uit patiënt studies blijkt dat de meeste hormoon-gerelateerde migraine aanvallen optreden wanneer oestradiol niveaus in het bloed dalen. Sommerville heeft echter 40 jaar geleden al aangetoond dat een daling van oestradiol alleen een migraine aanval veroorzaakte na een periode van aanhoudend hoge oestradiol niveaus in het bloed, zoals vlak voor de

menstruatie. Het effect van een daling van de oestradiol spiegels in het bloed op vasculaire functie is nog niet onderzocht. Bij vrouwen die lijden aan polycysteus ovarium syndroom (PCOS) treden minder schommelingen in oestradiol niveaus in het bloed op als gevolg van een hoge testosteronspiegel. Daarom zou men minder migraine zonder aura verwachten in deze vrouwen. **Hoofdstuk 5** beschrijft de vasculaire functie van een PCOS muismodel. In deze dieren, die behandeld zijn met testosteron, hebben we een verminderde endotheelfunctie aangetoond. Dit muismodel is ook een goed model om cardiovasculaire veranderingen die optreden bij vrouwen met PCOS te bestuderen. Verder is de verminderde vaatfunctie interessant voor migraine aangezien het voor migraine belangrijke neuropeptide CGRP een krachtige vaatverwijder is en de triptanen en ergot alkaloïden (veel gebruikte antimigraine medicijnen) een sterk vaatvernauwend effect hebben. Er is slechts een studie uitgevoerd naar de prevalentie van migraine bij patiënten met PCOS en hierbij is geen correlatie gevonden tussen migraine en PCOS. Helaas is in deze studie geen onderscheid tussen de verschillende vormen van migraine gemaakt. We zijn dus zeker van plan om deze relatie in de toekomst beter te onderzoeken.

Zoals reeds vermeld hebben de meeste specifieke antimigraine medicijnen een effect op het vaatstelsel. De oudste antimigraine medicijnen zijn de ergot alkaloïden, maar dit zijn ook de minst farmacologisch specifieke. Verondersteld wordt dat hun antimigraine effect wordt gemedieerd door binding aan 5-HT receptoren, maar ze kunnen ook binden aan dopamine$_{D2}$ en noradrenerge receptoren. Hoewel ze zeer effectief zijn tegen migraine, hebben ze veel bijwerkingen, vooral op het cardiovasculaire systeem. In de jaren '90 zijn de triptanen ontwikkeld. Deze zijn specifieker dan de ergot alkaloïden doordat ze voornamelijk aan 5-HT$_{1B/1D}$ receptoren kunnen binden. De antimigraine mechanismen achter binding aan 5-HT$_1$ receptoren worden besproken in **hoofdstuk 6**. De vasculaire effecten en bijwerkingen van zowel sumatriptan als dihydroergotamine worden onderzocht in **hoofdstuk 7 en 8**. We hebben aangetoond dat distale kransslagaders sterker vernauwd worden door sumatriptan dan proximale kransslagaders. Deze resultaten onderstrepen het belang van contra-indicatie in patiënten met cardiovasculaire aandoeningen.

Vanwege de cardiovasculaire bijwerkingen van de triptanen en ergot alkaloïden is er nog steeds een grote behoefte aan een nieuwe antimigraine middelen die geen invloed hebben op de bloedvaten. CGRP speelt een belangrijke rol in de pathofysiologie

van migraine en resultaten met de recent ontwikkelde CGRP receptor antagonisten, die geen directe vasculaire effecten hebben, waren veelbelovend. Helaas is de ontwikkeling van verschillende CGRP receptor antagonisten op het laatste moment beëindigd; als gevolg van slechte orale beschikbaarheid en verhoogde lever transaminase waarden bij sommige patiënten in een fase 3 klinische studie na tweemaal daagse toediening gedurende een langere periode is besloten met de ontwikkeling te stoppen. In **hoofdstuk 9** zijn verschillende technieken beschreven die gebruikt kunnen worden om werking en bijwerkingen van mogelijke CGRP receptor antagonisten te onderzoeken. In **hoofdstuk 10** hebben we aangetoond dat CGRP de binding van endotheline-1 , een krachtige vaatvernauwer, aan zijn receptor kan remmen. Vanwege de betrokkenheid van CGRP in verschillende fysiologische mechanismen is het dus zeer belangrijk om de bijwerkingen van geneesmiddelen die inwerken op de CGRP-erge systeem te bestuderen. In **hoofdstuk 11** hebben we het effect van CGRP en andere stoffen die potentieel in een experimenteel model migraine veroorzaken, onderzocht in meningeale bloedvaten van de mens en de rat. We hebben geen direct verband kunnen vinden tussen de effecten van deze middelen in migraine patiënten en het vaatverwijdende effect op de meningeaal arterie. Meer onderzoek is dus nodig om het verband tussen meningeale vasodilatatie en het ontstaan van migraine hoofdpijn te ontrafelen.

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About the author

Sieneke Labruijere, de schrijver van dit proefschrift, is geboren op 5 juni 1984 te Bergen op Zoom. Ze verhuisde kort na haar geboorte samen met haar familie naar Roosendaal waar ze opgroeide en in 2003 het VWO, atheneum heeft afgerond. Na het VWO is ze Biomedische Wetenschappen gaan studeren in Utrecht. Na een algemene bachelor van 3 jaar koos ze voor de master 'Biology of Disease' als afstudeerrichting. Na een werk en reis pauze van een jaar heeft ze de masteropleiding 'Science Communication and Education' gevolgd, wederom in Utrecht en ook deze met goed gevolg afgerond. Ze kon blijven werken bij het bedrijf waar ze haar afstudeerstage had gevolgd, waar ze gepersonaliseerd patiënten voorlichtingsmateriaal ontwikkelde. Door het missen van wetenschappelijke diepgang besloot ze om te gaan promoveren en dit bleek mogelijk te zijn bij het Erasmus MC, afdeling Interne Geneeskunde, bij de sector farmacologie. Hier heeft ze de afgelopen vier jaar met veel plezier onderzoek gedaan naar migraine en zich voornamelijk gericht of het effect van vrouwelijke hormonen op migraine, de rol van epigeneticsche mechanismen in migraine en de farmacologie van migraine medicatie. Dit proefschrift is hiervan het resultaat.

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S. Labruijere, R. de Vries, A.H.J. Danser, G.S. Cottrell, A. MaassenVanDenBrink. Endothelin-converting-enzyme 1 inhibition and CGRP receptor recycling in human coronary and middle meningeal arteries. The Journal of Headache and Pain, Volume 14, Supplement 1, February 2013

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coronary arteries. The Journal of Headache and Pain, Volume 14, Supplement 1, February 2013

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S. Labruijere, M.M.P.J. Verbiest, R. De Vries, A.H.J. Danser, A.G. Uitterlinden, L. Stolk, A. MaassenVanDenBrink. Methylation of Migraine-Related Genes in Different Tissues. Cephalalgia, Volume 33, Supplement, June 2013.

R.R. Klever, J.W. Rutten, **S. Labruijere**, K. Ibrahimi, L.A.M. Broos, N. Rieff, G.M. Terwindt, M.D. Ferrari, S.A.M.J. Lesnik-Oberstein, E.A.M.J. Tolner, A. Maassen-van den Brink, A.M.J.M. van den Maagdenberg. Novel Transgenic Mouse Models for Monogenic Cerebral Small Vessel Diseases Related to Migraine. Cephalalgia, Online Publication IHC 2013 Program Late-Breaking Abstracts. June 2013.

PhD Portfolio

Name PhD student:	Sieneke Labruijere
Erasmus MC Department:	Department of Internal Medicine, Division of Vascular Medicine and Pharmacology
Research School:	Coeur
PhD period:	2010-2014
Promotor(s):	Prof. Dr. A.H.J. Danser
Copromotor:	Dr. A. Maassen van den Brink

Education

2010-2014	Ph. D, Erasmus Medical Center, Rotterdam, The Netherlands. Title of Thesis: Neurovascular Pharmacology of Migraine; Epigenetics and Sex Hormones.
2009-2010	M.Sc. Science Communication and Education, Utrecht University
2003-2008	M.Sc. Biomedical Sciences, Biology of Disease, UtrechtUniversity

General Academic and Research Skills (5.7 ECTS)

2007	Laboratory animal science, University Medical Center, Utrecht, The Netherlands
2011	Statistics course: Classical methods for data analysis. Erasmus Medical Center, Rotterdam, The Netherlands

In-depth courses (e.g. Research school, Medical Training) (10.5 ECTS)

2010	Cardiovascular Pharmacology Clinical cardiovascular epidemiology Basic Data Analysis on Gene Expression Arrays (Molmed) Ensemble (Molmed)
2011	Molecular biology in atherosclerosis and cardiovascular research Heart failure research
2012	Intensive Care Research

Presentations (18.4 ECTS)

2010	Figon Dutch Medicine Days. The effect of DHT on vascular reactivity. (oral presentation)

2nd European Headache and Migraine Trust International Congress (EHMTIC) The effect of DHT on vascular reactivity (poster presentation).

2011 Wetenschapsdagen Interne Geneeskunde 2011, Antwerpen, Belgie. The effect of DHT on vascular reactivity (poster presentation).

6th International Meeting Steroids and Nervous System The role of 17beta-estradiol withdrawal in the neurovascular pathophysiology of migraine (Poster presentation)

2nd Coeur PhD day. Telcagepant and Sumatriptan in Human Middle Meningeal Arteries (Oral Presentation)

15th International Headache Congress. The effect of sumatriptan and telcagepant on CGRP-induced dilation in human isolated middle meningeal arteries. (Poster presentation)

Figon Dutch Medicine Days. Telcagepant and Sumatriptan in Human Middle Meningeal Arteries (oral presentation)

2012 NVF meeting. Methylation of migraine related genes in rat tissues

3th European Headache and Migraine Trust International Congress (EHMTIC) Endothelin-converting-enzyme 1 inhibition and CGRP receptor recycling in human coronary and middle meningeal arteries; Sumatriptan and dihydroergotamine in isolated distal human coronary arteries; 17β-estradiol and methylation of migraine-related genes. (2x poster, 2x oral presentation)

2013 Wetenschapsdagen Interne Geneeskunde 2013, Antwerpen, Belgie. Sumatritpan and DHE in human arteries (poster presentation).

7th International Meeting Steroids and Nervous System. 17β-estradiol and methylation of migraine-related genes. (Poster presentation)

23rd Anglo Dutch Migraine Association meeting. Methylation of migraine-related genes in different tissues of the rat (oral presentation)

16th International Headache Congress Methylation of migraine-related genes in different tissues of the rat (Poster presentation)

Wetenschapsdagen Interne Geneeskunde 2013, Antwerpen, Belgie. Methylation of migraine-related genes in different tissues of the rat

(oral presentation).

Werkbesprekingen afdeling Farmacologie (6x)

International Conferences (9 ECTS)

2010	20th Anglo Dutch Migraine Association meeting, London
	2nd European Headache and Migraine Trust International Congress (EHMTIC), Nice
2011	6th International Meeting Steroids and Nervous System, Torino
	21st Anglo Dutch Migraine Association meeting, Maastricht
	15th International Headache Congress, Berlin
2012	22nd Anglo Dutch Migraine Association meeting, Brighton
	3th European Headache and Migraine Trust International Congress (EHMTIC), London
	2012 Biannual International Forum. Frontiers in Drug Discovery, Maastricht
2013	7th International Meeting Steroids and Nervous System, Torino
	23rd Anglo Dutch Migraine Association meeting, Haarlem
	16th International Headache Congress, Boston

Seminars and Workshops (2.9 ECTS)

2010	Literature search + Endnote (Medical Library)
	Extracellular Matrix in Vascular Disease
2011	Identification of Novel Genetic Regulators of Vessel Formation
2010-2012	Coeur Lectures

Teaching Activities (1,35 ECTS)

2010-2014	VO Autonoom Zenuwstelsel, 1st year medical students
	Practical lessons cardiovascular pharmacology, 2nd year medical students
	Practical lessons Junior Med school, 5th year high school students (5 VWO)

Total ECTS: 47.85

Abbreviations

5-CT	5-carboxamidotryptamine
5-HT	5-hydroxytriptamine, Serotonin
Ach	Acetyl Choline
ADMA	Asymmetric dimethylarginine
AE	Adverse Event
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ar	Androgen receptor
BBB	Blood Brain Barrier
BH4	(6R)-5,6,7,8-tetrahydro-L-biopterin.2HCl
BOLD-fMRI	Blood Oxygenation Level-dependent functional MRI
Calca	Calcitonin related peptide alpha
Calcrl	Calcitonin receptor-like receptor
cAMP	Cyclic Adenosyl Mono Phosphate
CBF	Cerebral Blood Flow
CGRP	Calcitonin Gene-Related Peptide
CIMT	Carotid Intima Media Thickness
CLR	Calcitonin-like Receptor
CNS	Central Nervous System
COC	Combined Oral Contraceptive
CRC	Concentration Response Curve
Crcp	Calcitonin receptor component protein
CSD	Cortical Spreading Depression
DBF	Dermal Blood Flow
DHE	Dihydroergotamine
DHT	Dihydrotestosterone
DMSO	Dymethylsulfoxide
ECA	External Carotid Artery
eNOS	Endothelial NO Synthase
nNOS	Neuronal NO Synthase
ERα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
Esr1	Estrogen receptor 1
Esr2	Estrogen receptor 2
ET-1	Endothelin-1
ETA	Endothelin Type A receptor
FHM	Familial Hemiplegic Migraine
Gper	G-protein coupled estrogen receptor
GTN	Glyceryl Trinitrate
GWAS	Genome Wide Association Studies
GWMA	Genome Wide Methylation Array
HDL	High Density Lipoprotein
ICA	Internal Carotid Artery
ICHD-3 beta	International Classification of Headache Disorders 3rd edition, beta

IHS	International Headache Society
HCMA	Human Coronary Micro Artery
KCl	Potassium Chloride
LDL	Low Density Lipoprotein
L-NAME	N-Nitro-L-Arginine Methyl Ester
MA	Migraine with Aura
mGluR	Metabotropic Glutamate Receptor
MMA	Middle Meningeal Artery
MO	Migraine without aura
MRI	Magnetic Resonance Imaging
MRM	Menstrually Related Migraine
MTHFR	Methylene Tetrahydrofolate Reductase
NAC	N-acetyl cysteine
NMDA	N-methyl-D-aspartic acid
NO	Nitric Oxide
Nos3	Nitric oxide synthase 3
OVX	Ovariectomy
PACAP	Pituitary adenylate cyclase activating polypeptide
PCOS	Polycystic Ovary Syndrome
PET	Positron Emission Tomography
PGE_2	Prostaglandin E_2
PON1	Serum Paraoxonase 1
Ramp1	Receptor activity-modifying protein 1
RCP	Receptor Component Protein
RMA	Rat Mesenteric Artery
ROS	Reactive Oxygen Species
SD	Standard Deviation
SNP	Sodium Nitroprusside
SSS	Superior Sagittal Sinus
TCC	Trigemino Cervical Complex
TNC	Trigeminal Nucleus Caudalis
Trpv1	Transient receptor potential cation channel, subfamily V, member 1
U46619	9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$
Usf2	Upstream stimulating factor 2
VIP	Vasoactive Intestinal Peptide
VPM	Ventral Posteromedial thalamic Nucleus