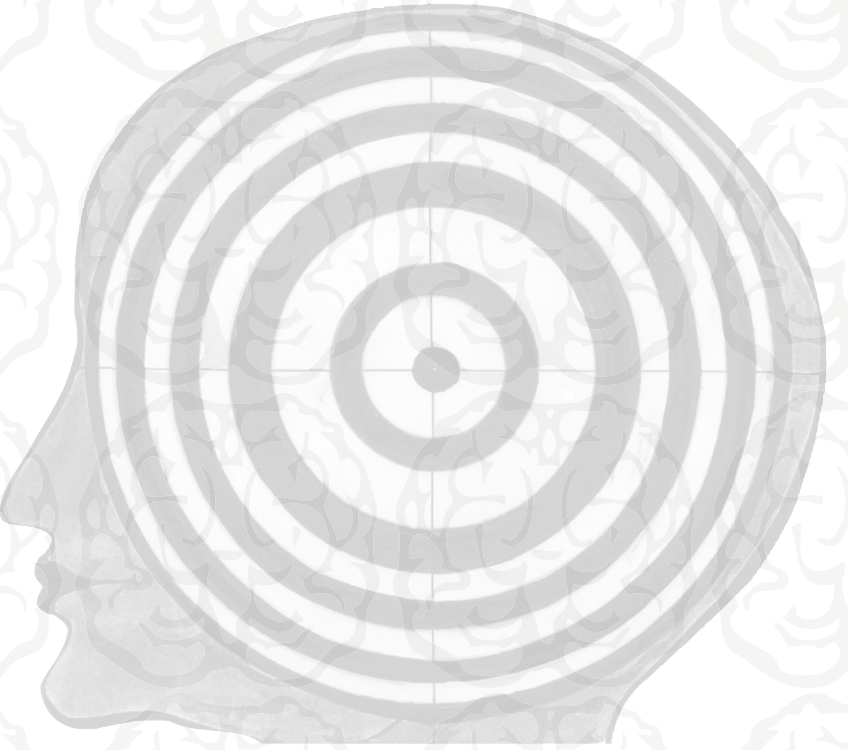
Characterization of vascular 5-hydroxytryptamine, adrenergic and CGRP receptors in relation to migraine



Suneet Mehrotra

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Characterization of vascular 5-hydroxytryptamine, adrenergic and CGRP receptors in relation to migraine

Karakterisering van vasculaire 5-hydroxytryptamine, adrenerge en CGRP receptoren in relatie tot migraine

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rectus magnificus
Prof. dr. S.W.J. Lamberts
and in accordance with the decision of the Doctorate Board

The public defence shall be held on
Wednesday, December 20, 2006 at 11.45 hours

by

Suneet Mehrotra
born in Lucknow, India

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Dedicated to my Father
Dr. B.N.Mehrotra
(1936-2005)

What appears to be the end may really be a new beginning

CONTENTS

Learn to speak little and to speak softly. That will reduce the chances of getting angry. Seek the good in others and the evil in yourself (Sai Baba)

Part I: Introduction

Chapter 1

The role of 5-hydroxytryptamine, adrenergic and CGRP receptor subtypes in migraine pathophysiology

Based on: **Suneet Mehrotra**, *Saurabh Gupta, David Centurión, Carlos M. Villalón, Pramod R. Saxena and Antoinette MaassenVanDenBrink*.
(Invited review; in preparation)

Abstract

Migraine affects a substantial fraction of the world population and is a major cause of disability in the work place. Migraine is a recurrent incapacitating neurovascular disorder characterized by attacks of debilitating pain associated with photophobia, phonophobia, nausea and vomiting. Though the pathophysiology of migraine is still unclear, it is believed to be a neurovascular disorder. The drugs used in the treatment of migraine affect vascular receptors. In earlier days, α-adrenoceptors agonists (ergotamine, dihydroergotamine, isometheptene) where used. The last decade has witnessed the advent of sumatriptan and the 'second generation triptans', which belong to a class of drugs known as 5-$HT_{1B/1D}$ receptor agonists. The triptans have a well-established efficacy in aborting migraine attacks. Current prophylactic treatments for migraine include 5-HT_2 receptor antagonists and β-adrenoceptor blockers. In view of the complexity of migraine etiology, the disease still remains under diagnosed, despite progress in migraine research and available therapies are underused. In this chapter, pharmacological targets, particularly those based on 5-HT, adrenergic and CGRP receptors, will be discussed in relation to migraine therapy.

1.1 INTRODUCTION

Five thousand years ago, Mesopotamian physicians viewed headache as a disease entity rather than a symptom and attributed it to an evil spirit named Tiu[1]. Since then, physicians have used a number of pharmacological and non-pharmacological interventions, to cure or prevent the patients' headaches. However, the major breakthrough in the understanding of headache came in the 17th century, when Thomas Willis published in 1664 his hypothesis that "megrim" was due to dilatation of blood vessels within the head[2]; this seems to be the first proposal for a vascular theory of migraine. Later on, Heyck[3] proposed that arterial blood flow during migraine is being shunted to the venous side due to dilatation of cephalic arteriovenous anastomoses (Figure 1.1). Afterwards, scientists started believing that migraine is a neurological dysfunction[2]. Towards the end of the 20th century attempts started to reconcile both the theories and the modern outlook towards the pathophysiology of migraine emerged as a neurovascular disorder[4].

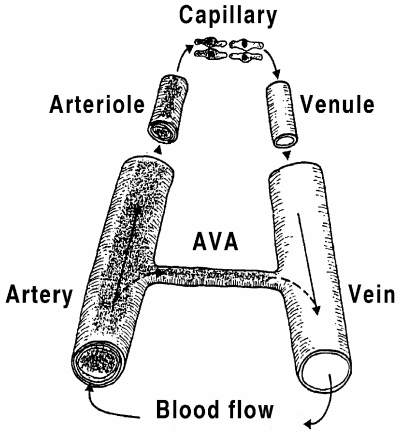

Figure 1.1: Schematic representation of arterio venous anastomoses (shunt model)

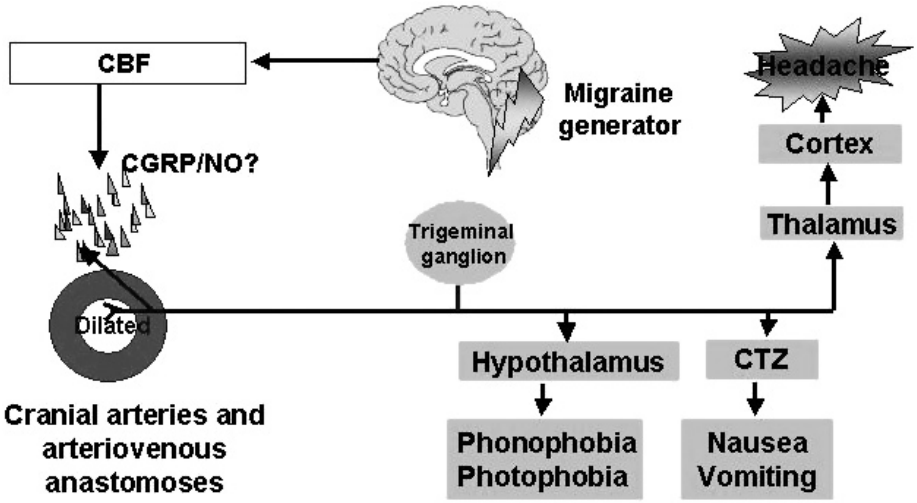

Figure 1.2: The pathophysiology of migraine (adapted from The Headaches[24]). CBF: cerebral blood flow, CTZ: chemo trigger zone

1.1.1 Pathophysiology of migraine

The pathogenesis of headache in migraine is not completely understood, but it is thought that there are three key component, namely: (i) the cranial blood vessels, (ii) the trigeminal innervation of these vessels, and (iii) the reflex connection of the trigeminovascular system (Figure 1.2) in the cranial parasympathetic outflow[4]. Although the brain is insensitive to pain, nociceptive stimulus can be generated by large cranial and proximal intracranial blood vessels as well as by the dura mater. In animals, stimulation of vascular afferents leads to neuronal activation in the superficial layers of the trigeminal nucleus caudalis in the region of the craniomedullary junction and the superficial layers of the dorsal horns C1 and C2 levels of the spinal cord trigeminocervical complex.

Furthermore, peripheral trigeminal activation in migraine is evidenced by release of CGRP, a vasodilator[5,6], even though the mechanism of generation of pain is not clear. Studies in animals suggest that the pain may be caused by a sterile neurogenic inflammatory process in the dura mater[7], but this mechanism has not been clearly established to correlate in humans[8]. The pain may be an amalgamation of an altered perception – as a result of peripheral or central sensitization of craniovascular input that is not usually painful[9] and the activation of a feed-forward neurovascular dilator mechanism that is functionally explicit for the first (ophthalmic) division of the trigeminal nerve[10]. It is also believed that nitric oxide is involved in migraine pathophysiology and inhibition of its synthesis seems to be of therapeutic relevance[11]. In any case, cranial vasodilatation leads to an enhanced blood volume following each cardiac stroke, with a resultant augmentation in pulsations within the affected blood vessels. The increased pulsations can then be sensed by "stretch" receptors in the vascular wall and the resultant boost in perivascular (trigeminal) sensory nerve activity provokes headache and other symptoms. This trigeminal stimulation may also release neuropeptides, thus reinforcing vasodilatation and perivascular nerve activity[12].

1.1.2 Epidemiology

Migraine affects a substantial proportion (16%) of the population[13] and is more prevalent in females than in males (15-18% vs. 6%[14]). The incidence of migraine begins earlier in males than in females and, in general, migraine with aura begins earlier than migraine without aura[15]. In 15% of patients, migraine attacks are usually preceded or accompanied by transient focal neurotic symptoms, which are usually visual; such patients have migraine with aura previously known as classic migraine[16]. In a large popula-

tion-based study, 64% of migraine patients have migraine without aura, 18% had it with aura and 13% had both types the remaining 5% had aura without headache[17].

1.1.3 Co-morbidity

Associations between migraine and a variety of somatic and psychiatric conditions have been reported since migraine was first described as a discrete syndrome. Although there is a dramatic variability in the methodology of studies on co-morbidity and migraine, this limits the conclusiveness of the findings. Migraine is considered as co-morbid in nature with a number of neurological and psychiatric disorders. To diagnose the patient with migraine, it is of importance to understand the co-morbidity related symptoms in migraineurs[18]. The high correlation between stroke attacks and migraine in young migraineurs is well considered in literature[19]; in addition, a high prevalence of epilepsy in migraine patients has been reported[20]. Migraine is also co-morbid with major depression, anxiety and panic disorders[21]. Taken together, for easy diagnosis of migraineurs, co-morbid symptoms are to be considered.

1.1.4 Diagnostic Criteria

Migraine is characterized by episodes of head pain that is often throbbing and frequently unilateral and severe. Migraine attacks without aura are usually associated with nausea, vomiting, sensitivity to light and sounds, or movement and, without treatment, the attacks typically last 4 to 72 h[22,23]. However, the migraine with aura is associated with attacks of reversible focal neurological symptoms that usually develop gradually over 5 to 20 min and last for less than 60 min[24]. Any severe and recurrent headache is most likely a form of migraine and should be responsive to antimigraine therapy[25]. The International Headache Society (IHS) has formally classified the headaches in order to improve clinical practice and research[26]. Presently, the IHS system recognizes six subtypes of migraine with two major varieties, namely, migraine without aura and migraine with aura[26].

1.2 SEROTONIN (5-HT) RECEPTORS

The hormone and neurotransmitter *serotonin* (5-hydroxytryptamine; 5-HT) was isolated from blood serum and named due to its origin and vascular function (sero=serum and tonin= vasoconstriction[27]). Since then, a number of 5-HT receptor subtypes have been discovered[28,29], which demonstrate a whole host of physiological functions. Migraine is regarded as a *"low-5-HT syndrome"*, indicating that 5-HT may play an important role in its pathophysiology and/or treatment[30]. For example, it has been reported that there is a reduction in urinary 5-HT and an elevation of its major metabolite 5-hydroxyindole acetic acid during migraine attacks[31], moreover, platelet serotonin levels were found to drop rapidly during the onset of a migraine attack[32]. Potent centrally active 5-HT-depleting agents (reserpine) can precipitate migraine attacks[33]. Intravenous injection of serotonin has been shown to lower headache in migraineurs[12]. Location of serotonergic cell bodies in central nervous system is depicted in Figure 1.3.

1.2.1 Physiological functions of 5-HT receptors and their role in migraine

Over the time, the classification of 5-HT receptors has undergone a considerable evolution, which emphasizes the integration of operational, transductional and structural criteria as a basis for classification[34]. 5-HT receptors belong to both the G protein-coupled and ligand-gated ion channel super-families. These have been divided into seven distinct families, or classes, according to structural diversity and the preferred effector mechanism[34]. Some of these classes comprise multiple receptors, which share similar structural and effector properties, but display very different operational profiles.

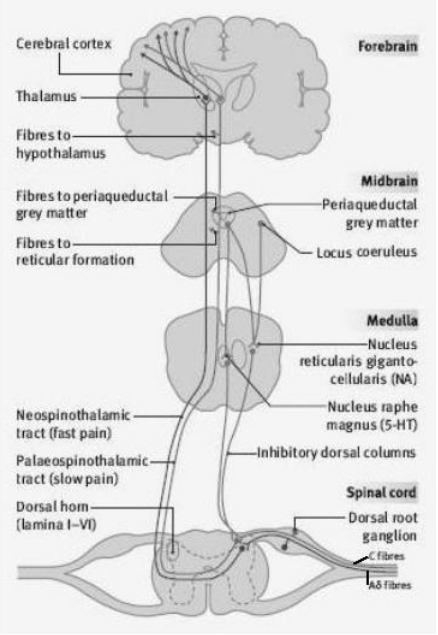

Figure 1.3: Location of serotonergic cell bodies and pathways in the CNS (internet google search).

1.2.2 5-HT$_{1A, 1B, 1D, 1e, 1F}$ receptors

The 5-HT$_1$ receptor class comprises five different receptors, which share 41–63% overall sequence identity and couple preferentially to G_i/G_o to inhibit cAMP formation[35] (Table 1.1). One of these, the 5-ht$_{1e}$ receptor, is given a lower-case appellation to denote that, although gene products encoding putative receptors have been identified, functional receptors with a definite physiological role have not yet been found.

A number of well-defined selective agonists and antagonists are available to probe the 5-HT$_{1A}$ receptor. Moreover, progress is now being made in the development of ligands that discriminate between 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors. Hitherto, unambiguous differentiation of 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor-mediated effects was hampered by the close pharmacological identity of these receptors. However, two antagonists can discriminate pharmacologically these two receptor subtypes, namely, BRL15572 (which has a 60-fold selectivity for 5-HT$_{1D}$ over 5-HT$_{1B}$ receptors[36] and SB224289 (which has a 75-fold selectivity for 5-HT$_{1B}$ over 5-HT$_{1D}$ receptors[36].

The 5-HT$_{1A}$ agonist buspirone, has been reported to have a prophylactic effect in migraine with anxiety disorder, which was not secondary to its anxiolytic effect[38]. Further lines of evidence support the hypothesis that migraine without aura is associated with a relative hypersensitivity of central 5-HT$_{1A}$ receptors. This is of relevance given the role of the 5-HT$_{1A}$ receptor in controlling raphé 5-HT tone and in the possible association between migraine, anxiety and depression[39]. Furthermore, most antimigraine drugs display high affinity for the 5-HT$_{1A}$ and/or 5-HT$_2$ receptor subtypes in human brain[40]. However, antimigraine efficacy cannot be explained by drug interactions with a single 5-HT receptor subtype[41]. For example, tertatolol, which is a β-blocker with 5-HT$_{1A}$ receptor agonist properties, induced carotid blood flow decreases, which were accompanied by similar decreases in vascular conductance in the porcine vasculature, indicating active arteriovenous anastomotic constriction. It has therefore been suggested that tertatolol may prove effective in the treatment of migraine[42]. However, several lines of evidence argue against 5-HT$_{1A}$ receptor involvement namely: (i) Ipsapirone, a selective 5-HT$_{1A}$ receptors

Table 1.1. Summary of 5-HT$_1$ receptor subtypes characteristics (IUPHAR[37])

	5-HT$_{1A}$	**5-HT$_{1B}$**	**5-HT$_{1D}$**	**5-ht$_{1e}$**	**5-HT$_{1F}$**
Transductional properties	$G_{i/o}$ preferentially inhibits cAMP formation	$G_{i/o}$ preferentially inhibits cAMP formation	$G_{i/o}$ preferentially inhibits cAMP formation	$G_{i/o}$ preferentially inhibits cAMP formation	$G_{i/o}$ preferentially inhibits cAMP formation
Selective agonists	N,N-di-n-propyl-5-CT 5-CT 8-OH-DPAT (+)-UH301	5-CT, 5-HT, sumatriptan RU24969 CP93129 8-OH-DPAT	PNU109291 sumatriptan L694247	5-HT RU24969 5-CT sumatriptan	N-dimethyl-5-HT 5-HT sumatriptan 5-CT LY334370
selective antagonist	(?) WAY100635 (pK=8,7)	GR55562 (pK=7,4) SB224289 (pK=8,5) methiothepin (pK=7,9)	BRL15572 (pK=7.9) methiothepin (pK=7,9) metergoline(pK=7,6)	methiothepin	-
receptor distribution	CNS: hippocampus septum, amygdala, raphe nuclei beningn and malignant prostrate tissue kidney cortex	CNS: hippocampus substantia nigra spinal cord thymus, peripheral blood lymphocyte	CNS: striatum nucleus accumbens dorsal raphé hippocampus trigeminal nerbe terminals prostrate tissue	CNS: caudate putamen entorhinal cortex frontoparietal motor cortex	CNS: cortex thalamus, olfactory bulb(rat) spinal cord claustrum (guinea pig) thymus, spleen
Tissue function	serotonergic somadendritic autoreceptor in hippocampus facilitates acetylcholine release in cortex, hippocampus, facilitation of noradrenaline release in cortex, hippocampus.	serotonergic terminal autoreceptor in hippocampus raphae nuclei, presynaptic heteroceptor in hippocampus (cholinergic) and vasculature (sympathetic) mediates contraction of vascular smooth muscle	serotonergic terminal autoreceptor in hippocampus raphae nuclei, GABAergic and cholinergic heteroreceptor in cerebellum mediates sympathoinhibition in autonomic neurones	not established	trigeminal (V) neuroinhibition in guinea-pig and rat. Physiological role in human unknown

Table 1.2. Summary of 5-HT_{2-6} receptor subtypes characteristics (IUPHAR[37])

	5-HT_{2A}	**5-HT_{2B}**	**5-HT_{2C}**	**5-HT_{4}**	**5-ht_{5a}**	**5-HT_{6}**
Transductional properties	$G_{q/11}$ preferentially increase PI hydrolysis and elevates Calcium	$G_{q/11}$ preferentially increase PI hydrolysis and elevates Calcium	$G_{q/11}$ preferentially increase PI hydrolysis and elevates Calcium	G_{s} preferentially increase cAMP formation	-	G_{s} preferentially increase cAMP formation
Selective agonists	5-HT, 5-CT sumatriptan BW723C86	5-HT, BW723C86, 5-CT, sumatriptan	Ro600175 (?) DOB (?) a-me-5-HT	SC53116, BIMU8 5-MeOT, ML10302	-	-
selective antagonist	ketanserin (pK=8.5-9.5) MDL100907 (pK=9.4)	RS127445 (pK=9.2) SB204741(pK=6.7) SB200646 (pK=7.5)	RS102221 (pK=8,6) SB242084 (pK=9.0) mesulergine (pK=8.8)	GR113808 (pK=9,0-9,5) SB204070 (pK=10.8)	-	Ro046790 (pK=7,3) Ro630563 (pK=7.9) SB271046(pK=7,8)
receptor distribution	CNS: cortex, hippocampus striatum olfactor bulb spinal cord	CNS: cerebellum lateral septum hypothalamus smooth muscle of ileum endothelium	choroid plexus, medulla, pons stiatum hippocampus hypothalamus spinal cord	CNS: striatum, brain stem, thalamus hippocampus olfactory bulb cardiac muscle vascular smooth muscle	CNS: hippocampus cortex cerebellum olfactory bulb habenula spinal cord	CNS: caudate putamen olfactory tubercle cortex, hippocampus superior cervical ganglion
Tissue function	vascular smooth muscle contraction platelet activation neuro-inhibition	endothelium dependent vasorelaxation via NO production vascular smooth muscle constriction medites mitogenic effect of 5-HT during embryogenesis	modulation of transferin production modulation of CSF volume	smooth muscle relaxation increased EEG amplitude(rat) facilitation of striatal dopamine release	-	modulation of CNS acetylcholine release

partial agonist was inactive to produce carotid vasoconstriction[43] (ii) Indorenate, another 5-HT_{1A} receptor agonist, produced porcine carotid vasoconstriction, however, metergoline a drug with higher affinity for 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} receptor, failed to significantly antagonize the responses to indorenate, thus it was concluded the effect of indorenate was not due to its agonist action on 5-HT_{1A} receptors[44]. Therefore it appears that 5-HT_{1A} receptors do not mediate vasoconstriction in the porcine carotid model and consequently the potential role of 5-HT_{1A} receptors in antimigraine action, if any, is rather limited.

5-$HT_{1B/1D}$ receptor agonists produce selective carotid vasoconstriction[29]. The 5-HT_{1B} receptor is primarily involved in sumatriptan-induced contractions of human cranial as well as peripheral blood vessels[45]. One such study reported that 5-carboxamidotryptamine (5-CT) and sumatriptan elicited a vasoconstriction that was antagonized by the 5-HT_{1B} receptor antagonist SB224289, whereas the 5-HT_{1D} receptor antagonist BRL115572 had no effect[46]. Thus, it appears that triptans induce vasoconstriction in arteries and veins, which is most likely mediated by activation of 5-HT_{1B} receptors[47]. The 5-HT_{1D}/5-HT_{1F} receptors induce presynaptic inhibition of the trigeminovascular inflammatory responses implicated in migraine[12]. Moreover, selective agonists at 5-HT_{1D} (PNU-142633) and 5-HT_{1F} (LY334370) receptors inhibit the trigeminovascular system without producing vasoconstriction[48]. Nevertheless, PNU-142633 proved to be ineffective in the acute treatment of migraine[49], whilst LY334370 did show some efficacy when used in doses, which may well interact with 5-HT_{1B} receptors[12,50]. Furthermore, activation of the trigeminovascular system excites cells in the nucleus tractus solitarius that can be inhibited by eletriptan and naratriptan (5-$HT_{1B/1D}$ agonists) via the activation of 5-$HT_{1B/1D}$ receptors[51]. Thus, these drugs acting in the trigeminal nucleus caudalis inhibit the relay of nociceptive information to the nucleus tractus solitarius. The patients having a migraine attack show activation of the trigeminovascular system, which in turn triggers nausea and vomiting, and the attenuation of these symptoms by antimigraine compounds may be via an action at 5-$HT_{1B/1D}$ receptors in the nucleus tractus solitarius[51]. In addition, inhibitory actions of 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors in the trigeminocervical complex of the cat have been suggested and 5-HT_{1B} receptor-mediated inhibitions are the most potent of the three in terms of inhibition of trigeminovascular nociceptive traffic[52]. Thus, the 5-HT_{1B} receptor subtype is highly explored in the drug development for migraine, and the present drugs for migraine (namely triptans) stimulate 5-HT_{1B} receptors, potently rather than to other receptor subtypes of 5-HT. However, the major disadvantage of triptans use is coronary vasoconstriction[53] as triptans are believed to narrow coronary arteries by 10% to 20% at clinical doses and should not be administered to patients with coronary or cerebrovascular disease[54].

In addition, the selective 5-HT_{1F} receptor agonist LY334370 was investigated assuming a central mechanism of action in blocking the transmission of nociceptive impulses within the trigeminal nucleus caudalis through which it has its antimigraine effect[55]. Furthermore it is believed that there occurs a high correlation between the potency of various 5-HT_1 receptor agonists in the guinea-pig dural plasma protein extravasation assay and their 5-HT_{1F} receptor binding affinity[56]. Also, as 5-HT_{1F} receptors are located on glutamate containing neurons and their activation might inhibit glutamate release[57], as glutamate excess may play a role in migraine, as one such study showed cerebrospinal fluid concentrations of glutamic acid were higher in migraineurs than in controls[58]. Moreover, triptans are believed to mediate their action via 5-HT_{1F} receptor as they show high pK_i values for 5-HT_{1F} receptors[52,59]; nevertheless, 5-HT_{1F} agonists are devoid of vasoconstrictor properties[60-62]. The 5-HT_{1F} receptor seems to mediate inhibition of dural plasma protein extravasation following trigeminal ganglion stimulation[48,63]. In addition LY334370 failed to demonstrate clinical efficacy in migraine[64]. However, it is worth emphasizing here that preclinical experiments and clinical observations argue for the affectivity of selective 5-HT_{1F} agonists in migraine.

1.2.3 5-$HT_{2A, 2B, 2C}$

The 5-HT_2 receptor class comprises three receptors, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}, which exhibit 46–50% overall sequence identity and couple preferentially to G_q/G_{11} to increase the hydrolysis of inositol phosphates and elevate cytosolic $[Ca^{2+}]$[65]. The anatomical distribution and physiological function of these

three receptors appear to be discrete. Furthermore, the 5-HT_{2B} and 5-HT_{2C} receptors display a higher affinity for 5-HT itself than the 5-HT_{2A} subtype. Although selective antagonists for each receptor are now available, only BW723C86 is known as a selective 5-HT_{2B} receptor agonist, this agonist produced hypertensive (rather than hypotensive) responses in vagosympathectomized rats[66].

Changes in 5-HT concentration has been found in patients with chronic headache, caused by mistreatment of analgesic substances as well as an up-regulation of 5-HT_2 platelet receptors, which has been correlated with chronicization of the headache[67]. Antagonists of 5-HT_2 receptors are most likely to be useful in the treatment of migraine[68]. The most commonly used 5-HT_2 receptor antagonist methysergide (which is also a 5-HT_1 partial agonist), used for migraine prophylaxis, is indicated for severe cases in which other migraine preventive drugs are not effective[69]. The 5-$HT_{2A/2C}$ receptor genes have been studied as candidate genes for migraine[70], but no mutations in the deduced amino acid sequence of either receptor in the sample of migraineurs was observed; thus, the authors concluded that DNA-based mutations in the 5-HT_{2A} and 5-HT_{2C} receptors are not generally involved in the pathogenesis of migraine. However, T102C polymorphism of the 5-HT_{2A} receptor gene has been reported to be related to migraine with aura[71] and thus a role of 5-HT_{2A} receptor in the pathophysiology of migraine or its aura should seriously be considered. Alternative splicing events yield non-functional isoforms of the 5-HT_{2C} receptor. Moreover as many as seven functional isoforms of the 5-HT_{2C} receptor are produced by adenine deaminase editing of the receptor mRNA. Although these receptor isoforms show no gross differences in their operational characteristics, they are differentially distributed throughout central and peripheral tissues. The purpose of these isoforms remains speculative, but they may be important in determining cell differences in receptor desensitization, cell-surface distribution or the trafficking of agonist responses through different effector pathways. Thus there is need to explore the functions of these isoforms that could or might explore new avenues for future antimigraine drugs.

Furthermore, activation of the 5-HT_{2A} receptor leads to an enhancement of NO production in the trigeminovascular pathway. This NO production may trigger migraine attacks by inducing cerebral vasodilation and sensitization of the perivascular nociceptors and central nociceptive neurons in the trigeminovascular system. Thus, the upregulation of this pronociceptive receptor can increase headache attacks and contributes to the development of chronic daily headache[72]. Also the role of 5-HT_{2B} receptors, located on endothelial cells of meningeal blood vessels, may trigger migraine headache through the formation of NO. Indeed, 5-HT_{2B} receptor stimulation induces relaxation of the pig cerebral artery via the release of NO[73]. Thus, if 5-HT_{2B} antagonists are used in migraine patients, this could lead to potential coronary side effects, as they in turn inhibit NO production which could lead to coronary artery vasoconstriction. Selective 5-HT_{2B} receptor antagonists have been described[74,75] and proven efficacious in blocking plasma protein extravasation[75]. The demonstration of 5-HT_{2B} receptor blockade in migraine prophylaxis is awaited to substantiate the presumed hypersensitivity of these receptors in migraine and, hence, their suspected role in the initiation of the neurogenic inflammatory response[73,76-78].

1.2.4 5-HT_3, 5-HT_4, 5-ht_{5a}, 5-HT_6, 5-HT_7

The 5-HT_3 receptor is unique among existing 5-HT receptors in being the only member to belong to the ligand-gated cation super-family of receptors[79]. It appears to be located exclusively in neuronal tissue where it mediates fast depolarization. In the periphery, 5-HT_3 receptors are found on autonomic neurons and on neurons of the sensory and enteric nervous system[80]. In the central nervous system, the 5-HT_3 receptor has been localized in the area postrema, nucleus tractus solitarii, nucleus vaudatus, nucleus accumbens, amygdala, hippocampus, entorhinal, frontal, cingulate cortex, and in the dorsal horn ganglia. Although the 5-HT_4, 5-HT_6 and 5-HT_7 receptors all coupled preferentially to G_s and promote cAMP formation, they are classified as distinct receptor classes because they exhibit <40% overall sequence identity with other 5-HT receptors[34].

The 5-ht$_5$ receptors are coupled to the G_i/G_0 family and causes adenylate cyclase inhibition[81,82]. 5-HT receptor diversity is increased by the existence of isoforms produced by post-translational modifications. Alternative splicing events yield four functional variants of the 5-HT$_4$ receptor (5-HT$_{4(a)}$-5-HT$_{4(d)}$)[83,84] and four functional variants of the 5-HT$_7$ receptor (5-HT$_{7(a)}$-5-HT$_{7(d)}$)[85]. The purpose of these isoforms remains speculative, but they may be important in determining cell differences in receptor desensitization, cell-surface distribution or the trafficking of agonist responses through different effector pathways.

These receptor types are not much explored for the development of antimigraine compounds. However some studies have assessed the role of these receptor types in the carotid circulation. In one such study, intravenously administered 5-HT was found to be a vasodilator *in vivo* in the cat dural circulation, and the authors showed that the dilation is not mediated by 5-HT$_1$, 5-HT$_2$, 5-HT$_4$ or 5-HT$_7$ receptors, but primarily mediated by a vagal reflex, initiated via 5-HT$_3$ receptor activation and brought about by an increase in parasympathetic tone to the middle meningeal artery as part of the Von Bezold-Jarisch reflex[86]. 5-HT$_3$ antagonists are used to prevent emesis[87]; hence, these compounds might be explored in patients with migraine. In addition, control of migraine associated nausea and vomiting is often achieved with the benzamide dopamine D$_2$ receptor antagonist metoclopramide[88]. This drug also has 5-HT$_3$ receptor antagonist activity and reproducibly stimulates gastric motility to increase the availability of orally administered drugs and is thus used in the treatment of migraine[88]. However, Ferrari[89] showed most 5-HT$_3$ receptor antagonists have proven to be toxic in man on chronic administration thereby preventing further trials in migraine with adjusted doses.

A number of 5-HT$_7$ receptor-selective antagonists, including SB-269970-A, SB-258741 and SB-656104-A have been developed[90,91]. There is evidence to suggest that this receptor type may play a role in central nervous system disorders including anxiety, cognitive disturbances, but also migraine, probably via both peripheral and central mechanisms[91]. Indeed, mRNA of 5-HT$_7$ receptors is present in trigeminal ganglia[92]. Since dilatation of cranial blood vessels has been proposed to play an important role in the pathogenesis of a migraine attack[12]. Thus 5-HT$_7$ receptor antagonists could be effective as prophylactic antimigraine agents[92], although clinical trials are awaited with interest.

1.3 ADRENERGIC RECEPTORS

The endogenous catecholamines noradrenaline and adrenaline, which are released upon activation of the sympathetic nervous system, play an essential role in the regulation of a host of physiological responses. Noradrenaline is a major neurotransmitter in the sympathetic nervous system, whereas adrenaline is the primary hormone secreted by the adrenal medulla.

Several decades ago, adrenoceptors were introduced to explain the difference in actions of noradrenaline and adrenaline[93]. Adrenoceptors are located in nearly all peripheral tissues and on many neuronal populations within the central nervous system. Both noradrenaline and adrenaline play important roles in the control of blood pressure, myocardial contractile rate and force, airway reactivity, and a variety of metabolic and central nervous system functions[94]. Agonists and antagonists interacting with adrenoceptors have proved useful in the treatment of a variety of diseases, including hypertension, angina pectoris, congestive heart failure, asthma, depression, benign prostatic hypertrophy, and glaucoma[94]. These drugs are also useful in several other therapeutic situations including shock, premature labour and opioid withdrawal, and as adjuncts to general anaesthetics.

1.3.1 Physiological functions of adrenoceptors and their role in migraine

Adrenoceptors can be subdivided into three major types, the α_1-, α_2-, and β-adrenoceptors[95]. Each of these types can be further subdivided into three subtypes, based on pharmacological characteristics. Molecular cloning techniques have supported this sub-classification. Recent data now suggest that

α-adrenoceptor subtypes identified by pharmacological and molecular techniques correspond well, although species orthologs of several adrenoceptor subtypes have been identified[96]. The secondary structure of the adrenoceptors has been elucidated and correlated with their interaction with second messenger molecules. α_1-adrenoceptors, β-adrenoceptors and α_2-adrenoceptors mediate their actions through stimulation of inositol phosphate release[97], stimulation of adenylate cyclase[98] and inhibition of adenylate cyclase[99], respectively (Table 1.3).

1.3.2 α_{1A}, α_{1B}, and α_{1D}-adrenoceptor subtypes

Cloning has identified three α_1-adrenoceptor subtypes (Table 1.3). The α_{1B}-adrenoceptor from the DDT cell line (hamster smooth muscle) was cloned first[100], followed by what was thought to be a novel α_1-adrenoceptor from a bovine brain cDNA library identified as the α_{1C}-subtype[101]. Subsequently, it was shown that this clone corresponded to the pharmacologically defined α_{1A} subtype[102,103]. A third α_1-adrenoceptor was cloned from rat cortex and designated as the α_{1A}-adrenoceptor[104]. However, an identical recombinant rat α_1-adrenoceptor subtype was independently identified by Perez *et al.*[105] and denoted the α_{1D}-adrenoceptor. This α_{1D}-subtype was subsequently characterized functionally in tissues[106,107]. A fourth α_1-adrenoceptor subtype has been postulated and is designated as α_{1L} based on its low affinity for prazosin[108]. The evidence for the existence of the α_{1L}-adrenoceptor is supported by pharmacological data in several tissues, including human prostate, bladder neck and periurethra longitudinal muscle[109,110]. It has been suggested that the α_{1L} subtype may represent a particular conformational state of the α_{1A}-adrenoceptor[111]. It is well known that α_1-adrenoceptors are G-protein-coupled receptors and mediate their responses via a $G_{q/11}$ mechanism, which involves activation of phospholipase C-dependent hydrolysis of phosphatidylinositol 4,5-diphosphate[103]. Activation of phospholipase C results in the generation of inositol (1,4,5)-triphosphate (IP_3), which acts on the IP_3 receptor in the endoplasmic reticulum to release stored Ca^{2+} and diacylglycerol that (together with Ca^{2+}) can activate protein kinase C. Production of these second messengers activates both voltage-dependent and independent Ca^{2+}-channels, leading to smooth muscle contraction in both vascular and non-vascular tissues (e.g. prostate, vas deferens, heart). α_1-adrenoceptor mRNA expression in mammary arteries double with age, which seems to be restricted for α_{1B}-adrenoceptor rather α_{1A}-adrenoceptor[112]. Also human heart failure is associated with a slightly increased cardiac α_1-adrenoceptor number[113]. Figure 1.4 summarizes the role of α_1-adrenoceptor subtypes.

In line with the finding that carotid arteriovenous anastomoses dilate and play a role in the pathogenesis of migraine[3], it is reasonable to believe that compounds, which produce a cranioselective vasoconstriction, may have potential therapeutic use in the treatment of migraine[114]. The α_{1A}-adrenoceptor is believed as the main subtype of α_1-adrenoceptors regulating systemic resistance and blood pressure[115,116]. In view of its widespread action, it is rather unlikely that a selective α_{1A}-adrenoceptor agonist would be useful in the treatment of migraine. Considering this standpoint, the α_{1B}-adrenoceptor is a motivating target for future antimigraine drugs, especially when considering that this receptor does not seem to be much involved in the constriction of the peripheral blood vessels[116,117] and predominantly α_{1A}-, but not α_{1B}- (or α_{1D}-), adrenoceptors mediate the hypertensive effect induced by intravenous administration of phenylephrine in anaesthetized pigs[118]. In addition, studies in mice have provided several clues to help elucidate subtype-specific physiological functions; for instance, α_{1A}-adrenoceptor and α_{1D}-adrenoceptor subtypes, play an important role in the regulation of blood pressure[119]; thus, these subtypes could not be explored for development of novel antimigraine drugs, however suggesting these subtype-selective antagonists might be desirable antihypertensive agents. Therefore, a selective α_{1B}-adrenoceptor agonist is believed to have advantages over the currently available acute antimigraine drugs, which all constrict the human isolated coronary artery[53,120]. In addition, using a closed cranial window migraine model, the dural blood vessels of rats were electrically stimulated to produce by local depolarization of nerves, dural vasodilatation. This response was not affected by pre-treatment with an α_1-adrenoceptor agonist

Table 1.3: Summary of 5-HT_7 and α_1-adrenergic receptors characteristics (IUPHAR[37])

	5-HT_7	**α_{1A}**	**α_{1B}**	**α_{1D}**
Transductional properties	G_s preferentially increase cAMP formation	$G_{q/11}$ Phospholipase C stimulation, Ca^{+2} activate MAPK, protein kinase C	$G_{q/11}$ Phospholipase C stimulation, Ca^{+2} activate MAPK, protein kinase C	$G_{q/11}$ Phospholipase C stimulation, Ca^{+2} activate MAPK, protein kinase C
Selective agonists	5-CT, 5-HT, 8-OH-DPAT sumatriptan	A61603, noradrenaline NS-49, oxymetazoline phenylephrine [^{125}I]-HEAT	[^{125}I]-HEAT oxymetazoline noradrenaline NS-49,(-)adrenaline	[^{125}I]-HEAT (-)-noradrenaline (-)adrenaline, NS-49 oxymetazoline
selective antagonist	SB258719 (pK=7,8) methiothepin (pK=8.4) spiperone (pK=7,2)	[^{125}I]BE-2254 (pK=9.88) (-)YM617 (pK=10.72) KMD-3213 (pK=10.72) prazosin (pK=9.5)	[^{125}I]BE-2254 (pK=9.92) (+) cyclazosin (pK=9.87) prazosin (pK=9.96) tamsulosin (pK=9.7)	[^{125}I]BE-2254 (pK=9.88) (-)YM617(pK=10.2) tamsulosin (pK=10.1-9.8) prazosin (pK=10.1-9.8)
receptor distribution	CNS: hippocampus hypothalamus thalamus superior colliculus raphae nuclei sympathetic ganglia thalamus	CNS: olfactory system, hypothalimic nuclei regions of brain stem spinal cord related to motor function cerebelum, liver, heart	highest levels found in the regions involved in functions, medial layer of the aorta and caudal, femoral, iliac, renal, superior mesenteric resistance arteries	protein is predominant in human bladder, olfactory bulb, cerebral cortex, hippocampus dentate gyrus, reticular thalamic nucleus, motor neurons.
Tissue function	smooth muscle relaxation circadian phase shifts	contraction of skeltol muscle resistance arteries, contraction of urethral smooth muscle stimulation of myocyte hypertrophy, activation of sarcolemmal Na^+-H^+ exchanger, hypotension in α_{1A}-KO mice	regulation of cardiac growth and contractile function. Contraction of mammary artery and saphenous ven, CNS stimulation by cocaine d-amphetamine contraction of umblical vein	contraction of mesenteric resistance arteries, nerve stimulated contraction of corpus cavernosa, coronary, femoralartery hypotension in α_{1D}-KO mice

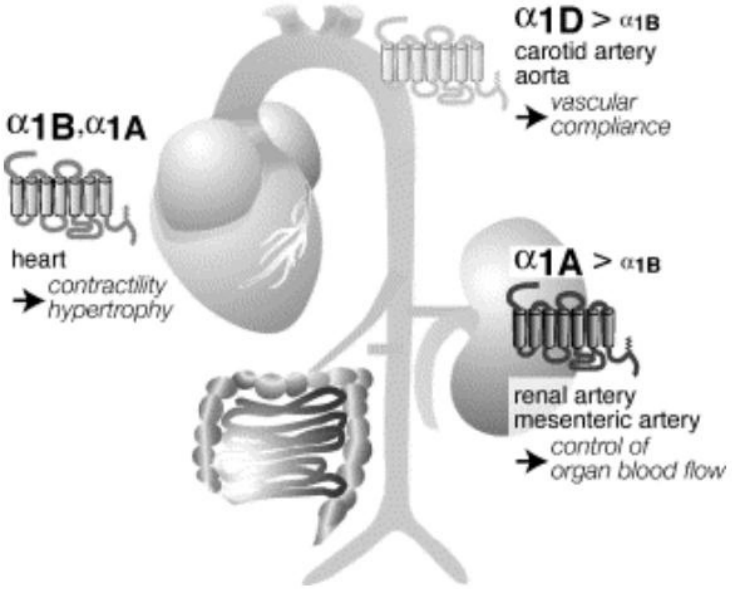

Figure 1.4: Distribution and function of α_1-adrenoceptor subtypes in the cardiovascular system[215] (with permission from publisher)

(phenylephrine), or its antagonist (corynanthine). The authors conclude that the adrenergic system does not play a significant role in neurogenic dural vasodilation[121]. Thus, it is reasonable to believe different migraine models showed variable results towards their view on the adrenergic system, and their involvement in migraine pathophysiology. However, some reports suggest that migraineurs have sympathetic hypofunction[122], and there occurs pressor hyperresponsiveness to phenylephrine that unmasks sympathetic hypofunction in migraine[123]. Thus, it appears that α_1-adrenoceptor activity is increased in migraineurs. Accordingly it is logical to believe that α_1-adenoceptor subtypes hold prospective targets for development of new antimigraine drug.

1.3.3 α_{2A}, α_{2B} and α_{2C}-adrenoceptor subtypes

The evidence for α_2-adrenoceptor subtypes has come from binding and functional studies in various tissues and cell lines, and more recently from cells transfected with the cDNA for the receptors[124]. On the basis of these studies, three genetic and four pharmacologically distinct α_2-adrenoceptor subtypes have been defined. The α_{2A}-adrenoceptor subtype, for which prazosin has a relatively low affinity and oxymetazoline a relatively high affinity, is found in human platelets and HT29 cells[125] and has been cloned from man[126]. The second subtype, the α_{2B}-adrenoceptor has been identified in neonatal rat lung and in NG108 cells[127]. This subtype has relatively high affinity for prazosin and a low affinity for oxymetazoline, and has been cloned from man[128]. A third subtype, the α_{2C}-adrenoceptor, has been identified in an opossum kidney (OK) cell line and cloned from human kidney[129,130]. Although this subtype has a relatively high affinity for prazosin and a low affinity for oxymetazoline, it is pharmacologically distinct from the α_{2B} subtype[131]. We have in our laboratory also cloned the porcine α_{2B}-adrenoceptor subtype (Genbank accession number DQ182110) and the partial porcine α_{2C}-adrenoceptor (Genbank accession number DQ225117). A fourth subtype, the α_{2D}, has been identified in the rat salivary gland[132] and in the bovine pineal gland[133]. This pharmacological subtype has been cloned from the rat[134]. On the basis of the predicted amino acid sequence, the α_{2D} is a species orthologue of the human α_{2A} subtype, and thus is not considered to be a separate subtype. α_2-adrenoceptors are considered as prejunctional (probably the α_{2A}- and/or

α_{2C}-subtypes, depending on species) by *Docherty*[115] and *Hieble*[135], these receptors are located on most adrenergic nerves and primarily mediate prejunctional inhibition. Since both presynaptic α_{2A}- and α_{2C}-adrenoceptors are targets for the neural release of noradrenaline, at least when the noradrenaline transporter is inhibited, it would be interesting to elucidate their individual pharmacological profiles (e.g. binding properties to known compounds), signal transduction pathways, second messengers and their potential differences in function[136]. A valuable tool in dissecting the role of these receptors in the regulation of a variety of physiological responses, e.g. blood pressure[137], is the use of genetically engineered mice deficient in each of the α_2-adrenoceptor subtypes[138-140]. The use of knock-out mice confirmed the earlier findings that the α_{2A}-adrenoceptor[115,135], which appears to be the major subtype in brain areas are involved in cardiovascular regulation[141], plays a critical role in regulating sympathetic outflow[140]. On the other hand, post-junctional α_2-adrenoceptors are located on the vascular smooth muscle and activation results in vasoconstriction[137], but the individual contribution of the three known α_2-adrenoceptor subtypes is poorly understood. However, the central distribution of α_{2B}-adrenoceptors is restricted to certain areas, such as the thalamus and the nucleus tractus solitarius[141], and it is abundant in arterial vascular smooth muscle, producing peripheral vasoconstriction[142-144].

Recent findings demonstrated that α_{2B}-adrenoceptor-deficient anephric mice are incapable of raising their blood pressure in response to an acute hypertonic saline stimulus[137,139]. These data are in agreement with studies using pithed rat preparations, where the α_{2B}-adrenoceptor is the main subtype mediating hypertension[135,145-147]. In contrast, Duka *et al*.[148] reported that vasoconstriction mediated by direct activation of vascular α_2-adrenoceptors in mice is attributable to the post-synaptic α_{2A}-adrenoceptor subtype. This is consistent with the finding that mRNA for the α_{2A}-adrenoceptor, but not α_{2B}-adrenoceptor, was detected in the arterial wall of rabbits[149]. In fact, the pressor response attributed to α_{2B}-adrenoceptor stimulation[142-144] could in fact be due to central, rather than peripheral, α_{2B}-adrenoceptors[148]. It has recently been demonstrated that endothelium of mouse aorta has an α_{2A}-adrenoceptor that responds to noradrenaline producing vasodilatation mediated by release of NO[150]. Knockout or genetic malfunction of this receptor should increase arterial stiffness, exacerbated by raised catecholamines, and contribute to heart failure[150]. Interestingly, the human isolated saphenous vein is a preparation in which the postsynaptic α_{2C}-adrenoceptor predominantly mediates contraction, whereas the involvement of α_1-adrenoceptors is limited[151]. It may be noted that so far only limited information exists on haemodynamic responses mediated by α_{2C}-adrenoceptors[115,135,140,143]. Figure 1.5 summarizes the role of α_2-adrenoceptor subtypes.

The potential role of these subtypes in the treatment of migraine has been elucidated in porcine and canine migraine models. Studies on these models indicated that the canine carotid vascular responses to BHT933 (α_2-adrenoceptor agonist) were markedly attenuated by BRL44408 (α_{2A}-adrenoceptor agonist) and MK912 (α_{2C}-adrenoceptor agonist), given either alone or in combination, while imiloxan (α_{2B}-adrenoceptor antagonist) remained ineffective[152]. Similarly, the carotid vasoconstrictor responses produced by BHT933 in anaesthetized pigs were markedly attenuated by MK912, while the other antagonists were ineffective[114]. These results suggest that mainly α_{2C}-adrenoceptors mediate vasoconstriction in the carotid circulation of both species[153]. In addition, we also demonstrated that α_{2C}-adrenoceptors are present in the isolated porcine meningeal artery as the α_{2C}-adrenoceptor antagonist (OPC28326) produced a rightward shift of the control curve of noradrenaline. Moreover, we also demonstrated the mRNA for α_{2C}-adrenoceptor in porcine meningeal artery[154]. Thus it is reasonable to believe the involvement of this receptor subtype in migraine and, hence, it should be considered as a prospective antimigraine target.

Additionally, studies have also shown the lack of a response to clonidine in menstrual migraine, where the authors speculate a postsynaptic α_2-adrenoreceptor hyposensitivity during the premenstrual period[155]. Moreover, we have demonstrated the increased function of α_2-adrenoceptor in placebo treated rats suggesting that if this condition occurs as such in humans, it can predict the decreased frequency of migraine in menopause[156]. Thus it appears a transient vulnerability of the neuroendocrine/neurovegeta-

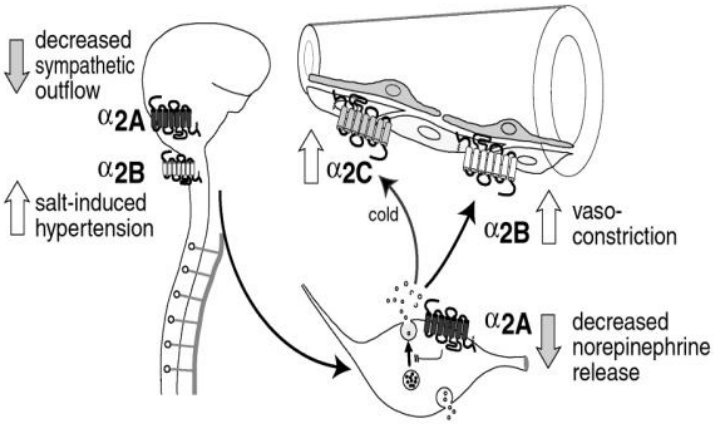

Figure 1.5: Physiological significance of α_2-adrenoceptor subtypes[157] (with permission from publisher).

tive systems in female migraine patients and hence this could be a factor facilitating the precipitation of both behavioural changes and migraine attacks. Therefore, based on the above findings, it would not be imperative to say that α_2-adrenoceptor subtype agonists may have a therapeutic potential in (menstrual) migraine patients. Clearly more studies are required in this direction and could possibly open new avenues for pharmaceutical industry to develop subtype selective agonists.

1.3.4 β (β_1, β_2 and β_3) adrenoceptors

In 1967, Lands et al.[158], comparing rank orders of potency of agonists in a manner similar to that of Ahlquist[159], concluded that there were two subtypes of the β-adrenoceptor. The β_1-adrenoceptor, the dominant receptor in heart and adipose tissue, was equally sensitive to noradrenaline and adrenaline, whereas the β_2-adrenoceptor, responsible for relaxation of vascular, uterine, and airway smooth muscle, was much less sensitive to noradrenaline in comparison with adrenaline[158]. Highly selective antagonists for both β_1- and β_2-adrenoceptors have been developed, as well as many potent and selective β_2-adrenoceptor agonists. Subsequently, it has become apparent that not all of the β-adrenoceptor-mediated responses can be classified as either β_1 or β_2, suggesting the existence of at least one additional β-adrenoceptor subtype[160,161]. This β_3-adrenoceptor is insensitive to the commonly used β-antagonists and has often been referred to as the 'atypical' β-adrenoceptor. It is unlikely that all of the atypical β-adrenoceptor responses observed have characteristics consistent with those of the β_3-adrenoceptor, and hence, the possibility of additional subtypes cannot be excluded. Pharmacological evidence has been accumulating for a fourth β-adrenoceptor localized in cardiac tissues of various species[162]. This β_4-adrenoceptor is activated with a low potency by noradrenaline and adrenaline, and is blocked by β-adrenoceptor antagonists such as bupranolol and CGP20712A[163,164].

Although some of the pharmacology overlaps with the β_3-adrenoceptor, the receptor-mediated response has recently been demonstrated in β_3-adrenoceptor 'knock-out' mice[165]. However, definitive evidence for this putative β_4-adrenoceptor is still lacking[166], and there is some evidence that it may be a 'state' of the β_1-adrenoceptor[167,168]. Two recent reports have suggested that the β_2-adrenoceptor may form homodimers[169] as well as oligomers with other receptors[170]. The β_2-adrenoceptor was cloned from man[171,172] using probes derived from the hamster β_2 receptor[173]. It proved difficult to clone the β_1-adrenoceptor, because the human β_2-adrenoceptor cDNA did not cross-hybridize with the β_1-adrenoceptor. A related receptor was isolated using the β_2-adrenoceptor cDNA as a probe[174], which proved to be the 5-HT_{1A} receptor[175]. Using the coding region of the 5-HT_{1A} receptor DNA to probe a human placental cDNA library, Frielle *et al.*[176] finally identified the β_1-adrenoceptor clone. The β_3-adrenoceptor was subsequently cloned

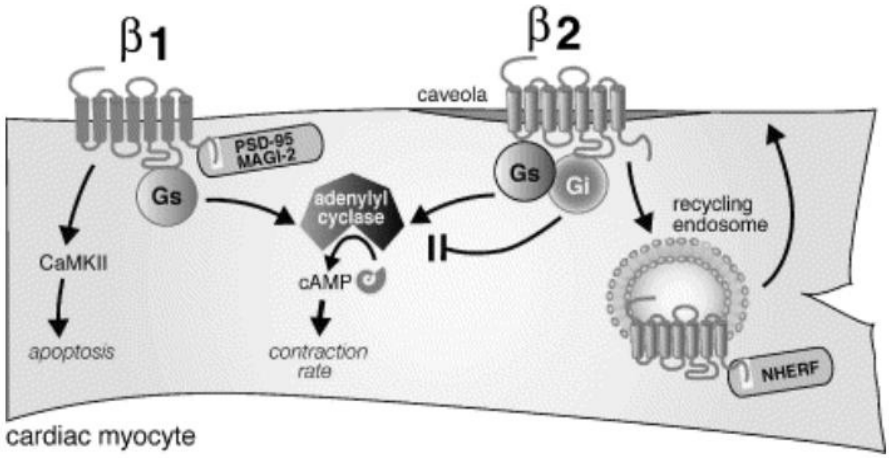

Figure 1.6: Distinct signalling pathway of $\beta_1$and β_2-adrenoceptor[215] (with permission from publisher)

from man[177]. Splice variants of the β_3-adrenoceptor have been reported[178]. The second messenger responses of β-adrenoceptor are depicted in Figure 1.6 and the characteristics of each subtype is depicted in Table 1.5.

Among all β-adrenoceptor blockers propranolol, and to a lesser extent metoprolol, underwent the most extensive clinical testing and served in many clinical trials as reference drugs when β-adrenoceptor blockers were compared with non-adrenergic drugs[179]. The efficacy of β-adrenoceptor blockers for the prophylaxis of migraine was discovered by chance when patients with migraine, who received β-adrenoceptor blockers for cardiac disorders, observed a significant reduction of migraine frequency[180]. On the other hand, it remains still controversial whether the prophylactic efficacy in migraine is a property of all β-adrenoceptor blockers or limited to individual members of this drug class with specific characteristics. β-Adrenoceptor blockers are typically classified according to factors such as selectivity for the β_1-adrenoceptor subtype; (i) β-blockade results in inhibition of noradrenaline release by blocking prejunctinonal β-receptors[135] and (ii) a delayed reduction in tyrosine hydroxylase activity, the rate-limiting step in noradrenaline synthesis, in the superior cervical ganglia[135]. It is also suggested that lipophilicity (penetration into the central nervous system), membrane-stabilizing effects and intrinsic sympathomimetic activity are relevant for antimigraine efficacy. Moreover, some β-adrenoceptor blockers have high affinity for certain 5-HT receptor subtypes[181]. β_1-adrenoceptor selectivity appears to play a major role in determining prophylactic efficacy since nonselective agents such as propranolol, moderately β_1-selective agents such as metoprolol and highly β_1-selective drugs such as bisoprolol[182,183] all are effective prophylactics. Thus, concomitant blockade of β_2-adrenoceptors does not appear to be required for effective migraine prophylaxis. While propranolol, metoprolol, oxprenolol and alprenolol are very lipophilic and hence penetrate well into the central nervous system. Whereas atenolol, nadolol, and practolol are only slightly or not at all lipophilic[181]. As several members of the latter group including atenolol[184,185] and nadolol[186,187] have demonstrated their efficacy in the prophylaxis of migraine attacks, high lipophilicity and hence penetration into the central nervous system does not appear to be required for prophylactic efficacy. The prophylactic efficacy of atenolol[184,185], nadolol[186,187] and timolol[188,189] demonstrates that membrane-stabilizing effects are required to reduce the frequency of migraine attacks.

Table 1.4. Summary of α_2-adrenoceptors and CGRP receptor subtypes characteristics. (IUPHAR[37])

	α_{2A}	α_{2B}	α_{2C}	$CGRP_1$
Transductional properties	$G_{i/o}$ family Adenylate cyclase inhibition Potassium channel Phospholipase A_2 stimulation	$G_{i/o}$ family Adenylate cyclase inhibition Potassium channel	$G_{i/o}$ family Adenylate cyclase inhibition Potassium channel calcium channel	$G_{i/o}$ family phospholipase C stimulation potassium channel
Selective agonists	adrenaline, brimonidine clonidine, guanfacine, xylazine oxymetazoline noradrenaline, dexmedetomidine	dexmedetomidine>clonidine> noradrenaline> brimonidine> guanfacine>oxymetazoline> xylazine=adrenaline	dexmetomidine> noradrenaline clonidine> oxymetazoline> adrenaline> guanfacine> xylazine	adrenomedullin α and β-CGRP [Cys (Et)2,7]-CGRP [Cys (ACM)2,7]-CGRP
selective antagonist	ARC-239, chlorpromazine phentolamine, rauwolscine, RX821002, WB4101 yohimbine	rauwolscine (pK=9.4)>RX821002 (pK=9.1), yohimbine (pK=8,9) phetolamine (pK=8,2) chlorpromazine (pK=8,3-7,2)	rauwolscine (pK=9.9) yohimbine (pK=9.5-8,7)> WB4101 (pK=9.4-8,4) > RX821002 (pK=9.2-8,2)	BIBN4096BS(pK= 10,7) α-CGRP 8-37 (pK=9,6-9,2)
receptor distribution	Brain>spleen>kidney>aorta=lung skeltal muscle> heart=liver absent in heart and liver in rat	spleen= kidney=aotra=lung= heart= skeletal muscle.	Brain=kidney>aorta=lung= skeltal muscle=heart=spleen absent in liver saphenous vein.	Spleen>liver, lung> whole brain> heart> kidney
Tissue function	α_{2A} knockout mice show an increase in sympathetic activity resting tachycardia, depletion of cardiac tissue, disruption of presynaptic inhibition of noradrenaline, α_{2A} is the principal autoreceptor in the presynaptic loop regulating noradrenaline release.	α_{2B} knockout mice are unable to develop salt-induced hypertension, involved in central hypotensive response to α_2 agonist, vasoconstriction in vascular smooth muscle.	α_{2C} knockout mice show no change in cardio vascular and sedative effects with a non- selective adrenoceptor agonist involved in central hypotensive response to α_2 agonists	vasodilation

Table 1.5. Summary of β-adrenoceptors subtypes characteristics (IUPHAR[37])

	β_1	β_2	β_3
Transductional properties	G_s family adenylate cyclase stimulation activation of protein kinase A	G_s family adenylate cyclase stimulation activation of protein kinase A	G_s family adenylate cyclase stimulation activation of protein kinase C
Selective agonists	CGP 12177 xamoterol, isoprenaline T-0509, dobutamine denopamine, prenalterol	isoprenaline, salbutamol salmeterol, fenoterol terbutaline, ephedrine	[^{125}I]-ICYP carazolol, BRL37344 CGP 12177A isoprenaline, SB 58611A
selective antagonist	(-)[^{125}I]-ICYP (pK=11.3) carvedilol (pK=9.52) propranolol (pK=8.89) betaxolol (pK=8.75)	[^{125}I]-ICYP (pK=11.09) timolol (pK=9.68) carvedilol (pK=9.89) CGP 12177A (pK=9.44)	carvedilol (pK=9.4) tertalol (pK=8.56) SR59230A (pK=8.38) bupranolol (pK=7.3)
receptor distribution	Heart, caudate cortex, cerebellum, hippocampus diencephalon, myocardium internal anal sphincter smooth mucle, renin release	Heart <lung, caudate, cortex, cerebellum, hippocampus, diencephalon lung>skeltalmuscle>spleen > kidney>heart>brain>liver (mouse)	brown adipose tissue endothelium of coronary microarteries, adipose> gallbladder> small intestine >stomach, prostrate>left atrium >bladder.
Tissue function	β_1 KO mice die prenatally, but those reach adulthood show reduced chronotropic and inotropic responses, a common polymorphism Gly389->Arg has been identified in humans resulting in difference in G_s binding properties.	muscle relaxation of internal anal sphincter smooth muscle Inhibition of apoptosis via a PTX-sensitive G-protein Hypotension, lowering of blood pressure presynaptic facilitation of noradrenaline release from sympathetic nerve.	lipolysis, relaxation of abdominal aorta smooth muscle, glucose uptake, vasodilatation via nitric oxide and vessel hyperpolarization relaxation of colon and oesophagus negative inotropic effect, decrease in gastrointestinal motility Try64->Arg polymorphism is associated with earleir onset of NIDDM

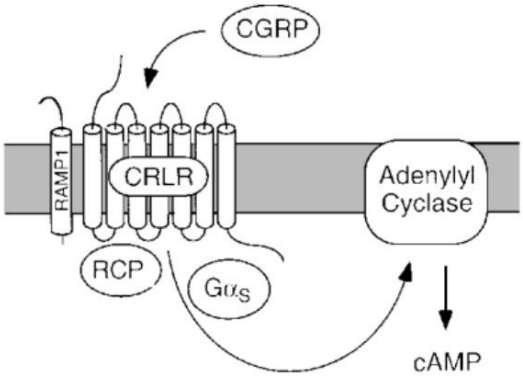

Figure 1.7: Model for functions of CGRP receptors. (Internet goggle search)

1.4 CGRP RECEPTORS

On the basis of pharmacological criteria, it is known that CGRP may act mainly on $CGRP_1$ and $CGRP_2$ receptors[5], with h-α$CGRP_{8-37}$ being a 10-fold more potent antagonist at $CGRP_1$ receptors than at $CGRP_2$ receptors[190]. While $CGRP_1$ receptors are widely distributed (Table 1.4), $CGRP_2$ receptors have only been described in rat vas deferens, and are more sensitive to the linear agonists [ethylamide-$Cys^{2,7}$]h-αCGRP ([$Cys(Et)^{2,7}$]h-αCGRP) and [acetimidomethyl-$Cys^{2,7}$]h-αCGRP ([$Cys(Acm)^{2,7}$]h-αCGRP)[191]. The structure of, $CGRP_1$ receptors consist of at least three main different entities, namely, the calcitonin receptor like receptor (CLR), receptor activity modifying protein-1 (RAMP-1)[192] and receptor component protein (RCP)[193,194] (see figure 1.7), whereas $CGRP_2$ receptors have not yet been molecularly characterised. The molecular components of $CGRP_1$ receptors that have been demonstrated in the human meningeal artery include CLR and the RAMPs 1, 2 and 3[195-197] and mRNA for RCP, which is required for the formation of a high-affinity G-protein-coupled receptor, thereby ensuring the signal transduction of CLR[198] was demonstrated in human meningeal artery in our laboratory[199]. The results from our laboratory also advocate the presence of $CGRP_1$ receptors in coronary artery segments investigated, while the human distal coronary artery seems to have an additional population of CGRP receptors not complying with the currently classified $CGRP_1$ or $CGRP_2$ receptors[200]. The characteristics of $CGRP_1$ receptor is illustrated in Table 1.4.

1.4.1 Physiological functions of CGRP receptors and their role in migraine.

In the recent past, there has been an upsurge in CGRP research and its notable role in migraine pathophysiology. CGRP immunoreactive fibres originating in the trigeminal ganglion innervate cranial cerebral blood vessels[201]. In animals, stimulation of these sensory nerve fibers has been shown to cause antidromic release of CGRP and subsequent vasodilatation in the cerebral vasculature[202,203]. Plasma concentrations of CGRP but not of other neuropeptides in the jugular venous blood are elevated during the headache phase of migraine[204]. Furthermore, in migraine patients: (a) a strong correlation was found between plasma CGRP concentrations and migraine headache as the changes in plasma CGRP levels during migraine attacks significantly correlated with the headache intensity[205-207] (b) infusion of CGRP produced a migraine-like headache (c) baseline CGRP levels were considerably higher in migraineurs. CGRP application induced a concentration-dependent relaxation in the middle cerebral artery when CGRP was applied, in the pressurized arteriography model in rat which suggest that CGRP-mediated vasodilatation is not caused by interaction with luminally situated receptors but more likely by receptors on the smooth muscle cells[208]. Hence, inhibition of CGRP or antagonism of CGRP receptors could be a viable therapeutic target for the pharmacological treatment of migraine[209].

An important breakthrough in the field of CGRP was the development of the potent CGRP receptor antagonist olcegepant[210] (BIBN4096). In *in vivo* animal models of migraine, olcegepant attenuated the vasodilation induced by trigeminal stimulation and capsaicin-induced anastomotic dilatation[205,209-211]. Data from a recently published clinical proof-of-concept study demonstrated the effectiveness and safety of olcegepant for the acute treatment of migraine[212], in which the response rate was found similar to oral triptans. As administration of olcegepant resulted in a lower pain free rate at 24 h than with triptans[212], it has been suggested that CGRP receptor antagonists could also be used as prophylactic drugs for migraine patients[213]. Since olcegepant does not seem to induce vasoconstriction, the CGRP receptor antagonist is believed to be safer than triptans. However, it must be highlighted that CGRP has a protective function during coronary ischemia. Indeed, the cardioprotective role of CGRP in cardiac preconditioning was demonstrated in a langendorff rat heart model in our laboratory[214]. Thus similar to triptans, CGRP receptor antagonists may be contraindicated in patients with coronary artery disease, suggesting a limited role of these drugs in patients with arteriosclerosis.

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Speech is Silver. Silence is Golden.

Chapter 2

Potential role of female sex hormones in the pathophysiology of migraine

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ABSTRACT

Clinical evidence indicates that female sex steroids may contribute to the high prevalence of migraine in women, as well as changes in the frequency or severity of migraine that are in tandem with various reproductive milestones in women's life. While estrogen and progesterone do not seem to be involved in the pathogenesis of migraine *per se*, they may modulate several mediators and/or receptor systems *via* both genomic and non-genomic mechanisms. These actions may be perpetuated at the central nervous system, as well as at the peripheral (neuro) vascular level. For example, estrogen enhances neuronal excitability by elevating Ca^{2+} and decreasing Mg^{2+} concentrations; this may act in concurrence with other mechanisms triggering migraine. Further, estrogen is reported to enhance the synthesis and release of nitric oxide and neuropeptides, such as calcitonin gene-related peptide; this in turn reinforces vasodilatation and activates trigeminal sensory afferents with a subsequent stimulation of pain centres. In addition, female sex steroids may increase the function of receptors mediating vasodilatation, while responses of receptors inducing vasoconstriction are attenuated. The serotonergic, adrenergic and GABAergic systems are also modulated by sex steroids, albeit to a varying degree and with potentially contrasting effects on migraine outcome. Taken together, female sex steroids seem to be involved in an array of components implicated in migraine pathogenesis. Future studies will further delineate the extent and the clinical relevance of each of these mechanisms, and will thus expand the knowledge on the femininity of migraine.

2.1 INTRODUCTION

Migraine is a common neurovascular syndrome, which is typified by intense, unilateral, throbbing and pulsatile headache attacks, lasting for 4-72 h and accompanied by anorexia, nausea, vomiting, photophobia and/or phonophobia[1]. In about 15% of patients, an aura may precede the migraine headache within about one hour (*migraine with aura*). Migraine prevalence peaks between the age of 35-45 years[2], a period, which is most productive in life. Thus, not only does migraine reduce quality of life, as evident from the fact that the WHO ranks it amongst the world's most disabling illnesses[3], but it also results in a considerable economic loss to the society in terms of lost workdays[4]. Although in the past one and a half decade constructive progress has been made in understanding migraine pathogenesis, the exact cause of this complex disorder has not yet been unravelled.

Since the prevalence of migraine is 2-3 fold higher in women than in men[2,5] (Figure 2.1), and reproductive milestones such as menarche, pregnancy and menopause are associated with changes in migraine frequency and/or severity, female sex steroids such as estrogen and progesterone have been implied to play a role in the migraine pathophysiology. This review elaborates the changes in migraine prevalence during females' life, as well as the potential mechanisms *via* which female sex steroids may play a role in the pathophysiology of migraine.

2.2 PREVALENCE OF MIGRAINE.

Migraine afflicts up to 15-20% of the general population[2,5]. The estimate of the prevalence of migraine across various studies shows large variations, as it may be influenced by age, gender, race, geography and socio-economic status[6,7]. Different stages in a woman's life are highlighted by changes in female hormone levels, especially estrogen and progesterone. As illustrated in the following sections, there is a concurrent change in the prevalence and severity of migraine during these different stages.

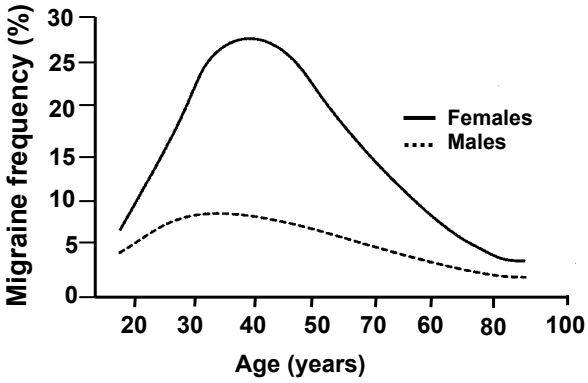

Figure. 2.1: Adjusted age-specific prevalence of migraine by sex. Adapted from Lipton *et al.*[2]

2.2.1 Before puberty

Paediatric migraine is one of the most common causes of headache in children, with a prevalence increasing from 3% at an age of 5 years to 14% at 15 years, with an overall prevalence of 11%[8]. Although children with migraine often remain suffering from this disease as an adult, about one quarter of children suffering from migraine have been reported to be migraine-free before the age of 25 years[9]. In very young children (≤7 yrs) the prevalence of migraine is higher in boys than in girls[10], while with increasing age the incidence in females starts rising.

2.2.2 After puberty

Starting at puberty, the migraine prevalence in women increases and considerably exceeds that in men. At its peak (between 35 and 45 years), the prevalence in women is almost three times higher than in men[11]. As many as 60% of female migraineurs report attacks during perimenstrual periods, i.e. two days before to three days after menstrual bleeding[12,13]. To address this phenomenon, the revised International Classification of Headache Disorders includes "candidate criteria" for two entities: *menstrually-related migraine* and *pure menstrual* migraine Headache Classification Committee of the International Headache Society,[1]. *Pure menstrual migraine,* which is not frequent (7-8%), is defined as migraine without aura that occurs exclusively on Day 1±2 of menstruation in at least 2 out of 3 menstrual cycles, while no migraine occurs at other times of the cycle. If such additional attacks, with or without aura, occur at other times of the cycle, it is defined as *menstrually-related migraine*. More than 50% women with migraine report an association between migraine and menstruation[14,15]. During menstruation, there is a rapid change in hormone levels and during the perimenstrual period there is an abrupt fall in female sex hormone levels towards the end of the luteal phase. MacGregor *et al*[16] reported a negative association between migraine and urinary estrogen levels across the menstrual cycle (Figure 2.2). The relative risk of a severe migraine increases by 3.4 fold on Days 1 to 3 of menstruation, as compared with other times of the month[13]. In addition, headache severity was positively correlated with premenstrual syndrome severity on Days -3 to +6 of the menstrual cycle in a post-hoc study[17]. Since studies suggested that the fall in female hormone levels is the trigger for migraine attacks, the use of estrogen supplements as preventive strategy has been assessed. In an elegant study, Somerville[18] showed that in 6 women suffering from menstrually-related migraine attacks could be postponed by a single injection of estradiol, and similarly Magos *et al*.[19] observed an improvement of more than 80% in women with menstrual migraine treated with subcutaneous estradiol implant. However, in a later study, Somerville[20] did not observe improvement of menstrual migraine with estradiol implant, although this negative result may have been related to the variable plasma concentrations of estradiol observed in this study. Interestingly, several days of high estrogen levels are thought to be a prerequisite before estrogen withdrawal can precipitate migraine attack[18].

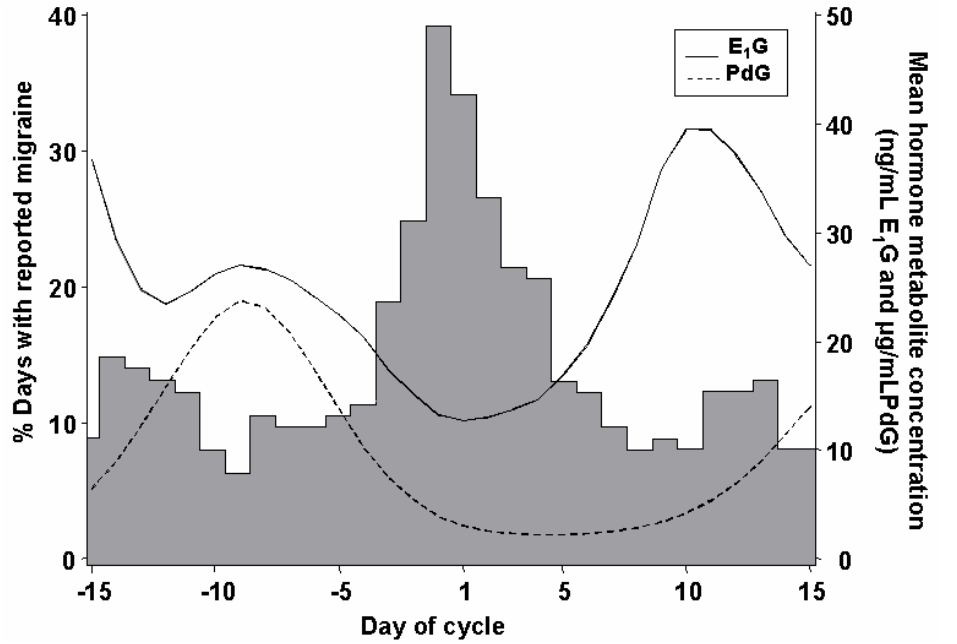

Figure 2.2: Migraine incidence and urinary estrone-3-glucuronide (E1G) and pregenandiol-3-glucuronide (PdG) levels recorded on each day of the menstrual cycle in 120 cycles from 38 women. Adapted from Massiou and MacGregor [293].

2.2.3 After hormonal contraceptives

Hormonal contraceptives are generally composed of an estrogen analogue, usually ethinyl estradiol, and a progestin analogue. Some decades ago, the risk for migraine attacks in women previously not suffering from migraine was reported to be about 10 times higher in those starting with an oral contraceptive than in those without these hormones[21]; the use of oral contraceptives in patients with pre-existing migraine increased the frequency of attacks[22]. However, since the dose of ethinylestradiol in oral contraceptives has been reduced from ≥50 µg to 35 µg or even less, the influence of oral contraceptives on migraine seems to have been diminished[23], although in a recent study migraine was still more likely among women using oral contraceptives containing estrogen[24]. Further, it is common that patients report either an improvement or worsening of their migraine attacks after starting oral contraceptives[25,26], but a continued use of oral contraceptives tends to improve headaches that occur at the start of oral contraceptives[27].

2.2.4 During pregnancy

During pregnancy, plasma levels of 17β-estradiol, estriol and progesterone increase to a level 10 to 100 times higher than in the nonpregnant state. The majority of women suffering from migraine without aura and menstrual migraine experience improvement during pregnancy[31]. In patients suffering from migraine without aura, Sances *et al.*[32] reported improvement in 47% during the first, 83% during the second and 87% during the third trimester, while complete remission was attained in 11%, 53%, and 79% of patients, respectively. It is worth mentioning that women suffering from menstrually-related migraine experience maximum improvement in their migraine attacks[33,34], probably due to constant levels of hormones. However, in some women attacks remain unchanged, worsen, or even appear for the first time during pregnancy, especially the attacks with aura[29,35]. Postpartum, the incidence of migraine may again increase[31]. Breast feeding seems to protect from migraine recurrence postpartum[32]; indeed headache during the first 3 months postpartum in breastfeeding mothers was reported to be similarly low as in the

second trimester of pregnancy[36]. The protective effect of breast feeding may be attributed to increased levels of oxytocin and vasopressin, which have anti-nociceptive properties[37,38] or alternatively to low levels of the estrogen and progesterone in this period.

2.2.5 During and after menopause

Migraine worsens in many cases just before or during the menopause, while it generally improves afterwards. Changing hormone levels in the perimenopausal years seem to be responsible for the increase in migraine prevalence during this period[20,39,40]. Further, low levels of estrogen and high levels of follicle-stimulating hormone may be associated with lower migraine prevalence[41]. Since migraine decreases in two third of women with spontaneous menopause and in only one third of women following surgical menopause, aging ovaries are thought to produce factors that may improve headache[42]. Hormone replacement therapy (HRT) has a variable effect on migraine prevalence; in this respect, HRT has been reported to improve migraine in 45% of women, worsen in 46%, have no effect in the rest of women[43]. Additionally, migraine attacks with aura may, similar to the situation in pregnancy, develop for the first time after the start of HRT[44]. HRT can be adjusted by reducing the dose, employing different estrogens or using less fluctuating hormone regimens[22,45].

Further, knowledge about the role of female sex hormones in migraine may be obtained from some special conditions in which hormonal interventions are required. For example, in a retrospective study, male to female transsexuals have been reported to have a similar migraine prevalence as genetic females[46], suggesting that the prevalence of migraine may have been increased by the administration of female sex hormones to these subjects. Obviously, a prospective study, including female-to-male transsexuals as a control group for excluding a stress-related mechanism as cause for the high prevalence of migraine in transsexuals, would be necessary to confirm this finding. Further, in women undergoing *in vitro* fertilization, where an analogue of gonadotropin-releasing hormone was administered to downregulate estrogen levels, the low levels of 17β-estradiol correlated with increased headache prevalence[47]. Interestingly, migraineurs have recently been reported to have a higher frequency of endometriosis and menorrhagia, possibly related to haematological, immunological, or genetic factors[48].

These epidemiological observations underline that there is a hormonal component in migraine pathophysiology, although the exact neurobiological mechanism has not yet been deciphered. In some conditions, such as the onset of puberty or menopause, when plasma levels of 17β-estradiol change[49], there is a concurrent change in migraine prevalence[2]; however, in menstrual migraine, it is the rapid decline of female hormones, in literature mostly referred to as 'withdrawal', that is thought be the trigger for migraine[49]. The fact that the correlation between plasma levels of female sex hormones and the prevalence of migraine is not simply positive or negative makes this correlation both complex and intriguing to investigate.

2.2.6 Genetics of migraine

The genes involved in the pathophysiology of migraine are difficult to search as migraine is an episodic disorder with a high prevalence, displaying both a clinical and genetic heterogeneity. However, three 'migraine genes' have now been identified in families with familial hemiplegic migraine (FHM), a rare form of migraine with an autosomal dominant inheritance pattern. These genes, CACNA1A[50], ATP1A2[51] and SCN1A[52], are all involved in ion transport and encode subunits of $Ca_v2.1$ (α_{1A}) voltage-dependent P/Q type calcium channels, Na^+/K^+ pumps, or neuronal voltage-gated $Na_v1.1$ sodium channels, respectively. Possibly, these genes could also be involved in more common forms of migraine[53]. It is not exactly known how these mutations could trigger migraine attacks, but it is believed that alterations in intracellular Ca^{2+} concentrations and abnormal K^+ levels, leading to increased neuroexcitability, play a critical role. Most intriguingly, as will be discussed in further detail later in this review, female sex steroids may also enhance neuronal excitability, and thus female sex steroids might enhance the effects of the ion channel or

pump mutations mentioned above. Indeed, very recently the threshold for cortical spreading depression in transgenic mice containing one of the CACNA1A mutations observed in FHM patients appeared to be lower in female mice than in their male counterparts, which also had a reduced threshold compared to the wild type mice[54], and the prevalence of FHM is 2.5 times higher in women than in men[55].

The genetic predisposition for migraine is similar in men and women[56,57], but the 3-fold higher incidence in females at peak prevalence, and the increased risk of migraine in first degree relatives of only male probands[58,59] suggests the possibility of an X-linked dominant gene. In accordance, Nyholt *et al.*[60,61] have reported that a locus on the X chromosome (Xq24-28) may be related to migraine susceptibility in some families, although it is unlikely that this locus completely accounts for the sex difference in migraine prevalence, even when one disregards the influence on migraine prevalence due to changes in hormone levels during life. Some genetic studies have tried to decipher the tentative relation between female sex hormone receptors and migraine. Individuals with a polymorphism at G594 (exon 8) of the estrogen receptor 1 (ESR1) gene are twice likely to suffer from migraine than those with the wild type gene[62]. Similarly, in a Spanish population, women carrying the C352 genotype were over 3 times more likely to suffer from migraine than those carrying the G325G genotype[63]. Further, individuals carrying the PROGINS inserts of the progesterone receptor gene polymorphism are twice as likely to suffer from migraine than control subjects[64]. However, it is worth mentioning that polymorphism in the case of estrogen receptor increases the expression of the receptor[62], whereas in the case of progesterone it negatively impacts its expression. Further, it is interesting that the PROGINS allele of the progesterone receptor acts synergistically with the 594A allele of ESR1 to increase the risk of migraine by 3.2 times[64]. Genetic studies investigating the CAG repeat of the androgen receptor revealed no correlation with migraine[64]. Similarly, the T/C *Pvu* II single nucleotide polymorphism in intron 1 of the ESR1 and the C325G single nucleotide polymorphism in exon 4 are not associated with migraine[65]. Taken together, these studies further underscore the relevance of hormonal factors in migraine pathogenesis, although genetic variation in the receptors for these hormones is unlikely to be a major cause for the higher prevalence of migraine in women.

2.3 PATHOPHYSIOLOGY AND TREATMENT OF MIGRAINE

Although the pathophysiology of migraine is largely unknown, migraine attacks most probably start in the brain, possibly within the brain stem[66,67]. Migraine is best understood as a neurovascular disorder; neuronal events are likely to result in the dilatation of blood vessels, which in turn induces pain and further nerve activation[68,69]. The basic mechanisms believed to be involved in migraine pathophysiology are: (i) neuronal hyperexcitability during the interictal phase[70,71], (ii) cortical spreading depression during the aura phase, and (iii) activation and sensitization of the trigeminal system at the peripheral and central levels that results in cranial vasodilatation. This trigeminal stimulation may involve the release of neuropeptides, in particular, calcitonin gene-related peptide (CGRP)[72,73] and nitric oxide (NO)[74], thus reinforcing vasodilatation and perivascular nerve activity[75]. While the early events during a migraine attack are unlikely to account for the headache, because the brain is largely pain-insensitive, trigeminal activation and vasodilatation are most probably responsible for the headache phase[69,76].

The pharmacologic treatment of migraine can be divided into drugs that are taken daily in order to reduce the frequency or severity of the attacks (prophylactic drugs) or those that are taken to abort attacks (acutely-acting drugs). Further, a distinction can be made between non-specific treatments (e.g., paracetamol and NSAIDS)[77] and specific treatments, such as ergot alkaloids (α-adrenoceptors and 5-HT receptor agonists)[78,79], the triptans (5-$HT_{1B/1D/(1F)}$ receptor agonists)[69,75]. The mechanism of action of prophylactic drugs in migraine is largely unknown, although it may depend, especially for the antiepileptics, on reduction of cortical excitability[80].

In addition, hormonal interventions have been explored as prophylactic treatment of menstrually related migraine or migraine experienced the in pill-free period. Estrogens in oral[20,81] and patch[19,82] form, as well as phyto-estrogens[83] have been utilized to prevent the rapid decline of estrogen levels during the menstrual cycle, which is thought be the main trigger of migraine in these patients. The reported results from these studies vary from no[20] or modest[82] improvement to an improvement up to eighty percent[19] in headache outcomes, although these results are based on a rather limited number of patients (n=11-24). The gonadotropin-releasing hormone agonists goserelin[84] and leuprolide acetate[85], which induce medical oophorectomy, have been studied in the prophylactic treatment of menstrual migraine as well. These gonadotropin-releasing hormone agonists have shown mixed results, and improvement was only observed when used in combination with estrogen. The results from these studies are promising, but the role of estrogen in the prevention of menstrual migraine seems inconsistent, as also described in a recent review by Brandes[86]. It is still not clear whether it is vital to keep the plasma concentration of estrogen above a certain threshold level, or whether the rapid decline of estrogen before the menstruation should be decelerated. Further, the value of progesterone in the prophylactic treatment of migraine should be explored.

In contrast to the prophylactic drugs, it is well known that the acutely acting specific antimigraine drugs may act *via* three different modes of action, namely: (i) constriction of dilated cranial arteries, (ii) inhibition of trigeminal neuropeptide release, and (iii) a central action. Since vasoconstrictor properties of antimigraine drugs may potentially lead to coronary vasospasms after their use, an attempt is currently being made to develop antimigraine drugs that are devoid of vasoconstrictor properties. One such approach is the development of the CGRP receptor antagonist olcegepant (BIBN4096)[87], which was recently demonstrated to be effective in the treatment of migraine[88]. Alternatively, in view of the involvement of NO in the pathophysiology of migraine, nitric oxide synthase (NOS) inhibitors have been successfully investigated as antimigraine treatment[89]. Blockade of CGRP receptors or inhibition of NOS does not induce vasoconstriction *per se*, and the use of olcegepant[88,90,91] or the NOS inhibitor L-NMMA[92,93] in humans did not induce any significant cardiovascular side effect. However, these studies were performed in a limited number of subjects, mostly healthy volunteers and the drugs were administered acutely. Especially in view of the possible prophylactic use of such drugs, it is important to study their cardiovascular side-effect potential, as they may inhibit the physiological protective properties of CGRP and NO[94,95].

In the following sections, we will focus on the (potential) action of sex steroids on several mechanisms and receptor systems that are involved in the pathophysiology of migraine (ion balance, transcription factors) and/or that are pharmacological targets for current (α-adrenoceptors, 5-HT receptors) or future (CGRP, NO) antimigraine drugs.

2.4 SEX STEROIDS AND THEIR CENTRAL AND VASCULAR MECHANISMS OF ACTION

It is widely documented that sex steroid hormones regulate several biological functions *via* two main mechanisms, which are triggered by genomic (transcription dependent) and/or non-genomic (non-transcription dependent) stimuli[96-98]. Ovarian steroids are capable of crossing the blood brain barrier by passive diffusion because of their lipophilic nature and low molecular weight[99]. Thus, the blood serum levels of the steroids are mirrored within the brain, and sex steroids induce a wide array of central effects. In addition, a number of enzymes involved in steroid production has been demonstrated in the human brain[100], suggesting that such hormones may also be produced within the central nervous system.

2.4.1 Central effects of sex steroids

The non-genomic effects of steroids in different excitable tissues have been extensively studied over the past decades. Initially, the non-genomic modulation by steroids in the central nervous system resulted

in numerous observations of steroid-induced changes in neuronal activity to induce anaesthetic[101-103], sedative/hypnotic[104] and anticonvulsant effects[105]; these are rapid effects, not mediated by intracellular receptor occupancy[106,107]. Although the receptors and mechanisms involved in their non-genomic action are not yet completely elucidated, at least three different mechanisms have been identified *via* which the steroids may non-genomically modulate the central nervous system for review, see[108]: (i) modulation of the coupling of a receptor to its first messenger system, such as uncoupling of μ-opioid and γ-aminobutyric acid B ($GABA_B$) receptors from their effector systems, which would result in increased neuroexcitability[109] (ii) altered conductance of ion channels by allosteric modulation, such as increase in the opening time of neuroinhibitory $GABA_A$ receptors[110-112], which, in contrast, would decrease neuroexcitability and (iii) enhanced neuronal excitability after acute exposure[113]. Most interestingly, the increase in neuronal excitability under normal, physiological conditions changes into a reduction in neuronal excitability after arterial occlusion[113], underlining the importance of an integrated neurovascular approach when studying the potential role of female sex steroids in the pathophysiology of migraine.

As mentioned above, the brain stem[66,67], possibly especially the periaqueductal grey[114,115], seems to be involved in the generation of migraine attacks. The facts that estrogen receptors are abundantly expressed in this brain area[116], and that the periaqueductal grey is an important antinociceptive center in the brain, make it tempting to suggest that abnormal activity of the brain stem, inducing migraine attacks, may be modulated via estrogen receptors located in this brain area.

The genomic mechanisms involved in the central action of sex steroids encompass two different receptors for estrogen (ERα and ERβ)[117], as well as two progesterone receptors (PR-A and PR-B)[118]. In contrast to the non-genomic effects, the central function of these nuclear receptors has not yet been well characterized, but they may alter receptor expression and may be implicated in the release and synthesis of various neurotransmitters and hormones. For example, CGRP (a facilitator in pain transmission)[119,120], galanin (a modulator of gonadotropin releasing hormone)[121], neuropeptide Y (a regulator of inflammation and central nociception)[122], as well as the neurotransmitters glutamate[123] and serotonin[124] have been reported to be genomically modulated by sex steroids. The genomic effects of estrogen may be mediated *via* extracellular signal-regulated kinase[125], by increased phosphorylation of cAMP response-element binding protein (CREB)[126], or by modulating the enzymes involved in the synthesis and metabolism of neurohormones and/or peptides[124]. Progesterone receptors are often co-localized with estrogen receptors, and the presence of estrogen may be required for expression of progesterone receptors in some brain areas. Remarkably, progesterone can act in a synergistic, antagonistic or neutral manner compared to the effects of estrogen[127-132].

2.4.2 Vascular effects of sex steroids

As previously described for their central actions, steroid hormones are capable of inducing non-genomic effects to regulate vasomotion, which occur at times too quickly to be explained by an altered gene expression[133]. In this respect, female sex steroids have been reported to possess a protective cardiovascular role and one of their most important responses is inhibition of vascular tone[134]. A large body of evidence has shown a marked vasorelaxation effect elicited by estrogens in a variety of vascular beds from different species[134-138], including humans[139-141]. Likewise, progesterone[139,142-145], as well as some natural[142] and synthetic[146,147] progestins also induce a relaxant effect on the vasculature.

On the other hand, it has been reported that 17β-estradiol induces vasorelaxation by both genomic and non-genomic mechanisms, *via*: (i) production of vasodilator agents, such as NO, cGMP, cAMP, adenosine and prostacyclin, (ii) alteration of receptor expression of these mediators, and (iii) alterations in ion channel activity[134].

Although endothelial factors might be involved[148], steroid-induced vasorelaxation is preserved in blood vessels without endothelium or pretreated with NOS inhibitors[140,142,143,149-151]. The complexity of mechanisms involved in steroid-induced vasorelaxation which, in turn, leads to divergent results could

be attributed to a myriad of factors, including species differences, the vascular bed under study and the experimental conditions, e.g. the presence of intact endothelium, specific agonists, blood vessels obtained from steroid-treated animals, etc.

Collaterally, as mentioned above for their central actions, several studies have provided evidence for a modulatory role of steroids on ion channel function. In this respect, it is suggested that two main mechanisms might help explain the vasorelaxant response elicited by male and female sex steroids, namely: i) the control of $[Ca^{2+}]$ homeostasis, including modulation of Ca^{2+} entry *via* inactivation of voltage-gated channels[143,144,152-154] and nonvoltage-gated pathways[142,143,150,155] or ii) activation of K^+ channels[135,137,156], particularly on the large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}).

The analysis of these findings leads to the conclusion that the relaxant action of steroids on vascular tone is through multiple cellular mechanisms; however, the actual mode of action in this process remains to be elucidated. Furthermore, it is interesting to note that sex steroids differ in their ability and potency to exert non-genomic activity on vascular tone[142,146,157]. Therefore, the different structural conformation of each steroid could be important for inducing vasorelaxation *via* activating different modes of action.

2.5 EFFECT OF FEMALE HORMONES ON VARIOUS MECHANISMS AND VASOACTIVE AGENTS INVOLVED IN MIGRAINE

As mentioned above, there is ample clinical evidence for a relationship between the occurrence of migraine attacks and changes in plasma levels of female sex hormones. Below, the effects of the sex hormones on different mediators that are most likely involved in the pathophysiology of migraine or that are a target of (future) antimigraine drugs will be discussed.

2.5.1 CGRP and its receptors

CGRP is a neurotransmitter that is widely distributed in the peripheral and central nervous systems, as well as in the cardiovascular system[158]. The wide distribution of CGRP-containing nerve fibres and CGRP receptors in the body suggests that this peptide plays an important role in the modulation of physiological functions. Indeed, CGRP has a number of functions in the cardiovascular system, amongst which potent vasodilatation[159] is probably the most vital effect of this peptide. In the central nervous system, CGRP modulates the motor, sensory and pain pathways[159] and may contribute to the maintenance of spontaneous activity in spinal trigeminal nucleus in rats[160]. Additionally, CGRP antagonists have been demonstrated to inhibit trigeminocervical activity evoked by superior sagittal sinus[161]. Thus, the central actions of CGRP appear to be a part of the pathophysiology of migraine[158].

CGRP is stored in perivascular nerve terminals surrounding most blood vessels at the junction of the adventitia and the media, passing into the muscle layer. Thus, this peptide is well capable of modulating vascular tone, including that of cranial blood vessels[162]. Because of the extensive presence of CGRP-containing neurons in the trigeminovascular system, the release of CGRP is thought to initiate cranial blood vessel dilatation, thus playing a key role in the pathophysiology of migraine[163]. CGRP levels in jugular venous plasma have been reported to increase during migraine and they are normalized after treatment with triptans[164], although this could not be confirmed recently[165]. Nevertheless, the newly developed CGRP receptor antagonist olcegepant[87,166] has been shown to be effective in the treatment of migraine[88], thus providing convincing evidence for the involvement of CGRP in migraine.

The homeostasis of CGRP in the central nervous system is strongly influenced by sex steroids. Indeed, both 17β-estradiol and progesterone may increase CGRP synthesis in dorsal root ganglion, the main site of CGRP synthesis in rats[167,168]. The role of CGRP in pain transmission[119,120] may be modified by 17β-estradiol, as was demonstrated by selective increase in sensory nociceptor vasodilator innervation of arterioles[169]. In accordance, using intravital microscopy on a closed cranial window in ovariectomized rats, we ob-

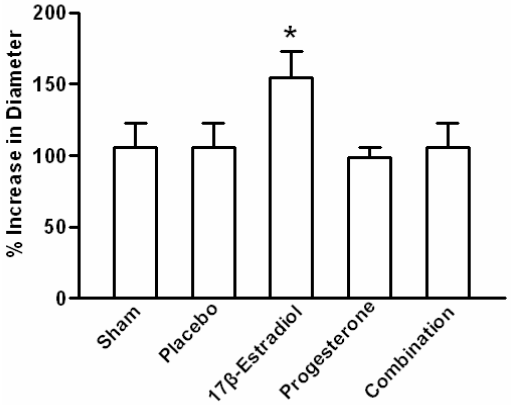

Figure 2.3: Effect of periarterial nerve stimulation (125 μA) on middle meningeal artery diameter in sham-operated or ovariectomized rats receiving placebo or hormone treatments (n=6-7). *, Significantly higher (P<0.05) than the corresponding value in the placebo group. Values are mean±SEM [170].

served that maximum relaxations of dural arteries in response to neurogenic periarterial stimulation were significantly increased in 17β-estradiol-treated rats compared to placebo-treated rats Figure 2.3; [170], which may be attributed to increased release of CGRP from sensory nerve endings [171]. In this context, it is important to note that human extracerebral intracranial arteries are innervated by sensory nociceptor nerves, and these pain sensors are of key importance in the headache phase of migraine[172].

Ovariectomy decreases CGRP plasma levels in rats, whilst subsequent treatment with 17β-estradiol, progesterone or their combination normalizes these levels[173], while the levels are even further increased during pregnancy[174]. In humans, there is a higher plasma level of CGRP in females as compared to their male counterparts[175]. Similar as reported in rats, plasma CGRP levels are elevated during pregnancy[176], as well as in postmenopausal women undergoing HRT[177,178]. Thus, the studies mentioned above provide substantial evidence that female sex steroids modulate levels of CGRP. Admittedly, in trigeminal ganglia of cycling female mice there was no significant change in mRNA expression of CGRP[179], although the actual peptide levels were not measured.

Not only the levels of CGRP are modulated by female sex steroids, but the mRNA levels of CGRP receptors, as well as CGRP binding sites are increased during pregnancy in rats[180]. Dexamethasone also increases the expression of RAMP1 and CL receptor in vascular smooth muscle cells[181] and the hypotensive responses to exogenously administered CGRP are enhanced in pregnant rats and ovariectomized rats treated with 17β-estradiol as compared to ovariectomized controls[182]. In our laboratory[183], as well as in other studies[173], an increased potency of CGRP in mesenteric and caudal arteries of pregnant rats or ovariectomized rats treated with 17β-estradiol was reported as compared to those treated with placebo, although this mechanism was not observed in basilar arteries. Interestingly, progesterone may attenuate the enhance responses to CGRP by 17β-estradiol[183]. Indeed, the two steroids may affect the CGRP receptor expression in opposite ways[184].

Taken collectively, these findings indicate that female sex steroids affect CGRP and its receptors in the central and peripheral nervous systems, as well as in blood vessels. Most findings point to a positive correlation between plasma levels of CGRP and 17β-estradiol, suggesting a higher prevalence of migraine in subjects exposed to relatively high levels 17β-estradiol. However, more research on the potentially opposing effects of 17β-estradiol and progesterone, as well as on the effects of estrogen withdrawal and CGRP are needed before a definite conclusion can be drawn on the relationship between female sex steroids and CGRP.

2.5.2 Noradrenaline and α-adrenergic receptors

The endogenous catecholamines, adrenaline and noradrenaline, which are released upon activation of the sympathetic nervous system, play an essential role in the regulation of a host of physiological responses *via* the activation of α- and β-adrenoceptors[185]. Adrenaline is the primary hormone secreted by the adrenal medulla, while noradrenaline is a major neurotransmitter released from sympathetic neurons. Since both participate in the maintenance of vascular tone mainly *via* α-adrenoceptors, these have been implicated to have a role in migraine pathophysiology[40,186,187].

α-Adrenoceptors can be divided into two major types, namely, the α_1- and α_2-adrenoceptors, and each of these types can be further subdivided into three subtypes, based on structural, transductional and operational (pharmacological) criteria[188]. Within this context, the high affinity of ergotamine and dihydroergotamine at α-adrenoceptors has been proposed to be one of the main reasons for their antimigraine action[78,79]. Indeed, activation of $\alpha_{2A/2C}$-adrenoceptor subtypes is partly involved in the canine external carotid (extracranial) vasoconstriction induced by ergotamine[189] and dihydroergotamine[190].

In the central nervous system, estradiol and progesterone may enhance hypothalamic noradrenaline release, leading to increased excitability of the ventromedial hypothalamus[191]. In addition, female mice have been reported to have lower pain thresholds on a tail flick test and to be less sensitive to the analgesia induced by the α_2-adrenoceptor agonist clonidine compared with their male counterparts[192]. The authors suggest that G protein-coupled inwardly rectifying potassium channels are responsible for these differences. Furthermore, estradiol has been reported to downregulate cortical α_{2A}-adrenoceptor by as much as 50% in ovariectomized rats[193], as well as uncouple α_{2A}-adrenoceptors from G proteins[194]. In addition, estrogen may attenuate α_2-adrenoceptor-mediated anti-nociception in the spinal cord[195]. Thus, it is likely that estrogen modulates the anti-nociceptive effects by compromising the expression and/or the function of α_{2A}-adrenoceptors, which are G-protein (G_i/G_o)-coupled receptors responsible for producing anti-nociception.

Female rats have higher plasma adrenaline levels than male rats, which decrease after ovariectomy[196]. In contrast, both 17β-estradiol[197] and progesterone[198] are known to decrease sympathetic tone. The latter seems to be in line with a decreased sympathetic tone in migraineurs[186], and with decreased plasma noradrenaline levels at menses in migraine patients compared to control subjects[199].

In addition to their neuronal effects, female sex hormones may affect (i.e., increase or decrease, depending on vessel system under study), vasoconstriction in response to α-adrenergic receptor agonists. For example, forearm blood flow responses to noradrenaline are higher in men than in women[200]. Consistent with this finding, Gisclard *et al.*[201], as well as our own group[202], have reported that 17β-estradiol administration after ovariectomy depresses contractions mediated by α_2-adrenergic receptors in the rabbit femoral and rat carotid artery, respectively. Thus, an increased α_2-adrenoceptor function may, at least partly, be responsible for the increased blood pressure in females after menopause[203]. In contrast, 17β-estradiol replacement in ovariectomized rats has been reported to enhance vasoconstriction induced by smooth muscle α_2-adrenergic receptor activation in rat mesenteric artery, although this effect was obscured due to an overriding influence of endothelial dilator substances, primarily NO[204]. Furthermore, it has been reported that vascular senstivity to noradrenaline in isolated arteries from the rat is regulated in opposite directions by female sex steroids, with senstivity being higher after treatment with 17β-estradiol than with progesterone[205].

Taken together, it appears that female sex hormones may centrally affect the noradrenergic system at the receptor level, as well as at the level of noradrenaline release in different brain regions. Both the increased cortical excitability and the lower pain threshold mediated by α-adrenergic receptors and female sex steroids may predispose women for migraine. Besides these central effects, female sex steroids in addition display an array of (sometimes contradictory) effects on blood vessels, which may be relevant in migraine pathogenesis.

2.5.3 5-HT and its receptors

In addition to being a central and peripheral neurotransmitter, serotonin (5-hydroxytryptamine; 5-HT) is capable of exerting complex cardiovascular effects, including hypotension or hypertension, vasodilatation or vasoconstriction and/or bradycardia or tachycardia; the eventual response primarily depends on the nature of the 5-HT receptors involved[206,207]. The conjunction of structural, transductional and operational criteria has led to classify 5-HT receptors into 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_7, recombinant (5-ht_5 and 5-ht_6) and 'orphan' receptors[207,208].

5-HT may be involved in the pathogenesis of migraine at different levels. Firstly, the brain of migraine patients seems to display an increased 5-HT synthesis compared to that of control subjects[209], which could lead to cortical hyperexcitability. Indirect evidence for the involvement of 5-HT in migraine pathogenesis is given by the fact that the 'triptans', which are all agonists at 5-$HT_{1B/1D}$ (and in most cases 5-HT_{1F}) receptors, are effective in the acute treatment of migraine[69,75]. Although their definite mode of action is still under debate, several mechanisms have been proposed, including: (i) inhibitory actions in the central nervous system, particularly in the *trigeminal nucleus caudalis*; (ii) prejunctional trigeminovascular inhibition at the level of cranial extracerebral arteries, with a corresponding inhibition of CGRP release; and/or (iii) a direct vasoconstrictor action on cranial extracerebral arteries[68,69,75].

Recent neuropharmacological evidence from a primate model of surgical menopause suggests a positive link between estrogen and 5-HT synthesis[124], which is most likely mediated by the ERβ subtype[210]. Such a possible link between estrogen and serotonergic signalling may be relevant in migraine, as illustrated by a small clinical study in women with status migrainosus occurring within 48 h of the discontinuation of oral contraceptives[211]. In this study, the neuroendocrine response to the direct central 5-HT agonist meta-chlorophenylpiperazine (m-CPP) was decreased in patients with status migrainosus, while this was restored after transdermal estradiol supplementation[211]. It remains, however, to be established whether these observed differences are causal in the development of migraine, or represent an epiphenomenon that is rather a consequence of the migraine attacks. The firing rate of serotonergic neurons in dorsal raphé nucleus is higher in male than in female rats, although, apparently in contrast, it increases during pregnancy[212]. The contractile responses to 5-HT in porcine isolated coronary artery decrease after acute exposure to physiologically relevant concentrations of 17β-estradiol[133]. Since classical ER antagonists, as well as *de novo* protein synthesis inhibitors did not inhibit this rapid effect, the authors concluded that this was mediated *via* a non-genomic pathway[133]. Further, the chronic exposure to 17β-estradiol in the rat isolated aorta[213], as well as the rabbit isolated coronary artery and thoracic aorta[214] decreases contractions to 5-HT. We observed an attenuation of 5-HT-induced contraction by 17β-estradiol in the rat isolated carotid artery and showed that this was also the case with progesterone as well as a combination of 17β-estradiol and progesterone (Figure 2.4;[202]). These actions of female sex

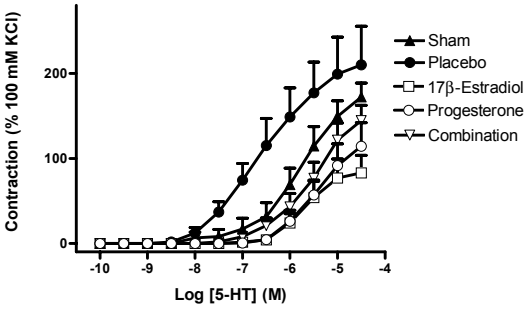

Figure 2.4: Effect of increasing concentrations of 5-HT in carotid artery obtained from sham-operated rats, as well as ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=5-7 each). Values are mean±SEM[202].

steroids seem to be, at least partly, mediated *via* a direct effect on vascular smooth muscle cells[215] and are thus not (completely) mediated by the endothelium.

In conclusion, similar as described above for CGRP and the α-adrenergic system, female sex steroids may modulate synthesis of and/or responses to 5-HT on both central and vascular levels. Whereas the vascular effects of 17β-estradiol and progesterone are opposite in the case of α-adrenoceptor-mediated contraction (see above), these hormones seem to display the same (inhibitory) effects on contractile responses to 5-HT.

2.5.4 Ion balance

The concentration of an array of ions, both intracellular as well as extracellular, is of key importance for neurotransmission and neuronal excitability[216]. As mentioned in the previous sections on Genetics and Pathophysiology of migraine, neuronal excitability is likely to play a crucial role in migraine pathophysiology, especially in the early phases of a migraine attack[217].

In addition, ion concentrations modulate vasoconstriction and vasorelaxation decreased magnesium concentrations are associated with cortical spreading depression in animals[218], and indeed there is evidence for low Mg^{2+} levels in the brain during[219] and between[220] migraine attacks. Patients suffering from migraine with or without aura or FHM all consistently showed decreased Mg^{2+} concentrations in the posterior part of the brain with increasing severity of neurological symptoms[221]. Mg^{2+} concentrations are decreased in blood, serum, red blood cells, mononuclear cells, as well as in salivary secretions of migraineurs, both interictally and during attacks[222]. Therefore these studies indicate that lowered Mg^{2+} concentration could be responsible for hyperexcitability of neuronal tissue in migraine patients. It is further substantiated by recent evidence which shows that in up to 50% of migraine patients there is lowered ionized level of magnesium and infusion of magnesium provided sustained relief from headache in these patients[223,224]. Oral magnesium has been demonstrated to be effective prophylactic in menstrual migraine[225]. Not only magnesium ions modulate excitability of brain there is evidence that may influence the vascular tone[226,227].

Calcium seems to be involved in the pathogenesis of migraine through multitude of pathways. As mentioned above: (i) mutations in the P/Q-type calcium channel are related to familial hemiplegic migraine[50] and possibly to migraines with and without aura[53]; and (ii) L-type calcium channels modulate the release of CGRP in neurons innervating trigeminal vasculature[228]. In keeping with these findings, Ca^{2+} channel blockers have shown a number of effects, for example: (i) the calcium channel blocker verapamil potentiates anti-nociception in rats[229]; and (ii) verapamil and flunarizine seem to be effective in migraine treatment[230,231]. Nevertheless, their putative mechanism of action is not understood, and many other calcium antagonists are ineffective in migraine[232,233].

Significantly, serum levels of Mg^{2+} and Ca^{2+} are affected by sex steroids (Figure 2.5) and vary throughout the menstrual cycle in women[234]. Interestingly, in a study in cerebral vascular smooth muscle cells the levels of both estrogen and progesterone were negatively correlated to cytosolic Mg^{2+} concentrations, whereas testosterone had no such effect[235]. Furthermore, the levels of Ca^{2+} are reported to rise in parallel with estrogen levels in women[236]; indeed 17β-estradiol modulates, either genomically or non-genomically, both the extracellular and intracellular Ca^{2+} concentrations[237].

Both Ca^{2+} and K^{+} channels are directly coupled to estrogen receptors, via pertussis toxin-sensitive G proteins. These G proteins are activated by steroids acting as a first messenger[238,239]. Transient exposure to estrogen results in a decreased potency of μ-opioid receptor agonists to hyperpolarise propiomelanocortin (POMC) neurons[240]. This action is mediated by activation of the G-protein-gated, inwardly-rectifying K^{+} channel subfamily known as GIRK1-4 (Kir3.1-3.4)[239,241,242]. Further, estrogen affects the rapid action on α_1-adrenergic receptor-mediated inhibition of the small-conductance, Ca^{2+}-activated K^{+} channels in GABAergic neurons[243]. Thus female sex steroid affect several physiological actions which eventually can influence migraine outcome, including amongst others: (i) increase in neuronal excitability with

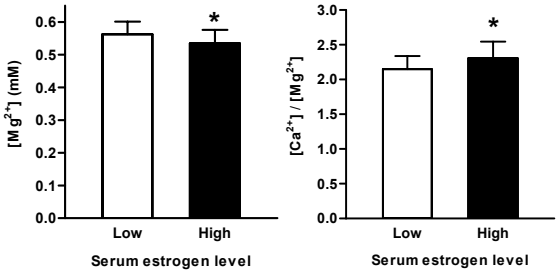

Figure 2.5: Effect of low and high concentration of estrogen in rat plasma concentration of Mg^{2+} and Ca^{2+}/Mg^{2+} concentration. *, Significantly different ($P<0.05$) from the corresponding low level of estrogen. Values are mean±SD Adapted from Muneyvirci-Delale *et al*.[234].

decreases in Mg^{2+} concentrations and modulation of Ca^{2+} concentrations (ii) neuropeptide (particularly CGRP) release; and/or (iii) postjunctional vascular actions. It remains to be established to what extent the modulation of various ion concentration and/or ion channels by female sex steroids is indeed relevant for the pathophysiology of migraine.

2.5.5 Nitric oxide

Nitric oxide (NO) displays a pronounced vasodilator effect and plays an important role in the physiological regulation of local blood flow and blood pressure[244,245]. It is synthesized from L-arginine and the reaction is catalyzed by nitric oxide synthase (NOS) in the endothelial, lungs, as well as neuronal cells[244]. Although NO can hyperpolarize vascular smooth muscle cells, activation of the endothelium can induce hyperpolarisation and vasodilatation by other means[245]. Amongst other functions, NO is involved in: (i) the neurogenic control of microcirculation through autonomic efferent nerves; (ii) the vasodilatation and inflammation associated with activation of sensory nerves; and (iii) the pathophysiology of several cardiovascular and other diseases[244,245].

Significantly, NO is able to differentially influence afferent fibres in the superficial laminae of rat spinal trigeminal nucleus caudalis, and estradiol modulates the basal expression of these transmitters and blocks the nitroglycerin effect[246]. In addition, estrogen may directly affect the vascular system by stimulation of NO release. The ERα increases NOS activity in endothelial cells[247] by direct activation of the protein phosphatidylinositol 3-OH kinase in a non-nuclear and perhaps membrane-associated compartment location[248]. Remarkably, the NO and platelet L-arginine pathway has been reported to exhibit a heightened activation in women with a history of menstrual migraine, especially during the luteal phase, compared to women with non-menstrual migraine and women without migraine[249]. In rats, estrogen is reported to decrease myogenic tone through a NO-dependent mechanism in rat cerebral arteries, as is clear from the smaller decrease in vessel diameter in response to increasing transmural pressure in females and ovariectomized females treated with estrogen compared to the responses in males and ovariectomized females without hormone replacement[250].

The interaction of sex hormones with cytosolic/nuclear receptors triggers long-term genomic effects that could stimulate endothelial cell growth while inhibiting smooth muscle proliferation[96]. Activation of plasmalemmal sex hormone receptors may trigger acute non-genomic responses that could stimulate endothelium-dependent mechanisms of vascular relaxation such as the nitric oxide-cGMP, prostacyclin-cAMP, and hyperpolarization pathways[96]. Thus, the effect of NO modulating the vasoconstrictor effect of vessels is non-genomic in nature. Vascular function in postmenopausal women may be improved through increasing the serum NO level after a 3-month oral intake of estradiol valerate, while estradiol valerate plus medroxyprogesterone acetate may attenuate the beneficial effects[251], suggesting a contrasting role of estrogen and progesterone towards the vascular tone. In summary, these findings

suggest a positive interaction between sex steroids and NO, which may thus augment the susceptibility to migraine.

2.5.6 Transcription factors

Nuclear factor-κB (NF-κB) has emerged as a critical and ubiquitous transcription factor underlying multiple cellular functions, e.g. induction of the inducible isoform of NOS (iNOS) and transcription of acute phase proteins[252,253]. Recently, Reuter *et al*.[254] demonstrated that nitroglycerin infusion, which triggers migraine attacks[255], increases iNOS expression and activates NF-κB within rodent dura mater several hours after its administration. Thus, it is believed that this transcriptional factor: (i) play a role in the pathophysiology of migraine; and (ii) provides a novel target for the development of antimigraine drugs.

Notwithstanding, the role of neurogenic inflammation in the pathophysiology of migraine- unlike that of neurogenic vasodilatation- should be considered with caution since plasma protein extravasation, one of the components of neurogenic inflammation, does not seem to be involved in the pathophysiology of migraine[256].

The transcription factor p65/relA, a member of the NF-κB family, plays a major role in inflammation and drives the expression of pro-inflammatory mediators[257]. Cell culture studies have shown that 17β-estradiol downregulates the expression of inflammatory genes, such as those coding for iNOS or matrix metalloprotease 9[258], enzymes directly involved in the progression of the inflammatory response[259], and inhibits the biochemical and morphological activation of macrophages[260]. Thus, evidence accumulated so far is uniformly concordant in identifying estradiol as a protective agent against the induction of inflammatory responses. The gene and protein expression of NF-κB are constitutive but ovarian hormones can decrease the nuclear location of NF-κB in dorsal raphé neurons and, thereby, decrease the ability of NF-κB to drive gene expression in response to cytokines[261]. Estrogen withdrawal has been reported to increase NF-κB DNA binding activity[262]. Further, 17β-estradiol modulates expression of Nrf-2[263], another transcriptional factor that might be involved in the pathophysiology of migraine, based on its increased expression in a mouse oligemia model[264], i.e. blood flow reduction without acute tissue damage that could occur in migraine.

In addition, a group of immediate early genes, including c-fos are expressed at higher levels in migraine patients[265]. C-fos has been widely used in migraine and pain research to trace the neuronal activity of pain-responsive neurons. As estrogen modulates expression of c-fos, depending upon the brain region understudy[266], c-fos may represent one of the mechanisms *via* which sex steroids modulate central aspects of migraine.

2.5.7 Miscellaneous

In addition to the receptor systems descibed above, other lines of evidences have also documented the effects of female sex hormones on other neurotransmitter receptors and its potential relevance in antimigraine therapy. As discussed below these include, amongst others, GABA, glutamate and opioid receptors.

GABA and its receptors

Although the GABAergic system does not seem to be primarily implicated in migraine aetiology, hyperexcitabilty of the brain is thought to be an important factor in its pathogenesis[217]. Hence, the potential role of GABA, a predominant neuroinhibitory transmitter, should not be underestimated. GABA is predominantly found in the brain, and accounts for 30% of neurotransmission at all synapses[267]. GABA acts mainly *via* two main receptors, namely: (i) the $GABA_A$ receptor, a ligand-gated postjunctional receptor[268]; and (ii) the $GABA_B$ receptor, a G-protein coupled receptor that is expressed both pre- and post-synaptically[269]. Activation of $GABA_A$ and $GABA_B$ receptors induces neuroinhibition, which is significantly influenced by female sex steroids. Indeed, the opening time of the $GABA_A$ receptor is increased by allopregnenolone,

a progesterone metabolite that does not act on progesterone receptors, thus leading to hyperpolarisation *via* an increased influx of Cl^- ions[112]. In contrast, estrogen uncouples the $GABA_B$ receptor from the GIRK potassium channels in rat hypothalamus, thus decreasing the neuroinhibitory effects of $GABA_B$ receptors[240,270,271]. Shughrue and Merchenthaler[272] have reported that estrogen increases the expression of glutamic acid decarboxylase, the rate-limiting step in GABA synthesis, as well as GABA release. More recently, it has been shown that estrogen and/or oxytocin can rapidly increase the formation of functional GABA synapses in the adult rat supraoptic nucleus[273]. In parallel to their central action, sex steroids may affect vascular GABA receptors, which appear to be localized only in certain blood vessels, such as the cerebral arteries[274-277]. Nevertheless, it is noteworthy that GABA has been shown to be one of the less potent endogenous cerebrovasodilators[278,279]. Considering the above lines of evidence, it is clear that sex steroids have contrasting effects on the GABAergic system homeostasis. Depending on the experimental conditions and the steroids under study, this balance may be tilted in either direction, and might thus have a relevant contribution to the excitation threshold of the neurons implicated in the pathogenesis of migraine.

Glutamate and its receptors

Glutamate may be involved in the cortical hyperexcitability in migraine[280]. The effects of glutamate on brain excitability are mediated via the ionotropic NMDA, AMPA and kainate receptors, which are being studied as potential antimigraine targets[281]. Interestingly, estradiol and progesterone have divergent effects on the ionotropic glutamate receptors, which depend on the location in the brain. For example, estrogen increases NMDA receptor-mediated excitatory responses in the hippocampus[282,283], but not in other brain regions[282]. In contrast, progesterone is likely to decrease NMDA receptors in frontal cortex[282]. The action of kainate receptors in hippocampal neurons is potentiated by estradiol[284]. Significantly, glutamate may, *via* AMPA and kainate receptors, contribute to the peripheral release of vasoactive neuropeptides such as CGRP[285], which, as described above, is modulated by female sex steroids.

Opioid receptors

The opioid system is also strongly influenced by sex-steroids. There are three major subtypes of opioid receptors, μ, κ and δ, which are further subdivided into subtypes. All these receptors are G-protein coupled, and analgesia is one of the major effects. Enkephalins and β-endorphin are endogenous ligands having high a affinity for μ and δ receptors, whereas dynorphins have a higher affinity for κ receptors. Gender differences in morphine-induced antinociception have been reported in a number of species, including rats[286], monkeys[287] as well as humans[288], with males generally showing greater antinociceptive effects than females. In pregnancy, spinal cord levels of enkephalin and dynorphins are increased, and high levels of oestrogen and progesterone are required to produce this analgesia[289]. The mRNA expression of the opioid precursor gene pro-opiomelanocortin is increased after treatment with estrogen and progesterone as compared to that in ovariectomized sheep[290]. Morphine increases the expression of c-Fos, one of the molecular marker of migraine, to a higher degree in males than in females, but these sexually dimorphic effects of morphine were independent of sex steroids[291]. Thus, the effects of female sex steroid on the opioid system do not always point to the same direction, and depend on the type of opioid receptor, the specific brain region and the type and or duration of hormone treatment.

2.6 IMPLICATIONS, FUTURE DIRECTION AND CONCLUSION

Both basic research and clinical studies suggest an intricate relation between female sex steroids and the occurrence and/or severity of migraine. At the onset of puberty, when plasma levels of estrogen increase, there is a concurrent increase in migraine frequency, while after menopause, when plasma

levels of estrogen decrease, there is a parallel decrease in migraine frequency. Intriguingly, the declining levels of estrogen during the menstrual cycle do cause an increase in migraine attacks, typically migraine without aura. Finally, in pregnancy there is generally an improvement of migraine without aura, whereas migraine with aura may increase, and some women experience attacks for with aura for the first time. As

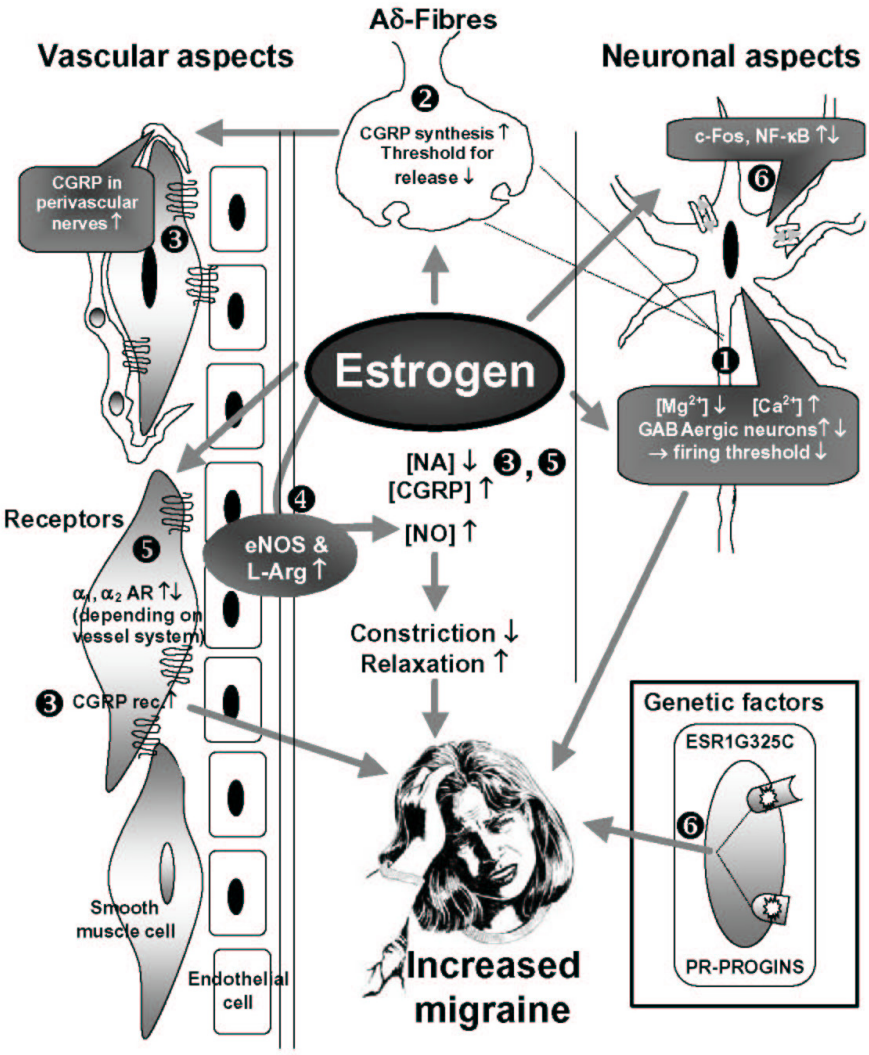

Figure 2.6: Schematic representation of main mechanisms by which estrogen may increase the incidence of migraine: ❶ Estrogen enhances neuronal excitability by decreasing levels of Mg^{2+} and increasing Ca^{2+} concentrations; ❷ Estrogen up-regulates the synthesis of calcitonin gene-related peptide (CGRP) in and its subsequent release from Aδ-fibres; ❸ On the vascular level, this increased CGRP release enhances vasodilatation, which acts in tandem with increased expression of CGRP receptors and an inhibition of the sympathetic nervous system; ❹ Further, estrogen enhances the production of nitric oxide (NO) *via* eNOS (endothelial nitric oxide synthase) from L-Arg (L-arginine) by the endothelium; ❺ The GABAergic and noradrenergic (α_1- and α_2-AR (adrenoceptors), as well as plasma concentrations of NA (noradrenaline)) systems may display both enhanced and attenuated effects in response to estrogen; and ❻ In addition, estrogen modulates the molecular markers c-fos and NF-κB, while mutations in both estrogen receptors (ESR1G325C) and progesterone receptors (PR-PROGINS) increase the risk for migraine.

elaborated earlier and summarized in (Figure 2.6), there is substantial evidence that estrogen enhances neuronal excitability as well as vasodilatation, which both lead to an increased propensity for migraine.

Obviously, there are certain methodical - and some inherent - limitations in the extrapolation of results from basic research to the clinical situation. Firstly, as the pathophysiology of migraine is not completely deciphered yet, experimental models employed in migraine research are based on only a few symptoms observed in the clinical situation (e.g., focus on only neuronal or only vascular aspects), and thus do not completely mimic all aspects of a migraine attack. Thus, the parameters obtained with these studies do not necessarily apply to a real migraine attack, as there is still no unequivocal neuronal, humoral or biochemical marker for migraine. Further, apart from obvious variables such as the species used, there are several experimental parameters that should considered, such as the route of administration and pharmacokinetic characteristics of the steroids in various studies, their dose, the duration and frequency of administration. As the effects of sex steroids may be mediated by classical genomic and/or non-genomic/rapid mechanisms, a lot of work is required in identifying non-genomic pathways in both the central nervous system and the cranial circulation. Further, while interpreting experimental results, it is important to take into account whether the sex hormones were given alone or in combination. Essentially, in most experimental models, stable levels of sex hormones are used, while clinical evidence indicates that not only do absolute levels of female sex steroids seem to be of relevance, but rather their (rate of) change. Therefore, future experimental studies should carefully take these considerations into account, and investigations focussing on the change in hormone levels, especially the decrease in 17β-estradiol after exposure to a high concentration, may reveal more insight into the pathophysiology of menstrual migraine.

Disregarding the limitations of basic research, we will inevitably need a multidisciplinary approach encompassing basic and clinical observations to understand the influence of female sex steroids on migraine. Such an integrated approach will be relevant to understand why migraine without aura seems to be predominantly triggered by a decline in estrogen levels, while migraine with aura seems to be prompted by high levels of estrogen. While migraine with and without aura are often considered as two entities of the same disease, the differential effect of female sex steroids on these two forms of migraine suggests that, at least in some aspects, they represent separate entities. Another interesting dimension to this ever increasing enigma is the so-called mismatch between the slow genomic and rapid non-genomic actions of sex steroids[292]. As the changes during menstrual cycle are drastic and rapid, the balance between excitatory and inhibitory stimuli might be disturbed, and could thus be attributed to increased susceptibility to migraine in females during this period.

Taken together, there is overwhelming clinical and experimental evidence for a relationship between migraine and levels of female sex hormones, which may influence the pathophysiology at a central, peripheral prejunctional and/or vascular levels. In-depth delineation of this relationship is an intriguing and challenging subject for future research.

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Chapter 3

Aims of the thesis

Based on the questions that were posed in the previous chapters, the objectives of this thesis were

1. to investigate whether the vasoconstriction/vasodilatation of the carotid circulation induced via 5-HT, adrenergic and CGRP receptors in pigs *in vivo* involves the meningeal artery (See part II of the thesis).

2. to characterize the 5-HT, adrenergic and CGRP receptors in porcine isolated meningeal arteries (See part II of the thesis).

3. to investigate the effect of female sex hormones on 5-HT, α-adrenergic, muscarinic and CGRP receptor responses in isolated blood vessels (See part III of the thesis).

4. to clone and characterize the porcine α_{2B}-adrenoceptor (See part IV of the thesis).

5. to assess whether there is a genetic basis for different clinical responses to sumatriptan (See part IV of the thesis).

Part II: Vascular effects mediated by 5-hydroxytryptamine, adrenergic and CGRP receptors

Chapter 4

Effects of current and prospective antimigraine drugs on the porcine isolated meningeal artery

Based on: **Suneet Mehrotra**, *Saurabh Gupta, Ingrid M. Garrelds, Carlos M. Villalón, Pramod R. Saxena, Ad J.J.C. Bogers and Antoinette MaassenVanDenBrink:* **Naunyn-Schmiedeberg's Archives of Pharmacology: In Press**

ABSTRACT

Vasoconstriction to agonists at serotonin (5-hydroxytryptamine; 5-HT) receptors and α-adrenoceptors, as well as vasodilatation induced by α-CGRP, have been well described in the porcine carotid circulation *in vivo*. The present study sets out to investigate the effects of current and prospective antimigraine drugs on the porcine meningeal artery segments *in vitro*. Unexpectedly, sumatriptan, ergotamine, dihydroergotamine, isometheptene and clonidine failed to contract the meningeal artery, but 5-HT, noradrenaline and phenylephrine induced concentration-dependent contractions. The contractions to 5-HT were competitively antagonized by the 5-HT_{2A} receptor antagonist ketanserin, whilst those to noradrenaline were antagonized by α_1- (prazosin), α_2- (rauwolscine and yohimbine) and $\alpha_{2C/2B}$- (OPC-28326) adrenoceptor antagonists. While dobutamine and salbutamol were ineffective, α-CGRP produced concentration-dependent relaxations that were antagonized by the $CGRP_1$ receptor antagonist olcegepant. In agreement with their lack of contractile effect, sumatriptan and ergotamine failed to influence forskolin-stimulated cyclic AMP accumulation in the porcine meningeal artery; in contrast, both compounds decreased forskolin-stimulated cyclic AMP accumulation in the human isolated saphenous vein, where they induced contractions. Finally, using RT-PCR, we could demonstrate the presence of mRNAs encoding for several 5-HT receptors (5-$HT_{1B,}$ 5-HT_{1D}, 5-$HT_{1F,}$ 5-HT_{2A} and 5-HT_7) and adrenoceptors (α_{1A}, α_{1B}, $\alpha_{1D,}$ α_{2A}, α_{2B}, α_{2C}, β_1 and β_2), as well as that for the calcitonin receptor like receptor, a component of the $CGRP_1$ receptor. These results suggest that (i) the porcine meningeal artery may not be involved in the vasoconstriction of the carotid vascular bed elicited by antimigraine drugs in anaesthetized pigs and (ii) the mismatch between the presence of receptor mRNA and the lack of response to sumatriptan, dobutamine and salbutamol implies that mRNAs for the 5-HT_{1B} receptor and β_1- and β_2-adrenoceptors are probably instable or their density is too low for being translated as receptor protein in sufficient quantities.

4.1 INTRODUCTION

The exact mechanisms involved in the pathophysiology of migraine remain elusive, but the headache in this neurovascular syndrome[1], involving central sensitization[2], seems to result from dilatation of cranial blood vessels following activation of the trigeminal system[3-5]. Indeed, several *in vivo* and *in vitro* vascular models[6-10] are based on meningeal or cerebral artery responses to antimigraine drugs.

An experimental pharmacological model that has proven highly predictive of antimigraine activity assesses constrictor effects on dilated carotid arteries and arteriovenous anastomoses in anaesthetized pigs[3,11-13]. The major advantages of this porcine model include that different vascular beds can be simultaneously studied to evaluate cranioselectivity of antimigraine drugs and that 5-HT receptor and α-adrenoceptor subtypes, often targeted for antimigraine action, show a very high (up to 90%) homology with their human counterparts[14-16]. However, despite the use of this model for many years, the involvement of the meningeal artery in the ergots- and triptans-induced porcine carotid (arteriovenous anastomotic) vasoconstriction remains unclear[17].

On this basis, the present study was designed to investigate the pharmacology of the porcine isolated meningeal artery with the aim of developing this vascular preparation as a convenient model for investigating compounds with putative antimigraine properties. Thus, we included in this study a number of standard agonists and antagonists, together with current antimigraine drugs acting as agonists at 5-$HT_{1B/1D}$ receptors sumatriptan;[4,18], α-adrenoceptors clonidine and isometheptene;[10,19,20] or both ergotamine and dihydroergotamine;[21,22] and a potential antimigraine drug, olcegepant(BIBN 4096BS[23]), which is a potent calcitonin gene-related peptide$_1$ ($CGRP_1$) receptor antagonist[24-26]. Lastly, as it turned out that all current antimigraine drugs proved ineffective in the porcine meningeal artery, we investigated their

effects on the human saphenous vein to establish their effectiveness in another tissue (positive control experiments).

The results of this study were presented at the XII congress of the International Headache Society, held in Kyoto, Japan on 9-12 October 2005[27].

4.2 MATERIALS AND METHODS

4.2.1 Tissue Preparation

Porcine meningeal artery

The heads of 63 female pigs (3-5 months) were obtained from a local slaughterhouse. Immediately after slaughter, the heads were stored in cold (0 to 4 °C) Krebs buffer solution (composition: 119 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11.1 mM glucose; pH 7.4) and transported to the laboratory. Upon arrival, the dura mater was removed from the skull and placed in cold Krebs buffer solution bubbled with 95% O_2 and 5% CO_2. Subsequently, branches of the middle meningeal artery (internal diameter: 100-250 µm) were dissected free and cut into 7-8 ring segments of 1-2 mm length.

Human isolated saphenous vein

The human saphenous veins were obtained postoperatively from 19 patients (15 men, 4 women; age: 58 to 83 years with a mean of 69±3 years) undergoing coronary bypass surgery at the cardiothoracic surgical unit of the Erasmus Medical Center. The tissue was immediately placed in cold (4 °C) physiological saline and transported within 15 min to the laboratory, where the vein was cleaned of connective tissue and placed in a cold, oxygenated Krebs solution (for composition, see above). Subsequently, 4-7 ring segments of 3-4 mm length were prepared. The medical ethics committee of the Erasmus Medical Centre Rotterdam approved the study.

4.2.2 Isometric tension measurements

Porcine meningeal artery segments were mounted in Mulvany myographs between two parallel titanium wires with a tension normalised to 90% of I_{100} (distance when transmural pressure equals 100 mmHg), thus achieving optimal conditions for active force development[28]. Human saphenous vein segments were suspended on stainless steel hooks in 15-ml organ baths and were stretched to a stable pretension of about 10 mN[7]. The myograph chambers and organ baths containing Krebs buffer solution (for composition, see above) were continuously aerated with 95% O_2 and 5% CO_2 at 37 °C. Vessel segments were allowed to equilibrate for at least 30 min and were washed every 15 min. Changes in tension were measured with isometric force transducers (Danish myotechnology A/S, Aarhus, Denmark for the porcine meningeal artery or Harvard, South Natick, MA, U.S.A. for the human saphenous vein) and recorded on a flatbed recorder (Servogor 124, Goerz, Neudorf, Austria).

Experimental procedures to construct concentration response curves were essentially those described earlier[8,29,30]. After the initial equilibration period, all segments were challenged with 30 mM KCl twice at 30-min intervals to verify the reproducibility of the responses. The integrity of the endothelium was then assessed by observing the relaxation to substance P (1-10 nM) in the porcine meningeal artery or to bradykinin (1 µM) in the human saphenous vein substance P is nearly inactive in this blood vessel;[7] after precontraction with the thromboxane A_2 receptor agonist U46619 (9,11-dideoxy-11α, 9α-epoxym ethano-prostaglandin $F_{2\alpha}$; 1 µM). After 30 min, 100 mM KCl was added to determine the reference contractile response in each segment. After washing, cumulative concentration response curves were constructed to the agonists in half-logarithmic steps. The concentration response curves to noradrenaline

were constructed in the presence of cocaine (1 μM) and corticosterone (3 μM) to prevent, respectively, neuronal and extra-neuronal re-uptake of catecholamines[31]. Relaxation to CGRP was studied in vascular segments precontracted by 30 mM KCl, which amounted to about 60% of the contraction to 100 mM KCl. Responses to dobutamine (β_1-adrenoceptor agonist) and salbutamol (β_2-adrenoceptor agonist) were studied in segments precontracted by 100 nM U46619. In experiments where an antagonist was used, the segments were incubated for 30 min with the antagonists before the concentration response curves to the agonists were performed. The control and antagonist experiments were paired and thus vessel segments for both experiments were used from the same animals. A list of agonists and antagonists with their target receptors is given in Table 4.2.

Each vessel segment from the porcine meningeal artery or human saphenous vein was exposed to a single agonist concentration response curve, either in the absence or presence of a particular antagonist concentration. The *n* refers to the number of pigs/human subjects from where the vessel segments were obtained; where multiple segments from the same blood vessel were used for a particular concentration response curve, the data obtained were averaged. However, segments from the same blood vessel were used for constructing concentration response curves to an agonist in the absence or presence of different antagonist concentrations.

4.2.3 Cyclic adenosine monophosphate (cAMP) measurements

Segments of porcine meningeal artery and saphenous vein were incubated in a Krebs buffer solution (same composition as above) containing isobutylmethylxanthine (IBMX, 0.5 mM) for 30 min. Forskolin (10 μM) was added with or without sumatriptan or ergotamine (10 μM each) and, after 5 min incubation, segments were snap-frozen in liquid nitrogen. The samples were stored at -80 °C until the cAMP assay was carried out, using an ELISA kit according to the instructions of the manufacturer (R&D Systems Europe Ltd., Abingdon, U.K.). It may be noted that only two compounds (*i.e.* sumatriptan and ergotamine) were used in this study because 15 mg of tissue, which is the total amount of meningeal artery that can be isolated from one pig, was needed to obtain one single cAMP measurement.

4.2.4 Reverse transcriptase-polymerase chain reaction (RT-PCR) studies

Porcine meningeal arteries were snap-frozen in liquid nitrogen and stored at -80 ˚C until use. The tissue was transferred to guanidium thiocyanate buffer, homogenised (Ultra-Turrax homogeniser, model T8; Janke & Kunkel Gmbh, Staufen, Germany) and the total RNA was extracted [32,33]. The RNA concentration was measured by UV absorbance at a wavelength of 260 nm using a Gene Quant RNA/DNA calculator (Pharmacia-LKB, Uppsala, Sweden) and the quality of RNA was assessed by formaldehyde agarose gel electrophoresis and DNA/protein ratio (OD_{260}/OD_{280}) ratio of >1.8. Prior to cDNA synthesis, RNA samples were treated with RNase-free DNase to eliminate contaminating genomic DNA (10 U 6 μg^{-1} RNA) for 25 min at 37 °C as per instruction of the manufacturer (Promega Benelux b.v., Leiden, The Netherlands). Total RNA (2 μg) was denatured at 65 °C and the first strand of cDNA was synthesised [14]. The cDNA thus synthesised was diluted 100-fold with autoclaved distilled water and stored at -20 °C until used as a PCR template. The quality of cDNA was verified by PCR amplification of β-actin (product size 625 bp) using specific oligonucleotide primers. For PCR amplification, a 20-μl reaction mixture containing the following components was prepared: 1.25 mM of each dNTP, 3 mM $MgCl_2$, PCR buffer (1 x PCR buffer: 10 mM Tris-HCl and 50 mM KCl; pH 8.3) Ampli Taq Gold™ enzyme (0.5 U), 0.5 μM of each of the forward and reverse primers and 2 μl of cDNA template. After a brief centrifugation, the enzyme was first activated for 5 min at 94 °C in a PCR thermocycler (model PTC-100™, MJ Research Inc., Watertown, MA, U.S.A.). PCR was carried out for 45 s at 94 °C, 45 s at 58 °C and 90 s at 72 °C with a total of 35 cycles. Finally, the reaction was extended for an additional 10 min. An aliquot of PCR reaction product was analysed on 2% agarose gel and visualized under UV light and digitally photographed. The forward and reverse primer sequences for the different mRNAs studied are listed in the Table 4.1.

Table 4.1. RT-PCR study to demonstrate the mRNA of the 5-HT, α- and β-adrenoceptor subtypes and $CGRP_1$ receptor in the porcine meningeal artery

Receptor	NCBI Gene bank Accession number	Length of nucleotide sequence	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Amplified Sequence	Size (Base pair)
5-HT_{1B}	NM214298	1170	CCTGCCCTGGAAAGTAGTAC	TGATGGGCATCACCAGGATG	135-316	180
5-HT_{1D}	NM214158	1134	GCCACTGTCCTTTCCAATGCCTTT	ACAGCCAGATGTCACACAGGAGT	151-346	196
5-HT_{1F}	NM214101	1101	CCAAGCAGGCTGGCATTATG	GCTTTGCGTTCTCTTGTGCC	410-872	462
5-HT_{2A}	NM214217	1413	ACCCTGCTTCTCCTTGCTTCATCT	CATGACAAGGAAACCCAGCAGCAT	189-384	196
5-HT_{7}	NM214085	1341	GCAGTGGCGTTCTACATCCC	CAAGGTGGTGGCTGCTTTCT	739-993	300
α_{1A}-AR	AJ251727	1401	AATCGGGTCTCAAGACCGACAAGT	TCACGGAGAAGTGCGTCTTGTTCT	656-778	123
α_{1B}-AR	AJ581297	976	TGGCGGTCATTCTGGTCATGTACT	TGAGGGTGTCCTCGTGAAAGTTCT	647-796	150
α_{1D}-AR	AJ250493	1428	TGCCCTTCTCTGCCATCTTTGAGA	AGCGGGTAGCTCACACCAATGTAT	254-410	157
α_{2A}-AR	NM214400	1353	CAACGTGCTTGTCATCATT	TGGGTGATGGACCAGTAA	150-410	261
α_{2B}-AR	DQ182110	1341	CTGCGGTCATCACCTTC	CAGCGCCAGGTACACTT	47-273	227
α_{2C}-AR*	NM000683	1386	TGCGCGCGCCACAGAACCTCTTCCT	ATGCAGGAGGACAGGATGTACCA	245-603	404
β_1-AR	AF042454	1407	AGTGCTGCGATTTCGTCACCAACA	AGCTGTCGATCTTCTTCACCTGCT	638-778	141
β_2-AR	AY526088	1257	TGGATTTCCATTGACGTGCTGTGC	ACACGACCCACACCATCAGAATGA	325-481	157
CALCRL	NM214095	1404	TCAAGAGCCTAAGTTGCCAAA	AATCAGCACAAATTCAATGCC	515-1107	560

AR, Adrenoceptor; CALCRL, Calcitonin receptor like receptor, a component of CGRP receptor. *, Human specific primer, because the porcine α_{2C}-adrenoceptor has not been cloned.

Table 4.2. List of compounds used and their pharmacological receptor target in the porcine meningeal artery and human saphenous vein.

Agonists		Antagonists	
Compound	**Receptor target**	**Compound**	**Receptor target**
5-HT[a]	5-HT_{1B}, 5-HT_{2A}	Ketanserin[a]	5-HT_{2A}
Sumatriptan[a]	5-HT_{1B}		
Noradrenaline[b]	α_1-, α_2-, β_1- β_2-adrenoceptors	Prazosin[b]	α_1-adrenoceptors
Phenylephrine[b]	α_1-adrenoceptor	Rauwolscine[b]	α_2-adrenoceptors
Clonidine[b]	α_2-adrenoceptor	Yohimbine[b]	α_2-adrenoceptors
BHT-933[b]	α_2-adrenoceptor	OPC28326[c]	$\alpha_{2C/2B}$-adrenoceptors
UK-14304[b]	α_2-adrenoceptor		
Isometheptene[d]	α_1- α_2-adrenoceptors		
Ergotamine[e]	α_1, α_2-adrenoceptors, 5-HT_{1B}		
Dihydroergotamine[e]	α_1, α_2-adrenoceptors, 5-HT_{1B}		
Dobutamine[b]	β_1-adrenoceptor		
Salbutamol[b]	β_2-adrenoceptor		
α-CGRP[f]	$CGRP_1$	Olcegepant[f]	$CGRP_1$

BHT-933, 6-ethyl-5, 6,7,8-tetrahydro-4H-oxazolo [4,5-d] azepin-2-amine dihydrochloride); UK-14304, 5-bromo-*N*-[4,5-dihydro-1*H*-imidazol-2-yl]-6-quinoxalinamine); OPC-28326, 4-(N-methyl-2-phenylethylamino)-1-(3,5-dimethyl-4-propionylamino benzoyl)piperidine.

[a], Hoyer *et al*.(1994), Tfelt-Hansen *et al*. (2000a); [b], Guimarães *et al.* (2001), Willems *et al.* (2003); [c], Orito *et al.* (2001), Sun *et al.* (2001) ; [d], Valdivia *et al.* (2004), Willems *et al.* (2001b); [e], Silberstein and McCrory (2003); Tfelt-Hansen *et al.* (2000b); [f], Doods *et al.* (2000).

4.2.5 Compounds

The compounds used in the present study (obtained from the sources indicated) were the following: BHT-933 (6-ethyl-5,6,7,8-tetrahydro-4H-oxazolo[4,5-d] azepin-2-amine dihydrochloride), clonidine, 5-hydroxytryptamine creatinine sulphate (serotonin; 5-HT), UK-14304 (5-bromo-6-(imidazoline-2-ylamino)quinoxaline), salbutamol, rauwolscine dihydrochloride, L-phenylephrine hydrochloride, yohimbine hydrochloride, dobutamine, noradrenaline, substance P acetate, U46619, isobutylmethylxanthine (IBMX), and corticosterone (all from Sigma, St. Louis, MO, U.S.A.); isometheptene mucate (Carnrick Laboratories, Cedar Knolls, NJ, U.S.A.); ketanserin tartrate (Janssen Pharmaceutica, Beerse, Belgium); ergotamine tartrate and dihydroergotamine tartrate (Novartis Pharma A.G., Basel, Switzerland); prazosin hydrochloride (Bufa Chemie b.v., Castricum, The Netherlands); sumatriptan succinate (GlaxoSmithKline, Ware, Herts, U.K.); h-αCGRP (Polypeptide, Wolfenbüttel, Germany); cocaine (Pharmacy Department, Erasmus Medical Center, Rotterdam); olcegepant (BIBN4096; Boehringer Ingelheim Pharma K.G., Biberach, Germany); KCl (Merck, Darmstad, Germany) and OPC-28326 ([4-(N-methyl-2-phenylethylamino)-1-(3,5-dimethyl-4-propionyl-aminobenzoyl)] piperidine hydrochloride monohydrate; Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd, Tokushima, Japan). Olcegepant was dissolved in acidified distilled water to obtain a 0.01 M stock solution and was then diluted with distilled water, while UK-14304, ergotamine, dihydroergotamine and corticosterone were dissolved in DMSO. All other compounds were dissolved in distilled water. Aliquots of stock solutions of all compounds were stored at -80 °C; these were thawed on the day of use, after which the remaining volume was discarded. The above vehicles had no effect on the agonist-induced responses.

4.2.6 Data and statistical analyses

Contractile responses elicited by the agonists are expressed as % contraction of the tone induced by 100 mM KCl. Relaxant responses are expressed as % of the tone induced by either U46619 (1 μM in the case of substance P; 100 nM in the case of salbutamol and dobutamine) or KCl (30 mM in the case of CGRP). The individual concentration response curves were analysed using non-linear regression analysis (software Graph Pad Prism 3.01; Graph Pad Software Inc., San Diego, CA, U.S.A.). The potency of the agonists was expressed as pEC_{50}. It is important to note that when the E_{max} of an agonist was not reached, the response at its highest concentration was considered as E_{max}, except for antagonist experiments, where the E_{max} in the presence of a given antagonist was assumed to be equal to the control E_{max} in case a plateau was not reached. The potency of the antagonists was calculated using the Schild equation[34]: pK_b=log(DR-1)-log[B], where DR is the ratio of agonist concentrations eliciting half maximal response with and without antagonist and [B] represents molar concentration of the antagonist. When the criteria for a competitive interaction between agonist and antagonist were met, i.e. parallel shifts (slopes of agonist curves are the same), E_{max} values being the same and Schild slopes not significantly different from one, the values obtained were considered as true pK_b. In all other cases, we used the term apparent pK_b.

Statistical significance for the functional experiments was determined by unpaired Student's *t*-test. Since the results obtained with the cAMP measurements were variable and a normal Gaussian distribution could not be assumed, these results were analysed using nonparametric statistical tests (Kruskal Wallis test for unpaired measurements or Friedman test for paired measurements in case of porcine meningeal artery and human saphenous vein, respectively, both followed by Dunn's post hoc test). Differences were considered to be significant at $p \leq 0.05$. All data are presented as means±SEM.

4.3 RESULTS

4.3.1 Functional studies

4.3.1.1 Porcine isolated meningeal artery

The thromboxane A_2 receptor agonist U46619 elicited contractions of the porcine meningeal artery (E_{max} 159.4±13, pEC_{50}: 6.83±0.13). The endothelium-dependent relaxant response to substance P was 31±4% of the precontraction induced by 1 μM U46619.

As shown in Figure 4.1, 5-HT (E_{max}: 95±17%; pEC_{50}: 7.44±0.22; *n*=5), noradrenaline (E_{max}: 154±21%; pEC_{50}: 5.83±0.20; *n*=6) and the α_1-adrenoceptor agonist phenylephrine (E_{max}: 163±10%; pEC_{50}: 5.63±0.02; *n*=6) contracted the porcine meningeal artery. On the other hand, none of the antimigraine drugs that we investigated, *i.e.* sumatriptan (5-$HT_{1B/1D}$ receptor agonist), ergotamine and dihydroergotamine (both $\alpha_{1/2}$-adrenoceptor and 5-$HT_{1B/1D}$ receptor agonists), isometheptene ($\alpha_{1/2}$-adrenoceptor agonist) and clonidine (α_2-adrenoceptor agonist), contracted the meningeal artery. Similarly, the α_2-adrenoceptor agonists UK-14304 and BHT-933 also failed to induce any discernible contraction (E_{max}: <2% of the contraction to 100 mM KCl). Since in some tissues the response to 5-$HT_{1B/1D}$ receptor agonists needs to be 'unmasked' by a threshold contraction by another agent[35-38], we also investigated the effects of sumatriptan in the presence of 10 nM U46619, eliciting a 'threshold' contraction of 3.3±0.7% of KCl-induced contraction. Even under this experimental condition, sumatriptan failed to induce a distinguishable contraction (Figure 4.1, upper left panel). This was also true for BHT-933, which failed to contract the blood vessel in the presence of 10 nM U46619 as well as a combination of 10 nM 5-HT and 10 nM phenylephrine (Figure 4.1, lower right panel).

To provide further background for the lack of contractile effect of sumatriptan and ergot alkaloids in the porcine meningeal artery, we pharmacologically characterized the contractions to the endogenous ligands, 5-HT and noradrenaline. 5-HT-induced contractions were competitively antagonized by

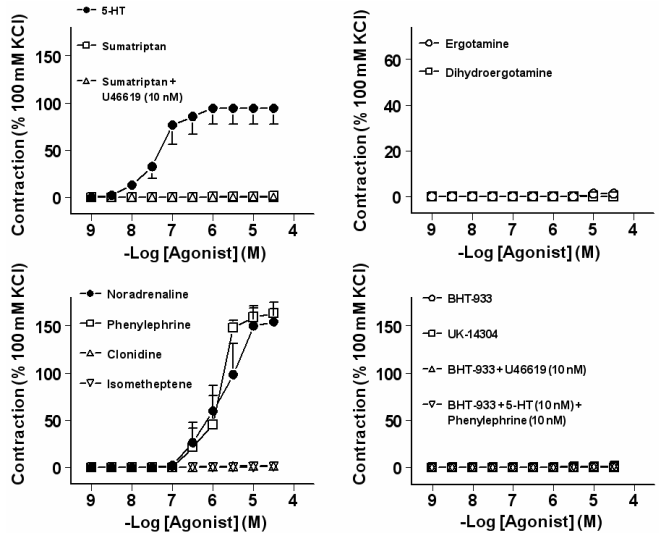

Figure 4.1: Concentration response curves in the porcine meningeal artery to different compounds. Upper left panel: 5-HT (*n*=5) and sumatriptan (5-HT_{1B} receptor agonist) alone (*n*=7) and in the presence of 10 nM U46619 (*n*=4); Upper right panel: ergotamine and dihydroergotamine (α-adrenoceptor and 5-HT_{1B} receptor agonists; *n*=5 each); lower left panel: noradrenaline (*n*=6), phenylephrine (α_1-adrenoceptor agonist; *n*=6), clonidine (α_2-adrenoceptor agonist; *n*=6) and isometheptene (α-adrenoceptors agonist; *n*=5); lower right panel: BHT-933 and UK-14304 (α_2-adrenoceptor agonists; *n*=6 and 7, respectively) and BHT-933 in the presence of 10 nM each of U46619 (*n*=4) or 5-HT and phenylephrine. (*n*=4).

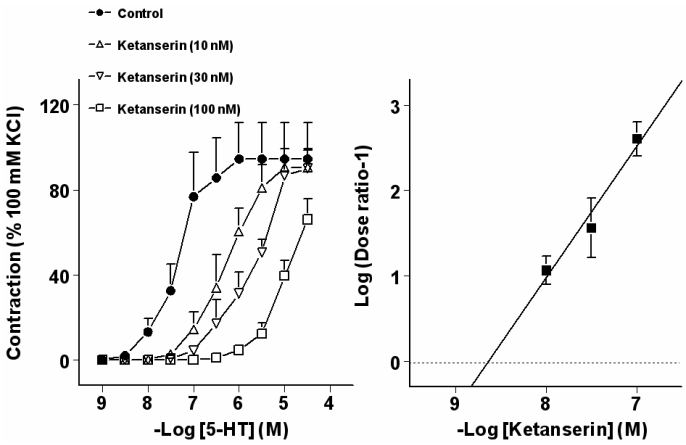

Figure 4.2: Concentration response curves in the porcine meningeal artery to 5-HT alone (control; *n*=5) and after incubation with the 5-HT_{2A} receptor antagonist ketanserin (10, 30 and 100 nM; *n*=5 each, left panel), as well as the corresponding Schild plot (right panel).

the 5-HT_{2A} receptor antagonist ketanserin (Figure 4.2). The pK_b of ketanserin was found to be 8.71±0.20, with a slope (1.54±0.20) not different from unity (*n*=5). The effects of α_1- (prazosin), α_2- (yohimbine and rauwolscine) and the relatively selective $\alpha_{2C/2B}$- (OPC-28326) adrenoceptor antagonists on the concentration-response curve obtained with noradrenaline in the meningeal artery are shown in Figure 4.3. In the case of prazosin, the lowest concentration (1 nM) did not affect noradrenaline-induced contractions, but higher prazosin concentrations caused either a rightward shift (10 nM) or a complete blockade (100 nM)

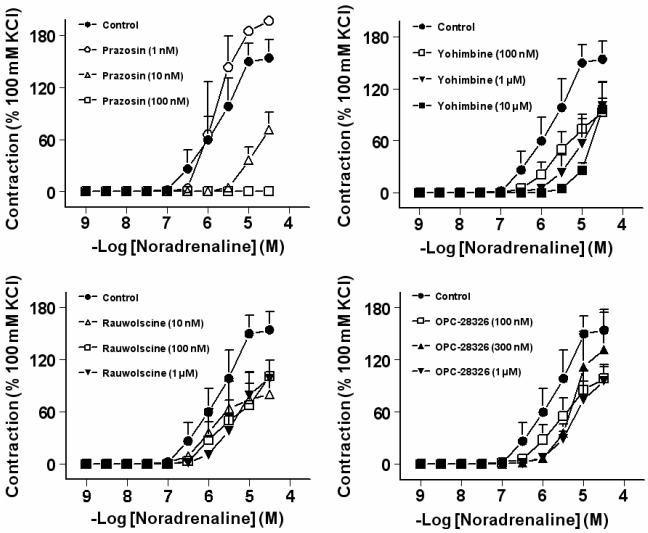

Figure 4.3: Concentration response curves to noradrenaline in the porcine meningeal artery. Contractions were induced by noradrenaline alone (control; *n*=6) and after incubation with the α_{1}-adrenoceptor antagonist prazosin (1, 10 and 100 nM; *n*=4, 4 and 5, respectively; upper left panel), the α_{2}-adrenoceptor antagonists, yohimbine (100 nM, 1 and 10 µM; *n*=6, 5 and 3, respectively; upper right panel) and rauwolscine (10, 100 nM and 1 µM; *n*=3, 6 and 6, respectively; lower left panel), and the $\alpha_{2C/2B}$-adrenoceptor antagonist OPC-28326 (100, 300 nM and 1 µM; *n*=5, 4 and 6, respectively; lower right panel).

of noradrenaline-induced contractions. Schild analysis using the 10 nM concentration of prazosin and slope constrained to unity revealed an apparent pK_b of 9.08±0.53 (*n*=5). For yohimbine, rauwolscine and OPC-28326, Schild analysis could not be performed on individual experiments, since the lowest of the three concentrations in some cases did not induce a sufficiently large shift. However, using the mean of individual EC_{50} values, the apparent pK_bs of yohimbine (100 nM), rauwolscine (100 nM) and OPC-28326 (1 µM) were found to be 8.05 (slope: 0.39), 8.25 (slope: 0.25) and 7.25 (slope: 0.87), respectively.

To study relaxant responses, porcine meningeal artery segments were precontracted and responses to β_{1}- (dobutamine) and β_{2}- (salbutamol) adrenoceptor agonists and to CGRP elicited (Figure 4.4). Neither dobutamine nor salbutamol induced any discernible relaxation of the meningeal artery, but CGRP caused concentration-dependent relaxations (E_{max}: 59±4%; pEC_{50}: 7.66±0.13; *n*=5). This relaxant response to CGRP was effectively antagonized by the $CGRP_{1}$ receptor antagonist olcegepant (apparent pK_b: 8.09±0.33, *n*=3).

4.3.1.2 Human isolated saphenous vein

U46619 (1 µM) contracted human saphenous vein segments (139±2 % of the contraction to 100 mM KCl) and the endothelium-dependent relaxation by bradykinin (1 µM) amounted to 33±5% of the precontraction induced by U46619.

Although sumatriptan, ergotamine, dihydroergotamine, UK-14304 and BHT-933 were ineffective in the porcine meningeal artery, all these compounds, as well as noradrenaline and phenylephrine, evoked concentration-dependent contractions in the human isolated saphenous vein (Figure 4.5). The E_{max} (% of the contraction to 100 mM KCl) and pEC_{50}, respectively, were: sumatriptan (65±14% and 7.13±0.33, *n*=4), ergotamine (145±54% and 8.06±0.38, *n*=4), dihydroergotamine (65±9% and 8.01±0.06, *n*=3), noradrenaline (152±22% and 7.10±0.37, *n*=6), phenylephrine (98±13% and 6.85±0.12, *n*=5), BHT-933 (80±17% and 7.06±0.21, *n*=6) and UK-14304 (81±5%and 7.69±0.21, *n*=6).

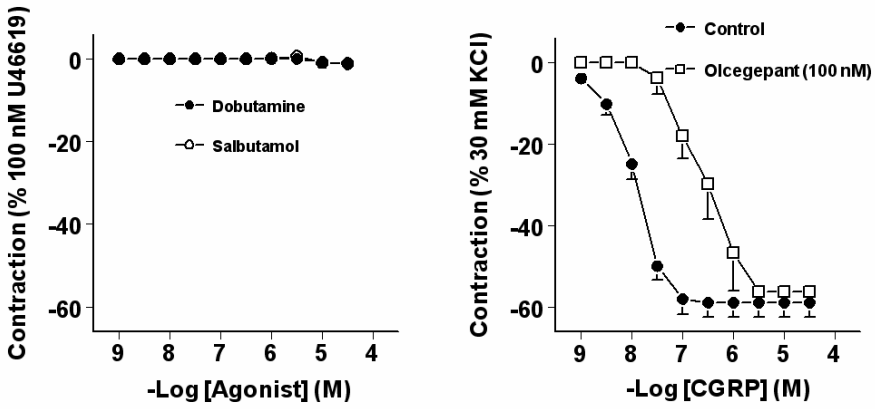

Figure 4.4: Concentration response curves in the porcine meningeal artery to β_1- (dobutamine; *n*=4) and β_2- (salbutamol; *n*=4) adrenoceptor agonists (left panel) and CGRP alone (control; *n*=5) and after incubation with the $CGRP_1$ receptor antagonist olcegepant (100 nM; *n*=3; right panel).

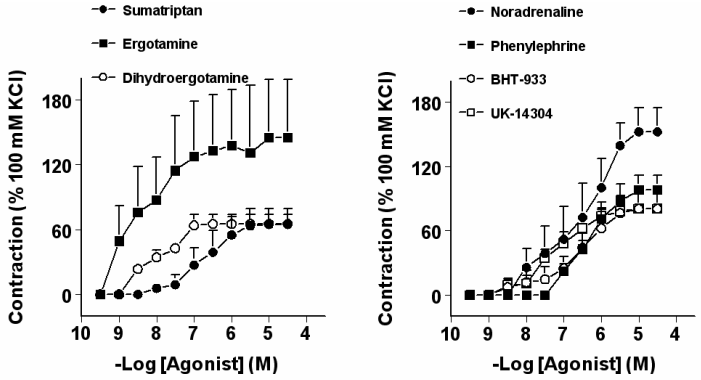

Figure 4.5: Concentration response curves in the human isolated saphenous vein to sumatriptan (*n*=4), ergotamine (*n*=4) and dihydroergotamine (*n*=3 each; left panel) as well as to noradrenaline (*n*=6), phenylephrine (*n*=5), BHT-933 (*n*=6) and UK-14304 (*n*=6; right panel).

4.3.2 cAMP measurements

In the porcine meningeal artery (Figure 4.6, left panel), forskolin (10 µM) increased the cAMP concentration. This increase was not significantly modified by either sumatriptan or ergotamine. In contrast, in the human saphenous vein (Figure 4.6, right panel), forskolin-induced cAMP accumulation was significantly reduced by both sumatriptan and ergotamine.

4.3.3 RT-PCR studies in porcine meningeal arteries

Using porcine-specific forward and reverse primers designed on the basis of porcine specific nucleotide sequences of the receptors reported in the NCBI Gene bank (except the α_{2C}-adrenoceptor; Table 4.1), PCR products corresponding in size were amplified in porcine meningeal artery (Figure 4.7). Thus, we could demonstrate the mRNA encoding for the subtypes of α_1- (α_{1A}, α_{1B}, α_{1D}), α_2- (α_{2A}, α_{2B}, α_{2C}) and β- (β_1, β_2) adrenoceptors and 5-HT receptors (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2A} and 5-HT_7), as well as for the calcitonin receptor like receptor (a part of the CGRP receptor) and the housekeeping gene β-actin. The negative control, where cDNA was replaced by water, did not show amplification, ruling out any contamination in PCR preparations.

Table 4.2. List of compounds used and their pharmacological receptor target in the porcine meningeal artery and human saphenous vein.

Agonists		Antagonists	
Compound	**Receptor target**	**Compound**	**Receptor target**
5-HT[a]	5-HT_{1B}, 5-HT_{2A}	Ketanserin[a]	5-HT_{2A}
Sumatriptan[a]	5-HT_{1B}		
Noradrenaline[b]	α_1-, α_2-, β_1- β_2-adrenoceptors	Prazosin[b]	α_1-adrenoceptors
Phenylephrine[b]	α_1-adrenoceptor	Rauwolscine[b]	α_2-adrenoceptors
Clonidine[b]	α_2-adrenoceptor	Yohimbine[b]	α_2-adrenoceptors
BHT-933[b]	α_2-adrenoceptor	OPC28326[c]	$\alpha_{2C/2B}$-adrenoceptors
UK-14304[b]	α_2-adrenoceptor		
Isometheptene[d]	α_1- α_2-adrenoceptors		
Ergotamine[e]	α_1, α_2-adrenoceptors, 5-HT_{1B}		
Dihydroergotamine[e]	α_1, α_2-adrenoceptors, 5-HT_{1B}		
Dobutamine[b]	β_1-adrenoceptor		
Salbutamol[b]	β_2-adrenoceptor		
α-CGRP[f]	$CGRP_1$	Olcegepant[f]	$CGRP_1$

BHT-933, 6-ethyl-5, 6,7,8-tetrahydro-4H-oxazolo [4,5-d] azepin-2-amine dihydrochloride); UK-14304, 5-bromo-*N*-[4,5-dihydro-1*H*-imidazol-2-yl]-6-quinoxalinamine); OPC-28326, 4-(N-methyl-2-phenylethylamino)-1-(3,5-dimethyl-4-propionylamino benzoyl)piperidine.

[a], Hoyer *et al.*(1994), Tfelt-Hansen *et al*. (2000a); [b], Guimarães *et al.* (2001), Willems *et al.* (2003); [c], Orito *et al.*(2001), Sun *et al.*(2001) ; [d], Valdivia *et al.* (2004), Willems *et al.* (2001b); [e], Silberstein and McCrory (2003); Tfelt-Hansen *et al.* (2000b); [f], Doods *et al.* (2000).

4.4 DISCUSSION

4.4.1 Pharmacological responses of the porcine meningeal artery

The most unexpected finding of this study was that a number of antimigraine drugs (sumatriptan, ergotamine, dihydroergotamine, isometheptene and clonidine) as well as UK-14304 and BHT-933 were found ineffective in the porcine isolated meningeal artery, particularly in view of the fact that these compounds are well known to constrict porcine cephalic arteries and arteriovenous anastomoses *in vivo*[4,10,11,22,39]. Moreover, triptans[4,7,40-43], ergot alkaloids[44] and as well as noradrenaline[45] contract human isolated meningeal and/or basilar arteries. One may thus argue that the ineffectiveness of these drugs in the porcine isolated meningeal artery is possibly due to extraneous factors, such as tissue handling destroying receptor protein or problems with shelf life of the compounds used. However, this is unlikely, porcine meningeal artery segments clearly responded to the endogenous ligands noradrenaline, 5-HT and CGRP as well as to phenylephrine demonstrating that, at least, α-adrenoceptors, 5-HT_{2A} and $CGRP_1$ receptors are functionally active. In addition, studies from our laboratory using pig hearts obtained from the same slaughter house have shown that coronary artery segments responded to a number of compounds, including angiotensin, angiotensin converting enzyme inhibitors, bradykinin, and nitric oxide donors[46-49]. Similarly, we have ensured that not only noradrenaline and phenylephrine, but also sumatriptan, ergotamine, dihydroergotamine, BHT-933 and UK-14304 did indeed contract another blood vessel, namely the human isolated saphenous vein (see Figure 4.5). The latter findings are in agreement with previous investigations with sumatriptan [7], noradrenaline and phenylephrine[50].

4.4.2 5-HT receptors

It is well known that in some blood vessels, including the human mammary[38] and coronary[51], guinea-pig iliac[35] and rabbit renal[36] arteries, the contractions to the 5-$HT_{1B/1D}$ receptor agonists, such as sumatriptan[4,18], manifest only in the presence of a threshold concentration of another vasoconstrictor (*e.g.* U46619). In the porcine meningeal artery (present study), sumatriptan failed to produce a discernible contraction of the porcine meningeal artery even in the presence of U46619. Since 5-HT-induced porcine meningeal artery contractions were antagonized by ketanserin with a pK_b of 8.71±0.20 and slope not different from unity (Figure 4.2), it seems that this vessel has predominantly 5-HT_{2A} rather than 5-HT_{1B} receptors.

4.4.3 Adrenoceptors

Noradrenaline induced concentration-dependent contractions, which were amenable to potent blockade by the α_1-adrenoceptor antagonist prazosin as well as the α_2-adrenoceptor antagonist rauwolscine. The relatively lower blocking potency of the classical α_2-adrenoceptor antagonist yohimbine observed in our study is consistent with its moderate affinities at α_1- (pK_i 6.39) and α_2- (pK_i 6.82) adrenergic binding sites[52]. Further, the $\alpha_{2C/2B}$-adrenoceptor antagonist OPC-28326[53,54] also clearly antagonized the contractions to noradrenaline. These findings suggest that the contractile effect of noradrenaline is mediated by both α_1- and α_2- (probably, $\alpha_{2C/2B}$-) adrenoceptors. However, isometheptene, which acts mainly as an indirect adrenoceptor agonist[10,20] and, in particular, the α_2-adrenoceptor agonists clonidine, UK-14304 and BHT-933[19,55] were inactive. The ergot alkaloids (ergotamine and dihydroergotamine), which have an agonist action at both 5-HT_{1B} and α-adrenoceptors[21,22,39,56,57], also failed to contract the porcine isolated meningeal artery. It would, therefore, appear that the density of α_2-adrenoceptors is such that it allows a response to noradrenaline, which apparently has a higher potency and efficacy compared to the other compounds used.

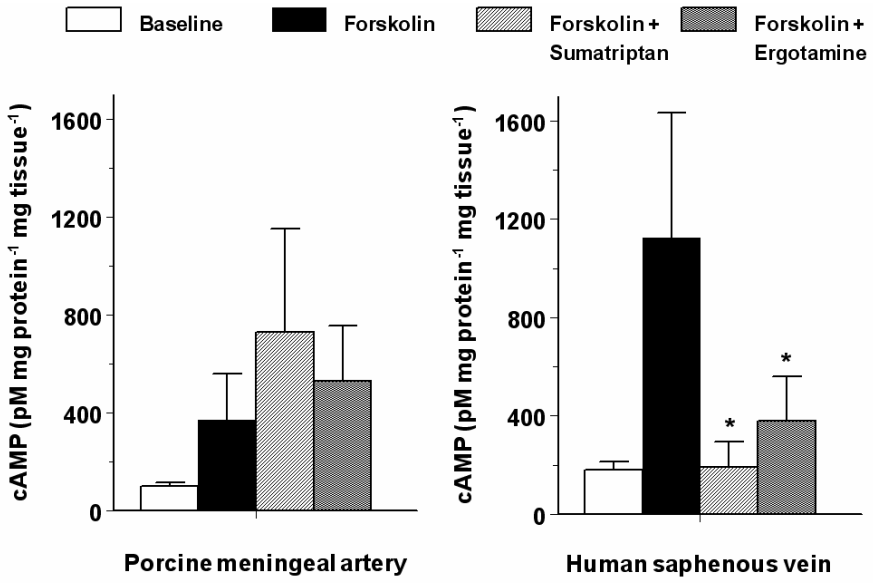

Figure 4.6: cAMP Effect of sumatriptan (10 μM) and ergotamine (10 μM) on forskolin (10 μM)-induced increases in cAMP in the porcine meningeal artery (*n*=3-6; left panel) and human saphenous vein (*n*=7 each; right panel). *, p<0.05 compared to forskolin.

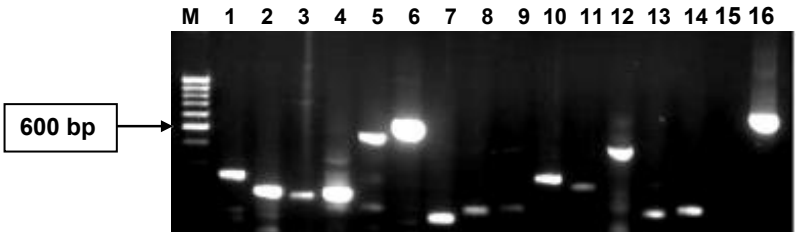

Figure 4.7: Agarose gel electrophoresis of PCR amplified products derived from cDNA obtained from the porcine meningeal artery. The different lanes marked on top of the panels denote: M, 100 bp DNA ladder marker; 1. 5-HT_7 receptor; 2. 5-HT_{2A} receptor; 3. 5-HT_{1B} receptor; 4. 5-HT_{1D} receptor; 5. 5-HT_{1F} receptor; 6. calcitonin receptor like receptor (a CGRP receptor component); 7. α_{1A}-adrenoceptor; 8. α_{1B}-adrenoceptor; 9. α_{1D}-adrenoceptor; 10. α_{2A}-adrenoceptor; 11. α_{2B}-adrenoceptor; 12. α_{2C}-adrenoceptor; 13. β_1-adrenoceptor; 14. β_2-adrenoceptor; 15. negative control (water); and 16. House-keeping gene β-actin. Please note that the corresponding individual receptor product sizes were as expected (see Table 3; *n*=3 for each receptor subtype).

As far as the β-adrenoceptors are concerned, no evidence for their presence was found since both β_1- (dobutamine) and β_2- (salbutamol) adrenoceptor agonists were devoid of any response in the porcine meningeal artery.

4.4.4 CGRP receptors

In accordance with previous observations in a number of other isolated blood vessels[25,58,59], α-CGRP induced concentration-dependent relaxations which were antagonized by olcegepant, a CGRP receptor antagonist[24,26] with prospective antimigraine action[23].

4.4.5 Transduction mechanisms

It is well known that the vasoconstrictor effect of sumatriptan[4,18] and ergotamine at least partly,[21,22] is mediated by the 5-HT_{1B} receptor, which is negatively coupled to cAMP formation[18]. Therefore, 5-HT_{1B} receptor agonists inhibit forskolin-stimulated cyclic AMP accumulation in cells and tissues expressing the 5-HT_{1B} receptor[18,40,60]. In conformity with their lack of vasoconstrictor action, both sumatriptan and ergotamine failed to reduce forskolin-stimulated cAMP accumulation in the porcine meningeal artery. On the other hand, these compounds significantly inhibited the cAMP accumulation in the human saphenous vein (Figure 4.6), which did contract in response to these compounds (Figure 4.5).

4.4.6 Molecular components of various receptors in the porcine meningeal artery

RT-PCR studies demonstrated the presence of mRNA for 5-HT (5-HT_{1B}, 5-HT_{1D}, 5-$HT_{2A,}$ 5-HT_7) receptors, α- (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}) and β- (β_1, β_2) adrenoceptors and the calcitonin receptor like receptor (a component of CGRP receptors) in the porcine meningeal artery. The presence of mRNA encoding the 5-HT_{1B} receptor[42,61], as well as the 5-HT_{1B} receptor protein[43], has been demonstrated in the human meningeal artery. In addition, the human blood vessel shows the presence of the mRNA for all essential components required for a functional $CGRP_1$ receptor[49], but, to the best of our knowledge, mRNA encoding for the adrenoceptor subtypes has not been demonstrated.

The presence of mRNA for the 5-HT_{2A} receptor, α-adrenoceptor subtypes and calcitonin receptors like receptor are in agreement with the observed contractile (5-HT, noradrenaline and phenylephrine) and relaxant (CGRP) effects in the porcine meningeal artery. There is, however, a mismatch between the presence of receptor mRNA and the lack of response to sumatriptan (5-HT_{1B} receptor), dobutamine (β_1-adrenoceptor) and salbutamol (β_2-adrenoceptor). Therefore, these mRNAs detected in the porcine meningeal artery are probably instable or their density is too low for being translated as receptor protein in sufficient quantities.

4.4.7 Apparent discrepancy with previous reports in anaesthetized pigs

The inability of sumatriptan, ergotamine, dihydroergotamine, clonidine and BHT-933 to contract porcine isolated meningeal artery segments seems to be at variance with our previous reports that sumatriptan[62], ergotamine and dihydroergotamine[57], clonidine[63] and BHT-933[39] elicit a potent constrictor response within the carotid vascular bed in anaesthetized pigs. In this respect, two possible explanations can account for their apparent discrepancy. Firstly, there is an obvious difference between *in vivo* and *in vitro* studies; thus, under *in vivo* conditions, the presence of a sympathetic tone and of circulating endogenous mediators may 'unmask' or augment the responses to exogenously injected compounds. However, this possibility seems unlikely, since U46619 failed to augment the response to sumatriptan and BHT-933. Secondly, the effects observed on the porcine isolated meningeal artery are typical of a 'conducting' vessel, whilst those observed in the carotid vascular bed of anaesthetized pigs basically reflect constriction of the corresponding arteriovenous anastomoses[62]. In keeping with this possibility, more than a decade ago our group reported that porcine dural arteriovenous anastomoses are not much affected by sumatriptan, ergotamine or dihydroergotamine[17]. Interestingly, another study has reported constriction of the porcine meningeal artery to sumatriptan *in situ*[40], which is apparently also in contrast with our findings. However, in this study the whole meningeal arterial bed was perfused, while we only studied a segment of the conducting portion of the meningeal artery.

Taken together, our results indicate that the 'conducting portion' of the porcine meningeal artery, as used in the present study, is not involved in the vasoconstriction within the carotid vascular bed elicited in anaesthetized pigs by sumatriptan and ergot alkaloids, as well as agonists acting at α_2-adrenoceptors, a potential antimigraine target. In contrast, the prospective antimigraine drug olcegepant did block relaxations to CGRP in the porcine meningeal artery. Thus, to identify potential antimigraine drugs, it seems appropriate to consider more distal portions of the meningeal artery and cranial arteriovenous anastomoses; in view of the high predictive value of the porcine carotid model for antimigraine efficacy, these blood vessels may eventually become important targets in the advancement of novel antimigraine drugs.

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Chapter 5

A61603-induced contractions of the porcine meningeal artery are mediated by α_1- and α_2-adrenoceptors

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ABSTRACT

It has recently been shown that A61603 (N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydro-naphthalen-1-yl]methane sulphonamide), a potent α_{1A}-adrenoceptor agonist, decreased carotid artery conductance in anaesthetized pigs by a novel non-adrenergic mechanism. In this study, we set out to pharmacologically characterize A61603-induced contractions of the porcine isolated meningeal artery. While the maximum contractile responses of the artery were similar, A61603 (E_{max}: 183±23% of 100 mM KCl; pEC_{50}: 7.25±0.18) was more potent than noradrenaline (E_{max}: 154±11%; pEC_{50}: 5.75±0.17) or phenylephrine (E_{max}: 163±26%; pEC_{50}: 5.63±0.02). Prazosin, as well as rauwolscine and yohimbine, antagonised the contractions to A61603. The 5-HT receptor antagonists, GR127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl) phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide) and ritanserin failed to affect the responses to A61603 but methiothepin, which, in addition, has a high affinity for α-adrenoceptors, proved an effective antagonist. The A61603-induced responses were suppressed by the cAMP stimulator forskolin, but not by the protein kinase C inhibitor chelerythrine. Our results suggest that the contraction of porcine isolated meningeal artery by A61603 is mediated via a mixed population of α_1- (probably α_{1A}) and α_2-adrenoceptors and that the adenylate cyclase, but not the diacylglycerol, pathway seems to be involved.

5.1 INTRODUCTION

The acutely-acting antimigraine drugs triptans[1-3] and ergot alkaloids[4] potently and selectively constrict cephalic arteriovenous anastomoses in anaesthetized pigs. Yet sumatriptan, ergotamine and dihydroergotamine failed to constrict the porcine isolated meningeal artery[5]. These drugs have an agonist action at the $5\text{-}HT_{1B}$ receptor [6,7], but the ergot alkaloids also act at α-adrenoceptors[2,8-10]. Both α_1- and α_2-adrenoceptors have been identified to mediate constriction of porcine cephalic arteriovenous anastomoses[11] and their subtypes are proposed as targets for novel antimigraine action[12,13].

A61603(N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methane sulphonamide) is a potent and selective α_{1A}-adrenoceptor agonist[14,15] which, like triptans and ergot alkaloids, constricted porcine cephalic arteriovenous anastomoses[16]. This effect of A61603 was, however, poorly modified by the adrenoceptor antagonists, 5-methylurapidil (α_{1A}) and prazosin (α_1), either alone or in combination with rauwolscine (α_2), to suggest the involvement of a novel non-adrenergic mechanism[16]. The present study in the porcine isolated meningeal artery was designed to further characterize the mechanism(s) of the contractile action of A61603 by using: (i) antagonists at α_1- (prazosin) and α_2- (rauwolscine and yohimbine) adrenoceptors as well as at $5\text{-}HT_{1B/1D}$ (GR127935, N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]- 2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide), $5\text{-}HT_{2A/2B/2C}$ (ritanserin) and $5\text{-}HT_{1B/1D/2A/2B/2C}$ and $\alpha_{1/2}$ (methiothepin) receptors (Table 5.1); and (ii) the protein kinase C inhibitor chelerythrine[17,18] as well as the cAMP stimulator forskolin[19,20].

5.2 MATERIALS AND METHODS

5.2.1 Tissue preparation and isometric tension measurements

The heads of 33 female pigs (3-5 months) were obtained from a local slaughterhouse. Immediately after slaughter, the heads were stored in cold (0 to 4 °C) Krebs buffer solution (composition: 119 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11.1 mM glucose; pH 7.4) and transported to the laboratory. Upon arrival, the dura mater was removed from the skull and placed in cold Krebs buffer solution bubbled with 95% O_2 and 5% CO_2. Subsequently, branches of the middle

meningeal artery (internal diameter: 100-250 µm) were dissected free and cut into 7-8 ring segments of 1-2 mm length. These segments were mounted in Mulvany myographs between two parallel titanium wires with a tension normalised to 90% of I_{100} (distance when transmural pressure equals 100 mmHg), thus achieving optimal conditions for active force development[21]. The myograph chambers and organ baths containing Krebs buffer solution (see above) were continuously aerated with 95% O_2 and 5% CO_2 at 37 °C. Vessel segments were allowed to equilibrate for at least 30 min and were washed every 15 min. Changes in tension were measured with isometric force transducers (Danish myotechnology A/S, Aarhus, Denmark) and recorded on a flatbed recorder (Servogor 124, Goerz, Neudorf, Austria).

5.2.2 Experimental procedures

Experimental procedures to construct concentration response curves were essentially those described earlier[22,23]. After the initial equilibration period, all segments were challenged with 30 mM KCl twice at 30-min intervals to verify the reproducibility of the responses. After 30 min, 100 mM KCl was added to determine the reference contractile response in each segment. After washing, cumulative concentration response curves were constructed to the agonists in half-logarithmic steps. The concentration response curves to noradrenaline were constructed in the presence of cocaine (1 µM) and corticosterone (3 µM) to prevent, respectively, neuronal and extra-neuronal re-uptake of catecholamines[24]. In antagonist experiments, segments were incubated for 30 min with the antagonist before an agonist concentration response curve was constructed. Each vessel segment was exposed to a single agonist concentration response curve, either in the absence or presence of a particular antagonist concentration. Except for the experiments on A61603 in the absence and presence of methiothepin, GR127935 and ritanserin, the control experiment and antagonist experiments were performed in a paired parallel set-up, i.e., segments from the same pig were used for concentration response curves with or without antagonist. The *n* refers to the number of pigs from which the vessel segments were obtained.

Contractile responses elicited by the agonists are expressed as % contraction of the tone induced by 100 mM KCl. The individual agonist concentration response curves were analysed using non-linear regression analysis (software Graph Pad Prism 3.01; Graph Pad Software Inc., San Diego, CA, U.S.A.). The agonist potency was expressed as pEC_{50}, i.e. the negative logarithm of the molar concentration eliciting half of the maximum response (E_{max}). In case a plateau was not reached, the response at the highest agonist concentration was considered as E_{max}, except for experiments in the presence of an antagonist, where the E_{max} was assumed to be equal to the respective control E_{max}, in case a stable plateau was not reached. The potency of the antagonists was calculated using the Schild equation[25]: pK_b=log(DR-1)-log[B], where DR is the ratio of agonist concentrations eliciting half the maximal response with and without an antagonist and [B] represents the molar concentration of the antagonist. In case of the concentration response curves to A61603 in the absence and presence of prazosin, three concentrations of antagonist that shifted the control concentration response curve were available, and thus the pA_2 and Schild plot slope were calculated[25].

5.2.3 Compounds

The compounds used in the present study (obtained from the sources indicated) were: A61603 (Tocris Cookson Ltd., Avonmouth, U.K.); cocaine (Pharmacy Department, Erasmus Medical Center, Rotterdam); prazosin hydrochloride (Bufa Chemie b.v., Castricum, The Netherlands); chelerythrine chloride (Calbiochem, Breda, The Netherlands), GR127935 (gift: GlaxoSmithKline, Stevenage, Herts, U.K.); methiothepin maleate (gift: Hoffman La Roche b.v., Mijdrecht, The Netherlands); KCl (Merck, Darmstad, Germany); 5-HT creatinine sulphate, corticosterone, forskolin, (-)-noradrenaline bitartrate, (-)-phenylephrine hydrochloride, rauwolscine dihydrochloride, ritanserin, substance P and yohimbine hydrochloride (all from Sigma Chemical Co., St. Louis, MO, U.S.A.). GR127935 was dissolved according to the instructions of the supplier

by heating the dispersion in distilled water to about 70 °C for 10 s and then allowing cooling down at room temperature.

5.3 RESULTS

5.3.1 Agonist responses

The concentration response curves in the porcine meningeal artery to A61603 (E_{max}: 183±23% of 100 mM KCl; pEC_{50}: 7.25±0.18; n=10), the endogenous ligand noradrenaline (E_{max}: 156±16%; pEC_{50}: 5.75±0.17; n=8) and the α_1-adrenoceptor agonist phenylephrine (E_{max}: 163±10%; pEC_{50}: 5.63±0.02; n=6) are shown in Figure 5.1. While the E_{max} of the three agonists was similar, the potency of A61603 was approximately 1.5 log units higher than that of noradrenaline and phenylephrine.

5.3.2 Effects of antagonist at α-Adrenoceptor and 5-HT receptor on the responses to A61603

The effects of α-adrenoceptor antagonists on the contractile response to A61603 in the porcine meningeal artery are shown in Figure 5.2. The α_1-adrenoceptor antagonist prazosin induced a rightward shift of the concentration response curves to A61603 with a pA_2 of 9.24±0.34 (Schild plot slope: 0.90±0.18, n=4). Likewise, the α_2-adrenoceptor antagonists rauwolscine and yohimbine also antagonized the contractions to A61603. The apparent pK_bs were, respectively, 6.69±0.20 (n=4) and 6.30±0.23 (n=4) for 100 nM and 1 μM rauwolscine, and 7.33±0.20 (n=3) for 100 nM yohimbine.

Since imidazole-containing compounds may act via a serotonergic mechanism[26], we decided to explore the possible involvement of 5-HT receptors in the contractile responses to A61603. Neither the 5-$HT_{1B/1D}$ receptor antagonist GR127935 (1 μM) nor the 5-$HT_{2A/2B/2C}$ receptor antagonist ritanserin (1 and 10 nM) affected the concentration response curve to A61603 (Figure 5.3, left panel). In contrast, the 5-HT-induced contractions were: (i) antagonized by GR127935 (1 μM, apparent pK_b: 7.61±0.07, n=4); and (ii) markedly (1 nM) or completely (10 nM) antagonized by ritanserin (Figure 5.3, middle panel). The concentration response curves to noradrenaline remained unaffected by 1 μM GR127935 as well as 100 nM ritanserin (Figure 5.3, right panel).

The effects of the mixed 5-HT receptor and α-adrenoceptor antagonist methiothepin on the responses to A61603 and noradrenaline are depicted in Figure 5.4. Methiothepin behaved as an extremely potent antagonist on the responses to A61603, inducing already at 1 nM a significant rightward shift of the concentration response curve to A61603 (apparent pK_b: 9.34±0.32, n=4) without affecting that to noradrenaline. At higher concentrations, methiothepin further attenuated (10 nM; pK_b: 9.34±0.15, n=5) or abolished (100 nM) the contractions to A61603, while it induced a seemingly insurmountable blockade of the contractions to noradrenaline (i.e. a reduction of the E_{max} without an apparent rightward shift of the concentration response curve, 10 nM) or, as observed with A61603, a complete blockade (100 nM). Finally, as expected from its high affinity for 5-HT receptors (Table 5.1), 10 nM methiothepin completely blocked the contractions to 5-HT.

5.3.3 Effects of chelerythrine and forskolin on the responses to A61603

The influence of chelerythrine and forskolin (100 nM each) on A61603-induced contraction of the porcine meningeal artery (E_{max}: 112±12%; pEC_{50}: 7.45±0.16; n=3) was studied to explore the involvement of diacyglycerol and adenylate cyclase pathways, respectively (Figure 5.5). Chelerythrine had no influence on the contractile response to A61603 (E_{max}: 130±31%; pEC_{50}: 7.33±0.32; n=4), but forskolin significantly reduced the maximum effect without affecting the potency of A61603 (E_{max}: 71±7%; pEC_{50}: 6.94±0.15; n=3).

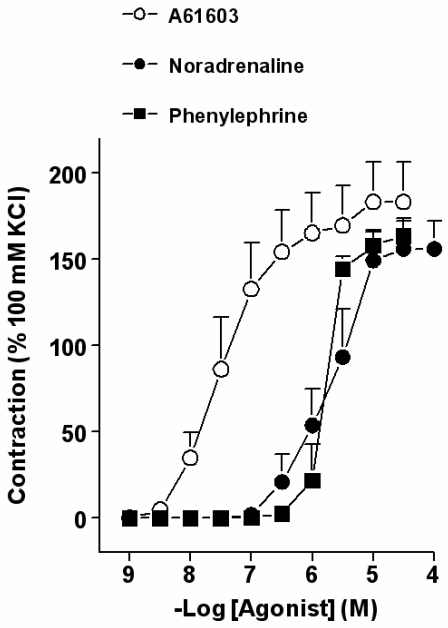

Figure 5.1: Porcine isolated meningeal artery contractions to A61603 (n=10), noradrenaline (n=8) and phenylephrine (n=6).

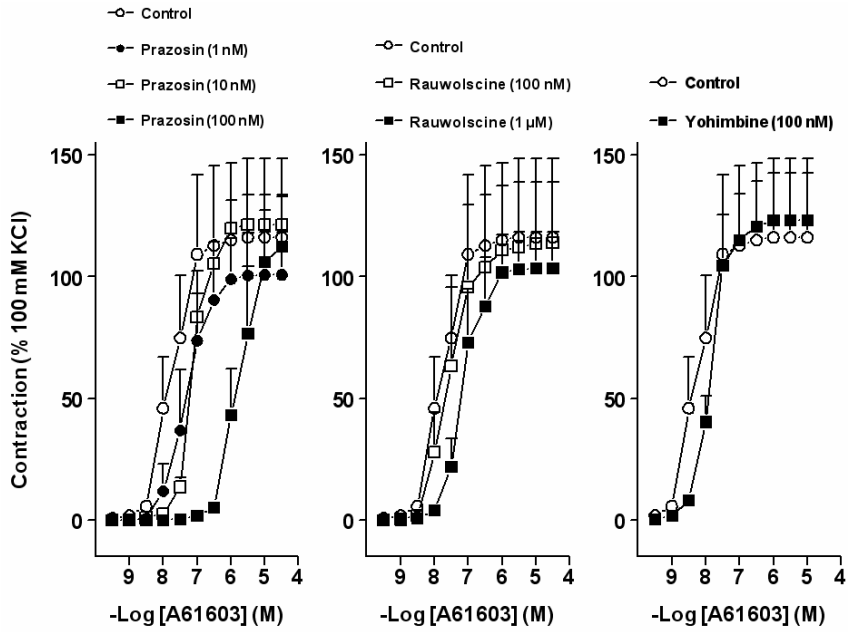

Figure 5.2: Porcine isolated meningeal artery contractions to A61603 in the absence (control) or presence of prazosin (n=4, left panel), rauwolscine (n=4, middle panel) and yohimbine (n=3, right panel).

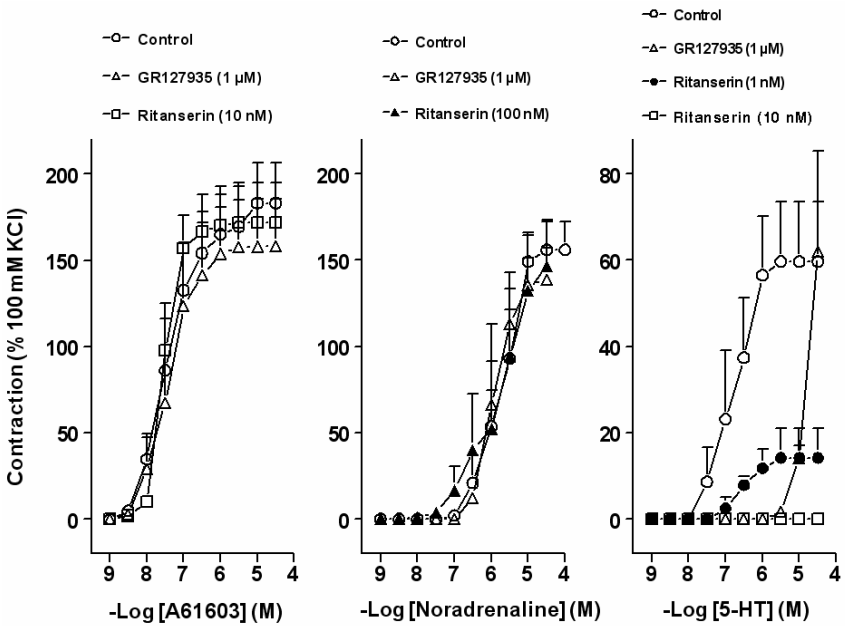

Figure 5.3: Contractions to A61603 (left panel), noradrenaline (middle panel) and 5-HT (right panel) in the absence (control, n=10, 8 and 6, respectively) or presence of GR127935 (5-$HT_{1B/1D}$ receptor antagonist, n=3-6) or ritanserin (5-$HT_{2A/2B/2C}$ receptor antagonist, n=3-5).

5.4 DISCUSSION

5.4.1 Effect of antagonists at α-adrenoceptors and 5-HT receptors on the responses to A61603

Our results clearly show that the contractile responses to A61603, which is reputed to be a selective and potent α_{1A}-adrenoceptor agonist[14,15], were antagonized by prazosin (α_1-adrenoceptor antagonist) as well as by rauwolscine and yohimbine (α_2-adrenoceptor antagonists). These findings suggest that a population of α_1- (probably α_{1A}-) and, possibly, also α_2-adrenoceptors is involved in the contractions to A61603. Indeed, this is in agreement with the binding affinity of A61603 at α_1- and α_2-adrenoceptors Table 5.1;[14], which also mediate the contractions to noradrenaline in the porcine meningeal artery[5].

In addition, our results suggest an apparent discrepancy between the *in vitro* cell binding data (Table 5.1) and functional studies (present results); accordingly, there is an almost 2-fold difference between the affinities and antagonist potencies of rauwolscine and yohimbine for α_2-adrenoceptors. Although, admittedly, we have no clear-cut explanation for this inconsistency, other studies have also reported a similar discrepancy[27].

In view of the possibility that imidazole compounds may act via a serotonergic mechanism[26], the effect of 5-HT receptor antagonists on the contractile response to A61603 was studied. Notwithstanding, neither the 5-$HT_{1B/1D}$ receptor antagonist GR127935, which also displays a moderate affinity for 5-HT_2 receptors Table 5.1;[28,29], nor the 5-HT_2 receptor antagonist ritanserin Table 5.1;[30], affected the contractions to A61603. These findings lead us to conclude that 5-$HT_{1B/1D}$ and 5-$HT_{2A/2B/2C}$ receptors are not involved in A61603-induced contractions. It may be noted that the concentrations of GR127935 and ritanserin used in our study were high enough to effectively block the contractions to 5-HT and yet were selective enough not to affect noradrenaline-induced contractions (Figure 5.3). Furthermore, as the 5-$HT_{1B/1D}$ receptor agonist sumatriptan[2,6] was devoid of any activity in the porcine meningeal artery, and the 5-HT-in-

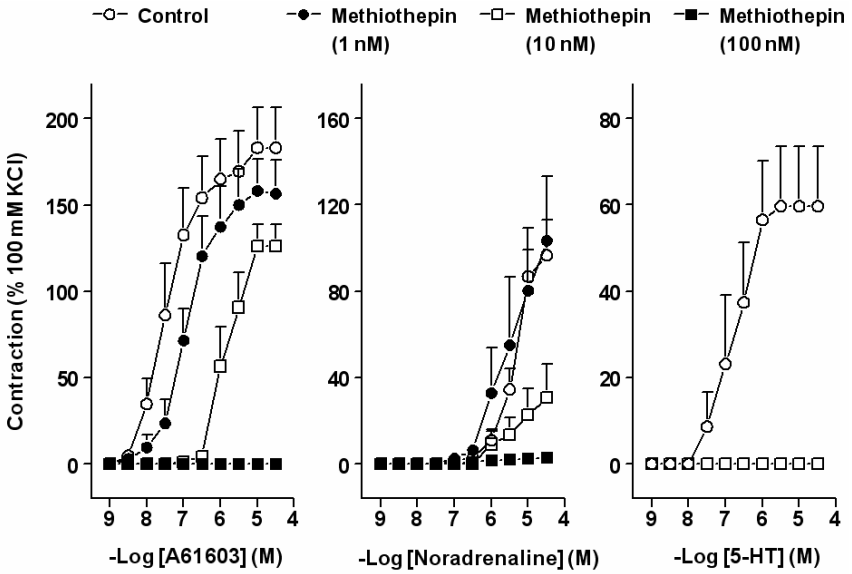

Figure 5.4: Porcine isolated meningeal artery contractions to A61603 (left panel), noradrenaline (middle panel) and 5-HT (right panel) in the absence (control, n=10, 8 and 5, respectively) or presence of the mixed 5-$HT_{1B/1D/2A/2B/2C}$ and $\alpha_{1/2}$ receptor antagonist methiothepin (n=3-6).

duced contractions were competitively antagonised by ketanserin with a pA_2 of 8.7[5], this blood vessel has a 5-HT_{2A}, but no functional 5-$HT_{1B/1D}$, receptor population. The antagonism of 5-HT by GR127935 (pK_b: 7.61) in our experiments indeed conforms more to its affinity at 5-HT_{2A} (pK_i: 7.8) than at 5-$HT_{1B/1D}$ (pK_i: 7.9-9.2) receptors (Table 5.1).

In keeping with the antagonism by prazosin, rauwolscine and yohimbine, the meningeal artery contractions to A61603 were blocked by the mixed 5-$HT_{1B/1D/2A/2B/2C}$ and $\alpha_{1/2}$-adrenoceptor antagonist methiothepin, which has a high affinity at α-adrenoceptors Table 5.1;[6]. Indeed, the antagonist potency of methiothepin against A61603 in our experiments (pK_b: 9.34±0.32 at 1 nM and 9.35±0.15 at 10 nM) matches with its pK_i at α_1-adrenoceptors (9.3; Table 5.1).

5.4.2 Effects of forskolin and chelerythrine on the responses to A61603

α_1-Adrenoceptors are coupled to the $G_{q/11}$-protein stimulating phospholipase C to hydrolyse phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol; IP_3 releases stored intracellular Ca^{2+}, while diacylglycerol activates protein kinase C and, thus, phosphorylation of target cellular proteins to alter their functions[31,32]. On the other hand, α_2-adrenoceptors are predominantly coupled to the heterotrimeric GTP-binding protein to decrease adenylate cyclase activity and, thus, inhibiting voltage-gated Ca^{2+} channels and activating K^+ channels[32]. In view that α_1- and α_2-adrenoceptors play a role in the contractile responses to A61603 (see above), we decided to further explore the second messenger mechanisms involved in these responses by analysing the effects produced by the protein kinase C inhibitor chelerythrine[17,18] and the cAMP stimulator forskolin[19,20]. The fact that the contractions to A61603 were attenuated by forskolin suggests the involvement of the adenylate cyclase pathway linked to α_2-adrenoceptors. Moreover, the ineffectiveness of chelerythrine in our study, which at the concentration used (100 nM) effectively blocks, for example, the negative inotropic effect of aldosterone in the human isolated atrial and ventricular trabeculae[33], indicates that the diacylglycerol pathway (linked to α_1-adrenoceptors) is not involved in the contractile response to A61603. Although it is tempting to

Table 5.1. pK_i values of agonists and antagonists used in this study at human recombinant (unless otherwise stated) α-adrenoceptors and 5-HT receptors.

Compounds	α_1-Adrenoceptors			α_2-Adrenoceptors			5-HT receptors		
	α_{1A}	α_{1B}	α_{1D}	α_{2A}	α_{2B}	α_{2C}	5-HT_{1B}	5-HT_{1D}	5-HT_{2A}
Agonists									
A61603[a]	7.1	4.8	4.9	7.3	6.5	6.2	5.2	5.6	<5.5
Noradrenaline	5.8-6.0	6.2	7.4	5.6-8.4	5.6-9.1	5.9-8.7	-	-	-
Phenylephrine	5.2-5.4	6.3-7.5 (pIC_{50})	5.9 (rat)	-	-	-	-	-	-
5-HT	4.5[b]	4.6[b]	4.6[b]	4.5 (pIC_{50}, rat brain)[c]			7.4-9.0; 7.8 (pig)[d]	8.0-9.0; 8.2, 8.4 (pig)[e]	6.0-8.4
Antagonists									
Prazosin	9.0-9.9	9.6-9.9	9.5-10.2	5.3-6.5	6.4-7.5	6.7-8.0	-	-	-
Rauwolscine	6.3 (pKi/nH, rat cortex)[f], pA_2 6.27±0.08[f] (rat vas deferens)			9.5 (pK_d)	9.4 (pK_d)	9.9 (pK_d)	6.5-7.4	7.8	-
				pA_2: 7.92±0.09[f] (rat vas deferens)					
Yohimbine	6.4 (rat brain)[c]			8.5-9.2	8.2-8.9	8.7-9.5	6.8-7.6	7.2-7.7	-
	pA_2 6.25±0.07[f] (rat vas deferens)			pA_2: 7.72±0.08[f] (rat vas deferens)					
GR127935	<6.0 (pIC_{50}, native receptor; rat brain)[g]			<6.0 (pIC_{50}, native receptor; rat brain)[g]			9.9[g]; 8.5 (pig)[d]	8.9[g]; 7.9 (pig)[e]	7.8
Ritanserin	8.4	8.0	7.8	6.2 (pIC_{50}, rat brain)[h]			6.0-6.5 (pIC_{50}); 6.7 (pig)[d]	7.6; 6.8, 7.3 (pig)[e]	9.4
Methiothepin	9.3 (rat brain)[c]			7.3 (rat brain)[c]			7.1-8.5; 8.3 (pig)[d]	7.3-8.2; 8.7, 8.8 (pig)[e]	8.5

The data are from IUPHAR[34], except when specified otherwise: [a]Craig[14], [b]Yoshio *et al.*[30], [c]Leysen[35], [d]Bhalla *et al.*[36], [e]Bhalla *et al.*[37], [f]Michel & Whiting[38], [g]Pauwels *et al.*[39], [h]Korstanje *et al.*[40].

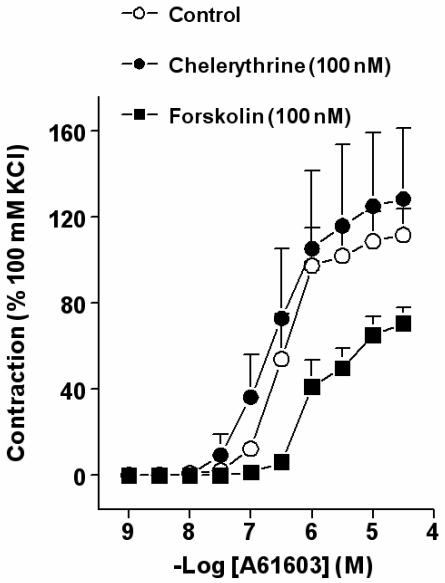

Figure 5.5: Porcine isolated meningeal artery contractions to A61603 in the absence (control, n=3) and presence of chelerythrine (protein kinase C inhibitor; n=4) or forskolin (cAMP stimulator; n=3).

suggest that the response via α_1-adrenoceptors may be mediated by the IP_3 pathway, other signalling pathways have also been shown to be activated via α_1-adrenoceptors see[31].

5.4.3 Comparison of the agonist responses in the meningeal artery and carotid vascular bed of pigs

A61603, which elicited concentration-dependent contractions of the porcine meningeal artery, was about 30-times more potent than noradrenaline or phenylephrine (Figure 5.1). This contractile response to A61603 is particularly interesting in view of the fact that sumatriptan and ergot alkaloids, like A61603[16], potently constrict carotid arteriovenous anastomoses in the anaesthetized pig[1-4]; yet, sumatriptan and ergot alkaloids failed to contract the porcine meningeal artery[5]. Thus, it appears that the porcine meningeal artery does not participate in the carotid vasoconstriction elicited by these compounds and that the non-adrenergic mechanism mediating carotid vasoconstriction by A61603 in the anaesthetized pig[16] is not operative in the porcine isolated meningeal artery.

In conclusion, our results suggest that A61603-induced contraction of the porcine isolated meningeal artery is mediated via a mixed population of α_1- (probably α_{1A}) and α_2-adrenoceptors. This response involves the adenylate cyclase, rather than the diacylglycerol, pathway. Interestingly, the porcine isolated meningeal artery does not show evidence of a major involvement of the non-adrenergic mechanism associated with constriction of porcine carotid arteriovenous anastomoses by A61603[16].

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Chapter 6

Potential vascular α_1-adrenoceptor blocking properties of an array of 5-HT receptor ligands in the rat

Based on: David Centurión, **Suneet Mehotra***, Araceli Sánchez-López, Saurabh Gupta, Antoinette MaassenVanDenBrink and Carlos M. Villalón,* **European Journal of Pharmacology 2006 Mar 27;535(1-3):234-42**

ABSTRACT

This study set out to analyse the potential ability of some 5-hydroxytryptamine (5-HT) receptor ligands widely used in cardiovascular experimental models to interact with vascular α_1-adrenoceptors in the pithed rat. These ligands included: methiothepin, methysergide and metergoline (5-HT_1/5-HT_2); WAY-100635, buspirone, ipsapirone and 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) (5-HT_{1A}); GR127935 (5-$HT_{1B/1D}$); ketanserin, ritanserin, spiperone and pizotifen (5-HT_2); granisetron and metoclopramide (5-HT_3); tropisetron (5-HT_3/5-HT_4); ergotamine (5-$HT_{1B/1D}$, 5-$ht_{5A/5B}$); clozapine (5-HT_6/5-HT_7); as well as LY215840 and mesulergine (5-HT_2/5-HT_7). For this purpose, the increases in diastolic blood pressure produced by the selective α_1-adrenoceptor agonist, phenylephrine, were analysed before and after the above antagonists or saline. The adrenoceptor antagonist properties of prazosin (α_1) and yohimbine (α_2) were also analysed for comparison. Thus, the phenylephrine-induced vasopressor responses were dose-dependently antagonised with the following apparent rank order of potency by:

prazosin≥methiothepin>ketanserin>clozapine≥lisuride>>buspirone; this potency correlates with the affinity of these compounds for α_1-adrenoceptor binding sites. In contrast, the other compounds were either devoid of any blocking effect on –or even potentiated (i.e. lisuride, methysergide, 8-OH-DPAT, granisetron and GR127935)- the responses to phenylephrine. These results show that methiothepin, ketanserin, clozapine, lisuride and buspirone can block α_1-adrenoceptors in the rat systemic vasculature.

6.1 INTRODUCTION

The operational characterization of receptors for 5-hydroxytryptamine (5-HT), particularly within the 5-HT_1, 5-HT_2, 5-$ht_{5A/5B}$, 5-HT_6 and 5-HT_7 types has often been hampered by the lack of selective agonists and antagonists. For example, methiothepin and ergotamine display affinity for these and other receptors including α_1-adrenoceptors (see Table 6.1) and dopamine D_2-like receptors[1-3]. The simplest hindrance, in this context, can be illustrated by the α_1-adrenoceptor blocking properties that some 5-HT_1, 5-HT_2 and/or 5-HT_7 receptor ligands (including methiothepin, ketanserin, lisuride, etc.) display on the cardiovascular system[4-6]. This situation worsens when characterizing, in pithed rats, the prejunctional 5-HT_1 receptors inhibiting the vasopressor responses induced by sympathetic stimulation[7,8] since such vasopressor responses involve an important α_1-adrenoceptor-mediated component[9].

Although selective agonists/antagonists at 5-$ht_{5A/5B}$ receptors are not available thus far, methiothepin and ergotamine have been used in the rat for characterizing these receptors, which seem to mediate: (i) inhibition of the cardiac sympathetic outflow[10]; and (ii) other effects in the spinal cord[11]. Although the above cardiovascular shortcomings could simply be based on the affinity that some 5-HT receptor ligands display for α_1-adrenoceptor binding sites[2], this is not always the case. For example, spiroxatrine, buspirone and ipsapirone, which have been reported as 5-HT_{1A} receptor ligands with negligible affinity for α_1-adrenoceptor binding sites (see Table 6.1), can act as: (i) partial or pure agonists at vascular α_1-adrenoceptors[6,12]; or (ii) "silent" α_1-adrenoceptor antagonists in other cases[5,13-15]. Clearly, binding data *per se* do not always allow to predict whether a compound will behave as an antagonist in pharmacological functional studies.

On this basis, the present study in pithed rats was designed to investigate the potential vascular α_1-adrenoceptor blocking properties of an extensive range of 5-HT receptor ligands widely used in cardiovascular experimental models; these ligands included: methiothepin, methysergide and metergoline (5-HT_1/5-HT_2); WAY-100635, buspirone, ipsapirone and 8-OH-DPAT (5-HT_{1A}); GR127935 (5-$HT_{1B/1D}$); ketanserin, ritanserin, spiperone and pizotifen (5-HT_2); granisetron and metoclopramide (5-HT_3); tropisetron (5-HT_3/5-HT_4); ergotamine (5-$HT_{1B/1D}$/5-$ht_{5A/5B}$); clozapine (5-HT_6/5-HT_7); as well as LY215840 and mesulergine (5-HT_2/5-HT_7)[1]. For this purpose, phenylephrine-induced vasopressor responses were analysed

Table 6.1. Binding affinities (pKi) of some compounds at α_1- and α_2-adrenoceptor binding sites.ND: not determined.

Compound	α_1	α_2	References
Prazosin	10.7	7.9	(Boyajian and Leslie, 1987[19] ; Jones *et al.*, 1987[20])
Yohimbine	6.6	7.5	(Millan *et al.*, 2000[21])
Methiothepin	9.3	7.3	(Leysen, 1985[2])
Methysergide	5.6	5.6	(Leysen, 1985[2])
Metergoline	7.4	6.4	(Leysen, 1985[2])
WAY100635	7.2 – 8.3	5.9	(Testa *et al.*, 1999[22])
Ergotamine	8.0	8.2	(Leysen, 1985[2]; Leysen and Gommeren, 1984[3])
Buspirone	5.1	4.9	(Rimele *et al.*, 1987[12])
Ipsapirone	6.2	5.7	(Rimele *et al.*, 1987[12])
8-OH-DPAT	5.3	6.7-6.9	(Blaxall *et al.*, 1991[23] ; Yoshio *et al.*, 2001[24])
GR127935	< 6.0	< 6.0	(Pauwels and Colpaert, 1996[25])
Ketanserin	7.1	6.4	(Boyajian and Leslie, 1987[19] ; Korstanje *et al.*, 1986[26])
Ritanserin	6.7	6.2	(Korstanje *et al.*, 1986[26])
Spiperone	7.4	6.2	(Richelson and Nelson, 1984[27] ; Schwinn *et al.*, 1995[28])
Pizotifen	ND	ND	-
Granisetron	ND	ND	-
Metoclopramide	4.5	6.3	(Hall *et al.*, 1986[29])
Tropisetron	ND	ND	-
LY215840	ND	ND	-
Mesulergine	ND	ND	-
Clozapine	7.8	6.9	(Hall *et al.*, 1986[29])
Lisuride	7.8-8.5	9.1-10.3	(Millan *et al.*, 2002[30])

Note: The binding affinities were obtained from competition studies with [^{3}H]prazosin and the corresponding serotonergic/adrenoceptor ligands

before and after administration of each of the above antagonists; the adrenoceptor antagonist properties of prazosin (α_1) and yohimbine (α_2) were also analysed for comparison.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Male Wistar normotensive rats (250-280 g) were used in the present experiments. The animals were maintained at a 12/12-h light-dark cycle (with light beginning at 7 a.m.) and housed in a special room at constant temperature (22±2°C) and humidity (50%), with food and water freely available in their home cages.

6.2.2 General methods

Experiments were carried out in a total of 115 rats. After anaesthesia with ether and cannulation of the trachea, the rats were pithed by inserting a stainless steel rod through the orbit and foramen magnum, and down the vertebral foramen[16]. The animals were artificially ventilated with room air using an Ugo Basile pump (56 strokes/min and a stroke volume of 20 ml/kg), as previously established[17]. After bilateral vagotomy, catheters were placed in: (i) the right and left femoral vein for the administration of phenyl-

ephrine and the antagonists; and (ii) the left carotid artery, connected to a Grass pressure transducer (P23 XL), for the recording of blood pressure. Heart rate was measured with a tachograph (7P4, Grass Instrument Co., Quincy, MA, U.S.A.) triggered from the blood pressure signal. Both blood pressure and heart rate were recorded simultaneously by a model 7D Grass polygraph (Grass Instrument Co., Quincy, MA, U.S.A.).

6.2.3 Experimental protocol

After a stable haemodynamic condition for at least 30 min, baseline values of diastolic blood pressure and heart rate were determined. Then, dose-vasopressor response curves were elicited by subsequent intravenous (i.v.) bolus injections of phenylephrine (0.1, 0.3, 1, 3 and 10 μg/kg); when diastolic blood pressure had returned to the pre-administration levels (5-10 min), the next dose was given. This procedure was systematically performed until the dose-response curve was completed (about 30 min); at this point, the animals were divided into 23 groups. Then, the responses to phenylephrine were elicited again after i.v. administration of each dose of either physiological saline (1 ml/kg given three times; n=6), prazosin (3, 10 and 30 μg/kg; n=6), yohimbine (300, 1000 and 3000 μg/kg; n=4), methiothepin (10, 30 and 100 μg/kg; n=4), methysergide (300, 1000 and 3000 μg/kg; n=4), metergoline (3000 μg/kg; n=6), WAY100635 (100, 300 and 1000 μg/kg; n=5), buspirone (300, 1000 and 3000 μg/kg; n=6), ipsapirone (300, 1000 and 3000 μg/kg; n=6), 8-OH-DPAT (300, 1000 and 3000 μg/kg; n=4), GR127935 (100, 300 and 1000 μg/kg; n=5), ketanserin (300, 1000 and 3000 μg/kg; n=4), ritanserin (300, 1000 and 3000 μg/kg; n=4), spiperone (10, 30, 100 and 300 μg/kg; n=5), pizotifen (300, 1000 and 3000 μg/kg; n=6), granisetron (300, 1000 and 3000 μg/kg; n=4), metoclopramide (300, 1000 and 3000 μg/kg; n=6), tropisetron (300, 1000 and 3000 μg/kg; n=4), ergotamine (0.1, 0.3 and 1 μg/kg; n=5), LY215840 (100, 300 and 1000 μg/kg; n=5), mesulergine (300, 1000 and 3000 μg/kg; n=5), clozapine (300, 1000 and 3000 μg/kg; n=5) and lisuride (100, 300 and 1000 μg/kg; n=6). It is important to note that when certain doses of antagonists blocked the responses to phenylephrine, higher doses of this agonist were subsequently given (see results). The interval between the different doses of phenylephrine depended on the duration of its vasopressor effects (i.e. between 5 and 10 min); since these were short-lasting effects returning to baseline values, we decided to use sequential, rather than cumulative, doses of phenylephrine. For the antagonists, a period of 10 min was allowed to elapse before the dose-response curves to phenylephrine were elicited again. The dosing with all drugs used was sequential.

The protocol of the present investigation was approved by the Ethical Committee of our institution (CICUAL), which deals with the appropriate use of animals in scientific experiments.

6.2.4 Data presentation and statistical evaluation

All data in the text and figures are presented as mean ± S.E.M. The peak changes in diastolic blood pressure produced by the different doses of phenylephrine were determined. The increases in diastolic blood pressure just before and after each dose of a particular compound were compared by using Duncan's new multiple range test, once an analysis of variance (randomized block design) had revealed that the samples represented different populations [18]. Statistical significance was accepted at $P<0.05$ (two-tailed). The dose of phenylephrine to produce an increase of 50 mmHg in diastolic blood pressure ($ED_{50\,mmHg}$) was calculated by fitting the data to the four-parameter logistic equation with Graph Pad Prism 4.0. Then, the dose-ratio ($ED_{50\,mmHg}$ ratio) was calculated by dividing the $ED_{50\,mmHg}$ of phenylephrine in the presence of each dose of every compound against the $ED_{50\,mmHg}$ in the absence (control) of the corresponding compound. The 95% confidence intervals for $ED_{50\,mmHg}$ were also estimated. Moreover, the dose of the compound needed to raise the $ED_{50\,mmHg}$ of phenylephrine by a factor of 2 (AD_2), (in μg/kg and nmol/kg), was calculated by using the method developed by Lew and Angus[31]. This method basically consists in fitting in, by nonlinear regression, the data of each compound (at all doses used) with the following equation: $pED_{50\,mmHg} = -\log([B]^S + 10\text{-}AD_2{}^{*S}) - \log C$; where pED_{50} is $-\log ED_{50\,mmHg}$; [B] is the dose of the

compound; S is a logistic slope factor analogous to the Schild slope; -log C is a fitting constant; and AD_2 was defined above for further details see Motulsky et.al.,[32,33]. These values have been previously used as an apparent index of antagonist potency[34].

6.2.5 Compounds

Apart from the anaesthetic (diethyl ether), the drugs used in the present study were obtained from the sources indicated: ketanserin and ritanserin (gift: Janssen Pharmaceutica, Beerse, Belgium); methiothepin maleate (gift: Hoffman-La Roche, Ltd., Basel Switzerland); methysergide hydrogen maleate, mesulergine maleate, pizotifen hydrogen maleate, clozapine, and 3α-tropanyl-1H-indole-3-carboxylic acid ester (tropisetron) (gift: Novartis Pharma A.G., Basel, Switzerland); metergoline (gift: Farmitalia, Milan, Italy); (±)-8-hydroxy-dipropylaminotetraline hydrobromide (8-OH-DPAT), prazosin, yohimbine, phenylephrine, N-{2-[4-(2-methoxy- phenyl)-1-piperazinyl]ethyl}-N-(2-pyridinyl)cyclohexane carboxamide trihydrochloride (WAY100635), lisuride hydrogen maleate, spiperone hydrochloride, buspirone hydrochloride and metoclopramide hydrochloride (Sigma Chemical Co. St. Louis, MO, U.S.A.); ipsapirone hydrochloride (gift: Troponwerke, Köln, Germany); ergotamine tartrate (gift: Novartis Pharma de Mexico, Mexico City, Mexico); granisetron hydrochloride (gift: SmithKline Beechman Pharmaceuticals, Harlow, U.K.); N-[4-methoxy- 3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'- (5-methyl-1,2,4- oxadiazol- 3-yl) [1,1,- biphenyl]-4-carboxamide hydrochloride monohydrate (GR127935; gift: GlaxoSmithKline, Stevenage, Hertfordshire, U.K.); and cis-n-(2-hydroxycyclopentyl)-6-methyl-1-(1-methylethyl)ergoline-8-carboxamide (LY215840; gift: Eli Lilly & Co., Indianapolis, IN, U.S.A.). All compounds were dissolved in physiological saline. When needed, 5% propylene glycol (methiothepin, methysergide and ergotamine), 5% dimethyl sulfoxide (clozapine and lisuride) or 1% ascorbic acid (ritanserin, ketanserin, metergoline, tropisetron and LY215840) was added. GR127935 was dissolved according to the instructions of the supplier by the dispersion in distilled water to about 70 ºC for 10 s and then allowing to cool down to room temperature. Fresh solutions were prepared for each experiment, and the vehicles had no effect on either basal heart rate or blood pressure (not shown). The doses mentioned in the text refer to the salts of substances except in the case of phenylephrine, where it refers to the free base.

6.3 RESULTS

6.3.1 Systemic haemodynamic variables

The baseline values of diastolic blood pressure and heart rate in the 115 rats were, respectively, 54±2 mmHg and 283±5 beats/min, as previously reported [10]; moreover, these values did not significantly differ from the corresponding values in each of the 23 groups of animals (not shown). It must be emphasised that the above haemodynamic variables were not modified by treatment with the different doses of saline, prazosin, methiothepin, ketanserin, clozapine, lisuride, yohimbine, mesulergine, metergoline, methysergide, ritanserin, granisetron, metoclopramide, GR127935, tropisetron, spiperone or LY215840 (not shown). In contrast, other drugs displaying moderate affinity at α_1-adrenoceptors (see Table 6.1) produced, at the doses used (see experimental protocol section), short-lasting increases in diastolic blood pressure; these drugs included buspirone (52±4, 45±4 and 37±7 mmHg), ipsapirone (29±4, 34±7 and 36±7 mmHg), 8-OH-DPAT (24±5, 46±4, 53±3 mmHg), WAY100635 (8±1, 13±2, 23±5 mmHg), pizotifen (17±1, 21±2, 43±5 mmHg) and ergotamine (9±1, 11±2, 18±4 mmHg).

6.3.2 Effects of phenylephrine on diastolic blood pressure and heart rate

I.v. bolus injections of phenylephrine (0.1, 0.3, 1, 3 and 10 µg/kg) produced dose-dependent increases in: (i) diastolic blood pressure (control vasopressor responses) which, in all cases, were statistically significant when compared to baseline values ($P<0.05$; see Figure 6.1 and 6.2); and (ii) heart rate (1±0.4, 2±0.5,

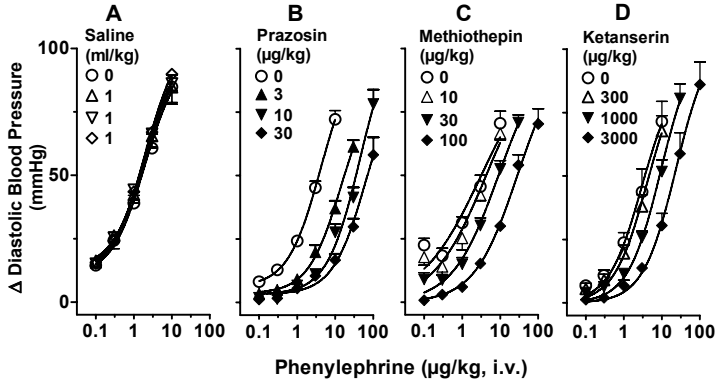

Figure 6.1: Effect of i.v. saline (0.1, 0.3 and 1 ml/kg), prazosin (3, 10 and 30 µg/kg), methiothepin (10, 30 and 100 µg/kg) or ketanserin (300, 1000 and 3000 µg/kg) on the vasopressor responses induced by i.v. bolus injections of phenylephrine. For the sake of clarity, empty symbols depict either control responses (○) or non-significant responses vs. control (△▽◇); solid symbols (▲▼◆) represent significantly different responses (P<0.05) vs. control.

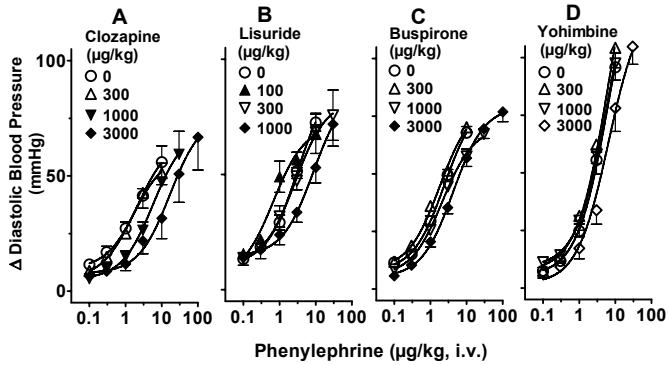

Figure 6.2: Effect of i.v. clozapine (300, 1000 and 3000 µg/kg), lisuride (100, 300 and 1000 µg/kg), buspirone (300, 1000 and 3000 µg/kg) or yohimbine (300, 1000 and 3000 µg/kg) on the vasopressor responses induced by i.v. bolus injections of phenylephrine. For the sake of clarity, empty symbols depict either control responses (○) or non-significant responses (△ ▽ ◇) vs. control; solid symbols (▲ ▼ ◆) represent significantly different responses (P<0.05) vs. control.

5±0.8, 12±1* and 27±2* beats/min; n=115) which were significantly different from baseline (P<0.05) at 3 and 10 µg/kg of phenylephrine.

6.3.3 Effects of saline and several compounds on the vasopressor responses to phenylephrine

Figure 6.1 and 6.2 also show that the vasopressor responses to phenylephrine were: (i) highly reproducible as they remained unaltered after three i.v. bolus injections of physiological saline (Figure 6.1A); (ii) antagonised by prazosin, methiothepin and ketanserin (Figure 6.1B, 6.1C and 6.1D, respectively) as well as by clozapine, lisuride and buspirone (see Figure 6.2A, 6.2B and 6.2C, respectively); and (iii) unaffected by yohimbine (Figure 6.2D). In order to establish the potency of blockade of these ligands, the dose of compound needed to raise the $ED_{50\ mmHg}$ of phenylephrine by a factor of 2 (AD_2)[31] was calculated (see the section referring to data presentation and statistical evaluation). Hence, Table 6.2 shows that the apparent rank order of potency to block the phenylephrine-induced vasopressor responses was: prazosin>methiothepin>>ketanserin≥clozapine>lisuride>>buspirone. Accordingly Figure 6.3, it was not possible to calculate AD_2 here since the above compounds did not significantly antagonize the vasopressor responses to phenylephrine (i.e. $AD_2 \rightarrow \infty$) shows that the binding affinity (pKi) of the above compounds

for α_1-adrenoceptor binding sites significantly correlates ($P<0.05$) with their respective apparent potency to block the phenylephrine-induced vasopressor responses (pAD_2).

As shown in Table 6.3, the rest of compounds (methysergide, metergoline, WAY100635, ipsapirone, 8-OH-DPAT, GR127935, ritanserin, spiperone, pizotifen, granisetron, metoclopramide, tropisetron, ergotamine, LY215840 and mesulergine) failed to block the vasopressor responses to phenylephrine. Indeed, their respective $ED_{50\ mmHg}$ ratio was equal to (or lower than) unity, indicating no blockade by the above compounds. In fact, the $ED_{50\ mmHg}$ ratio for several compounds being lower than unity indicates a potentiation of the vasopressor responses to phenylephrine; the potentiating doses of these compounds include: 100 µg/kg lisuride; 300 µg/kg methysergide; 300 and 1000 µg/kg granisetron; 300 and 1000 µg/kg GR127935; and 300 µg/kg 8-OH-DPAT.

6.4 DISCUSSION

6.4.1 General

To the best of our knowledge, the α_1-adrenoceptor blocking properties of some 5-HT receptor ligands have been investigated in functional studies mainly *in vitro*, or in limited number *in vivo*. These properties may certainly produce misleading results, particularly when used in cardiovascular –and other- preparations. Hence, the present study set out to systematically analyse in pithed rats the potential α_1-adrenoceptor blocking properties of a wide range of 5-HT receptor ligands frequently used to characterise 5-HT receptors in the cardiovascular system

The doses used of these compounds were high enough to block/stimulate their respective functional 5-HT receptors. Apart from the implications discussed below, our study shows that the 5-HT receptor ligands used (including agonists, partial agonists and antagonists) are capable of interacting with vascular α_1-adrenoceptors, even when they do not display high affinity at α_1-adrenoceptor binding sites (i.e. buspirone, ipsapirone and 8-OH-DPAT; see Table 6.1;[5]). Conversely, our findings also show that several compounds displaying moderate affinity at α_1-adrenoceptor binding sites are not capable of blocking vascular α_1-adrenoceptors (i.e. metergoline, spiperone and WAY100635), whereas others displaying variable affinity (i.e. methysergide, lisuride, granisetron, 8-OH-DPAT and GR127935; see Table 6.1) even potentiated these responses.

Interestingly, ergotamine, which displays high affinity for α_1-adrenoceptor binding sites (see Table 6.1), apparently failed to block the responses to phenylephrine (see Table 6.3) probably due to the use of low doses of this ergot derivative. In favour of this view, another study using higher doses of ergotamine in pithed rats has shown a blockade of the vasopressor responses to phenylephrine[35]. It must be emphasised that doses of ergotamine higher than 1 µg/kg (which are not shown in the present study) induce significant increases in blood pressure that may produce a physiological, rather than a pharmacological, antagonism due to a reduced window in blood pressure. The above findings, taken together: (i) lead us to suggest that binding affinity constants do not always correlate with their pharmacological properties *in vivo*; and (ii) are consistent with previous *in vitro* observations in the rat caudal, canine carotid, and rat thoracic arteries[24].

6.4.2 Validity of the experimental model

Our experiments, in which three i.v. bolus injections of physiological saline were given to pithed rats, show that no substantial changes occurred in baseline diastolic blood pressure or heart rate over the duration of the experiments. Therefore, we can conclude that no time-dependent changes are seen in the above haemodynamic variables during the experimental period in the animal model used here. Moreover, no evidence of tachyphylaxis was observed on the vasopressor responses to phenylephrine as they remained unchanged after repeated administration of saline (Figure 6.1A).

Table 6.2. Doses and apparent potency indexes of several compounds (µg/kg) to block the vasopressor responses to phenylephrine in pithed rats. For comparison, these parameters are also given as nmol/kg in parenthesis.

Compound	[a]Dose (µg/kg, i.v.)	[b]Phenylephrine $ED_{50\ mmHg}$ (µg/kg)	95% confidence intervals (µg/kg)	$ED_{50\ mmHg}$ ratio	[c]AD_2 (µg/kg)
Saline	0	2.4 (11.8)	1.2 – 4.9 (5.9-24)	1.0	-
(n=6)	1	1.8 (8.8)	0.9 – 3.6 (4.4-17.7)	0.8	
	1	1.6 (7.9)	0.8 – 3.2 (3.9-15.7)	0.7	
	1	2.0 (9.8)	1.0 – 3.7 (4.9-18.2)	0.8	
Prazosin	0	4.2 (20.6)	2.7 – 6.5 (13.3-14)	1.0	1.54 (3.7)
(n=6)	3 (7.1)	14.5 (71.2)	9.0 – 23.2 (44.2-114)	3.5[d]	
	10 (23.8)	45.6 (224)	27.3 – 76.2 (134-374)	10.9[d]	
	30 (71.4)	64.4 (316.2)	28.6 - 145.1 (140-721)	15.3[d]	
Methiothepin	0	3.2 (15.7)	2.1 – 4.8 (10.3-23.6)	1.0	13.0 (27.5)
(n=4)	10 (21.2)	4.3 (21.1)	3.0 – 6.0 (14.7-29.5)	1.3	
	30 (63.5)	9.0 (44.2)	7.4 – 11.0 (36.3-54)	2.8[d]	
	100 (211.6)	28.4 (139.5)	23.6 – 34.2 (116-168)	8.9[d]	
Ketanserin	0	3.0 (14.7)	1.4 – 6.3 (6.9-31)	1.0	418.8 (768)
(n=4)	300 (550)	4.1 (20.1)	2.2 – 7.4 (10.8-36.3)	1.4	
	1000 (1833)	9.5 (46.6)	6.9 – 14.4 (33.4-70.7)	3.2[d]	
	3000 (5500)	23.6 (115.9)	14.4 – 38.6 (70-7-190)	7.9[d]	
Clozapine	0	2.3 (11.2)	0.8 – 6.5 (3.9-32)	1.0	435.5 (1332.6)
(n=5)	300 (918)	1.5 (7.4)	0.5 – 4.4 (2.5-21.6)	0.7	
	1000 (3060)	5.8 (28.5)	1.9 – 17.4 (9.3-85)	2.5[d]	
	3000 (9180)	16.8 (82.5)	4.4 – 64.7 (21.6-317.7)	7.3[d]	
Lisuride	0	3.2 (15.7)	1.8 – 5.6 (8.8-27.5)	1.0	603.9 (1328.6)
(n=6)	100 (220)	0.7 (3.4)	0.2 – 2.1 (1-10.3)	0.2[d]	
	300 (660)	2.7 (13.3)	1.4 – 5.2 (6.9-25.5)	0.8	
	1000 (2200)	17.9 (87.9)	9.0 – 35.5 (44.2-174)	5.6[d]	
Buspirone	0	2.4 (11.8)	1.5 – 3.9 (7.4-19.2)	1.0	3184.2 (7547)
(n=6)	300 (711)	1.8 (8.8)	1.3 – 2.5 (6.4-12.3)	0.8	
	1000 (2370)	2.2 (10.8)	1.4 – 3.2 (6.9-15.7)	0.9	
	3000 (7110)	4.2 (20.6)	2.9 – 6.2 (14.2-30.4)	1.8[d]	

[a] The doses of saline are expressed in ml/kg. [b] $ED_{50\ mmHg}$, dose of phenylephrine to increase blood pressure by 50 mmHg. [c] AD_2, dose of antagonist needed to raise the $ED_{50\ mmHg}$ of phenylephrine by a factor of 2. Calculations were based on the method developed by Lew and Angus (1995). [d], Statistically different from 1.0 ($P<0.05$).

As expected, the vasopressor responses to phenylephrine were significantly and potently blocked by the α_1-adrenoceptor antagonist prazosin (see Figure 6.1B), but not by the α_2-adrenoceptor antagonist yohimbine (at doses up to 3000 µg/kg; Figure 6.2D). Interestingly, stimulation of vascular 5-HT_2 receptors also produces vasopressor responses[36], and some of the ligands used in the present study (including mesulergine, methysergide, metergoline, ergotamine, GR127935, ritanserin, spiperone, pizotifen, LY215840, yohimbine, etc.; see Table 6.3) display moderate/high affinity for 5-HT_2 binding sites[37,1]. However, these ligands failed to block phenylephrine-induced vasopressor responses (see Table 6.3) and, to the best of our knowledge, phenylephrine does not interact with 5-HT_2 receptors. Therefore, these findings reconfirm that phenylephrine-induced vasopressor responses are mainly mediated by vascular α_1-adrenoceptors[38,39], but not by α_2-adrenoceptors or 5-HT_2 receptors. These phenylephrine-sensitive α_1-adrenoceptors have previously been reported to resemble the pharmacological profile of the α_{1A}- and α_{1D}-adrenoceptor subtypes[38,39]

Table 6.3. Potency index of several 5-HT receptor antagonists against the vasopressor responses to i.v. bolus injections of phenylephrine in pithed rats. Note that all compounds failed to block the responses to phenylephrine.

Compound	Dose (µg/kg, i.v.)	[a]Phenylephrine $ED_{50\ mmHg}$ (µg/kg)	95% Confidence intervals (µg/kg)	$ED_{50\ mmHg}$ ratio
Mesulergine (n=5)	0	2.2	1.0 - 4.8	1.0
	300	1.7	1.1 - 3.0	0.8
	1000	1.9	1.0 - 3.5	0.9
	3000	3.7	1.6 - 8.6	1.7
Methysergide (n=4)	0	8.5	2.2 - 33.3	1.0
	300	2.1	0.4 - 10.8	0.3*
	1000	3.5	0.6 - 20.6	0.4
	3000	4.6	0.5 - 44.5	0.5
Metergoline (n=6)	0	2.0	1.3 - 3.1	1.0
	3000	2.4	1.5 - 3.7	1.2
Ergotamine (n=5)	0	4.5	2.9 - 6.9	1.0
	0.1	5.5	2.8 - 10.7	1.2
	0.3	3.2	1.6 - 6.3	0.7
	1.0	2.3	0.9 - 5.9	0.5
WAY100635 (n=5)	0	3.6	1.7 - 7.6	1.0
	100	2.4	1.4 - 4.1	0.7
	300	2.3	1.4 - 3.8	0.6
	1000	3.2	1.8 - 5.8	0.9
8-OH-DPAT (n=4)	0	3.3	1.0 - 10.6	1.0
	300	0.9	0.3 - 2.6	0.3*
	1000	1.4	0.4 - 4.4	0.4
	3000	2.8	0.6 - 12.9	0.8
Ipsapirone (n=6)	0	4.8	1.7 - 13.7	1.0
	300	3.5	1.0 - 12.5	0.7
	1000	2.1	0.3 - 13.9	0.4
	3000	4.4	0.7 - 28.8	0.9
GR127935 (n=5)	0	6.4	2.6 - 15.8	1.0
	100	4.3	2.4 - 7.7	0.7
	300	2.2	1.0 - 4.7	0.3*
	1000	2.0	1.2 - 3.3	0.3*

[a]$ED_{50\ mmHg}$, dose of phenylephrine to increase blood pressure by 50 mmHg.
*, Statistically different from 1.0 ($P<0.05$).
It was not possible to calculate AD_2 here since the above compounds did not significantly antagonize the vasopressor responses to phenylephrine (i.e. $AD_2 \rightarrow \infty$).

It is noteworthy that the vasopressor responses to phenylephrine were accompanied with tachycardic responses, which may be probably due to the stimulation of cardiac β-adrenoceptors (sensitive to propranolol) and α_1-adrenoceptors (sensitive to prazosin), as previously suggested[40].

Admittedly, the magnitude of the control vasopressor responses to phenylephrine differed somewhat in the different groups of animals (compare Figure 6.1 and 6.2). This could be due to biological variability related to several factors including the particular sensitivity of each animal to phenylephrine, but not to differences in baseline diastolic blood pressure, as this variable was not significantly different in the 23 groups (see Results section). Consequently, this biological variability would not influence our results

Table 6.3 (cont.). Potency index of several 5-HT receptor antagonists against the vasopressor responses to i.v. bolus injections of phenylephrine in pithed rats. Note that all compounds failed to block the responses to phenylephrine.

Compound	Dose (µg/kg, i.v.)	[a]Phenylephrine $ED_{50\ mmHg}$ (µg/kg)	95% Confidence intervals (µg/kg)	$ED_{50\ mmHg}$ ratio
Ritanserin	0	4.2	2.1 - 8.5	1.0
(n=4)	300	3.4	1.9 - 6.0	0.8
	1000	3.1	2.2 - 4.4	0.7
	3000	2.8	1.5 - 5.0	0.7
Spiperone	0	4.7	2.5 - 9.1	1.0
(n=5)	10	3.5	1.9 - 6.6	0.7
	30	3.3	1.9 - 5.6	0.7
	100	3.1	1.2 - 8.5	0.7
	300	7.6	2.5 - 22.9	1.6
Pizotifen	0	3.5	2.1 - 5.9	1.0
(n=6)	300	2.5	1.4 - 4.3	0.7
	1000	1.8	1.0 - 3.1	0.5
	3000	2.7	1.8 - 4.2	0.8
Granisetron	0	4.6	2.1 - 10.3	1.0
(n=4)	300	1.6	0.9 - 2.9	0.3*
	1000	1.9	1.2 - 2.9	0.4*
	3000	2.7	1.5 - 4.9	0.6
Metoclopramide	0	3.6	2.2 - 6.2	1.0
(n=6)	300	2.6	1.9 - 3.5	0.7
	1000	2.0	1.5 - 2.7	0.6
	3000	1.8	1.1 - 3.0	0.5
Tropisetron	0	4.7	2.2 - 9.9	1.0
(n=4)	300	2.0	1.1 - 3.6	0.4
	1000	2.1	1.1 - 4.0	0.4
	3000	3.9	2.3 - 6.7	0.8
LY215840	0	3.2	0.9 - 11.5	1.0
(n=5)	100	1.7	0.5 - 5.7	0.5
	300	2.2	0.5 - 10.2	0.7
	1000	1.8	0.4 - 7.7	0.6
Yohimbine	0	5.5	3.0-10.0	1.0
(n=4)	300	4.5	1.9-10.6	0.8
	1000	5.3	1.9-14.3	1.0
	3000	7.3	4.4-12.3	1.3

unduly as the responses to phenylephrine were analysed before and after administration of a particular compound in the same group of animals.

6.4.3 α_1-adrenoceptor blocking properties of methiothepin, ketanserin, clozapine, lisuride and buspirone

The fact that methiothepin and ketanserin antagonised the responses to phenylephrine (Figure 6.1C and 6.1D, respectively) undeniably reveals a direct blockade of vascular α_1-adrenoceptors, which clearly correlates with their binding affinities (Table 6.1; Figure 6.3). Consistent with this view: (i) methiothepin[41] and ketanserin[42] decreased mean blood pressure in anaesthetised dogs; and (ii) ketanserin also decreased blood pressure in anesthetised rats and blocked the vasopressor responses to noradrenaline in pithed rats[43]. In addition, the fact that clozapine and lisuride (displaying high affinity at 5-HT_6 and 5-HT_7 receptors;[1]) produced a moderate blockade of the responses to phenylephrine seems to correlate with

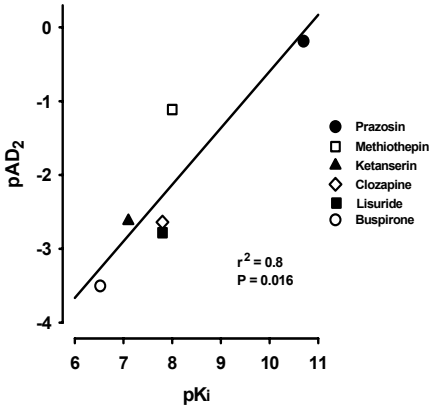

Figure 6.3: Linear regression between binding affinities (pK_i) at α_1-adrenoceptor binding sites and functional potency (pAD_2) of various 5-HT receptor ligands to block phenylephrine-induced vasopressor responses. pK_i and pAD_2 (negative logarithm of AD_2 expressed in µg/kg) values were obtained from Tables 6.1 and 6.2, respectively.

the moderate affinity of these compounds at α_1-adrenoceptor binding sites (Table 6.1; Figure 6.3). The α_1-adrenoceptors involved in the vasopressor responses to phenylephrine have previously been shown to correlate with the α_{1A} and α_{1D} subtypes[38,39].

6.4.4 Possible interactions of buspirone, ipsapirone, 8-OH-DPAT, WAY100635, pizotifen and ergotamine with vascular α_1-adrenoceptors

The short-lasting increases in diastolic blood pressure produced by of buspirone, ipsapirone, 8-OH-DPAT, WAY100635, pizotifen and ergotamine, in conjunction with their affinities at α_1-adrenoceptor binding sites (Table 6.1), suggest that these drugs may stimulate vascular α_1-adrenoceptors. This suggestion is reinforced by previous findings showing the role of α_1-adrenoceptors blocked by prazosin in: (i) the vasopressor responses to buspirone and ipsapirone in pithed rats[44]; and (ii) the canine external carotid vasoconstriction to 8-OH-DPAT[41], ipsapirone and buspirone[6], as well as to ergotamine[45].

Admittedly, we have no clear-cut explanation for the vasopressor responses to pizotifen (a non-selective 5-HT_2 receptor antagonist;[1]) since its affinity at α_1-adrenoceptor binding sites has not been determined (see Table 6.1). Moreover, the fact that the vasopressor responses to buspirone and ipsapirone were not dose-dependent probably suggests that these compounds behaved as partial agonists at vascular α_1-adrenoceptors; clearly, additional experiments will be required to further explore this possibility.

In connection with the above, the fact that WAY100635 failed to block the vasopressor responses to phenylephrine (Table 6.3), despite displaying affinity at α_1-adrenoceptor binding sites (Table 6.1), is apparently contradictory. In this respect, Villalobos-Molina *et al.*[46] have shown in rats (weighing 450 g) that WAY100635 dose-dependently: (i) decreased diastolic blood pressure; and (ii) blocked the vasopressor responses to phenylephrine. This apparent discrepancy with our study (using rats of 250-280 g) may be explained in terms of differences in the age of the rats used in each study. Thus, although aging might be related with a reduction in the adrenergic influence associated with changes in the expression of α_1-adrenoceptors[47], other findings imply an age-dependent functional expression of α_{1D}-adrenoceptors in the rat vasculature[48].

6.4.5 Lack of blockade of several 5-HT receptor ligands at vascular α_1-adrenoceptors

In contrast to methiothepin, ketanserin, clozapine, lisuride and buspirone (Figure 6.1 and 6.2), other ligands failed to block the vasopressor responses to phenylephrine at doses high enough to block/stimulate their corresponding 5-HT receptors.

The compounds include:

(i) Mesulergine, methysergide, granisetron, metoclopramide, GR127935, tropisetron, pizotifen and LY215840 (Table 6.3). The lack of blockade of these compounds could be easily explained in terms of their low affinity at α_1-adrenoceptors (see Table 6.1).

(ii) Metergoline, ritanserin and spiperone (Table 6.3). The lack of blockade of these compounds could similarly be explained by their low/moderate affinity at α_1-adrenoceptors (Table 6.1), although we cannot categorically exclude a potential α_1-adrenoceptor blockade if higher doses of these compounds had been used. It must be pointed out that the dose of spiperone (300 µg/kg) used, a 5-HT_{2A} receptor antagonist (pKi=9.0), was ten-fold higher than that used to block cardiac 5-HT_{2A} receptors[49]. Consistent with these findings, ritanserin also failed to block the noradrenaline-induced vasoconstrictor responses in the canine external carotid circulation[50]. Admittedly, we have no clear-cut explanation for the low blocking potency shown by metergoline, ritanserin and spiperone despite displaying moderate/high affinity at α_1-adrenoceptors.

(iii) Lisuride (100 µg/kg), methysergide (300 µg/kg), granisetron (300 and 1000 µg/kg), GR127935 (300 and 1000 µg/kg), and 8-OH-DPAT (300 µg/kg) (Table 6.3). Remarkably, not only did these compounds fail to block the vasopressor responses to phenylephrine, but even significantly potentiated these responses (at the doses indicated). A possible explanation for this potentiation could be that the above ligands may have activated multiple signalling pathways through vascular α_1-adrenoceptors. Indeed, the activation of α_1-adrenoceptors causes: (i) calcium influx; (ii) release of arachidonic acid by stimulation of phospholipase A_2 or phospholipase D; and (iii) accumulation of inositol phosphates by activation of phospholipase C[51]

In conclusion, the present study demonstrates that several 5-HT receptor ligands, including methiothepin, ketanserin, clozapine, lisuride and buspirone, can block vascular α_1-adrenoceptors. Moreover, it is suggested that some 5-HT_{1A} receptor ligands such as 8-OH-DPAT, buspirone, ipsapirone (agonists) as well as WAY100635 (antagonist) can stimulate vascular α_1-adrenoceptors. On this basis, and in order to avoid conflicting results, the pharmacological properties of these compounds (i.e. interactions with α_1-adrenoceptors) should be considered with caution when characterizing 5-HT receptors in the cardiovascular system.

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"You have to grow from the inside out. None can teach you, none can make you spiritual. There is no other teacher but your own soul" – Swami Vivekananda

Chapter 7

The role of calcitonin gene-related peptide (CGRP) in ischemic preconditioning in isolated rat hearts

Based on: Wenxia Chai, **Suneet Mehrotra***, A.H.J. Danser and Regien G. Schoemaker,* **European Journal of Pharmacology, 2006 Feb 15; 531(1-3): 246-53.**

ABSTRACT

Brief coronary artery occlusion (CAO) can protect the heart against damage during subsequent prolonged CAO; ischemic preconditioning (PC). The role of calcitonin gene-related peptide (CGRP) in PC is investigated in isolated perfused rat hearts, by measuring CGRP release during PC and mimicking this by exogenous CGRP infusion, either in the absence or presence of the CGRP antagonist olcegepant. CGRP increased left ventricular pressure (LVP) and coronary flow (CF) in a concentration dependent manner, which was effectively antagonized by olcegepant. Rat hearts (n=36) were subjected to 45 min CAO and 180 min reperfusion, which was proceeded by: (1) sham pretreatment, (2) olcegepant infusion (1 μM), (3) PC by 15 min CAO and10 min reperfusion, (4) as 3, but with olcegepant, (5) 15 min CGRP infusion (5 nM) and 10 min washout, (6) as 5, but with olcegepant. Cardiac protection was assessed by reactive hyperaemia (RH), creatine kinase (CK) release, infarct size related to the area at risk (I/R%), and LVP recovery. PC increased CGRP release into the coronary effluent from 88±13 to 154±32 pg/min/g, and significantly protected the hearts by decreasing RH (35%), reducing CK release (53%), limiting infarct size (48%), and improving LVP recovery (36%). Exogenous CGRP induced preconditioning-like cardioprotection. Olcegepant completely abolished the cardioprotection induced by PC as well as by exogenous CGRP. In conclusion, since cardioprotection of PC-induced CGRP release can be mimicked by exogenous CGRP, and a CGRP antagonist can block both, results indicate an important role for CGRP in ischemic preconditioning.

7.1 INTRODUCTION

The phenomenon that brief ischemia/reperfusion protects the heart from injury during a subsequent extended ischemic period was first recognized in dogs[1] and later firmly established in almost all animal models[2-4] as well as in human hearts [5]. This ischemic preconditioning induced cardioprotection includes reduction of infarct size and apoptosis[6]; decrease in the incidence of ventricular arrhythmias during reperfusion [7] and improved post-ischemic myocardial function[8]. The mechanism of preconditioning appears to be complex and involves signal transduction pathways such as the ATP-sensitive potassium (K_{ATP}) channel[9] and specific isoforms of protein kinase C (PKC)[10]. Endogenous substances, locally released during brief ischemia/reperfusion, activate second messenger pathways for preconditioning. Calcitonin gene-related peptide (CGRP) has recently been suggested as one of the mediators for this ischemic cardiac preconditioning[11].

Previous studies have shown that CGRP concentration in the coronary effluent of isolated rat hearts increased during brief global ischaemia, and that cardioprotection induced by this preconditioning was abolished after pretreatment with a selective $CGRP_1$ receptor antagonist $CGRP_{8-37}$[12], implicating a role for CGRP in global ischemic preconditioning. However, these studies were mainly focused on global cardiac ischemia. Since clinically myocardial infarction develops after single vessel occlusion rather than due to global ischemia, local myocardial ischemia may provide a more appropriate model for the clinical situation. In addition, the release of CGRP in the coronary effluent was gradually decreased with increasing number of cycles of PC stimulus (3 times 5 min ischemia). Moreover, the reperfusion times (30-45 min) in previous studies are relatively short since a full transmural myocardial infarction develops within 2 hours in rat[13,14].

In the present study, we first examined the pharmacological profile of CGRP by constructing concentration response curves for CGRP both on left ventricular pressure and coronary flow, using the isolated perfused rat heart. Secondly, to address whether CGRP plays a role in local ischemic preconditioning, we investigated the endogenous CGRP release in cardioprotection due to a single 15-min left descending coronary artery occlusion (CAO) and 10 min reperfusion, as a stimulus prior to 45 min CAO followed

by 180 min of reperfusion in isolated rat hearts. Subsequently, we mimicked this preconditioning by exogenous CGRP. We evaluated CGRP dependency by measuring cardioprotection in ischemic as well as mimicked preconditioning, both in the absence or presence of a selective and potent $CGRP_1$ antagonist olcegepant[15].

7.2 MATERIALS AND METHODS

Male Wistar rats (Harlan; Zeist, the Netherlands) weighing 300-340 g were used in the experiments. Experiments were performed in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" and with approval of Erasmus Medical Centre Rotterdam Animal Care Committee.

7.2.1 Perfusion of the isolated heart

Rats were anaesthetized with sodium pentobarbital (60 mg kg^{-1}, i.p.) and the hearts were rapidly excised and cooled in iced-cold Krebs-Henseleit solution. The hearts were mounted in a Langendorff apparatus at a constant pressure of 80 mmHg and perfused with oxygenated (95% O_2 / 5% CO_2) Krebs-Henseleit solution of the following composition (mM): NaCl 125, KCl 4.7, $NaHCO_3$ 20, NaH_2PO_4 0.43, $MgCl_2$ 1.0, $CaCl_2$ 1.3 and D-Glucose 9.1; pH 7.4 (37°C). A water-filled latex balloon was placed in the left ventricle via the left atrium, and connected to a pressure transducer. The volume of the balloon was adjusted to achieve a stable left ventricular end-diastolic pressure of 5 mmHg during initial equilibration and was maintained throughout the experiments. Hearts were paced at 350 beats min^{-1} (4 V, 2 ms). LVP was obtained from the latex balloon and CF was measured by an in-line flow probe (Transonic Systems, Ithaca, NY USA). The heart rate (HR), LVP and CF were recorded on a Grass polygraph. The hearts were equilibrated for 15 min before starting the experiments.

7.2.2 Experimental Protocol

7.2.2.1 Protocol I: Effects of CGRP

To characterize the pharmacological profile of CGRP, concentration-response curves to CGRP (n=6; 1 nM-10 µM) were constructed. For that, 100 µl bolus injections were administered into the perfusion buffer just before entering the coronary arteries. The time between the injections was 5-8 min or when the maximal response to the previous dose was reached. To examine tachyphylaxis, a second CGRP concentration response curve was constructed, after 15 min washout. Concentration response curves to CGRP were constructed in the absence or presence of olcegepant (100 nM and 1 µM, respectively; *n*=6 each, incubated for 15 min) to confirm that the effects were mediated by the CGRP receptor. The concentration of olcegepant that was used for the preconditioning study is based on the results of the above experiment. Stock olcegepant was dissolved in DMSO (100% dimethylsulphoxide), and was diluted with perfusion buffer to the required concentrations for the experiments. The final concentration of DMSO was 0.01 % and 0.001% for 100 nM and 1 µM olcegepant, respectively. DMSO at these concentrations had no effect on any of the parameters (pilot studies).

7.2.2.2 Protocol II: The role of CGRP in ischemic preconditioning

Rats were divided randomly over six experimental groups (n=6 each). All hearts were subjected to 45 min left anterior descending coronary artery occlusion (CAO) followed by 3 hours reperfusion. This was proceeded by: (1) Control: sham pretreatment; (2) olcegepant: infusion of olcegepant (1 µM) started 40 min before CAO; (3) PC: preconditioning (PC) by 15 min CAO and 10 min reperfusion; (4) olcegepant + PC: as group 3, but infusion with olcegepant (1 µM) started 15 min before PC; (5) CGRP: 15 min infusion with

CGRP (5 nM) and 10 min washout; (6) olcegepant + CGRP: as group 5, but infusion with olcegepant (1 μM) started 15 min before CGRP infusion (see figure 7.1).

7.2.3 Experimental measurements

Coronary effluent was collected at different time points: (1) Last min of stabilization for baseline CGRP and creatine kinase (CK) measurements. (2) First min of reperfusion after preconditioning for CGRP release measurement. (3) First 5 min of the 3 hours reperfusion for CK measurement. Samples were collected and stored at -80°C until analysis. Cardiac protection was measured by reactive hyperaemia (RH, maximal coronary flow during reperfusion), the incidence of left ventricular fibrillation (a period of >1 min without individually recognizable cardiac contractions) during reperfusion, CK release, infarct size and LVP recovery (the LVP after 20 min reperfusion compared to the baseline value before 45 min CAO).

7.2.4 CGRP measurement

CGRP was extracted from coronary effluent using a C_{18}SEPCOLUMN, dried by lypholisation, and measured by radioimmunoassay[16] as per the protocol of the Peninsula Laboratories, Inc (Belmont, CA, U.S.A.). All samples were coded and the estimation of CGRP levels were performed blinded.

7.2.5 CK measurement.

The activity of CK in the coronary effluent at different time points was assayed spectrophotometrically by ELAN analyzer from eppendorf MERCK (Germany) and compared with baseline values[17].

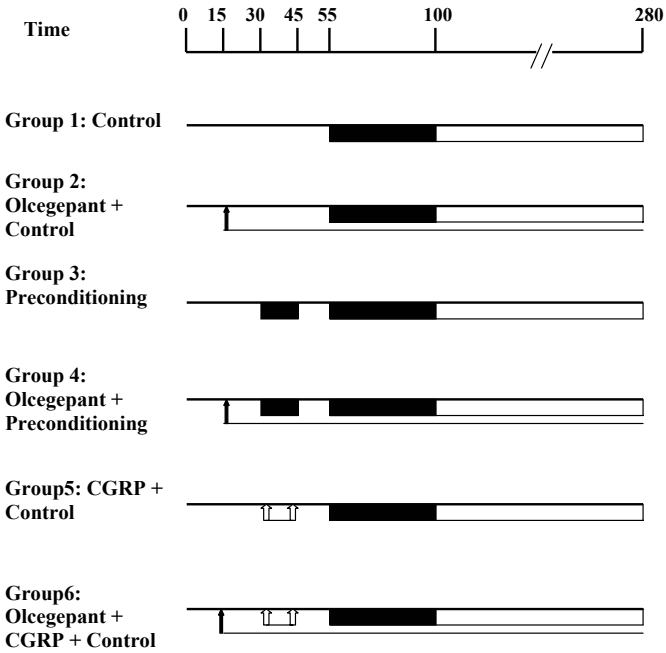

Figure 7.1: Experimental group. *Group1*: Hearts were subjected to 45 min occlusion of the left anterior descending coronary artery followed by 3 hours reperfusion; *Group 2*: 40 min perfusion with olcegepant (1 μM) + as in group 1; *Group 3*: 15 min occlusion and 10 min reperfusion of the left anterior descending coronary artery + as in group 1; *Group 4*: 15 min perfusion with olcegepant (1 μM) + as in group 3; *Group 5*: 15 min perfusion with CGRP (5 nM) + as in group 1; *Group 6*: 15 min perfusion with olcegepant (1μM) + as in group 5. *Solid bar:* Coronary occlusion; *Open bar:* Coronary reperfusion; *Solid arrow:* olcegepant perfusion; *Open arrow:* CGRP perfusion.

7.2.6 Measurements of area at risk and infarct area

At the end of each experiment, the ligature was retightened and the perfusion was stopped. The heart was infused with trypan blue (0.4%, Sigma Chemical) by injection for 30 seconds-1 min until a clear deliniation between perfused (blue) and non-perfused (pink) tissue was visible. The heart was taken off from the perfusion apparatus. Large vessels and atria were removed and the ventricles were placed at –80°C for 10 min before cutting into slices (1mm thick) from the apex to the base. From each slice, the right ventricle was removed and the left ventricle including the septum was divided into the area at risk and the remaining left ventricle. The area at risk was then incubated for 10 min at 37°C with nitro-blue tetrazolium (Sigma chemical: 1 mg ml^{-1} Sorensen buffer, pH 7.4), which stained the viable tissue purple but left the irreversible damaged tissue unstained (the infarct area). After the infarct area was separated from the non-infarct area, the different areas of the heart were dried overnight and weighed individually. The area at risk was expressed as percentage of the left ventricle (including the septum) and the infarct size was expressed as percentage of the area at risk; I/R.

7.2.7 Chemical compounds

Human α-CGRP and olcegepant were kindly provided by Dr. Henri Doods, Boehringer Ingelheim Pharma KG, Biberach, Germany. Calcitonin gene-related peptide kit was purchased from Peninsula (Sweden).

7.2.8 Data analysis

Concentration response curves to CGRP were characterized by the maximal response, E_{max}, and negative logarithm of the concentration eliciting 50% of the maximal contractile response, pEC_{50}, as determined with Graphpad Prism software (Graphpad Prism Inc., San Diego, California, U.S.A.). The maximum response observed at the highest concentration of CGRP was considered as E_{max} when a complete concentration-response curve could not be obtained. The CF and LVP before 45min CAO were considered as the baseline for group 2, group 4 and group 6, because olcegepant incubation decreased baseline of LVP and CF. RH and LVP recovery were expressed as a percentage changes from baseline. CGRP and CK release were expressed as the release of per gram total ventricular dry weight (pg $min^{-1}g^{-1}$ and mU min^{-1} g^{-1}, respectively). The risk area was expressed as the percentage of the left ventricle. The infarct size was expressed as percentage from the area at risk (I/R %)[18]. All data are presented as mean ± s.e.m. Differences in E_{max}, pEC_{50} values of the CGRP concentration response curve in the absence and presence of olcegepant, and the difference in CGRP release between the baseline and PC were analyzed by paired t-test. Differences in other values between two groups were determined by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison t-test. The difference of incidence of left ventricular arrhythmias between groups was determined by Chi square test. $P<0.05$ was considered as statistically significant.

7.3 RESULTS

7.3.1 Pharmacological profile of CGRP

Baseline values of LVP and CF were 62.0±5.8 mmHg and 11.4±1.0 ml min^{-1}, respectively. CGRP increased the LVP and CF in a concentration dependent manner (Figure 7.2a and 7.2b); the maximum response was obtained at 10 μM; 83.2±9.5 mm Hg (146±11% of baseline) for LVP and 19.3±1.0 ml min^{-1} (179±13% of baseline) for CF. The second concentration response curve to CGRP, which was constructed after 15 min wash-out, was not different from the first one. The maximum response amounted to 90.0±18.1 mmHg (154±14% of baseline) for LVP and 18.1±1.5 ml min (163±14% of baseline) for CF.

Incubation with olcegepant did not significantly affect baseline LVP (90±10% from baseline at 100 nM and 81±18% from baseline at 1 μM). However, as shown in Figure 7.2c and 7.2d, incubation with

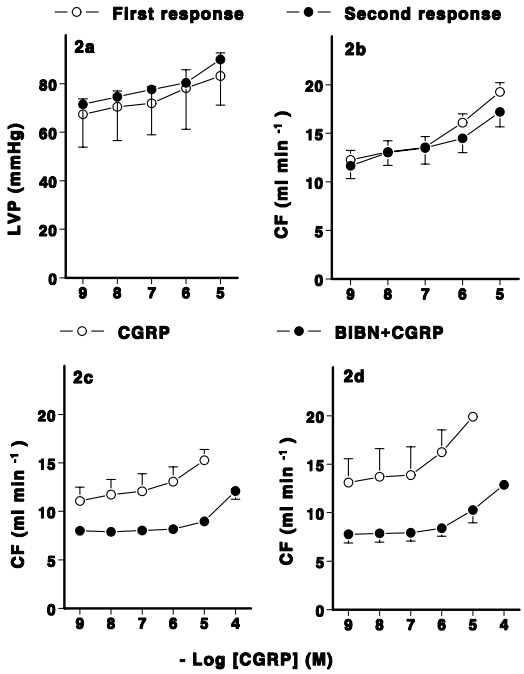

Figure 7.2: Concentration response curves of LVP as well as CF to CGRP (2a and 2b) and concentration response curves of CF to CGRP in the absence or presence of 100 nM olcegepant (2c) as well as 1 μM olcegepant (2d). The changes were expressed in mmHg for LVP and ml min^{-1} for CF. Data are shown as mean±s.e.mean. LVP: left ventricular pressure; CF: coronary flow.

olcegepant caused a significant reduction of baseline coronary flow of 20±5% at 100 nM and 32±4% at 1 μM. In the presence of olcegepant, the concentration response curves induced by CGRP were shifted to the right. The pEC_{50} values changed from 5.86±0.3 to 6.97±0.4 ($p<0.05$) in the presence of 100 nM olcegepant and from 5.54±0.2 to 6.96±0.4 ($p<0.05$) in the presence of 1 μM olcegepant. As a result, we used the concentration of 1 μM olcegepant to antagonise the CGRP responses in the PC study.

7.3.2 The role of CGRP in ischemic preconditioning

Baseline values of six groups. As shown in the Table 7.1, there were no significant differences in whole body weight, dry heart weight, and baseline values of LVP as well as CF among the six groups.

CGRP release. The baseline release of CGRP into the coronary effluent of hearts of the PC experiment was 87.9±12.8 pg $min^{-1}g^{-1}$, which did not differ from control hearts (88.2±15.3 pg $min^{-1}g^{-1}$). The release of CGRP significantly increased up to 154.2±32.4 pg $min^{-1}g^{-1}$ during brief CAO (PC period). The actual concentration of CGRP at PC was 0.93 ng/ml (which is in the same range as the CGRP concentration used to mimic precondition in group 5).

Cardiac protection. CF was significantly reduced (to 58.5±3.1% of baseline) during 45 min of regional CAO. CF increased immediately after reperfusion. This increase peaked during the first 5 min of reperfusion and declined to the stable level about 20 min of reperfusion. Ventricular arrhythmias, mostly ventricular fibrillation, occurred mainly in the first 10 min of reperfusion. The risk area was similar in all groups (see Table 7.2). However, the incidence of ventricular fibrillation is lower in PC and the exogenous CGRP group compared to the other groups. As shown in figure 7.3, RH, CK release, I/R and LVP recovery were 136±10%, 11390±960 (mU min^{-1} g^{-1}), 66±2% and 45±3%, respectively, in the control group. These parameters were not significantly affected by olcegepant itself. PC significantly protected the heart, as

Table 7.1. Baseline values of the six groups; group identification as in Figure 7.3

	Number	Body weight (g)	Heart dry weight (mg)	LVP (mmHg)	CF (ml min^{-1})
Control	6	349 ± 6	174.5 ± 6.3	83.3 ± 2.8	12.9 ± 0.9
olcegepant	6	326 ± 9	177.0 ± 2.9	88.2 ± 5.6	11.7 ± 0.7
PC	6	323 ± 3	167.2 ± 2.2	82.1 ± 2.3	12.8 ± 0.9
olcegepant +PC	6	326± 5	169.9 ± 6.9	89.1 ± 5.1	13.0 ± 0.7
CGRP	6	325± 4	168.8 ±2.9	83.6 ± 3.5	12.6 ± 0.4
CGRP+ olcegepant	6	328 ± 4	168.9 ± 3.3	82.7 ± 6.1	12.5 ± 0.6

LVP: Left ventricular pressure, CF: Coronary flow

Table 7.2. Incidence of ventricular fibrillation; group identification as in Figure 7.3

	Number	Risk area (% To LV)	Ventricular fibrillation
Control	6	49.8 ± 4	5/6
Olcegepant	6	52.6 ± 6	6/6
PC	6	51.4 ± 5	2/6**
Olcegepant +PC	6	51.1 ± 5	5/6
CGRP	6	53.5 ± 2	2/6**
CGRP+ olcegepant	6	52.6 ± 2	6/6

LV: Left ventricular ** p<0.01

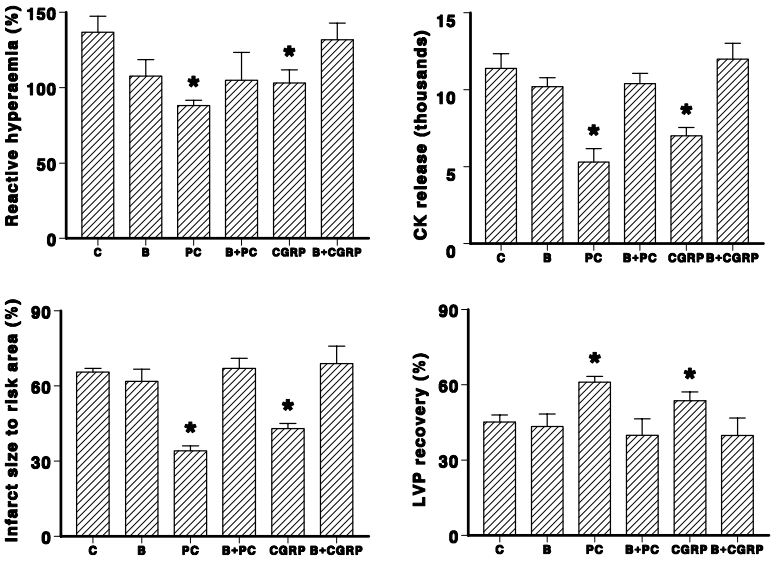

Figure 7.3: Reactive hyperaemia, CK release, infarct size in relation to the area at risk and LVP recovery in six groups of rats (*n*=6 each). All hearts were subjected to a 45 min left anterior descending coronary artery occlusion (CAO), followed by a 3 hr reperfusion. *Control:* no further treatment (group 1); olcegepant: infusion of 1 μM olcegepant (group 2); *PC:* preconditioning the heart by a 15 min CAO and 10 min reperfusion (group 3); olcegepant +*PC:* infusion with olcegepant + Preconditioning (group 4). *CGRP:* infusion with 5 nM CGRP before 45 min CAO. Olcegepant +*CGRP*: infusion with olcegepant (1 μM) started 15 min before CGRP infusion till the end of experiments. Data are shown as mean ± s.e.mean. * P<0.05 *vs.* control. **C:** Control group; **B:** olcegepant group; **PC:** preconditioning group; **B+PC:** olcegepant +PC group; **CGRP:** CGRP group; **B+CGRP:** olcegepant +CGRP group.

indicated by decreasing RH by 35%, reducing CK release by 53%, limiting infarct size by almost 50%, and improving LVP recovery by 36%. Infusion of CGRP had similar effects as PC; RH was decreased to 103±18%, CK release was reduced to 6915±712 mU min^{-1} g^{-1}, infarct size was limited to 43±4% and LVP recovery was improved to 53±4%.

The effects were significant although the cardioprotection was not as profound as PC. All measured cardiac protective effects of PC as well as CGRP infusion were significantly abolished by olcegepant.

7.4 DISCUSSION

7.4.1 CGRP and its effects

In the present study, CGRP increased LVP and CF in a concentration dependent manner, which is consistent with previous studies showing that CGRP caused positive inotropic effect and coronary vasodilatation in isolated rat hearts[19,20]. Furthermore, the second CGRP response curve after 15 min washout was similar to the first CGRP response curve, suggesting that CGRP acts as a coronary vasodilator without tachyphylaxis. olcegepant, a highly selective α-$CGRP_1$ receptor antagonist, dose-dependently antagonized the effects of CGRP, confirming that the vasodilatory effects are indeed mediated by CGRP receptors. Since the final concentration of CGRP in the perfusion buffer was roughly 100 times lower than the concentration of the bolus injection when considering the coronary flow is around 10 ml/min and the volume in cannulation tube was 10 ml. In this way, EC_{50} will be changed by 2 units and is in agreement with the EC_{50} in many other systems. Coronary vasodilatation induced by CGRP is probably mediated by endothelium-dependent as well as endothelium-independent mechanisms[21,22]. In patients with stable angina pectoris, it has been shown that intracoronary infusion of CGRP not only delayed the onset of myocardial ischaemia but also increased the work tolerance during treadmill exercise[23]. These beneficial effects of CGRP in myocardial ischemia are due to its ability to dilate the epicardial coronary arteries at the site of atheromatous stenoses.

Olcegepant decreased baseline coronary flow in a concentration dependent manner, indicating that endogenous CGRP may play a role in maintaining vasoactive tone in isolated rat hearts. However, a study in conscious dogs with CGRP antagonist CGRP (8-37) indicated that endogenous CGRP does not play a role in regulating the regional haemodynamics under resting conditions[24]. Our results, which were obtained from *in vitro* rat hearts, do not necessarily parallel the fact that CGRP (8-37) does not affect either LVP or CF in intact hearts, where many factors are responsible for the regulation of these parameters *in vivo*. Thus, whether endogenous CGRP plays a role in maintaining vasoactive tone remains to be determined.

7.4.2 The role of CGRP in preconditioning

The present study shows that brief regional CAO and reperfusion significantly reduced CK release, limited infarct size, decreased the incidence of arrhythmia during reperfusion and improved LVP recovery after consecutive prolonged CAO, confirming that local ischemic PC protects the heart during subsequent prolonged ischemia in isolated rat hearts. This is in accordance with previous studies[25-27]. One study performed in rats[28] showed that CGRP infusion 10 min before occlusion until the end of reperfusion reduced infarct size by 89±5 %; while infusion of the same dose of CGRP commencing from the start of reperfusion until its end induced a 40±3 % reduction of the infarct size, and both of these benefit effects were antagonized by olcegepant. These results favour our conclusion that CGRP play an important role during preconditioning since CGRP protects heart from reperfusion injury as well.

The concentration of CGRP in coronary effluent increased significantly during PC. Moreover, in the present study, ischemic PC could be mimicked by a timed short-term infusion of CGRP and resulted in a similar degree of cardioprotection. In patients with acute myocardial infarction, it has also been

observed that plasma CGRP levels increased, indicating that CGRP is released in response to the reduction of myocardial perfusion. In patients with coronary artery disease, administration of CGRP delayed the onset of myocardial ischemia during exercise testing. This may be attributed to the beneficial effect of coronary vasodilatation[29]. In the present study, olcegepant completely abolished the cardioprotection induced both by ischemic PC as well as by CGRP infusion, indicating that both effects were indeed mediated by CGRP receptors. Thus the observation that the CGRP concentration in the coronary effluent increases during ischemic PC, that exogenous CGRP has a preconditioning-like cardiac protection, and that both effects can be abolished by a selective CGRP antagonist, strongly suggest an important role for CGRP in ischemic PC.

Though the present study was performed *in vitro*, CGRP mediated PC may represent a phenomenon present *in vivo*. Previous studies have shown that CGRP can mediate delayed or remote cardioprotection, by using intestinal or gastric PC in rats *in vivo*[30-32]. Moreover, Kallner *et al.* reported[33] that exogenous CGRP could protect the heart, but that the injury was exacerbated when pigs were pretreated with high-dose capsaicin to deplete endogenous CGRP.

Whether CGRP may play a similar role in patients needs further investigation. Studies in patients with coronary artery disease made it feasible that the phenomenon of PC may also exist in the human myocardium. Although CGRP may be released during pathophysiological conditions such as heart failure as well as myocardial infarction[23,34], and may act as a potent vasodilator in the human coronary vasculature[24], whether CGRP mediated by CGRP receptors plays a role in ischemic PC in the human heart is not fully studied. On the other hand, a study[35] have shown that acute ischemic chest pain is associated with neither increased CGRP levels in peripheral plasma nor in the coronary circulation, suggesting that CGRP may be released in the response of the inflammation rather than ischemia.

Besides the involvement of CGRP in classic preconditioning as described above, there is evidence that delayed cardiac protection[30-32] or remote cardiac protection by intestinal and gastric preconditioning is mediated by CGRP, indicating a rather general mechanism of action. Until now, the exact mechanism of the cardiac protection induced by CGRP remains unknown. Studies indicated that CGRP enhances the activity of protein kinase C[11,36] and activates K_{ATP} channels[37] in vascular smooth muscle. However, another study showed that CGRP-mediated preconditioning could not be abolished by glibenclamide, a K_{ATP} channels blocker[38]. It has been reported that capsaicin-sensitive local sensory innervation is involved in pacing-induced preconditioning[39], as well as in remote ischemic preconditioning in rat hearts[18]. The effect of pacing-induced preconditioning, which included CGRP and NO release from capsaicin-sensitive nerves, was found to be sensitive to the K_{ATP} blocker glibenclamide. Endogenous NO could regulate the release of CGRP[40] and probably was involved in endogenous CGRP induced preconditioning. NO is known to activate K_{ATP} and other potassium channels [41] and may mediate CGRP related delayed preconditioning[42]. Obviously, further insights are required to understand the precise mechanism of CGRP mediated preconditioning.

In conclusion, in isolated perfused hearts CGRP act as a coronary vasodilator without tachyphylaxis. Since the CGRP antagonist olcegepant was able to reduce baseline coronary flow, CGRP may play a role in regulating vascular tone. Ischemic cardiac preconditioning is associated with increased release of endogenous CGRP, while exogenous CGRP could mimick the cardioprotective effect of preconditioning. The observations that the effects of both ischemic preconditioning and exogenous CGRP could be abolished by olcegepant provide strong evidence that indeed CGRP plays an important role via CGRP receptor stimulation. The mechanism behind these effects is still requiring further study. Similarly, additional study is necessary to evaluate the consequences of these findings for the clinical situation.

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Life is a challenge - meet it. Life is a gift - accept it.
Life is an adventure - dare it. Life is a sorrow - overcome it.
Life is a tragedy - face it. Life is a duty - perform it.
Life is a game - play it. Life is a mystery - unfold it.
Life is a song - sing it. Life is an opportunity - take it.
Life is a journey - complete it. Life is a promise - fulfill it.
Life is a beauty - praise it. Life is a struggle - fight it.
Life is a goal - achieve it. Life is a puzzle - solve it.
Life is eternal - believe it.

Part III: Effect of female sex hormones on vascular pharmacology of migraine

Chapter 8

Rat carotid artery responses to α-adrenergic receptor agonists and 5-HT after ovariectomy and hormone replacement

Based on: **Suneet Mehrotra***, *Saurabh Gupta**, *Carlos M. Villalón, Frans Boomsma, Pramod R. Saxena and Antoinette MaassenVanDenBrink,* **Headache, In Press (* the first two authors had an equal contribution)**.

ABSTRACT

To compare the contractile responses to α-adrenergic receptor agonists and 5-HT in the rat carotid artery after ovariectomy and subsequent hormone replacement with 17β-estradiol, progesterone or the combination of 17β-estradiol and progesterone. The prevalence of migraine is higher in women than in men, and changes in 17β-estradiol levels seem to affect the frequency of attacks in female migraineurs. However, the underlying mechanisms are not yet completely understood. After one week of acclimatization (Day 0), female Sprague-Dawley rats were either sham-operated or bilateral ovariectomized. One week later (Day 7), the ovariectomized rats were subcutaneously implanted with a pellet releasing over a 21-day period either placebo, 0.25 mg 17β-estradiol, 50 mg progesterone or the combination of the two hormones. Blood samples were collected on Days 0, 7 and 21 to measure plasma noradrenaline and adrenaline. On day 25-28, the animals were killed to isolate carotid artery and mount its segments in Mulvany myographs. Cumulative concentration response curves to α-adrenoceptor agonists and 5-HT were constructed in the absence or presence of suitable antagonists. The potency of noradrenaline in ovariectomized rats was significantly reduced in animals treated with progesterone as compared to those with placebo. In placebo-treated ovariectomized animals there was a noticeable response mediated by α_2-adrenoceptors, in contrast to that in sham-operated or ovariectomized rats treated with 17β-estradiol and progesterone, either alone or in combination. The plasma levels of noradrenaline and adrenaline were not significantly affected by either ovariectomy or the subsequent hormone replacement. The potency of 5-HT was significantly reduced in animals having circulating sex hormones as compared to that in placebo-treated ovariectomized animals. Taken together, our results indicate that circulating progesterone and/or 17β-estradiol may reduce the contraction of the rat carotid artery in response to noradrenaline and 5-HT. This effect of female sex hormones might be one of the factors through which these hormones aggravate migraine in women.

8.1 INTRODUCTION

Migraine is three times more common during reproductive years in women than in men[1]. This gender difference in the prevalence of migraine appears to be related to fluctuating levels of female sex hormones during the menstrual cycle[2]. Plasma levels of 17β-estradiol and progesterone in women are at their lowest just before periods[3], and there seems to be an association between 17β-estradiol "withdrawal" and migraine attacks, which tend to cluster just before or during menstruation in a majority of afflicted women[4-8]. In contrast, the sustained high concentration of 17β-estradiol during pregnancy frequently results in headache relief[9], although migraine with aura seems to have a higher prevalence[10]. The frequency of migraine attacks increases after child birth, when 17β-estradiol levels decline[11]. Moreover, in two controlled trials, percutaneous 17β-estradiol reduced the frequency of migraine headaches[12,13]. Thus, migraine severity correlates with changes in estrogen levels and it is reasonable to assume that there may be a causal relationship.

It is now well recognized that migraine is a neurovascular syndrome, where the headache phase seems to be associated with cranial extracerebral vasodilatation.[14-17]. Although the meningeal artery is thought to play a major role in the pathophysiology of migraine[18,19], animal experimental models based on carotid vasoconstriction in the anesthetized animals, including the cat[20,21], dog[16,22], pig[23,24] and rabbit[25], clearly show the effectiveness of antimigraine compounds, particularly those acting via 5-hydroxytryptamine or α-adrenoceptors. Moreover, *in vitro* studies show that the rat[26,27] as well as rabbit[28,29] isolated carotid artery contracts in response to both 5-HT[26,28] and α-adrenoceptor agonists[27,29]. Interestingly, female sex hormones are known to influence the vasoconstriction elicited by these agonists. For instance: (i) the contractile responses to 5-HT and α-adrenoceptor agonists in the rat[30] and rabbit[31] isolated aorta decrease after treatment with 17β-estradiol; (ii) in ovariectomized rats, 17β-estradiol replace-

ment reduces the vasoconstriction to α_2-adrenoceptor activation[32]; and (iii) in ovariectomized rabbits, 17β-estradiol depresses the contractions mediated by α_2-adrenoceptors in the femoral artery, but not in the saphenous vein[33]. Furthermore, 17β-estradiol[34] and progesterone[35] have been shown to decrease the sympathetic tone, and it has been suggested that migraineurs have a low sympathetic tone[36].

In the light of these observations, the present study sets out to investigate the influence of female sex hormones on contractile responses to noradrenaline, 5-HT and the α_1-adrenoceptor agonists phenylephrine and A61603 in isolated carotid arteries obtained from sham-operated and ovariectomized rats; the latter were subsequently treated with either placebo, 17β-estradiol, progesterone or the combination of both hormones.

8.2 MATERIALS AND METHODS

8.2.1 Animal groups

Female Sprague-Dawley rats (body weight: 190-260 g, age: 8-11 wks) were purchased from Harlan Netherlands (Horst, The Netherlands). All animals were given free access to food and water. After one week of acclimatization (Day 0), the rats were anesthetized with isoflurane anesthesia for either a sham-operation (exteriorization but not the removal of ovaries; Group 1) or bilateral ovariectomy. One week later (Day 7) and under the same anesthesia, the ovariectomized rats were subcutaneously implanted with a pellet releasing over a 21-day period either placebo (Group 2), 0.25 mg 17β-estradiol (Group 3), 50 mg progesterone (Group 4) or the combination of the two hormones (Group 5). As reported recently[37], in these animals the concentrations of both 17β-estradiol and progesterone (Day 0; 25±6 pg ml^{-1} and 24±6 pg ml^{-1}, n=36 each, respectively) decrease following ovariectomy (Day 7; 23±4 pg ml^{-1} and 6±2 pg ml^{-1}, n=26 each, respectively) and increase after implantation of hormones pellets in ovariectomized animals; for example, on Day 21 (i.e. 14 days after treatment with respective hormones pellets), plasma concentrations of 17β-estradiol and progesterone were 187±45 pg ml^{-1} and 17±4 pg ml^{-1}, n=5-7 each, respectively.

8.2.2 Measurements of plasma concentration of noradrenaline and adrenaline

Blood samples were collected from the tail vein under isoflurane anesthesia three times: Day 0 (before ovariectomy), Day 7 (only in ovariectomized animals) and Day 21 (21 days after sham-operation or 14 days after pellet implantation in ovariectomized animals). After collection in chilled heparinized tubes containing 3 mg glutathione S-transferase, blood was immediately centrifuged (3000 g, 15 min, 4 °C) to separate plasma, which was stored at -80 °C until the catecholamines were assayed using high performance liquid chromatography with fluorimetric detection[38]. The values obtained in the different groups of animals on Day 0 (before sham operation or ovariectomy) and on Day 7 in ovariectomized animals (before pellet implantation) were combined, while the values obtained on Day 21 in the 5 groups are presented separately.

8.2.3 Isolation of the carotid artery

Rats were sacrificed on Day 25-28 (i.e. 25-28 days after sham operation or 18-21 days after pellet implantation in ovariectomized animals) by intraperitoneal injection of sodium pentobarbital (100 mg kg^{-1}). The right carotid artery was isolated and kept in Krebs buffer solution (composition: 119 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11.1 mM glucose; pH 7.4; 4 °C). Immediately after removal, the carotid artery was cut into 1-2 mm ring segments that were mounted in Mulvany myographs (Danish myo technology a/s, Aarhus, Denmark) between two parallel titanium wires with tension normalized to 90% of I_{100} (distance when transmural pressure equals 100 mmHg), thus achieving optimal conditions for active force development[39]. The myograph chambers contained Krebs buffer solution (see above), which was aerated with 95% O_2 and 5% CO_2 at 37 °C. Changes in tension of

the rat carotid artery segments were measured with isometric force transducers (Danish myo technology a/s, Aarhus, Denmark) and recorded on a flatbed recorder (Servogor 124, Goerz, Neudorf, Austria).

The carotid artery segments were allowed to equilibrate for at least 30 min and were washed every 15 min. They were then challenged at 30-min intervals twice with 30 mM KCl to verify the reproducibility of responses and then with 100 mM KCl to obtain a reference contractile response for each vessel segment (see data analysis). Subsequently, the vessel segments were stabilized and concentration (10^{-10} M to $3x10^{-5}$ M) response curves to noradrenaline (α_1-, α_2- and β_1-adrenoceptor agonist), 5-HT (vascular 5-HT_{1B} and 5-HT_2 receptor agonist), phenylephrine and A61603 (both α_1-adrenoceptor agonists) were constructed to investigate the involvement of their respective receptors. Only one concentration response curve was constructed in each arterial segment. The concentration response curves to noradrenaline were also made in arterial segments incubated for 30 min with either: (i) vehicle, (ii) the α_1-adrenoceptor antagonist prazosin (100 nM), or (iii) the α_2-adrenoceptor antagonist rauwolscine (100 nM). Moreover, it may be noted that all experiments with noradrenaline were performed in the presence of cocaine (1 μM), corticosterone (3 μM) and propranolol (100 nM) to eliminate, respectively, the neuronal and extra-neuronal reuptake and the possible vasorelaxation via β-adrenoceptors.

8.2.4 Ethical approval

The ethical committee of Erasmus MC dealing with animal experiments approved this study protocol

8.2.5 Data analysis

As described earlier[40,41], the contractile responses to all agonists were expressed as percentage of the contraction induced by 100 mM KCl in the respective segments and the data were analyzed (Graph Pad Prism 3.01, Graph Pad Software Inc., San Diego, CA, U.S.A.) to obtain, in each case, agonist E_{max} (maximum plateau response) and pEC_{50} (negative logarithm of the molar concentration eliciting 50% of the E_{max}). It must be highlighted that when a plateau was not reached with an agonist, the response at its highest concentration was considered as E_{max}, except for the experiments in the presence of an antagonist, where the E_{max} in the presence of an antagonist was assumed to be equal to the control E_{max} in case a plateau was not reached. The E_{max} and pEC_{50} values represent, respectively, the *efficacy* and *potency* of an agonist to contract the rat carotid artery. Assuming a slope of unity, the potency of the antagonists was expressed as apparent pK_b (negative logarithm of the antagonist concentration, whereby the agonist concentration eliciting a certain response is increased by a factor of 2). Statistical significance was determined by ANOVA followed by Dunnett's multiple comparison tests with differences considered significant at $P \leq 0.05$. All data are presented as means±S.E.M. and *n* represents the number of carotid artery rings used, each obtained from a different animal.

8.2.6 Compounds

The compounds used in the present study (obtained from the sources indicated) were the following: 5-hydroxytryptamine creatinine sulphate (serotonin; 5-HT), L-phenylephrine hydrochloride, noradrenaline, corticosterone, glutathione S-transferase and heparin sulfate sodium salt (all from Sigma, St. Louis, MO, U.S.A.), prazosin hydrochloride (Bufa Chemie b.v., Castricum, The Netherlands), cocaine (local pharmacy at Erasmus Medical Center, Rotterdam, The Netherlands), propranolol hydrochloride (Sigma, St. Louis, MO, U.S.A.) and rauwolscine dihydrochloride (RBI, Natick, MA, U.S.A.). Corticosterone was dissolved in dimethyl sulfoxide and prazosin hydrochloride in ethanol, while all other compounds were dissolved in distilled water. The above vehicles had no effect on the agonist responses at the concentrations employed. The placebo and hormone pellets were purchased from Innovative Research of America (Sarasota, FL, U.S.A.).

8.3 RESULTS

8.3.1 Contraction to KCl

All segments contracted in response to 100 mM KCl. There were no significant differences in KCl-induced contractions between sham-operated (2.8±0.9 mN) and ovariectomized animals treated with placebo (3.3±0.6 mN), 17β-estradiol (4.3±0.7 mN), progesterone (3.7±0.6 mN) or the combination of these hormones (2.6±0.3 mN).

8.3.2 Contractions mediated by α-adrenoceptors

In all carotid artery segments investigated, the endogenous ligand noradrenaline induced concentration-dependent contractions (Figure 8.1). The potency (pEC_{50}) of noradrenaline to induce contractions was significantly less in vessel segments obtained from rats treated with progesterone as compared to those treated with placebo (Figure 8.1, Table 8.1).

Figure 8.2 shows the concentration response curves to noradrenaline on carotid artery segments obtained from sham-operated rats as well as from ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones, in the absence (control) or presence of the adrenoceptor antagonists, prazosin (α_1) and rauwolscine (α_2). As compared to control responses, prazosin induced a significant rightward shift of the concentration response curve to noradrenaline in all groups; the corresponding pK_b values in the different groups, which were not significantly different from each other, were: sham-operated rats (8.38±0.35) and ovariectomized rats treated with either placebo (9.28±0.23), 17β-estradiol (9.34±0.29), progesterone (9.09±0.24) or the combination of these hormones (9.00±0.21). In contrast to the results obtained with prazosin, rauwolscine failed to induce a significant shift of the concentration response curves to noradrenaline in animals with endogenous (sham-operated) or exogenous (ovariectomized, but treated with hormone pellets) female sex steroids; interestingly, only in ovariectomized animals treated with placebo did rauwolscine significantly antagonize the contractions to noradrenaline (pK_b: 8.21±0.58; Figure 8.2).

As shown in Figure 8.3, phenylephrine induced concentration-dependent contractions that were not different between the groups, although its potency tended to be somewhat lower in progesterone-treated animals than in the other groups (Table 8.1). Likewise, the contractions to A61603 were also not significantly different between the groups (Figure 8.3; Table 8.1).

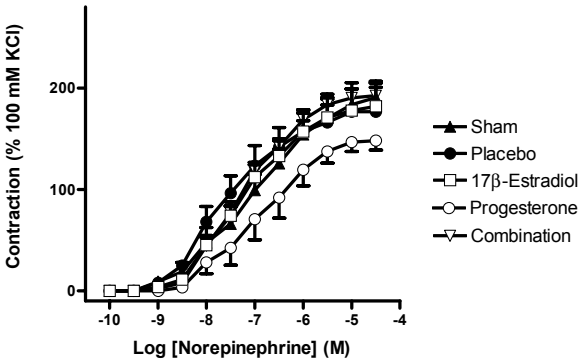

Figure 8.1: Contractions to noradrenaline in isolated carotid arteries obtained from sham-operated rats, as well as ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=5-7 each).

Table 8.1. E_{max} and pEC_{50} values of noradrenaline, phenylephrine, A61603 and 5-HT in contracting isolated carotid artery segments obtained from rats after different interventions.

Pharmacological parameters		Sham-ovariectomized rats (*n*=5)	Ovariectomized rats treated with			
			Placebo (*n*=5)	17β-estradiol (*n*=5-6)	Progesterone (*n*=5-6)	Progesterone + 17β-estradiol (*n*=7)
Noradrenaline	E_{max}	189±15	146±24	182±23	147±11	193±8
	pEC_{50}	7.1±0.2	7.7±0.1	7.3±0.1	7.0±0.2*	7.3±0.1
Phenylephrine	E_{max}	195±49	244± 90	104±28	103±18	158±21
	pEC_{50}	7.2±0.2	6.8±0.1	7.0±0.5	6.0±0.2	7.1±0.2
A61603	E_{max}	88±4	108±15	66±23	78±26	133±24
	pEC_{50}	5.9±0.1	5.8±0.1	5.5±0.2	5.5±0.10	6.0±0.2
5-HT	E_{max}	172±16	210±45	83±20*	148±18	145±18
	pEC_{50}	5.8±0.2*	6.6±0.2	5.7±0.1*	5.6±0.2*	5.7±0.2*

8.4.3 Contractions to 5-HT

The contractile responses induced by 5-HT on the carotid artery rings from different groups of animals are shown in Figure 8.4. The pEC_{50} (potency) of 5-HT was greater in ovariectomized animals treated with placebo than in those treated with 17β-estradiol, progesterone, the combination of these hormones, or the sham-operated animals (Table 8.1). The E_{max} of 5-HT was significantly smaller in the ovariectomized animals treated with 17β-estradiol than in the placebo-treated group, while the E_{max} values obtained in the other groups were not different from those in the placebo group (Table 8.1).

8.4.4 Plasma concentrations of noradrenaline and adrenaline

The plasma concentrations of noradrenaline and adrenaline were not significantly different before (Day 0: 324±45 pg ml^{-1} and 242±34 pg ml^{-1}, respectively) or after (Day 7: 361±27 pg ml^{-1} and 344±54 pg ml^{-1}, respectively) ovariectomy. Similarly, the plasma levels of noradrenaline and adrenaline were not affected by treatment with 17β-estradiol and/or progesterone pellets (Figure 8.5).

8.4 DISCUSSION

The results of our study demonstrate that ovariectomy followed by the subsequent treatment with female sex hormones affected the contractile responses of the rat isolated carotid artery to both α-adrenoceptor agonists and 5-HT. This effect is unlikely to be due to differences in muscle mass of the carotid artery, since we corrected for differences in muscle mass and, moreover, the contractile responses to 100 mM KCl were similar across all treatment groups. Furthermore, our results suggest that the influence of female sex hormones is dependent on the agonist used (i.e. noradrenaline or 5-HT); thus, it seems improbable that an aspecific mechanism may account for the effects that we observed in our study. Admittedly, the plasma levels of 17β-estradiol reached after pellet implantation were about twice as high as the highest levels reached in a normal menstrual cycle; future studies may shed more light on the influence of different estrous levels of 17β-estradiol on vasoactive responses.

17β-estradiol promotes vasodilatation via both genomic and non-genomic mechanisms that: (i) generate agents, such as nitric oxide, cGMP, cAMP, adenosine and prostacyclin[42,43]; and (ii) alter ion channel activity[44]. Although endothelial factors might be involved, some lines of evidence suggest that steroids-induced vasodilatation is also mediated by endothelium-independent mechanisms[45-47]. Furthermore, sex steroids may induce vasodilatation by inhibiting Ca^{2+} currents[48] and by activating K^+ channels[49,50]. However, the effects of sex hormones on contraction and dilatation are divergent, which may be

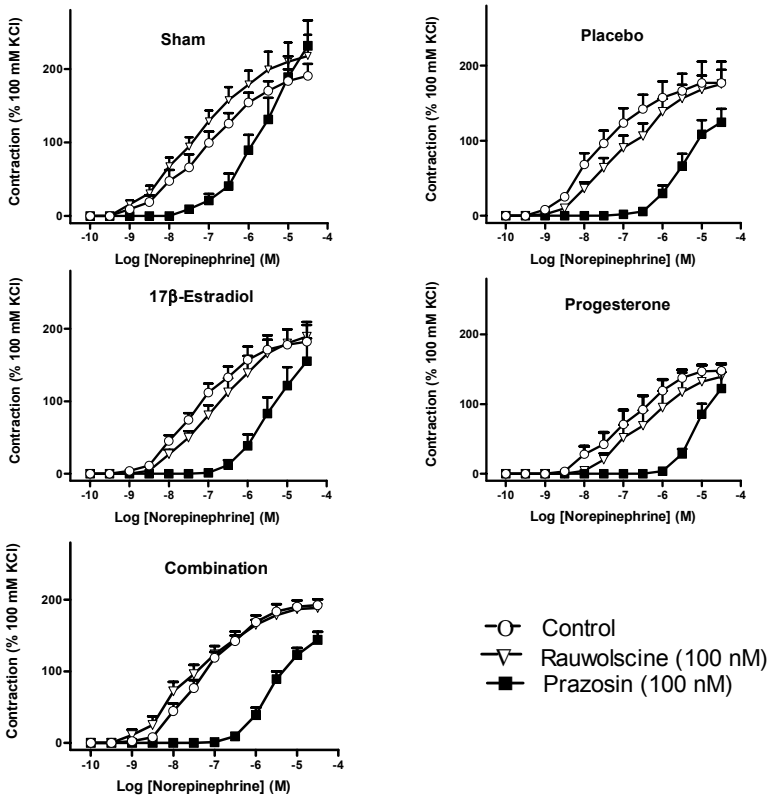

Figure 8.2: Contractions to noradrenaline in the absence (control) and in the presence of prazosin or rauwolscine (both 100 nM) in sham operated rats, as well as ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=5-7 each).

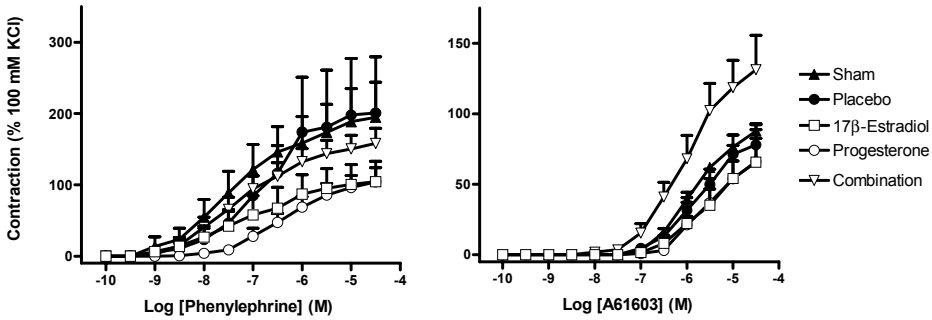

Figure 8.3: Contractions to phenylephrine and A61603 in sham operated rats, as well as ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=5-7 each).

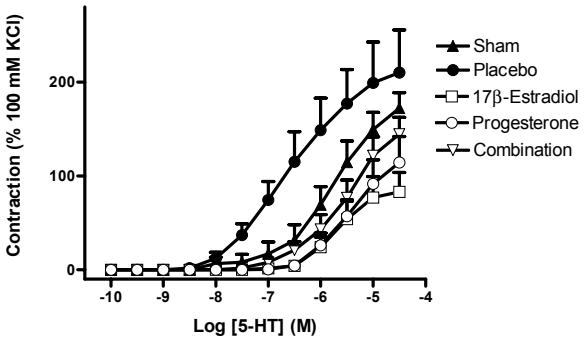

Figure 8.4: Contractions to 5-HT in sham operated rats, as well as ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=5-7 each).

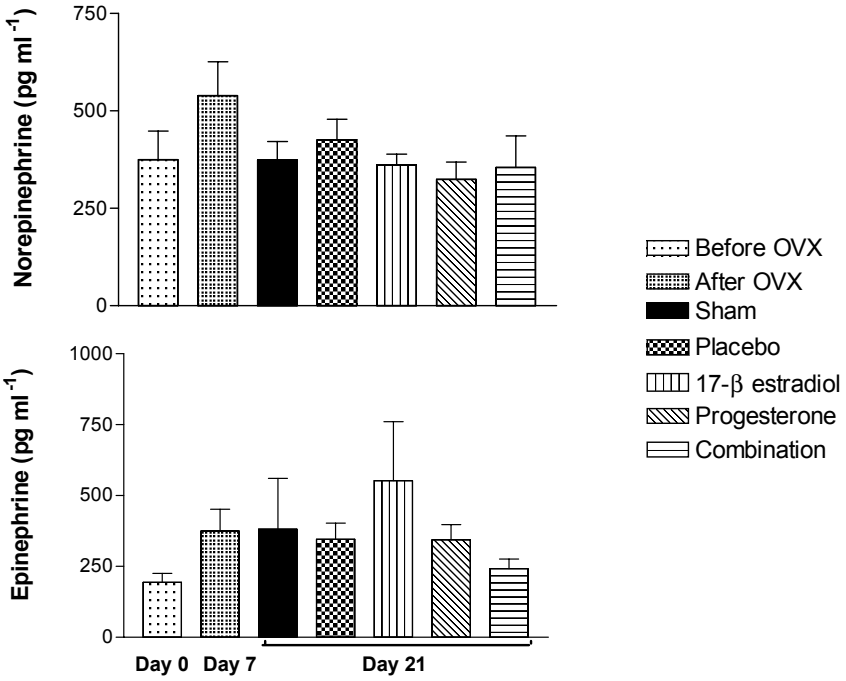

Figure 8.5: Plasma concentrations of noradrenaline and adrenaline in sham-operated or ovariectomized (OVX) rats on Day 0 (just before sham-operation or ovariectomy, *n*=7), Day 7 (7 days after ovariectomy before pellet implantation, *n*=10), and Day 21 (21 days after sham operation, *n*=4; or 14 days after implantation in ovariectomized animals of pellets containing placebo, *n*=8; 17β-estradiol, n=7; progesterone, *n*=8; or their combination, *n*=4). No value differed significantly from that in placebo-treated rats.

due to several factors, including species differences, the vascular bed under study and the experimental conditions (e.g. a specific agonist, duration and dose of steroid pretreatment).

The vasoconstrictor potency of noradrenaline in ovariectomized rats treated with progesterone, but not 17β-estradiol, was significantly attenuated. This observation seems to differ from the report that rat aorta contraction via α_1-adrenoceptor remains unaffected by both 17β-estradiol and progesterone[51]. However, 17β-estradiol and progesterone may have opposing actions[52-56]. Indeed, our results are in ac-

cordance with the finding showing that pretreatment with progesterone, but not estrogen, inhibited the contractions to noradrenaline in the rat tail artery[57]. The decrease in the sensitivity to noradrenaline in ovariectomized rats treated with progesterone, suggesting less availability of postsynaptic α-adrenoceptors, may lead to cranial vasodilatation and, therefore, proneness to migraine headache[15-17]. This is in line with a recent investigation showing that higher urinary progesterone metabolites were positively correlated with headache outcome measures during the luteal phase of the menstrual cycle[9]. In addition, it has been reported that plasma progesterone levels show an irregular pattern in migraine patients[58]. Intriguingly, it has been suggested that migraineurs have a reduction in sympathetic function as compared to nonmigraineurs[36]. In addition, migraine with aura patients have a resting supine sympathetic hypofunction[59], which seems to play an important role in the maintenance of headaches[60].

Interestingly, our results also point to an increased function of α_2-adrenoceptors after ovariectomy without hormonal replacement (placebo treatment). This is in agreement with observations that α_2-adrenergic responsiveness decreases in blood vessels obtained from ovariectomized rabbits treated with 17β-estradiol[33]. As placebo-treated animals do not have circulating female sex hormones, these animals might be considered as a model for the menopause conditions in women. Keeping this in mind, this increased function of α_2-adrenoceptors may, at least partly, account for the increased blood pressure in females after menopause[61]. Admittedly, this interesting finding should be explored further before any definite conclusions can be drawn. Both 17β-estradiol[34] and progesterone[35] are known to decrease the sympathetic tone, a fact that may strengthen the effects of female sex hormones on the expression of α-adrenoceptors. Unfortunately, our study could not confirm differences in the plasma levels of noradrenaline or adrenaline amongst the different groups, probably due to the relatively small number of animals used and the variability in the measurements.

It is noteworthy that, in contrast to our findings with noradrenaline, the contractile responses to the α_1-adrenoceptor agonists, phenylephrine and A61603 did not differ in the different groups. Although this seems to be in contrast to the report that 17β-estradiol attenuated the vasoconstrictor action of phenylephrine in the rat mesenteric artery[62], others have reported an upregulation of the α_1-adrenoceptor in mandibular glands[63] as well as no change (saphenous vein) or a decrease (femoral artery) in the contractile response to noradrenaline[33] in the rat. In agreement with Gisclard *et al*.[33], our findings also suggest that changes in female sex hormones mainly perpetuate their effect via altered α_2-adrenoceptor function in the rat blood vessels. Admittedly, additional studies using selective α_2-adrenoceptor agonists such as BHT-933[64], which clearly fall beyond the scope of the present investigation, may further clarify the mechanisms involved.

With regards to 5-HT, we found that the contractile response in the carotid artery was reduced in the presence of both 17β-estradiol and progesterone. This is in agreement with other studies demonstrating that the aortic contraction to 5-HT was decreased after treatment with 17β-estradiol in rats[30] as well as rabbits[31].

The difference between the responses mediated by activation of α_1/α_2-adrenoceptors in the presence of progesterone and of 5-HT receptors in the presence of both sex hormones may be due to several factors, including differences in: (i) the density of α_1/α_2-adrenergic and 5-HT_{1B}/5-HT_{2A} receptors in the carotid artery segment; and (ii) the second messenger signaling of α_1-adrenergic and 5-HT_{2A} receptors (both involve activation of G_q proteins and phospholipase C with a resulting increase in phosphoinositol turnover and elevation of intracellular Ca^{2+}), as well as of α_2-adrenergic and 5-HT_{1B} receptors (both involve activation of $G_{i/o}$ proteins and inhibition of adenylyl cyclase with a resulting decrease in c-AMP levels). Accordingly, differences in the responsiveness of these receptors in ovariectomized animals could be mediated via the same signaling pathways.

Taken together, our results suggest that: (i) circulating progesterone, but not 17β-estradiol, reduces the potency of noradrenaline to contract the rat isolated carotid artery, while the absence of progesterone and 17β-estradiol most probably leads to an increased function of α_2-adrenoceptors; and (ii) pro-

gesterone, 17β-estradiol as well as their combination attenuate the contraction to 5-HT. These findings, if true for human cranial blood vessels, could possibly provide a mechanism through which female sex hormones may aggravate migraine in women.

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"You have to dream before your dream can come true" (Dr. Abdul Kalam, President of India)

Chapter 9

Effects of female sex hormones on responses to CGRP, acetylcholine and 5-HT in rat isolated arteries

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ABSTRACT

Female sex hormones are implicated in the modulation of reactivity of a wide range of blood vessels in physiological as well as pathological conditions. Migraine, a neurovascular syndrome, is three times more prevalent in women during their reproductive period than in men. This study sets out to investigate the effects of the female sex steroids, 17β-estradiol and progesterone (separately and in combination) on vasoactive responses to calcitonin gene-related peptide (CGRP), acetylcholine and 5-hydroxytryptamine (5-HT) in rat isolated mesenteric, caudal and basilar arteries. Female Sprague-Dawley rats were ovariectomized (Day 0) and 7 days later subcutaneously implanted with pellets releasing over a 21-day period 17β-estradiol (0.25 mg), progesterone (50 mg), their combination or placebo. On Day 25-28, the animals were killed, arteries isolated and mounted in Mulvany myographs and cumulative concentration response curves to CGRP, acetylcholine and 5-HT were constructed. The relaxant responses to CGRP were significantly potentiated in mesenteric and caudal arteries from rats treated with 17β-estradiol as compared to the placebo-treated rats. Acetylcholine-induced relaxations were potentiated in the caudal artery from rats treated with the combination of 17β-estradiol and progesterone, as compared to that from placebo-treated rats. The 5-HT-induced contractions in the three arteries were not significantly different in efficacy or potency. Our results show that 17β-estradiol potentiates CGRP-induced relaxations in the mesenteric and caudal arteries, whilst the combination treatment enhances acetylcholine-induced relaxations in the caudal artery. If similar mechanisms operate in the human intracranial extracerebral vasculature, this may, at least partly, explain how increased levels of 17β-estradiol can lead to a higher incidence of migraine in women.

9.1 INTRODUCTION

Migraine, a neurovascular syndrome, is about three times more common in women than in men at peak prevalence[1,2] and its incidence fluctuates according to reproductive milestones in female life[3-5]. Interestingly, the occurrence of migraine before puberty is higher in men than in women, while it reverses after puberty[6]. In women, the migraine attacks are aggravated during menstruation[3], while most migraineurs experience improvement during pregnancy[4] and after menopause[5]. These observations suggest a role for female hormones in migraine pathogenesis.

Calcitonin gene-related peptide (CGRP) is implicated in the pathophysiology of migraine as a dilator of cranial blood vessels and, hence, activation of pain-sensitive nociceptors in these blood vessels[7-10]. Accordingly, the CGRP receptor antagonist, olcegepant [11], has been reported to be effective in the acute treatment of migraine[12]. Moreover, in the past one and half decade, triptans (5-$HT_{1B/1D}$ receptor agonists) have been developed and proven effective in alleviating migraine headache[7,13,14]. The antimigraine activity of triptans is, at least partly, attributed to constriction of dilated intracranial extracerebral arteries[7,13,14]. These observations provide evidence for a strong vascular component in the pathophysiology of migraine.

Vascular tone is controlled by an interplay of a number of factors and female sex hormones are one of the significant determinants[15,16]. In this respect, 17β-estradiol and progesterone cause vasorelaxation *per se* at high non-physiological concentrations (rapid non-genomic pathways), but they may also manifest their actions via slow genomic actions[15,17,18]. In addition, sex steroids modulate the synthesis of and/or sensitivity to various vasoactive agents. In fact, it seems that CGRP homeostasis is strongly influenced by sex steroids as: (i) ovariectomy in rats decreases, whilst subsequent treatment with female hormones increases CGRP plasma levels[19], (ii) the expression of CGRP receptors is augmented during pregnancy, while it decreases after parturition[20], (iii) pregnancy in women augments myometrium sensitivity to CGRP[21], and (iv) there is a positive correlation between plasma levels of CGRP and 17β-estradiol

in postmenopausal women undergoing hormone replacement therapy[22]. These responses are thought to be mediated by classical genomic effects as a result of chronic exposure to 17β-estradiol. Moreover, the enhancement of the relaxant responses by female sex steroids in various vascular beds is suggested to be due to an increased activity of endothelium-dependent pathways, such as nitric oxide or cGMP[15,17]; indeed, acetylcholine-induced endothelium-dependent relaxations are augmented in rat isolated cerebral arteries[23] as well as in aortic rings from ovariectomized rats treated with 17β-estradiol[24]. In contrast to CGRP and acetylcholine, the reactivity to 5-HT has been reported to increase after ovariectomy, and to normalize after treatment with female sex hormones in primate[25] and rabbit[26] arteries; indeed, both 17β-estradiol and progesterone can attenuate the increase in intracellular Ca^{2+} and protein kinase C translocation in vascular smooth muscle cells by 5-HT[27]. Similarly, acute incubation with 17β-estradiol decreases the maximum response to contractile agents, including 5-HT, in porcine and human arteries[28,29]. On this basis, the present study has examined the effect of 17β-estradiol and progesterone, separately and in combination, on the responses to CGRP, acetylcholine and 5-HT in isolated segments of mesenteric, caudal and basilar arteries of female rats.

9.2 MATERIALS AND METHODS

9.2.1 Animal groups and isolation of blood vessels

Female Sprague-Dawley rats (body weight: 190-260 g, age: 8-11 wks) were purchased from Harlan Netherlands (Horst, The Netherlands). All animals were given free access to food and water. After one week of acclimatization (Day 0), the rats were anesthetized with isoflurane for either a sham-operation (exteriorization but not the removal of ovaries; Group 1) or bilateral ovariectomy. One week later (Day 7) and under the same anesthesia, the ovariectomized rats were subcutaneously implanted with a pellet releasing over a 21-day period either placebo (Group 2), 0.25 mg 17β-estradiol (Group 3), 50 mg progesterone (Group 4) or the combination of the two hormones (Group 5). As reported recently[30], in these animals the concentrations of both 17β-estradiol and progesterone (Day 0; 27±2 pg ml^{-1} and 21±2 pg ml^{-1}, n=43 each, respectively) decreased following ovariectomy (Day 7; 22±2 pg ml^{-1} and 7±1 pg ml^{-1}, n=35 each, respectively) and increased after implantation of hormones pellets in ovariectomized animals; for example, on Day 21 (i.e. 14 days after treatment with respective hormones pellets), plasma concentrations of 17β-estradiol and progesterone were 201±36 pg ml^{-1}, n=11 and 17±4 pg ml^{-1}, n=9, respectively.

The rats were killed on Day 25-28 under anesthesia using sodium pentobarbital (100 mg kg^{-1}, i.p.). Subsequently, second order mesenteric, caudal and basilar arteries were isolated and placed in cold oxygenated Krebs bicarbonate solution (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; 4 °C). The surrounding tissue was removed and the arteries were cut into ring segments of 1-2 mm length (internal diameters: mesenteric, 300-500 µm; caudal, 300-400 µm; and basilar, 100-125 µm). Subsequently, the segments were mounted in Mulvany myographs (Danish myo technology a/s, Aarhus, Denmark) between two parallel titanium wires with tension normalized to 90% of I_{100} (distance when transmural pressure equals 100 mmHg), thus achieving optimal conditions for active force development[31]. The myograph chambers containing Krebs buffer solution (see above) was aerated with 95% O_2 and 5% CO_2 at 37 °C. In the case of basilar artery segments, 0.1 µM indomethacin (cyclooxygenase inhibitor) was added to the Krebs buffer to inhibit small spontaneous contractions often observed in this vessel[32]. Changes in tension of the arterial segments were measured with an isometric force transducer (Danish myo technology a/s, Aarhus, Denmark) and recorded on a flatbed recorder (Servogor 124, Goerz, Neudorf, Austria).

9.2.2 Concentration response curve

After an initial equilibration period of 30 min, two successive challenges to KCl (30 mM) were performed to check the reproducibility of the responses in blood vessels obtained from all five groups of rats. After another 30-min period, 100 mM KCl was added to determine the reference contractile response in each segment. Then, a stable contraction was obtained with 1 μM 9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619; mesenteric), 0.1 μM U46619 (caudal) or 5 nM endothelin-1 (basilar); U46619 did not induce a stable precontraction in this tissue. In preconstricted arterial segments, cumulative concentration response curves to r-αCGRP and acetylcholine were constructed. Concentration response curves to 5-HT were constructed in the absence of any precontraction. Only one concentration response curve was performed in each artery segment and all the concentration response curves were made in half log steps.

9.2.3 Ethical Approval

The ethical committee of Erasmus MC dealing with animal experiments approved this study protocol.

9.2.4 Data Analysis

The contractile response to 100 mM KCl, expressed in mN, was used to compare the contractile force developed by arteries from different experimental groups. The relaxant responses to r-αCGRP and acetylcholine are expressed as percentage relaxation of the precontraction induced by U46619 or endothelin-1. The contractile responses to 5-HT are expressed as percentage of the contraction induced by 100 mM KCl. All values are expressed as mean±S.E.M. and *n* represents the number of vessel segments, each of which was obtained from a different rat.

The concentration response curves for all agonists were analyzed using nonlinear regression analysis to determine the maximal response (E_{max}: maximum plateau response reached with an agonist) and potency (pEC_{50}: negative logarithm of the molar concentration eliciting 50% of the E_{max}) of each agonist. ANOVA was conducted between all the groups, followed by post hoc Dunnett's multiple comparisons using E_{max} or pEC_{50} values obtained from placebo-treated ovariectomized rats as the control group with differences considered significant at $P<0.05$.

9.2.5 Compounds

Apart from the anesthetic (sodium pentobarbital), the compounds used in the present study (obtained from the sources indicated) were: 5-hydroxytryptamine creatinine sulphate, U46619 and acetylcholine chloride (Sigma Chemical Co., Steinheim, Germany); endothelin-1 and rat-αCGRP (NeoMPS S.A., Strasbourg, France). All compounds were dissolved in distilled water and stored in aliquots at -80˚C, until required. Just before use, the stock solutions were further diluted to the appropriate concentration in distilled water. The hormone pellets were obtained from Innovative Research of America (Sarasota, FL, U.S.A.).

9.3 RESULTS

9.3.1 Contractions induced by KCl, U46619 and endothelin-1

All segments obtained from mesenteric, basilar and caudal arteries contracted in response to KCl, U46619 and endothelin-1. There were no significant differences in contractions induced by these agents between placebo-treated animals and other treatment groups in any of the three arteries (Table 9.1).

9.3.2 Relaxations induced by αCGRP

αCGRP induced concentration-dependent relaxations in mesenteric, caudal and basilar artery segments (Figure 9.1). αCGRP was more potent in relaxing the mesenteric and caudal artery segments from ovariectomized rats treated with 17β-estradiol than those from placebo-treated rats, but the other groups did not differ significantly from placebo (Table 9.2). In basilar artery, no differences in potency were observed between the groups. Similarly, the maximal responses to αCGRP were not different between the groups in either mesenteric, caudal arteries or basilar artery.

9.3.3 Relaxations induced by acetylcholine

Acetylcholine, like CGRP, caused concentration-dependent relaxations in all arterial segments studied. In mesenteric and basilar arteries, there were no significant differences in the E_{max} or potency of acetylcholine (Figure 9.2, Table 9.3). Further, in the case of the basilar artery, the intra-group variations were relatively high and maximal responses were less as compared to the mesenteric and caudal arteries. In the caudal artery, the responses to acetylcholine were significantly potentiated in rats treated with the combination of 17β-estradiol and progesterone ($P<0.05$) and possibly, albeit not significantly, in rats treated with 17β-estradiol alone ($P=0.06$), as compared to the placebo-treated group. There were no significant differences in the maximal response to acetylcholine in caudal artery segments (Figure 9.2, Table 9.3).

9.3.4 Contractions induced by 5-HT

Since 2-3 mesenteric artery segments from each treatment group did not respond to 5-HT (maximal response <5% of KCl 100 mM), the data from these animals were excluded from further analysis. In the rest of the arterial segments, 5-HT produced a concentration-dependent contractile response, where neither the E_{max} nor the potency (pEC_{50}) differed amongst the study groups (Figure 9.3, Table 9.4). Similarly, there were no significant differences in the E_{max} or potency of the responses to 5-HT in the caudal and basilar artery segments (Figure 9.3, Table 9.4).

9.4 DISCUSSION

Our results demonstrate that ovariectomy and subsequent treatment with 17β-estradiol and progesterone affect the vasoactive responses in the arteries studied. The observed changes were not generalized with respect to a particular agonist, a specific type of blood vessel or even the kind of effect being

Table 9.1. Contractile responses (mN) to KCl (used as reference) and to U46619 and endothelin-1 (used for precontraction) in isolated artery segments obtained from sham-ovariectomized rats or ovariectomized rats that were subcutaneously implanted with either placebo pellets or pellets releasing 17β-estradiol, progesterone or their combination

Arterial segments	Contractile agent (concentration)	Sham-ovariectomized rats (*n*)	Ovariectomized rats treated with			
			Placebo (*n*)	17β-Estradiol (*n*)	Progesterone (*n*)	17β-Estradiol + Progesterone (*n*)
Mesenteric	KCl (100 mM)	9.5±1.1 (8)	11.1±1.2 (15)	11.0±1.5 (12)	9.1±0.8 (12)	8.3±0.7 (12)
	U46619 (1 μM)	9.1±0.9 (8)	9.2±1.13 (14)	9.2±1.4 (10)	7.2±1.0 (9)	7.9±1.0 (10)
Caudal	KCl (100 mM)	6.0±0.9 (7)	7.6±0.8 (13)	8.5±1.2 (11)	6.5±1.3 (8)	8.4±0.6 (10)
	U46619 (100 nM)	7.7±1.0 (6)	8.4±1.5 (11)	8.0±0.9 (10)	6.0±2.4 (7)	7.4±0.5 (10)
Basilar	KCl (100 mM)	4.7±0.7 (9)	6.2±0.8 (12)	5.9±0.9 (14)	6.0±0.5 (10)	5.8±0.7 (11)
	Endothelin-1 (5 nM)	4.3±0.6 (9)	5.76±1.04 (12)	4.7±0.8 (13)	5.3±0.7 (10)	5.2±1.0 (10)

Table 9.2. E_{max} and pEC_{50} values of CGRP in relaxing precontracted isolated artery segments obtained from sham-ovariectomized rats or ovariectomized rats that were subcutaneously implanted with either placebo pellets or pellets releasing 17β-estradiol, progesterone or their combination

Arterial segments	Pharmacological parameters	Sham-ovariectomized rats (*n*)	Ovariectomized rats treated with			
			Placebo (*n*)	17β-Estradiol (*n*)	Progesterone (*n*)	17β-Estradiol + Progesterone (*n*)
Mesenteric	E_{max}	98±1 (9)	93±2 (11)	97±2 (8)	91±4 (9)	99±1 (8)
	pEC_{50}	9.53±0.22 (9)	9.76±0.20 (11)	10.84±0.34 (8)*	9.48±0.27 (9)	9.80±0.22 (8)
Caudal	E_{max}	83±3 (6)	71±4 (8)	84±5 (8)	81±8 (7)	81±5 (9)
	pEC_{50}	8.17±0.10 (6)	8.23±0.05 (8)	8.69±0.17 (8)*	8.33±0.14 (7)	8.25±0.04 (9)
Basilar	E_{max}	40±5 (8)	38±7 (9)	48±5 (11)	50±6 (9)	48±6 (6)
	pEC_{50}	8.30±0.09 (8)	8.25±0.07 (9)	8.18±0.23 (11)	8.37±0.11 (9)	8.45±0.11 (6)

E_{max}, Maximum response, expressed as % of precontraction induced by U46619 (mesenteric and caudal arteries) or endothelin-1 (basilar artery)

pEC_{50}, Concentration required to produce 50% of maximal response

*Significantly different ($P < 0.05$) from the corresponding value in placebo-treated rats

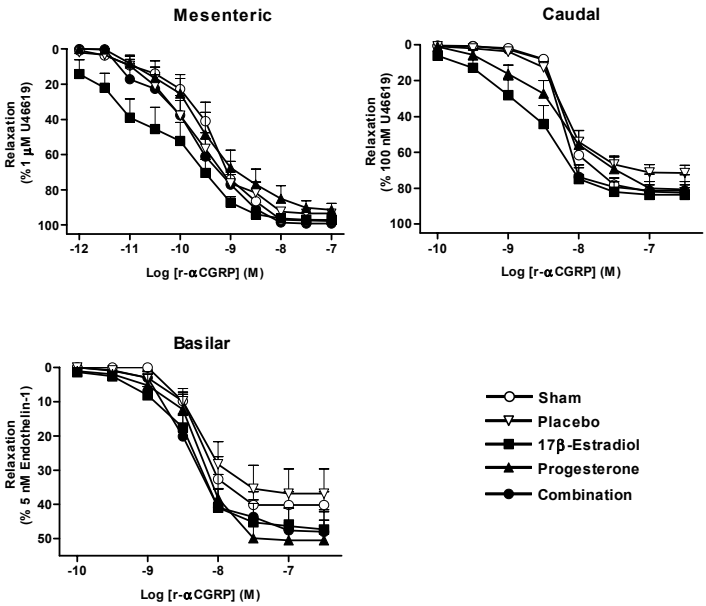

Figure 9.1: Relaxations induced by αCGRP in isolated precontracted mesenteric, caudal and basilar artery segments obtained from sham-ovariectomized rats, as well as from ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=8-11).

investigated (relaxant or contractile responses). The reference contractile responses to 100 mM KCl and the contractions induced by U46619 or endothelin-1 were not significantly different amongst the various treatment groups in the mesenteric, caudal or basilar artery. Thus, the observed differences in the responses to the agonists cannot be attributed to differences in the blood vessel muscle mass or a general, non-specific mechanism.

The maximum relaxation induced by CGRP was highest in the mesenteric artery, followed by that in the caudal and basilar arteries. Similarly, CGRP was a more potent relaxant (i.e., it induced relaxations at

Table 9.3. E_{max} and pEC_{50} values of acetylcholine in relaxing precontracted isolated artery segments obtained from sham-ovariectomized rats or ovariectomized rats that were subcutaneously implanted with either placebo pellets or pellets releasing 17β-estradiol, progesterone or their combination

Arterial segments	Pharmacological parameters	Sham-ovariectomized rats (*n*)	Ovariectomized rats treated with			
			Placebo (*n*)	17β-Estradiol (*n*)	Progesterone (*n*)	17β-Estradiol + Progesterone (*n*)
Mesenteric	E_{max}	82±6 (6)	89±3 (9)	91±3 (7)	94±2 (8)	89±5 (8)
	pEC_{50}	7.74±0.29 (6)	7.46±0.27 (9)	7.50±0.13 (7)	7.71±0.21 (8)	7.69±0.13 (8)
Caudal	E_{max}	48±7 (7)	65±5 (8)	67±10 (6)	72±4 (6)	76±6 (8)
	pEC_{50}	6.39±0.23 (7)	6.22±0.17 (8)	6.93±0.33 (6)	6.54±0.23 (6)	7.10±0.18 (8)*
Basilar	E_{max}	38±7 (5)	37 ±10 (7)	37±8 (5)	48±11 (7)	59±13 (5)
	pEC_{50}	7.50±0.52 (5)	7.21±0.35 (7)	6.78±0.16 (5)	6.57±0.25 (7)	7.42±0.33 (5)

E_{max}, Maximum response, expressed as % of precontraction induced by U46619 (mesenteric and caudal arteries) or endothelin-1 (basilar artery)

pEC_{50}, Concentration required to produce 50% of maximal response

*Significantly different (P<0.05) from the corresponding value in placebo-treated rats

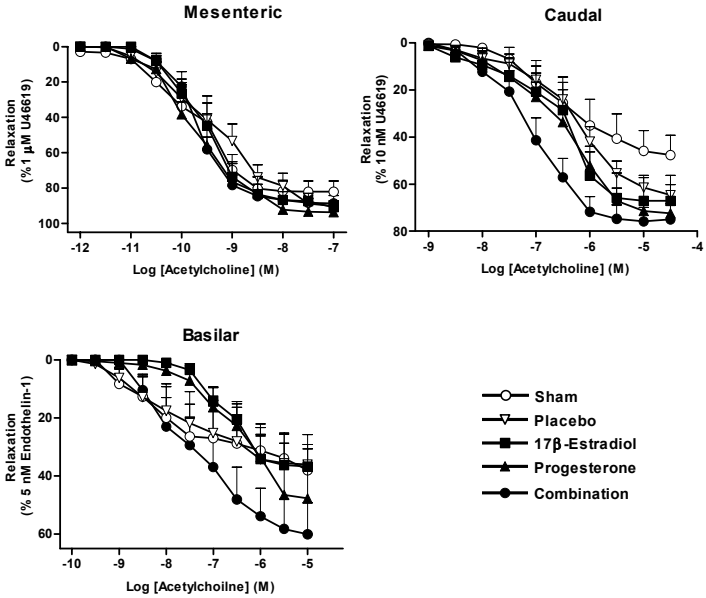

Figure 9.2: Relaxations induced by acetylcholine in isolated precontracted mesenteric, caudal and basilar artery segments obtained from sham-ovariectomized rats, as well as from ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=5-9).

lower concentrations) in the mesenteric artery as compared to the other two arteries. This may be due to the fact that CGRP is reported to be more efficacious and potent in resistance blood vessels[33], such as the second order mesenteric arteries, which are vital in regulating total vascular tone, than in conducting blood vessels like caudal and basilar arteries. The potency of CGRP in both mesenteric and caudal arteries was significantly increased in 17β-estradiol-treated rats as compared to placebo-treated rats. In keeping with these findings, Gangula *et al.*[34] have also reported that ovariectomy decreases the CGRP potency; moreover, in pregnancy, where 17β-estradiol levels are increased, the potency of CGRP is augmented.[34] It is important to highlight that the CGRP-induced relaxations are endothelium-independent in the rat

Table 9.4. E_{max} and pEC_{50} values of 5-HT in contracting isolated artery segments obtained from sham-ovariectomized rats or ovariectomized rats that were subcutaneously implanted with either placebo pellets or pellets releasing 17β-estradiol, progesterone or their combination

Arterial segments	Pharma-cological parameters	Sham-ovariectomized rats (*n*)	Ovariectomized rats treated with			
			Placebo (*n*)	17β-Estradiol (*n*)	Progesterone (*n*)	17β-Estradiol + Progesterone (*n*)
Mesenteric	E_{max}	157±8 (7)	187±14 (9)	140±11 (10)	174±15 (7)	158±24 (9)
	pEC_{50}	6.32±0.07 (7)	6.33±0.14 (9)	6.39±0.16 (10)	6.42±0.22 (7)	6.35±0.16 (9)
Caudal	E_{max}	199±15 (7)	190±15 (10)	178±15 (9)	200±13 (7)	168±19 (8)
	pEC_{50}	6.96±0.13 (7)	6.95±0.15 (10)	7.12±0.10 (9)	6.53±0.20 (7)	7.07±0.11 (8)
Basilar	E_{max}	140±13 (6)	128±9 (9)	145±17 (8)	139±13 (8)	95±11 (6)
	pEC_{50}	6.88±0.15 (6)	7.21±0.17 (6)	7.14±0.09 (6)	6.88±0.18 (6)	7.21±0.23 (6)

E_{max}, Maximum response as percentage of the contraction induced by 100 mM KCl.
pEC_{50}, Concentration required to produce 50% of maximal response
*Significantly different (P<0.05) from the corresponding value in placebo-treated rats

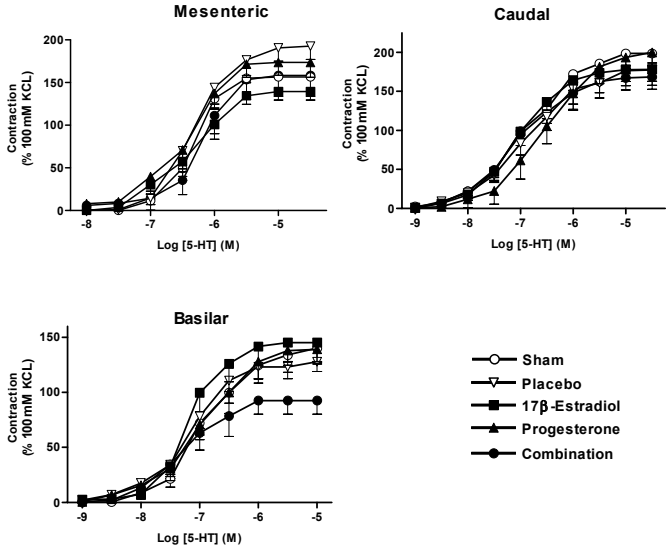

Figure 9.3: Contractions to 5-HT in mesenteric, caudal and basilar arteries segments obtained from sham-ovariectomized rats, as well as from ovariectomized rats treated with placebo, 17β-estradiol, progesterone or the combination of these hormones (*n*=6-10).

mesenteric[35] and caudal[36] arteries, similar to the observations in the human cerebral[37], meningeal[37] and coronary[38] arteries. Interestingly, unlike the mesenteric and caudal arteries, the basilar artery did not exhibit any increase in the responses to CGRP, although the potency of CGRP is in line with the previous reports in the rat basilar artery[39]. Indeed, the enhanced responses to CGRP by 17β-estradiol do not seem to be a systemic phenomenon, as estrogen is reported to selectively increase the sensory nociceptor innervation of mesenteric arterioles in female rats[40]. In a recent study in human vascular smooth muscle cells, dexamethasone was reported to increase mRNA expression of RAMP1 and CRLR[41], which are essential components of $CGRP_1$ receptors. Hence, steroidal hormones may regulate the synthesis as well as the receptor expression for CGRP. These findings clearly suggest that 17β-estradiol increases the responses to CGRP. Further, when the rats were treated with the combination of 17β-estradiol and progesterone,

the effect of the latter was reversed, whilst the sham-operated rats or those treated only with progesterone did not significantly differ from the placebo-treated animals. Similarly, other authors have also reported opposing actions of 17β-estradiol and progesterone[42,43]. Also, the CGRP receptor expression in rats is regulated in divergent ways by these female sex steroids[44].

The responses to acetylcholine in mesenteric and basilar arteries were not significantly different amongst various treatment groups. This seems contrary to the fact that female sex hormones have been reported to (specifically) affect the vasoactive responses in blood vessels by enhancing the synthesis of nitric oxide, and by a direct Ca^{2+} antagonistic effect on vascular smooth muscle[45-47], hence facilitating endothelium-dependent responses. However, it has recently been shown in the rat mesenteric artery that relaxations mediated by nitric oxide, which is released by acetylcholine, are decreased and, in contrast, there is an increase in the part of the acetylcholine response mediated by endothelial-derived relaxing factor (EDRF). Hence, overall, no changes were observed in acetylcholine-induced relaxations[17], as also reinforced in the present study. Further, as the potency of CGRP was enhanced in the mesenteric artery, the mechanism involved seems to be independent of endothelium-dependent relaxations. In the case of the caudal artery, there was a significant increase in the potency of acetylcholine in the rats treated with the combination of 17β-estradiol and progesterone. In fact, a similar tendency was observed in the rats treated with 17β-estradiol alone; this could be due to endothelium-dependent mechanisms.

In mesenteric arteries, the potency of 5-HT amongst the different treatment groups was in line with the previously reported values in rats[48]. But, no significant differences were observed in the E_{max} and the potency of 5-HT in the various groups. In the basilar artery, there were no significant differences in the efficacy or potency of 5-HT in the rats exposed to different hormonal interventions; this is in accordance with previous observations in the rat isolated anterior cerebral arteries, where female sex steroids did not alter the functional activity of 5-HT[23]. In contrast, in the rabbit basilar artery estrogen withdrawal may result in hypersensitivity to 5-HT[49], and an acute incubation with estrogen is reported to decrease the sensitivity to 5-HT and other contractile agents[28,29].

The plasma levels of 17β-estradiol in the ovariectomized rats treated with 17β-estradiol pellets were ~2.5 times the highest levels encountered in normal estrous cycle in rats; this may be a potential limitation of this study. Ideally, potentiation of CGRP responses in dural arteries, like meningeal arteries, would have provided a more direct evidence, but these arteries are too small to mount in organ baths, but nevertheless these observations provide a putative mechanism of how 17β-estradiol may modulate migraine outcome.

It is important to note that several studies addressing the effect of sex steroids on vascular responses have used acute incubation with these hormones in arteries obtained from pigs[28], rhesus monkeys[27] and even humans[29], where suppression of the contractile effects and potentiation of the vasodilator responses was observed[50]. These effects are attributed to rapid non-genomic mechanisms, whereas in our experimental set-up the arteries were isolated and suspended in Krebs solution free of female sex hormones. Therefore, we assume that the effects observed in the present study are the result of a chronic treatment with the female sex hormones, mediated by a classical genomic mechanism. In this respect, it is essential to distinguish between the genomic and non-genomic actions of sex steroids; as the non-genomic actions are induced rapidly, it can thus not be excluded that in the absence of these hormones the effects will revert to the basal state[51,52].

Overall, the treatment with 17β-estradiol augments the vasorelaxation potency of CGRP in the mesenteric and caudal arteries, and of acetylcholine in the caudal artery. Nevertheless, if 17β-estradiol correspondingly potentiates the CGRP-induced dilatation of dural resistance arteries, this might be one of the mechanisms explaining how 17β-estradiol can aggravate migraine attacks in women.

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Part IV: Molecular pharmacology and genetic studies in migraine

Chapter 10

Cloning of the porcine α_{2B}-adrenoceptor: Tissue distribution and pharmacological characterization

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ABSTRACT

Constriction of carotid arteriovenous anastomoses in the pig serves as a predictive model for assessing the antimigraine efficacy of α_2-adrenoceptors-based drugs. To gain further insight in to α_2-adrenoceptors mediated mechanisms, we amplified, cloned and sequenced a full-length cDNA encoding porcine α_{2B}-adrenoceptor, assessed its tissue distribution and pharmacological characteristics. Sequence analysis of the porcine cDNA clone (1341 bp) revealed an open reading frame of 446 amino acids encoding α_{2B}-adrenoceptor, which showed 88% and 85% similarity with the human and guinea pig sequences, respectively. The porcine α_{2B}-adrenoceptor showed 7 transmembrane domains and differed with mouse and rat, both of which have 5 amino acids extension at the N-terminal. The expression of α_{2B}-adrenoceptor mRNA was demonstrated in renal, femoral, mesenteric, carotid, pulmonary and coronary arteries, external jugular and saphenous veins as well as in the brain cortex, cerebellum and trigeminal ganglion. In transient transfection system based on HEK293 cells, receptor binding with the endogenous ligand, noradrenaline showed pEC_{50} ≥7.67±0.4 and with the antagonist, rauwolscine pK_b 9.76±0.6. Stimulation of transfected HEK293 as well as COS-7 cells with noradrenaline, in dual luciferase assay, we found significant (P≤0.05) elevation of serum response element (SRE) and activator protein-1 (AP-1), while nuclear factor of κB cells (NFκB) and nuclear factor of activated T-cells (NFAT) remained unchanged. In conclusion, we have cloned a full-length cDNA and established the amino acid sequence, ligand binding profile and signalling pathway and have demonstrated the tissue distribution of the porcine α_{2B}-adrenoceptor. This information may be useful in exploring the role of α_{2B}-adrenoceptor in pathophysiological processes relevant for novel drug discovery in diseases.

10.1 INTRODUCTION

The central and peripheral actions of noradrenaline in the body are mediated via adrenoceptors. Based on pharmacological properties, distinct signal transduction and molecular cloning, adrenoceptors have been divided into three major subfamilies: α_1, α_2 and β[1]. The α_2-adrenoceptors are part of the large family of cell surface receptors which are characterized as autoreceptors[2] transducing intracellular regulatory signals through interactions with $G_{i/o}$ proteins[3]. Studies on signalling mechanisms revealed that all three α_2-adrenoceptor subtypes couple to the same intracellular signalling cascade, which include inhibition of adenylyl cyclase[4], activation of receptor-operated K^+ channel[4] and inhibition of voltage-sensitive Ca^{2+} channel[5]. Additionally, coupling to mitogen activated protein kinase[6,7], phospholipase C[8-10], phospholipase A_2[11] and phospholipase D[12] has been described in various cell systems expressing cDNAs encoding α_2-adrenoceptor subtypes.

Using different pharmacological antagonists, the α_{2A}-adrenoceptor has been shown to be the major inhibitory presynaptic receptor regulating release of noradrenaline from sympathetic neurons as part of the feedback loop[13]. However, in some tissues, α_{2C}-adrenoceptors exhibit presynaptic inhibitory action[14]. Apart from their presynaptic role, α_2-adrenoceptors, in particular, α_{2B} subtype has also been shown to function postsynaptically[15,16]. The α_{2B}-adrenoceptor has been implicated in initial stages of hypertension, whereas, long-lasting hypotension is mediated by α_{2A}-adrenoceptor[17,18]. Recently, α_{2B}-adrenoceptor antisense oligonucleotide injection into the lateral brain ventricle initiated salt-dependent hypertension[19]. Zou and Cowley[20] showed that the activation of renal vascular α_{2B}-adrenoceptor resulted in increased medullary nitric oxide production that counteracts the vasoconstrictor effects of noradrenaline, thus maintaining the blood flow and oxygen supply in the renal medulla. The α_{2B}-adrenoceptor (**IUPHAR code 2.1:ADR: 5:A2B**) homologues have been cloned from human[21], rat[22], mouse[23], and guinea pig[24]. Genomic DNA sequence analysis have shown that the human gene for the α_{2B}-adrenoceptor is intronless and lo-

cated on chromosome 2[25]. Recent studies have shown that mice lacking α_{2B}-adrenoceptor were born with altered Mendelian ratios, indicating that this receptor plays a pivotal role during embryogenesis[26].

Investigations from our laboratory have established that constriction of carotid arteriovenous anastomoses in the anaesthetized pigs can serve as predictive model for antimigraine efficacy[27], where we have reported that α_2-adrenoceptor subtypes could be of importance in the development of antimigraine drugs[28-30]. To gain further insight into the mechanisms involved in drug actions as well as the disease, it is important to study trigeminal neural control of porcine arteriovenous anastomoses and its potential modification by α_2-adrenoceptor ligands. However, one difficulty in undertaking such studies is the limited knowledge of the molecular pharmacology of the porcine α_2-adrenoceptor sub-types. Hence, the present study was undertaken to clone a full-length porcine cDNA encoding α_{2B}-adrenoceptor, to examine its tissue distribution as well as to investigate the pharmacological and second messenger signalling properties of the recombinant receptor by transient transfection in HEK293 cells.

10.2 MATERIALS AND METHODS

10.2.1 Reagents

Oligonucleotide primers and pCR2.1-Topo vector system were procured from Invitrogen b.v. (Breda, The Netherlands), Wizard® PCR prep and maxi-, mini-prep DNA purification systems and Dual-Luciferase® reporter assay system were purchased from Promega Benelux b.v. (Leiden, The Netherlands). Pure Gene™ DNA Purification Kits were from Gentra systems (Minneapolis, Minnesota, USA), whereas AmpliTaqGold® was bought from Perkin Elmer Applied Biosystem Benelux (Nieuwerkerk a/d Ijssel, The Netherlands). PolyFect Transfection Reagent (QIAGEN Benelux b.v., Venlo, The Netherlands), Mercury™ pathway profiling system was procured from BD Biosciences Benelux N.V. (Woerden, The Netherlands). Other molecular biology grade reagents were obtained either from Sigma-Aldrich Chemie b.v. (Zwijndrecht, The Netherlands) or from Roche Diagnostics Nederland b.v. (Almere, The Netherlands).

10.2.2 Isolation of total RNA and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted from different porcine tissues as described earlier[31,32] and the quality of RNA was assessed by $OD_{260/280}$ ratio>1.8 as well as by formaldehyde agarose gel electrophoresis. Prior to cDNA synthesis, RNA samples were treated with RNase-free DNase to eliminate contaminating genomic DNA. An aliquot of total RNA (2 µg) was processed for cDNA synthesis using MMLV-Reverse transcriptase as previously described[33,34]. A negative control reaction was always included where MMLV-reverse transcriptase was omitted. The cDNA samples were aliquoted and stored at -20 °C until used as a PCR template. The quality of cDNA was verified by PCR amplification of the porcine β-actin cDNA using human specific oligonucleotide primers[35].

10.2.3 Amplification of a cDNA encoding a partial α_{2B}-adrenoceptor sequence

The porcine specific α_{2B}-adrenoceptor cDNA was amplified using a combination of RT-PCR and inverse-PCR techniques. Forward Oligonucleotide primer (5'-CTGCGGTCATCACCTTC-3'; nucleotides 47-63) was designed from a known partial porcine sequence (Gene Bank Accession number **AJ251177**), whereas, reverse (5'-TCACCAGGCTGTCTGGGTCC-3'; nucleotide 1328-1347; Gene Bank Accession number **U25723**) was designed from guinea pig α_{2B}-adrenoceptor. The amplified product of expected size was analysed by agarose gel electrophoresis and further purified using a commercial PCR purification kit. The pure DNA fragment was ligated into a pCR2.1-Topo vector, transformed into competent DH5α cells and grown overnight at 37 °C on LB agar plates containing ampicillin and X-gal. White over blue colonies was selected which were then grown in LB-medium and plasmid DNA was isolated to screen positive clones containing the insert of expected size. The plasmid DNA was isolated using mini-prep DNA purification

systems and processed for sequencing by dideoxy nucleotide chain termination method using an automated fluorescence based DNA sequencer (ABI Prism™ 310 Genetic analyzer, Perkin Elmer Applied Biosystem Benelux, Nieuwerkerk a/d Ijssel, The Netherlands). The nucleotide sequences were compared and a consensus sequence derived (DNAMAN sequence analysis program, Version 3.2, Lynnon Biosoft© 1994-1997). The final partial cDNA sequence was compared with those in the GenBank (BLAST search National Centre for Biotechnology Information, Bethesda, MD, USA).

10.2.4 Identification of 5′ and 3′ ends of the porcine α_{2B}-adrenoceptor by inverse PCR

Inverse PCR was performed to establish the porcine-specific sequence of 5′ and 3′ ends [36]. The porcine genomic DNA was isolated using the Pure Gene™ DNA Purification Kit as per the manufacturer's instructions and was digested with Hind III restriction enzyme, in view of the previously cloned human α_{2B}-adrenoceptor (**NM_000682**) did not show any restriction site for this enzyme. After purification, the restricted DNA was ligated overnight at 16 °C in the presence of T_4-DNA ligase in order to obtain DNA circles. The ligated DNA circles were subjected to inverse PCR using primers specific for the porcine α_{2B}-adrenoceptor forward (5′-CGTCGAAAGTACCAGTAGCCCAG-3′) and reverse (5′-TTGTGCTCTGCTGGTTC-3′). The amplified products were separated on 1% agarose gel, purified, cloned and sequenced, as described above.

10.2.5 Amplification and cloning of the full-length porcine α_{2B}-adrenoceptor

For the amplification of full-length porcine α_{2B}-adrenoceptor cDNA, forward (5′-ATGGACCACCAGGAGCCCTAC-3′) and reverse (5′-TCACCAGGCTGTCTGGGTCC-3′) oligonucleotide primers were designed from the sequence generated from the inverse PCR. After PCR amplification of cDNA, samples from porcine pulmonary artery, product of expected size was purified, ligated into pCR2.1-Topo vector, transformed in competent DH5α cells, plasmid DNA isolated and sequenced. The full-length cDNA sequence of the porcine α_{2B}-adrenoceptor was derived from two independent PCR-amplified products and further verified by multiple partial sequences derived from cDNAs. Furthermore, in analogy to the human gene being intronless, we amplified the porcine genomic DNA using forward (5′-ATGGACCACCAGGAGCCCTAC-3′) and reverse (5′-TCACCAGGCTGTCTGGGTCC-3′) oligonucleotide primers to obtain the full-length α_{2B}-adrenoceptor exonic sequences representing the entire coding region. The PCR products were cloned into pCR2.1-Topo vector and sequenced. The obtained genomic DNA sequence was compared with the porcine cDNA sequence, which was found to be 100% identical. The final sequence was translated as a peptide sequence and compared with those in the GenBank (BLAST search National Centre for Biotechnology Information, Bethesda, MD, USA; website: http://www.ncbi.nlm.nih.gov/BLAST/). The hydrophobic regions (indicating putative transmembrane domains) and sequence similarity with known α_{2B}-adrenoceptor from other species were determined.

10.2.6 Expression of α_{2B}-adrenoceptor mRNA in porcine tissues

For the isolation of total cellular RNA, renal, femoral, mesenteric, carotid, pulmonary and coronary arteries, external jugular and saphenous veins, brain cortex, cerebellum and trigeminal ganglion were obtained from pigs (Yorkshire x Landrace, female, 12 kg) sacrificed after acute haemodynamic experiment. The ethical committee of the Erasmus MC, University Medical Centre Rotterdam approved the experimental protocols conducted on pigs. Three separate experiments where performed to verify the reproducibility of mRNA detection in the tissue. The tissue samples were snap frozen in liquid nitrogen and stored at -80 °C until used. The tissues were dissected, cleaned and the total RNA was isolated[31,32]. The residual DNA contamination was removed by treatment with RNase-free DNase (10U 6 μg^{-1} RNA) for 25 min at 37 °C as per instruction (Promega Benelux b.v. Leiden, The Netherlands). The purified total RNA samples were reverse transcribed into cDNA, and used for PCR amplification of a 211 bp DNA fragment encoding α_{2B}-adrenoceptor. For the PCR amplification, porcine α_{2B}-adrenoceptor specific forward (5′-CTGCGGTCAT-

CACCTTC-3'; nucleotides 46-62) and reverse (5'-CCAGCGCCAGATACACT-3'; nucleotide 257-273) primers were used along with other components of PCR. A DNA fragment of 625 bp was amplified by PCR in each cDNA samples as an internal control. The PCR amplified product were separated on 2% agarose gel containing ethidium bromide, visualized under UV-light and photographed.

10.2.7 Transient transfection and dual luciferase assay

The purified full-length cDNA insert of α_{2B}-adrenoceptor was directionally cloned (with Xho I and Hind III) into the eukaryotic expression vector pcDNA 3.1$^{(-)}$ (Invitrogen b.v. Breda, The Netherlands) and the reaction mixture was transformed into DH5α competent cells. The generated plasmid (pSM2B) was purified and sequenced to verify the CMV promoter in front of the reading frame of α_{2B}-adrenoceptor. HEK293 cells (Approximately 2x10^6 per well) cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal calf serum, 100U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin at 37 °C in a humidified incubator with 5% CO_2 were transiently transfected by PolyFect transfection reagent using the reaction mixture containing 25 ng of either NFAT or NFκB or AP-1 or SRE transcription factors in Firefly-TA-LUC vector™, 25 ng of pRLTK™ (*Renilla*) and 50 ng pSM2B plasmid DNA and 150 ng of pcDNA3.1$^{(-)}$ empty vector. The control plasmid mix was prepared by replacing 50 ng of pSM2B plasmid DNA with the empty vector. Transfected cells were washed after 16 h and were further incubated with noradrenaline (10^{-10} to 10^{-5} M) in 0.5% DMEM in a concentration dependent manner to investigate agonist-mediated signalling. Concentration response curve of noradrenaline was constructed with or without the antagonist rauwolscine (10^{-7} M). After 24 h of transfection (8 h after stimulation), cells were lysed, strictly following the supplier's protocol and 10 µl of lysate was analysed using a dual luciferase reporter assay system (Promega Benelux b.v. Leiden, The Netherlands). The assays for *firefly* and *Renilla* luciferase activities were assessed sequentially in one reaction well by measuring luminescent signal in a luminometer (Top-Count NTX™, Packard, Groton, CT, USA). For the calculation of the transcription factor activity, fluorescence values of the firefly were normalized with the *Renilla* value. The transcription factor activation was reflected by the luciferase activity, which was expressed as X-fold to the untreated transfected cells.

10.2.8 Data analysis

The concentration response curves for noradrenaline were analysed using non-linear regression analysis and the potency of agonists was expressed as pEC_{50} using Graph Pad Prism 3.01 (Graph Pad Software Inc., San Diego, CA, U.S.A.). The agonist-mediated changes were expressed as fold increase with respect to untransfected HEK293 cells set as 1. It is important to note that when the E_{max} of an agonist was not reached, the response at its highest concentration was considered as E_{max} and the pEC_{50} was expressed as the value obtained. For the experiments in the presence of an antagonist, where the E_{max} in the presence of a given antagonist was assumed to be equal to the control E_{max} in case a plateau was not reached. The potency of the antagonists was expressed as apparent pK_b values, assuming a slope of unity. For the dose response curve two tailed student *t-test* whereas, for luminescence intensity analysis for various transcription factors One-Way ANOVA with Dunnett post hoc were applied in order to maintain stringent statistics, with differences considered significant at $p \leq 0.05$. All data are presented as means ± S.E.M. and *n* represents number of experiments performed in different plates and combinations on one plate were run in triplicate.

10.3 RESULTS

10.3.1 Cloning and sequence analysis of the porcine α_{2B}-adrenoceptor

The porcine α_{2B}-adrenoceptor was amplified using a combination of PCR, RT-PCR and inverse PCR techniques. The 5' and 3' ends of the mature mRNA encoding porcine α_{2B}-adrenoceptor were identified

by inverse PCR using genomic DNA circles as templates. The sequencing data of the RT-PCR amplified cDNA fragment was compared with that of genomic DNA amplified α_{2B}-adrenoceptor and it was found to be 100% identical, establishing the fact that the porcine gene for α_{2B}-adrenoceptor is intronless. The sequence analysis of the full-length cDNA of porcine α_{2B}-adrenoceptor clones disclosed a nucleotide sequence of 1341 bp encoding for a 446 amino acid long polypeptide (Figure 10.1). The mRNA sequence comparison of porcine α_{2B}-adrenoceptor revealed 88% and 85%, whereas the amino acid sequence analysis demonstrates 82% and 87% similarity with known human and guinea pig α_{2B}-adrenoceptors, respectively. There were 7 hydrophobic regions, indicating 7 transmembrane domains, a typical characteristic of G protein-coupled receptors (Figure 10.1). Furthermore, differences in amino acid sequences were mainly localized to the third intracellular loop, as reported for other cloned adrenoceptors [37]. The amino acid sequence of the receptor was compared with other species (Figure 10.2). Interestingly, the porcine α_{2B}-adrenoceptor amino acid sequence differed with mouse and rat, both of which have 5 amino acids extended at N-terminal (Figure 10.2).

10.3.2 Tissue distribution of α_{2B}-adrenoceptor mRNA

To examine the mRNA expression of α_{2B}-adrenoceptor in various porcine tissues, cDNA templates were processed for RT-PCR to amplify a DNA fragment of 211 bp located between 46-273 bp in our porcine cDNA clone (pSM2B). Results shown in Figure 10.3 demonstrate specific amplification of the expected size DNA fragment in the renal, femoral, mesenteric, carotid, pulmonary and coronary arteries, external jugular and saphenous veins, brain cortex, cerebellum as well as the trigeminal ganglion. The negative control, where cDNA was replaced by water, did not show any amplification ruling out any contamination in PCR preparations. Additionally, using oligonucleotide primers located on two separate consecutive exons of the β-actin gene, a single band was amplified in all tissue preparations verifying no genomic DNA contamination.

10.3.3 Activation of second messenger systems via α_{2B}-adrenoceptor

The α_{2B}-adrenoceptor -mediated activation of second messenger systems was assessed using a HEK293 cell-based *in vitro* mammalian expression system. Activation of particular transcription factors (AP-1, NFAT, NFκB or SRE) by receptor stimulation was examined using Firefly-TA-LUC vector that contain a specific *cis*-acting DNA sequence (enhancer element) *(Mercury™ pathway profiling constructs)*. Transcriptional responses of the porcine α_{2B}-adrenoceptor were compared to untransfected HEK293 cells. We monitored the binding of transcription factors to enhancer elements by measuring luminescence (Figure 10.4). The SRE and AP-1 at increasing concentrations of noradrenaline (0.1, 10 nM and 1μM) showed significant increase in luciferase activity, while NFAT and NFκB remained unchanged in cells harbouring α_{2B}-adrenoceptor. Maximal induction by agonist stimulation was observed in case of SRE (5.8±1.0 folds of luciferase activity) as compared to non-transfected HEK293 cells.

10.3.4 Pharmacological characterization of recombinant α_{2B}-adrenoceptor

After establishing that noradrenaline activated the reporter SRE, we further characterized the receptor functionally by constructing concentration response curves to the endogenous ligand noradrenaline (10^{-10} M to 10^{-5}M) in the absence or presence of the selective α_2-adrenoceptor antagonist rauwolscine (10^{-7}M). The data, shown in Figure 10.5, depict that noradrenaline significantly increased luciferase activity in a concentration-dependent manner ($pEC_{50} \geq 7.67 \pm 0.4$) and that rauwolscine (apparent pK_b: 9.76±0.6) clearly antagonized the responses to noradrenaline.

```
1       ATGGACCACCAGGAGCCCTACTCCGTGCAGGCCACTGCGGCCATCGCTGCGGTCATCACC
1        M  D  H  Q  E  P  Y  S  V  Q  A  T  A  A  I  A  A  V  I  T

61      TTCCTCATCCTGTTCACTATCTTCGGCAACTCCCTGGTCATCTTGGCTGTGCTGACAAGC
21       F  L  I  L  F  T  I  F  G  N  S  L  V  I  L  A  V  L  T  S

121     CGCTCGCTGCGCGCGCCGCAAAACCTGTTCCTAGTGTCGCTGGCCGCAGCCGACATCCTG
41       R  S  L  R  A  P  Q  N  L  F  L  V  S  L  A  A  A  D  I  L

181     GTGGCCACACTCATCATCCCTTTTTCGCTGGCCAATGAGCTGCTGGGCTACTGGTACTTT
61       V  A  T  L  I  I  P  F  S  L  A  N  E  L  L  G  Y  W  Y  F

241     CGACGCACCTGGTGTGAAGTGTACCTGGCGCTGGACGTGCTTTTCTGCACCTCCTCCATC
81       R  R  T  W  C  E  V  Y  L  A  L  D  V  L  F  C  T  S  S  I

301     GTGCACCTGTGTGCCATCAGCCTGGACCGCTACTGGGCAGTGAGCCGCGCGTTGGAGTAC
101      V  H  L  C  A  I  S  L  D  R  Y  W  A  V  S  R  A  L  E  Y

361     AACTCCAAGCGCACCCCGCGGCGCATCAAGTGCATCATCCTCACCGTGTGGCTCATCGCA
121      N  S  K  R  T  P  R  R  I  K  C  I  I  L  T  V  W  L  I  A

421     GCTGTCATCTCGCTGCCGCCTCTTATCTACAAGGGCGACCCGGGTCCCCAGCCCCGGGGG
141      A  V  I  S  L  P  P  L  I  Y  K  G  D  P  G  P  Q  P  R  G

481     CGCCCTCAATGCAAGCTCAATCAAGAGGCCTGGTACATCCTGGCCTCCAGCATTGGCTCT
161      R  P  Q  C  K  L  N  Q  E  A  W  Y  I  L  A  S  S  I  G  S

541     TTCTTTGCACCCTGCCTTATCATGATCCTTGTTTACCTGCGCATCTACTTGATCGCTAAG
181      F  F  A  P  C  L  I  M  I  L  V  Y  L  R  I  Y  L  I  A  K

601     CGCAGCCACCGCAGAGGCCCTAGGGCTAAGGGGGGCCCTGGGAAGGGGGAGTCTAAGCAG
201      R  S  H  R  R  G  P  R  A  K  G  G  P  G  K  G  E  S  K  Q

661     CCCCGCCCAGTCCCTGGGGAAGTTTCAGCCTCAGCCAAGTTGCCAACCTTGGTCTCTCAA
221      P  R  P  V  P  G  E  V  S  A  S  A  K  L  P  T  L  V  S  Q

721     CTGGCCACTGCTGGAGAGACCAATGGGTGCTCTCAGCACACTGGGGAGAAGGATGAAGGG
241      L  A  T  A  G  E  T  N  G  C  S  Q  H  T  G  E  K  D  E  G

781     GAGACCTCTGAAGACACTGGGACCCCCGCTTTGCCACCCAGCTGGCCTGCCCTTCCCAGC
261      E  T  S  E  D  T  G  T  P  A  L  P  P  S  W  P  A  L  P  S

841     TCGGGCCAGGGTCAGAAGGAAGGTGTTTGTGGAACATCACCAGAGGAGGAGGCTGAAGAG
281      S  G  Q  G  Q  K  E  G  V  C  G  T  S  P  E  E  E  A  E  E

901     GAGGAGGAGGAGGAGTGTGAGCCTCAGACTTTGCCAGCCTCTCCTGCCTCGGCTTGCAGT
301      E  E  E  E  E  C  E  P  Q  T  L  P  A  S  P  A  S  A  C  S

961     CCACCCCTGCAGCAGCCACAGGGTTCCCGGGTGCTGGCAACCCTACGGGGCCAGGTGCTC
321      P  P  L  Q  Q  P  Q  G  S  R  V  L  A  T  L  R  G  Q  V  L

1021    CTGGGCAGGGGCATGGGCACTTCGAGTGGGCAGTGGTGGCGTCGGCGGGCGCAGCTGACC
341      L  G  R  G  M  G  T  S  S  G  Q  W  W  R  R  R  A  Q  L  T

1081    CGGGAGAAGCGGTTCACGTTCGTGCTGGCTGTGGTCATTGGCGTGTTTGTGCTCTGCTGG
361      R  E  K  R  F  T  F  V  L  A  V  V  I  G  V  F  V  L  C  W

1141    TTCCCCTTCTTCTTCAGCTATAGCCTGGGTGCCATCTGCCCTCAGCACTGCAAGGTGCCC
381      F  P  F  F  F  S  Y  S  L  G  A  I  C  P  Q  H  C  K  V  P

1201    CATGGCCTATTCCAGTTCTTCTTCTGGATCGGCTACTGCAACAGCTCGCTGAACCCTGTC
401      H  G  L  F  Q  F  F  F  W  I  G  Y  C  N  S  S  L  N  P  V

1261    ATCTACACCATCTTCAACCAGGACTTTCGTCGTGCCTTCCGAAGGATCCTGTGCCGCCAG
421      I  Y  T  I  F  N  Q  D  F  R  R  A  F  R  R  I  L  C  R  Q

1321    TGGACCCAGACAGCCTGGTGA
441      W  T  Q  T  A  W  *
```

TM I — TM II — TM III — TM IV — TM V — TM VI — TMVII

Figure 10.1: Nucleotide and deduced amino acid (in bold) sequences of the recombinant cDNA derived from mRNA isolated from porcine pulmonary artery. Numbering of nucleotide and amino acids is shown on the left. DNA sequence analysis (software DNAMAN, version 3.2, Lynnon Biosoft©) predicted a typical G protein-coupled receptor with transmembrane domain I-VII (TM; underlined).

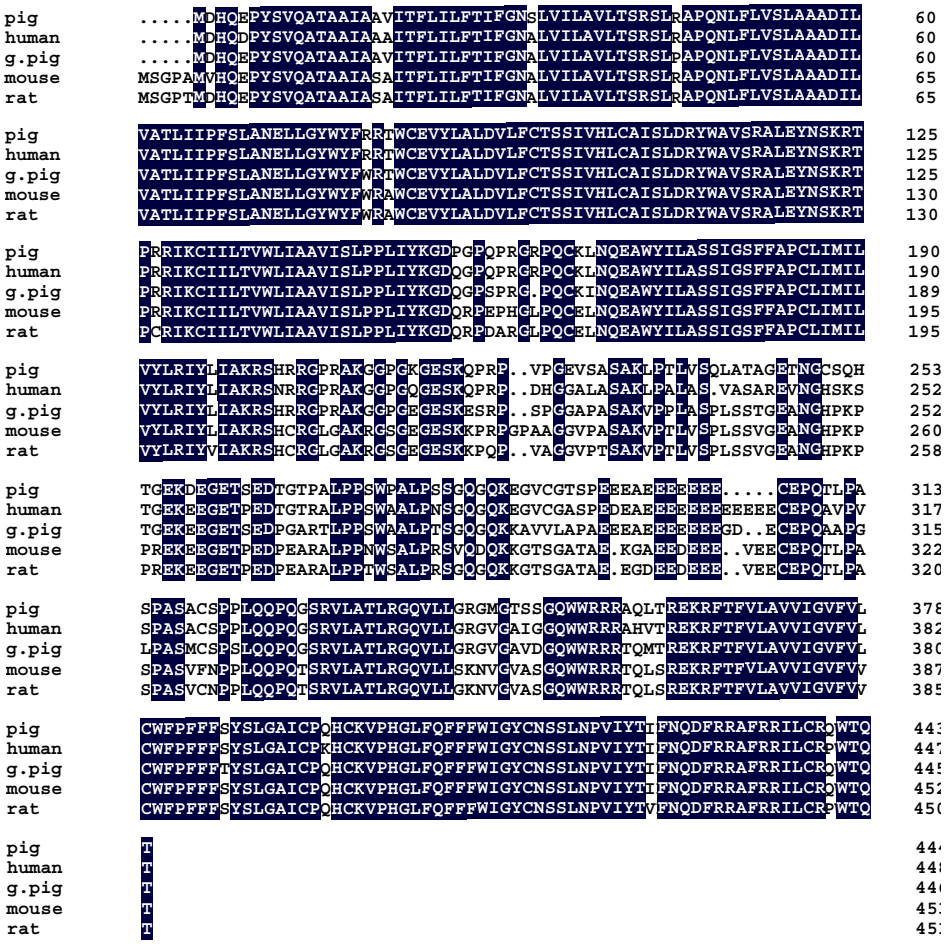

Figure 10.2: Comparison of amino acid sequence of the porcine α_{2B}-adrenoceptor with the human, guinea pig (g.pig), mouse and rat (software DNAMAN, version 3.2, Lynnon Biosoft©). The areas shaded in black show the amino acid identity across the different species.

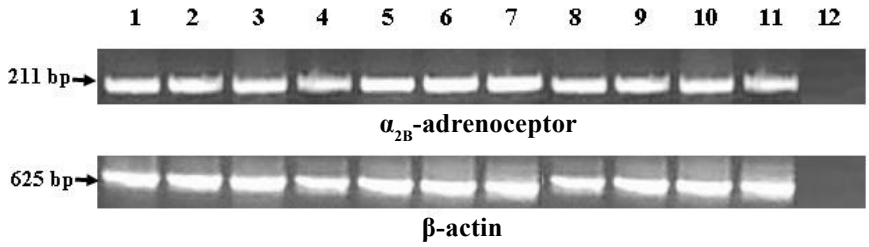

Figure 10.3: Demonstration of α_{2B}-adrenoceptor mRNA in porcine tissues (n=3). Lanes 1-12 depict: 1) renal artery, 2) femoral artery, 3) mesenteric artery, 4) brain cortex, 5) carotid artery, 6) external jugular vein, 7) cerebellum, 8) pulmonary artery, 9) trigeminal ganglion, 10) saphenous vein, 11) coronary artery and 12) negative control water. Amplification of a DNA fragment of 625 bp for β-actin was performed for reference purposes.

10.4 DISCUSSION

10.4.1 Molecular cloning and sequence analysis of the porcine α_{2B}-adrenoceptor

In this study, we have cloned and characterized a cDNA encoding the porcine α_{2B}-adrenoceptor using combination of RT-PCR and inverse PCR. The nucleotide sequence of the generated 1341 bp DNA fragment was compared to the previously known partial sequence of porcine α_{2B}-adrenoceptor (**AJ251177**); only one nucleotide difference was found at position 264 (T to C), which does not alter the deduced amino acid sequence. Nucleotide sequence of the full length porcine α_{2B}-adrenoceptor cDNA revealed an open reading frame of 446 amino acids, showing high similarity with the corresponding human and guinea pig cDNA and protein sequences. Comparing the α_{2B}-adrenoceptor sequences from different species, we found that most residues in the seven transmembrane domains, in the intracellular loops 1 and 2, and in the extra cellular loop 1 were strictly conserved[37]. Amino acid sequence differences across different species were noticed in the intracellular loops 3. Notably, residues in transmembrane domains 3, 5 and 6 that are thought to be involved in the ligand binding were all conserved in the porcine α_{2B}-adrenoceptor[38-40]. In analogy to human and guinea pig α_{2B}-adrenoceptor amino acid sequences, the porcine sequence shows no extended N-terminal sequence, which is evident in rat and mouse. Furthermore, the DRY motif after third transmembrane domain, a typical characteristic of G protein-coupled receptors, was conserved in the porcine α_{2B}-adrenoceptor. As reported earlier for other previously cloned α_{2B}-adrenoceptor from different species[37], the porcine receptor also showed a very long third intracellular loop that contains a unique glutamate residue repeat between amino acid 294 to 305. The porcine sequence also revealed the conserved amino acid at cysteine^{164}, found in most G protein-coupled receptors, which forms a structurally important disulfide bond with cysteine^{85} at the extracellular end of transmembrane domain 3.

10.4.2 Tissue distribution of α_{2B}-adrenoceptor mRNA

Using RT-PCR, we demonstrated ubiquitous expression of α_{2B}-adrenoceptor mRNA in various porcine blood vessels and cranial tissues. Expression of mRNA for α_{2B}-adrenoceptor in the renal, femoral, pulmonary and mesenteric arteries suggest that the α_{2B}-adrenoceptors may contribute to the peripheral regulation of vascular tone[16]. Recently, Kanagy *et al*.[41] reported that α_{2B}-adrenoceptor is a major postsynaptic receptor causing vasoconstriction. However, despite the presence of α_{2B}-adrenoceptor mRNA in porcine coronary artery, there are reports suggesting minimal or no constriction in porcine pressurized as well as quiescent coronary microvessels[42]. Even in the presence of β-adrenoceptor antagonist propranolol, these vessels dilate to noradrenaline via endothelial release of, presumably, by nitric oxide[42]. The presence of mRNA for α_{2B}-adrenoceptor in porcine coronary arteries in this study though contradicts the lack of constrictor response to noradrenaline; this may be attributed to either low receptor density and/or a fast turnover of the α_{2B}-adrenoceptor (mRNA instability) in these vessels. The presence of mRNA for α_{2B}-adrenoceptor in saphenous vein indicates that these receptor may contribute in contractile responses to noradrenaline in addition to, as shown earlier for α_{2C}-adrenoceptor mediating contractions[43]. The presence of α_{2B}-adrenoceptor in porcine carotid artery is in line with *in vitro* studies on isolated carotid arteries showing contractions via the α_2-adrenoceptor[44]. Investigations from our laboratory also showed the importance of α_2-adrenoceptor subtypes in the carotid vasculature of anaesthetized pigs[27]; however, due to the paucity of subtype selective compounds it was difficult to predict the involvement of specific α_2-adrenoceptor subtypes in the carotid circulation. Studies in mice have shown the expression of α_{2B}-adrenoceptor in the central nervous system (brain cerebral cortex, anterior olfactory nucleus, hypothalamus, brainstem, and cerebellar Purkinje cells) during embryonic development, suggesting the role of these receptors in brain development and antinociception[45,46]. Using *in vitro* pharmacological tools, we have recently demonstrated the presence of α_2-adrenoceptor in porcine meningeal arteries. Our data on the ubiquitous mRNA expression of α_{2B}-adrenoceptor in peripheral blood vessels support our previ-

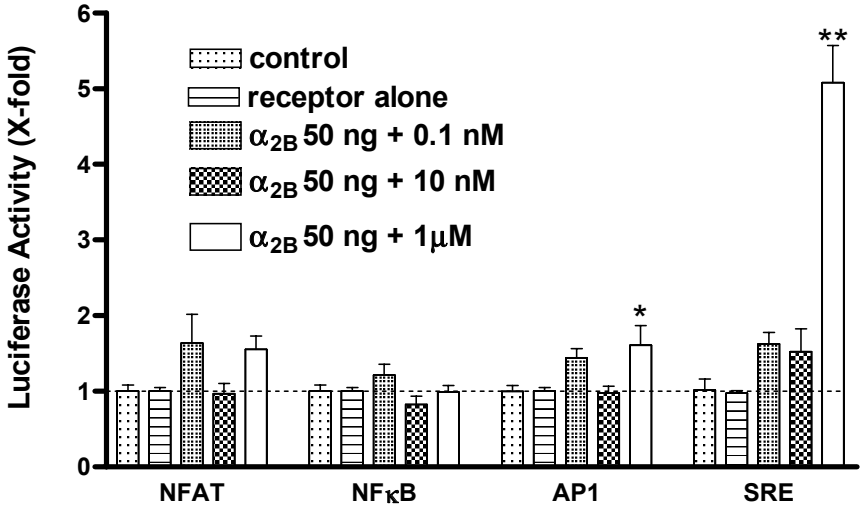

Figure 10.4: Demonstration of fold increase (with respect to untransfected HEK293 cells set as 1; control) in the luminescence intensity towards various transcription factors combined with recombinant porcine α_{2B}-adrenoceptor (n=3x3). The concentrations of noradrenaline used were 0.1nM-1μM. AP-1, activator protein 1; NFAT, Nuclear factor of activated T-cells; NFκB, Nuclear factor of κB cells; SRE, Serum response element. * $p<0.05$ ** $p<0.01$.

ous pharmacological findings of carotid vasoconstriction in pigs[27] and further warrant to explore other adrenoceptor subtypes such as α_{2C} as potential targets for the development of antimigraine drugs.

10.4.3 Second messenger systems and pharmacology

G protein-coupled receptors play a fundamental role in regulating the activity of virtually every cell in the body. Upon binding with extra-cellular ligands, G protein-coupled receptors interact with a specific subset of heteromeric proteins that can in their activated form inhibit or activate various effecter enzymes, ion channels and transcription factors. To evaluate the functionality of the porcine α_{2B}-adrenoceptor, we used Clontech's *cis*-acting enhancer element vectors in the Mercury Pathway Profiling System™ to test for the activation of four potential transcription factors involved in receptor signalling. Each Mercury vector contains a specific *cis*-acting DNA binding sequence located upstream to a TATA box, and a luciferase gene as a reporter[47]. Our data demonstrate that noradrenaline activated luciferase reporter constructs for SRE and AP-1 transcription factors in α_{2B}-adrenoceptor expressing HEK293 cells. This suggests that the α_{2B}-adrenoceptor transmit signalling information via at least these two transcriptions factors, although activation of other, here not investigated, factors cannot be ruled out. To our knowledge, this is the first study of its kind using the *cis*-acting enhancer element vectors to characterize the functionality of a new cloned receptor. The maximum elevation of *cis*-acting signal by SRE suggests that the porcine α_{2B}-adrenoceptor acts via Elk-1 or SRF transcription factor and signalling transduction pathway, which predictably leads either via mitogen activated protein kinase (MAPK) or c-Jun NH_2-terminal protein kinase (JNK)[48]. Notably, we also established the fact that the elevation of SRE is not a HEK293 cell dependent phenomenon; with COS7 cells, we also found elevation of SRE transcription factor. As α_2-adrenoceptors are cell surface receptors that interact via $G_{i/o}$ proteins for transducing intracellular signals[3], further studies perhaps by using e.g. pertussis toxin could provide information whether the porcine α_{2B}-adrenoceptor also inhibits responses mediated by G_i-proteins.

The concentration response curve for the α_{2B}-adrenoceptor was constructed using the endogenous ligand noradrenaline, both in the absence and presence of the selective α_2-adrenoceptor antagonist rauwolscine, in the dual luciferase assay. The previously reported antagonist potency of rauwolscine in

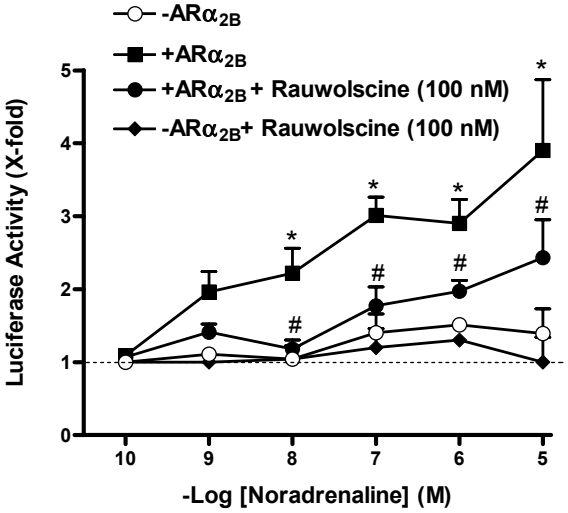

Figure 10.5: Increase in luciferase activity by serum response element. Stimulation in HEK293 cells harbouring recombinant α_{2B}-adrenoceptor treated with increasing concentrations of endogenous ligand, noradrenaline 10^{-10} M-10^{-5}M in the absence or presence of the antagonist rauwolscine (100 nM). -ARα_{2B}, HEK293 cells without recombinant porcine α_{2B}-adrenoceptor; +ARα_{2B}, HEK293 cells with recombinant porcine α_{2B}-adrenoceptor. (n=3 x3 each). *p<0.05 for each concentration of noradrenaline, # p<0.05 vs. noradrenaline treatment.

recombinant human α_{2B}-adrencoceptor (pK_i: 9.4)[49] is in line with the potency that we observed for the porcine α_{2B}-adrenoceptor (pK_b: 9.76±0.6).

In conclusion, we cloned and characterized the cDNA sequence of porcine α_{2B}-adrenoceptor, which showed high similarity to the human homologue. We further showed that the gene for α_{2B}-adrenoceptor is intronless, and demonstrated the presence of its mRNA in various porcine blood vessels and cerebral structures. Using transient transfection system based on HEK293 cells, we showed that the generated porcine recombinant α_{2B}-adrenoceptor is functional and mediates noradrenaline activated down stream signalling as shown by luciferase reporter constructs of SRE and AP-1 transcription factors. Furthermore, the binding profile to endogenous ligand and antagonist characterized the pharmacology of the newly identified porcine α_{2B}-adrenoceptor.

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ABSTRACT

The 5-$HT_{1B/1D}$ receptor agonist sumatriptan is highly effective in the treatment of migraine. However, some patients do not respond to sumatriptan or experience recurrence of the headache after initial relief. In addition, some patients report chest symptoms after the use of sumatriptan. To assess, whether two genetic variants (F124C changing a phenylalanine for a cysteine and polymorphism A/T at nucleotide position -161 in the 5' regulatory region) of the 5-HT_{1B} receptor play a major role in the therapeutic response to sumatriptan. The 5-HT_{1B} receptor most likely mediates the therapeutic action and coronary side effects of sumatriptan, and both F124C and A-161T have relevant functional consequences on either the affinity of sumatriptan to bind to the 5-HT_{1B} receptor or on receptor expression level itself, respectively. Genomic DNA of a relatively small but very well-characterized set of migraine patients with consistently good response to sumatriptan (*n*=14), with no response (*n*=12), with recurrence of the headache (*n*=12), with chest symptoms (*n*=13), and patients without chest symptoms (*n*=27) was available for the genetic analyses and screened for the F124C variant and the A-161T polymorphism in the human 5-HT_{1B} receptor gene. F124C was not detected in any of the patients studied. In addition, we did not observe drastic changes in allele frequencies of the A-161T polymorphism that might hint to a causal relation with the therapeutic effect of sumatriptan. We have not obtained any evidence that variants F124C and A-161T of the 5-HT_{1B} receptor are major determinants in the clinical response to sumatriptan.

11.1 INTRODUCTION

Migraine is a common neurovascular disorder[1]. Over the years several pharmacological interventions are used either to abort or to prevent migraine attacks. The 5-$HT_{1B/1D}$ (5-HT: 5-hydroxytryptamine, serotonin) receptor agonist sumatriptan is highly effective in the acute treatment of migraine attacks[2]. However, about 15% of migraine patients do not respond to sumatriptan, even when administered subcutaneously, and up to 40% of responders consistently experience recurrence of the headache within 24 hours after initial headache relief[3-5]. In addition, about 40% of the patients experience one or more 'chest symptoms', including chest pressure, heaviness in arms, shortness of breath and chest pain, shortly after the use of subcutaneous sumatriptan[6,7].

Demographic, clinical and pharmacokinetic characteristics may contribute to the inter-patient differences in clinical response to drugs, but these factors do not seem to be responsible for the different clinical responses to sumatriptan[8-10]. We and others hypothesize that genetic variation may contribute to the different clinical responses to drugs[11,12]. The clinical effect of sumatriptan is likely to be mediated via the 5-HT_{1B} receptor, which mediates vasoconstriction in cranial arteries[13]. In addition, this receptor may well be responsible for chest symptoms experienced after the use of sumatriptan[14] since sumatriptan can constrict coronary arteries[15]. This warrants a genetics investigation of variation of the 5-HT_{1B} receptor in relation to the clinical response to sumatriptan. In an earlier study we could not show a drastic effect of two variants G861C and T261G of the 5-HT_{1B} receptor on clinical response to sumatriptan[16]. Recently, the variant F124C in the same gene[17] was described to affect binding of sumatriptan to the 5-HT_{1B} receptor[18]. The amino acid change is caused by a T to G transversion at nucleotide position 371 of the coding region of the 5-HT_{1B} receptor[17]. In a heterologous cell system, variant Cys^{124} caused a 2.8 to 3.6-fold higher potency, albeit combined with a 50-65% lower maximal response, of sumatriptan than in cells expressing the wild-type receptor. It was suggested that sumatriptan-induced coronary vasospasm might be related to the expression of Cys^{124}, especially in patients with additional pathogenetic factors such as coronary heart disease[19]. Indeed, a recent study indicated that the contribution of the 5-HT_{1B} receptor to the mediation of the effects of 5-HT is increased in temporal arteries obtained from subjects with the Cys/Phe genotype compared to those with the Phe/Phe genotype[20]. Of note, non-naturally occurring Ala^{327} (e.g.

normally a tryptophan is present at this position) resulted in a 60-fold decreased affinity of sumatriptan to the same receptor.

Recently, single nucleotide polymorphism A-161T, located in the 5′ regulatory region of the 5-HT$_{1B}$ gene, was described[21] and shown to be related to alcohol dependence in Taiwanese Han. Functional studies suggest that this polymorphism is involved in 5-HT$_{1B}$ receptor expression[21], with the highest expression observed for the 'T' variant. Similarly to what was suggested for F124C, this polymorphism may also be related to clinical responses to sumatriptan, especially the occurrence of chest symptoms.

Based on the genetic and functional studies of variants F124C and A-161T we decided to investigate whether they have a major role in the clinical response to sumatriptan.

Since we included patients that fulfilled very stringent clinical criteria, the number of patients included in our study was small. As a consequence the low statistical power allowed us only to test for drastic effects (e.g. large changes in allele frequencies). We present results for variants F124C and A-161T and their potential role as major determinants of the clinical response to sumatriptan.

11.2 MATERIALS AND METHODS

11.2.1 Patients and statistical analysis

Forty unrelated migraine patients (35 female, 5 male; age 20-69 years) from the outpatient database of the Department of Neurology of Leiden University Medical Centre were included in this study. All patients fulfilled the International Headache Society criteria[22]. Patients were divided into five groups according to their clinical response to 6 mg subcutaneous (s.c.) sumatriptan: *i) Responders* were defined as patients who had headache relief within 2 hours after 6 mg s.c. sumatriptan in at least 4 out of 5 migraine attacks and who experienced headache recurrence within 24 hours in less than 1 out of 5 successfully treated attacks; *ii) Patients with headache recurrence* were defined as those who responded to s.c. sumatriptan in at least 4 out of 5 migraine attacks, but in whom the headache recurred within 24 hours in at least 4 out of 5 successfully treated attacks; *iii) Non-responders* were defined as patients who had headache relief in no more than one out of 5 migraine attacks treated with s.c. sumatriptan, or in none of the 3 consecutively treated migraine attacks; *iv) Patients with chest symptoms* (chest pressure, heaviness in arms, shortness of breath or chest pain) had treated at least 3 migraine attacks with s.c. sumatriptan and had experienced one or more chest symptoms in each of these attacks (the use of the minimal number of 3 rather than 5 attacks in the latter groups emerged from practical reasons since patients with severe adverse events or no response usually refrained from continuing sumatriptan use); and *v) Patients without chest symptoms*, derived from the other groups, were compared with patients with chest symptoms. Clinical and demographic data of the patients are summarised in Table 11.1 The local ethics committee of Leiden University Medical Centre, The Netherlands approved the study and informed consent was obtained from all the patients.

In statistical analyses, responders, non-responders, and patients with recurrence of the headache were compared with each other. Patients with chest symptoms were compared with patients without chest symptoms. Power analysis was performed using the DSS Research program (www.dssresearch.com) to determine the potential difference in allele frequency that could be detected (α=0.05, β=0.80). No deviations from the Hardy–Weinberg equilibrium using were observed for the genotypes of the total set that was tested using the HWE program[23], thus excluding potential confounders such as severe population stratification or errors in genotyping. *P* values <0.05 were considered to indicate significant differences.

11.2.2 Blood Samples

EDTA venous blood samples were collected from patients. Leukocyte DNA was isolated by salting out procedure[24].

Table 11.1. Demographic data of the study groups and allele frequencies of the human 5-HT_{1B} variant A-161T in migraine patients with different clinical responses to sumatriptan.

	n	Sex (M/F)	Mean age (range)	Allele frequency	
				A	T
Responders	14	1 M, 13 F	49 (23-69)	0.75	0.25
Recurrence	12	1 M, 11F	50 (34-66)	0.80	0.20
Non-responders	12	3 M, 9 F	44 (20-60)	0.62	0.38
Chest symptoms	13	0 M, 13 F	48 (23-59)	0.80	0.20
No chest symptoms	27	5 M, 22 F	47 (20-69)	0.70	0.30
Total (all patients)*	40	5 M, 35 F	47 (20-69)	0.74	0.26
Reference group[§]				0.77	0.23

*Most patients were included in several (non-mutually excluding) groups, e.g. patients in the group with chest symptoms could also be included in the groups with responders. M and F refer to male and female, respectively; age is expressed in years.

[§]Allele frequency information of this sequence variant in the European population was available from the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP, Ref SNP rs130058, population EUR panel).

11.2.3 Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out in a 15-µl reaction volume. For genotyping of polymorphism F124C we used 7.5 pmol of forward primer IDB5-F (5′-CCCTACCCTGGAAAGTACTGC-3′) and reverse primer IDB5_RM (5′ - TGATGTCCGACGACAGCTAG-3′). For analysing polymorphism A-161T we used 7.5 pmol of Forward primer (5′- CAGCGCTGCTCCTAGACTTCACC-3′) and Reverse primer (5′-GTTCCTCCATGGCTCTCCTCG-3′). The reaction mixture also contained 1× superTaq PCR Buffer I (HT Biotechnology Ltd., Cambridge, UK), 1.3 M betaine (ICN Biomedical Inc., Irvine, CA, US), 3 mM dNTP, 0.25 U Silverstar (Eurogentech, Liège, Belgium), and 50 ng of genomic DNA. PCR was carried out in a PTC-200 apparatus (MJ Research Inc, Waltham, MA, US). Following an initial denaturation step of 10 min at 94°C, samples were amplified for 33 cycles of 40 s at 94°C, 30 s at 60°C, and 40 s at 72°C, followed by a final extension step of 10 min at 72°C. Thus PCR products were obtained of 258 bp and 606 bp for F124C- and A-161T-specific PCRs, respectively.

11.2.4 Restriction enzyme assay

The T→G and A→T substitutions at nucleotides 371 and -161 were assessed with restriction enzyme analysis. Subsequently, F124C-PCR products were digested with restriction enzyme *Nhe*I as published before[25]. For the T-allele (Cys^{124}) and the G-allele (Phe^{124}) digestion products were obtained of 258 bp and 238 bp & 20 bp, respectively. Digestion of the A-161T-PCR products was performed with restriction enzyme *Nla*III. The restriction patterns are more complicated because of two internal *Nla*III restriction sites. The A- and T-allele produce allele-discriminative bands of 438 & 120 bp and 558 bp, respectively. In addition, for both alleles, smaller products are produced of 41 and 7 bp. For both digests, digestion products were separated on a 3% agarose gel, and bands were visualized by UV transillumination of gels stained with ethidium bromide.

11.3 RESULTS

11.3.1 Patient Characteristics

Patient groups did not differ significantly in age and gender distribution (Table 11.1); also they did not differ in age of onset of migraine (mean ± s.e.m: 21 ± 12 years), duration of migraine history (26 ± 2 years), and attack frequency (2.7 ± 0.3 attacks/month). The number of attacks treated with s.c. sumatriptan was

lower in non-responders than in responders and patients with recurrence of the headache (6 ± 1 *vs.* 72 ± 21 and 67 ± 26, resp.; $P<0.001$), and tended to be lower in patients with chest symptoms (36 ± 15 *vs.* 54 ± 15 for patients without chest symptoms). The mean number of attacks treated with subcutaneous sumatriptan was 48 ± 11 for all patients.

11.3.2 Polymorphism Analysis

In none of the patients, the F124C variant was detected. Therefore it can be concluded that Cys^{124} is not a major determinant in the clinical response to sumatriptan. In contrast, both alleles of the A-161T were observed as well as all three possible genotypes: A/A, A/T and T/T. In the reference group allele frequencies of 0.77 and 0.23 were observed for the A- and T-allele, respectively (Table 11.1).

11.3.3 Power of the study

Power analysis showed that our patient groups were sufficiently large to detect an allele frequency difference of 35%. Thus, we have power to determine whether the functional A-161T polymorphism is a major determinant in the clinical response to sumatriptan if the allele frequencies for the A- and the T-allele change as drastically as <0.42 and >0.58, respectively. However, from Table 11.1 it is clear that neither in the total group nor in the subgroups such allele differences were observed. Depending on which (sub) group of patients is studied, the allele frequencies for the T-allele range between 0.20 – 0.38, well below the threshold level. We can conclude that the A-161T variant does not explain the clinical response to sumatriptan.

11.4 DISCUSSION

In the present study, we investigated whether the differences in the clinical response to subcutaneous sumatriptan were largely determined by one of two variants: (i) F124C amino acid change in 5-HT$_{1B}$ receptor and (ii) variant A-161T in the 5′-flanking region of the same receptor 5-HT$_{1B}$ gene. Since both these variants were shown to affect the action of sumatriptan on this receptor (either by increased binding affinity in case of Cys^{124} or by increased expression of the 5-HT$_{1B}$ receptor by the T-allele of promoter polymorphism A-161T) our *a priori* hypothesis was that one or both of these variants might explain the clinical response to sumatriptan. Especially the chest symptoms that patients experience after sumatriptan (and which may be related to coronary vasoconstriction), recurrence of the headache, or non-response after sumatriptan, may be explained by gene variants in the 5-HT$_{1B}$ receptor. Of interest, the A-161T polymorphism was demonstrated to be related to alcohol dependence in a population of Taiwanese Han[26].

In the present study, we did not detect Cys^{124} and can exclude it as a major factor in the clinical response to sumatriptan in our patient group. In fact this Cys^{124} variant was identified only once in 46 unrelated healthy subjects in a study by Nöthen et al.[17], and three times in 98 subjects studied by Verheggen et al.[20]. We also provided evidence that the A-161T polymorphism does not seem to be a major determinant; no drastic changes in allele frequencies were observed between the various (sub) groups and the European reference population. However, it should be stated that our study lacks power to study smaller effects on the clinical outcome.

We chose to study genetic variation in the 5-HT$_{1B}$ receptor and not in the 5-HT$_{1D}$ and 5-HT$_{1F}$ receptors, despite the fact that these receptors also show affinity for sumatriptan and thus may be responsible for the therapeutic efficacy of sumatriptan[27]. It is unlikely that these receptors mediate the therapeutic action of sumatriptan, because (i) the selective 5-HT$_{1D}$ receptor agonist PNU-142633 was not effective in the treatment of migraine[28], and (ii) the 5-HT$_{1F}$ receptor agonist LY344864 was only effective in the treatment of migraine at doses at which may well interact with 5-HT$_{1B}$ receptors[29], and (iii) genetic variation in the 5-HT$_{1F}$ receptor gene as determinant of the clinical response to sumatriptan was not studied since we

are not aware of variants that may have a functional effect, and we previously did not demonstrate any genetic diversity in this set of patients[30].

A possible restriction of this study is that patients who are non-responders to sumatriptan are not necessarily non-responders to other triptans[31], which are also agonists at the 5-HT_{1B} receptor. Although this finding suggests that the factor determining whether a person is a non-responder to sumatriptan would rather be dependent on pharmacokinetics than on the 5-HT_{1B} receptor gene, we already mentioned above that there are no pharmacokinetic differences between responders and non-responders to sumatriptan[8].

In conclusion, we have demonstrated that the F124C and A-161T variants of the 5-HT_{1B} receptor are not major determinants of the clinical response to sumatriptan in our set of well-characterized migraine patients.

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Chapter 11

The Phe-124-Cys and A-161T variants of the human 5-HT_{1B} receptor are not major determinants of the clinical response to sumatriptan

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Part V: General Discussion and Summary

Chapter 12

General Discussion, Conclusions and Summary

12.1 MIGRAINE MODELS

12.1.1 Porcine Model

The pathophysiology of migraine headache is still not clear. However, it is believed that this episodic syndrome of pain involves intracranial structures associated with other neurologic disturbances,[1] like aura symptoms and photo- and phonophobia. Based on the neurovascular hypothesis several animal models[2] have been proposed to study the effect of potential antimigraine compounds and their action on the systemic and cranial circulation. A number of migraine models are either based on the reversal of cranial vasodilatation in migraine, inhibition of cranial vasodilatation (carotid, dural, cortical) induced by trigeminal stimulation, central trigeminal neuronal inhibition and inhibition of plasma protein extravasation after stimulation of the trigeminal nerve. However, none of the models available studies all the aspect of migraine pathophysiology.

The concept of the *in vivo* porcine model for migraine, which suggests a decrease in total carotid blood flow after the administration of various potential and prospective antimigraine compounds, was developed on the basis of involvement of cranial vasodilatation in migraine. Wolff (1963)[3] proposed that the vasodilatation of extracranial arteries supplied by the external carotid artery is the primary cause of the migraine attacks. Later on, Heyck (1969)[4] performed preliminary experiments in migraine patients and suggested that arterial blood during migraine is being shunted to the venous circulation due to dilatation of the cephalic arteriovenous anastomoses (AVA). Under physiological conditions, carotid AVAs are under a sympathetic constrictor tone, shunting only <5% of arterial blood back to the right side of the heart. This results in a difference in oxygen saturation at the arterial and venous side ($AVSO_2$ difference). Moreover, Heyck showed that in migraine patients the $AVSO_2$ difference was lower as compared to control subjects. However, the possible locus of action in this porcine model[5] was largely unknown over the years. Keeping this in mind we conducted studies on the isolated meningeal artery of the pig to determine the possible locus of action of current and prospective antimigraine compounds.

Thus, in our study we assessed on cranial vasodilation and constriction in the porcine isolated meningeal artery. It is worth emphasizing here that the isolated blood vessel system may provide the mechanistic approach for a ligand under study, but lacks the *in vivo* conditions. The *in vivo* setup involves the influence of a sympathetic tone, as well as circulating endogenous mediators that may 'unmask' or augment the responses to exogenously injected compounds. Thus, an isolated blood vessel system does not provide a comprehensive view of a ligand under study. Another major drawback of our model using isolated blood vessels is that a drug in the organ bath set-up reaches both the intraluminal and extraluminal side of the blood vessel. This is different in the *in vivo* setup, where drugs initially only reach the intraluminal side of blood vessels, but may later diffuse to the extraluminal side. Thus, experiments on perfused meningeal arteries may reveal whether intraluminal exposure to drugs differs from exposure to drugs on both sides of the blood vessel. However, the major advantage of an organ bath study is that one can simultaneously investigate a large number of blood vessel segments from the same human subjects or animals and can concurrently screen like a 'high through-put' several compounds and their effect on the vessel under study. In addition, the advantage of using organ baths is that we can study the agonist as well as antagonist responses in paired fashion, i.e. arteries from the same subject. Moreover, organ-specific drugs can be studied; we studied the meningeal arteries in our study as they are implicated in migraine pathophysiology, whereas drugs for hypertension can be studied in coronary or renal arteries.

We found in our study that none of the current antimigraine compounds as sumatriptan or ergot alkaloids constrict the porcine meningeal artery, suggesting the possible locus of action of these drugs is more towards the small resistance arteries and AVAs but not in the 'conducting' part of the porcine meningeal artery.

12.1.2 Ovariectomized Rat Model

Migraine is more prevalent in females as compared to their male counterparts[7]. Clinical evidence indicates that female sex steroids may contribute to the high prevalence of migraine in women, as well as to changes in the frequency or severity of migraine that are in tandem with various reproductive milestones in women's life. While estrogen and progesterone do not seem to be involved in the pathogenesis of migraine *per se*, they may modulate several mediators and/or receptor systems. We ovariectomized female rats and administered pellets of placebo, estrogen, progesterone or both, or the rats were sham (ovaries exteriorized but not removed) operated. We studied the effect of female sex hormones on receptor-mediated responses, namely via α_1- and α_2-adrenoceptors, 5-HT receptors and CGRP receptors in the isolated blood vessel system. A major limitation of our model was that blood levels of 17β-estradiol were about two times higher than the physiological levels. Another shortcoming of our model is that it does not take into consideration that the menstrual cycles of rats and women vary. However a study by Staley and Scharfman[8] suggests that the proestrus phase of rats could be considered as the luteal phase in humans (Figure 12.1).

Further, the segments used in our *in vitro* model where either from carotid, caudal, basilar or mesentric arteries, that are all not directly involved in migraine pathogenesis. Nevertheless, although the carotid artery is probably not directly involved in the trigeminal pathway, the experimental models involving carotid vascular responses in several animal species, including the cat[9,10], dog[11,12], pig[13,14] and rabbit[15], have proven highly predictive of antimigraine efficacy. In addition, extracranial arteries are the source of the pain in some migraine sufferers[16] and it has been reported that migraine is associated with lower levels of blood pressure and with smaller values of carotid wall thickness[17]. Thus, changes in the reactivity to α-adrenergic and 5-HT receptors in the carotid artery by female sex hormones may indirectly influence migraine sensitivity. We used these arteries instead of dural arteries, since the latter are too small to be studied in myographs. However, it is emphasized here that the blood vessels used in our study have similarity to meningeal arteries, as they are (i) densely innervated by CGRP-containing fibres (mesenteric arteries[18]), (ii) display an endothelium-independent relaxation to CGRP (mesenteric[19] and caudal[20] arteries), in addtion, we used also (iii) cranial blood vessels (basilar artery). However our assumption on CGRP receptor reactivity changes in the 17β-estradiol group and the placebo group appears to be quite leap. Thus, future studies should focus on the effect of α-adrenoceptors, CGRP receptors and 5-HT receptors in female rats at different levels of the female sex hormones using different concentrations of

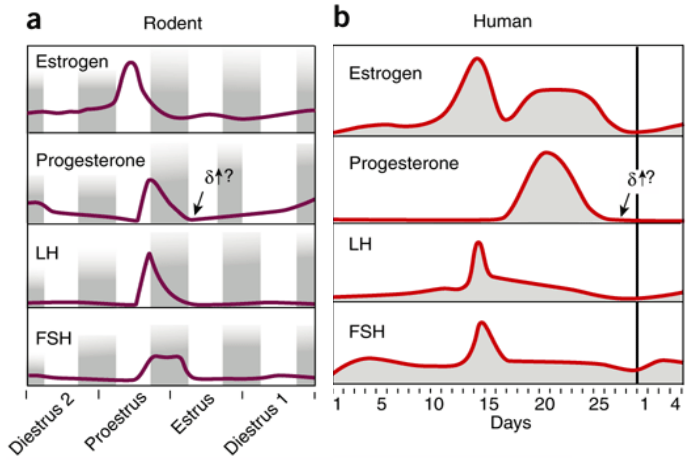

Figure 12.1: Comparing the menstrual cycle of rats and human (from *Staley and Scharfman*[8])

hormone-releasing pellets. This type of studies will give insight on the role of these receptors at different levels of female hormones, although, this could still only partially mimic the menstrual cycle of females, where hormone levels fluctuate over a period of 28 days. However, it will definitely give insight into the receptor activity and expression (mRNA and protein) due to variation in the hormone levels.

Essentially, in our experimental models, stable levels of sex hormones are used, while clinical evidence indicates that not only do absolute levels of female sex steroids seem to be of relevance, but rather their (rate of) change. As it is evident from the fact that in conditions like menstruation and the perimenopausal period where, the hormonal levels are very unstable, migraine severity is also enhanced. Therefore, future experimental studies should carefully take these considerations into account, and investigations focussing on the change in hormone levels, especially the decrease in 17β-estradiol after exposure to a high concentration, may reveal more insight into the pathophysiology of menstrual migraine. Also studies on isolated arteries from transsexual humans undergoing hormonal treatments might give better insight into the effect of female sex hormones and on receptor activity. In addition, future studies might make use of arterial segments cultured in medium with different levels of female hormones, either acutely or chronically. Afterwards, vascular function could be studied functionally (Mulvany myographs) as well as at the receptor level (mRNA expression and receptor protein expression). Moreover, a microarray analysis on the different groups of ovariectomized rats will give a comprehensive view of mRNA expression (upregulation or downregulation) of various receptor and transcription factors.

12.2 α-ADRENOCEPTOR AND MIGRAINE

The role of α-adrenoceptors in the pathophysiology of migraine has been explored in various clinical studies[21]. It has been suggested that sensitization of blood vessels, reinforced by a direct vasoconstriction through activation of smooth muscle α-adrenoceptors, may contribute to the mechanism of action of antimigraine drugs targeting α-adrenoceptors, such as clonidine[22] and ergots[23]. The advent of α-adrenoceptor subtypes[24] opened the gate for researchers to explore the role of each subtype and their possible implications for the development of novel antimigraine drugs. Antimigraine drugs should be devoid of systemic vasoconstrictor properties leading to an increase in arterial blood pressure and coronary artery constriction[25]. Thus, there is a need to study for each receptor subtype of both α_1- and α_2-adrenoceptor, whether it is devoid of systemic vasoconstrictor and specifically constricts cranial blood vessels. Studies on a porcine model of migraine have depicted a potential role for the α_{2C}-adrenoceptor subtype[26]. However, the possible implication of this study was not tangible until the porcine receptor structure was known. It seems evident that particular pharmacological characteristics of a compound targeted for a specific receptor in one species, in this case the pig, cannot always be extrapolated to another species like humans. Thus, drug-receptor interactions should not be extended as such from animal to human without verification of molecular structure of the receptor and its pharmacological profile. Therefore, a precise comparison of recombinant expression studies with *in vivo* and *in vitro* experiments may eventually lead to better understanding of the physiological role(s) of a specific receptor in a particular tissue or vascular bed[27]. Hence, we set out to clone and characterize the porcine specific α_{2B}- and α_{2C}-adrenoceptor. The full-length α_{2B}-adrenoceptor of the pig showed 90% homology to the human α_{2B}-adrenoceptor. The mRNA of this receptor subtype was ubiquitously present throughout the porcine vasculature, suggesting that an agonist of this subtype should not be explored for the development of novel antimigraine drugs, as it would involve potential side effects such as systemic vasoconstriction. We also studied the signalling mechanism of this receptor subtype and found that the transcription factor 'serum response element' is responsible for its signalling downstream inside the cell. This was the first study of its kind using a dual luciferase assay to characterize this new receptor. We cloned the partial sequence of the porcine α_{2C}-adrenoceptor (DQ225117; http://www.ncbi.nlm.nih.gov), which also

showed up to 90% homology with the human receptor. Future studies should focus on the localization of other subtypes of α_1- and α_2-adrenoceptors by real-time PCR, as this will give insight into the number of copies of mRNA of the receptor subtype present in the particular tissue. This will provide knowledge about the receptor presence, which in turn will help in developing subtype specific agonists with lesser side effects.

In addition, further studies are required in order to confirm the involvement of the α_{2C}-adrenoceptor in the pathophysiology of migraine. Even disregarding its involvement in migraine, agonists specific for α_{2C}-adrenoceptor could be of therapeutic importance because the agonist for α_2-adrenoceptor is being reported to decrease the total carotid conductance in the porcine *in vivo* model[26]. Thus, there is a need for the development of subtype-specific agonists both for α_1- and α_2-adrenoceptor subtypes. In this thesis, we also explored the role of the α_{2C}-adrenoceptor in the porcine isolated meningeal artery using the specific antagonist OPC28326[28], which shifted the control curve to the endogenous ligand noradrenaline to the right, suggesting the presence of this subtype in the meningeal artery of the pig. However, its role may be limited because the α_2-adrenoceptor agonists BHT-933 and UK14304 did not induce constriction in isolated porcine meningeal artery. Future studies should now focus on the presence of this receptor subtype in the human meningeal artery. In addition, in order to avoid potential coronary side effects of a α_{2C}-adrenoceptor agonist, the role of this receptor in the coronary vascular bed should be investigated. This could be done by constructing concentration response curves to noradrenaline in the absence or presence of OPC28326 in human and porcine coronary arteries and by assessing the shift induced by this antagonist.

In this thesis we found a decreased sensitivity to noradrenaline in ovariectomized rats treated with progesterone, suggesting a prominent role of postsynaptic α-adrenoceptors, which may lead to cranial vasodilatation and, therefore, proneness to migraine headache[11,29,30]. This is in line with a recent investigation showing that higher urinary progesterone metabolites during the mid-luteal time periods are associated with worse headache outcome measures during that period[31]. In addition, it has been reported that plasma progesterone levels show an irregular pattern in migraine patients[32]. Thus there appears to be a positive correlation between progesterone levels and headache. We also observed an increased function of α_2-adrenoceptors in the carotid artery of ovariectomized rats treated with placebo pellets. This finding may at least partly explain the decreased prevalence of migraine in female patients after their menopause. Additionally, *in vivo* experiments on ovariectomized and hormone-treated rats with a closed cranial window should assess the differences in contractility of middle meningeal artery to α_1- and α_2-adrenoceptor responsiveness, in different groups of animals. Furthermore, the human isolated meningeal artery contractility to α_2-adrenoceptor agonists in postmenopausal women should be compared to that in men and premenopausal women. This may give insight into the increased function of α_2-adrenoceptor in postmenopausal females.

We also characterized the α_{1A}-adrenoceptor agonist-, A61603-, in the porcine isolated meningeal artery. Previously, contraction to A61603 in the porcine *in vivo* model[33] was reported to be mediated via a non-adrenergic mechanism. As described in the previous paragraph, the exact locus of action of this drug was not demonstrated, as the authors measured decreases in total carotid conductance. The selective α_1-adrenoceptor antagonist prazosin and the α_2-adrenoceptor antagonists rauwolscine and yohimbine blocked the A61603-induced contraction. Thus, the contraction by A61603 in porcine isolated meningeal artery involves both α_1- and α_2-adrenoceptors. In addition, the 5-HT_1 receptor antagonist GR127935 and 5-HT_2 receptor antagonist ritanserin did not affect the A61603-mediated contraction. Further, contractions to A61603 were attenuated by the mixed α-adrenergic and 5-HT receptor antagonist methiothepin, and forskolin also decreased the contraction to A61603 suggesting the involvement of a cAMP pathway, which in turn again supports α_2-adrenoceptor-mediated contraction of A61603. Thus, we did not observe the involvement of a non-adrenergic mechanism in A61603 mediated contraction in the isolated porcine meningeal artery as previously described by Willems *et al*.[33]. However, to explore further the

discrepancy between our results and *in vivo* porcine model findings, future studies should also focus on A61603-mediated contraction in other vessels of the pig as there is a possibility that A61603 invariably binds with high potency to porcine α_{1A}-adrenoceptors. Furthermore, the affinity of A61603 for recombinant porcine α-adrenoceptor subtypes should be assessed.

12.3 5-HT RECEPTORS AND MIGRAINE

After synthetic serotonin (5-hydroxytryptamine 5-HT) became available in the early 1950s, attempts were soon under way to study the nature of 5-HT receptors[34]. The cardiovascular effects of 5-hydroxytryptamine (5-HT) consist of bradycardia or tachycardia, hypotension or hypertension, and vasodilatation or vasoconstriction; three main types of receptors (5-HT_1, 5-HT_2, and 5-HT_3 receptors) mediate these effects. 5-HT elicits a short-lasting bradycardia, accompanied by hypotension, via stimulation of 5-HT_3 receptors located on sensory vagal nerve endings in the heart (Bezold-Jarisch reflex). The nature of 5-HT receptors mediating tachycardiac responses is species dependent.

Sumatriptan (a 5-HT_1 receptors agonist) reduces the cranial arteriovenous anastomotic shunting in porcine vasculature[35]. Further studies have shown sumatriptan constricts porcine carotid arteriovenous anastomoses primarily via 5-HT_{1B}, but not via 5-HT_{1D} receptors[36]. However, vascular contraction by 5-HT is generally mediated by 5-HT_2 receptors (located primarily on the large conducting vessels), though in some instances (e.g., dog saphenous vein, dog and human basilar artery, and porcine arteriovenous anastomoses) the contractile response is (also) mediated via 5-HT_1 receptors[37]. In our study in porcine meningeal 'conducting artery' the responses towards agonist 5-HT were also primarily mediated by 5-HT_2 receptors. This was based on the findings that 5-HT_1 receptor agonist sumatriptan did not induce contraction in these blood vessels, and the 5-HT_2 receptor antagonist ketanserin and ritanserin shifted the concentration response curve to 5-HT to the right.

In addition, we also studied the function of 5-HT receptor under the influence of female sex hormones, as it is believed that migraine without aura is associated with a central 5-HT receptor hypersensitivity[38]. Moreover, an action of female sex hormones on endogenous vasoactive agents might well be involved in cranial vasodilatation leading to migraine. Thus, we studied the effect of 5-HT receptors in the isolated carotid artery obtained from ovariectomized rats. We found that in placebo-treated ovariectomized rats; there was an increased contractility to 5-HT as compared to animals with normal circulating female sex hormones. Thus if placebo treated rats are compared to menopausal state this could easily explain the decrease incidence of migraine attacks after menopause. If such a mechanism is also present in human dural arteries, this could provide a possible explanation for the relation between female sex hormones and migraine. Additionally, *in vivo* experiments studying the trigeminal pathway using a closed cranial window will shed further light on our *in vitro* findings in isolated blood vessels. Further, future studies are required to investigate which 5-HT receptor subtype is influenced by the female sex hormones. Moreover, future studies should focus on mRNA expression to learn more about receptor regulation and to analyze the up-/ or down-regulation of each 5-HT receptor subtype involved.

We also studied the potential ability of some 5-hydroxytryptamine (5-HT) receptor ligands, which are widely used in cardiovascular experimental models, to interact with vascular α_1-adrenoceptors in the pithed rat. We observed that methiothepin, ketanserin, clozapine, lisuride and buspirone could block α_1-adrenoceptors in the rat systemic vasculature, suggesting differences between data from binding studies in cell systems with those obtained under *in vivo* conditions in animals. Thus, future studies should also focus on this apparent discrepancy, which could be due to heteromeric proteins (in this case a crosstalk between 5-HT receptor subtype and α_1-adrenoceptor subtype). Thus, recombinant receptors of each subtype (both α_1-adrenoceptor and 5-HT receptor subtype) should be transfected in different

combinations, and subsequently binding characteristics and coupling to second messenger system pathways should be investigated.

12.4 CALCITONIN GENE RELATED PEPTIDE (CGRP) RECEPTORS AND MIGRAINE

Calcitonin gene-related peptide (CGRP) is a potent neuromodulator[39] that is expressed in the trigeminovascular system and that is released into the cranial circulation in various primary headaches[40]. The search for a potent small molecule CGRP antagonist has been successful and such an agent has been tested in preclinical and clinical studies. Olcegepant (BIBN4096BS) is a specific and potent CGRP receptor antagonist in humans[41]. CGRP receptor blockade has been shown to be an effective acute antimigraine strategy and is non-vasoconstricting in terms of the mechanism of action[42]. Studies have shown that CGRP is a major sensory neuronal messenger in the trigeminovascular system, the pathway conveying intracranial pain[43]. In the porcine model of migraine olcegepant has been reported to decrease the total carotid blood flow[44], although the exact locus of action of this drug in the porcine model was largely unknown. In our study we demonstrated the effect of olcegepant in isolated meningeal artery, which shifted the control curve of CGRP to the right suggesting the presence of this receptor in these blood vessels. We also showed the mRNA for a component of the CGRP receptor, the calcitonin receptor like-receptor (CALCRL) in these blood vessels.

In addition, we studied relaxant responses to CGRP in ovariectomized rats treated with female sex hormone pellets and placebo pellets. The responses were studied in isolated mesenteric and caudal arteries. We observed a significant difference between ovariectomized rats treated with placebo pellets and those treated with estrogen pellets. If such responses are also present in cranial vasculature, this could explain the higher prevalence of migraine in females as compared to males. Thus, future studies should focus on the effects of female sex hormones using the human isolated meningeal artery, and investigate the differences in relaxation to CGRP in postmenopausal women compared to men and premenopausal women. This could give insight into the increased function of CGRP receptors in females with circulating female sex hormones and thus more migraine.

Safety and tolerability studies report that olcegepant is well tolerated with no or only mild side effects[45]. As CGRP plays a protective role in preconditioning of the heart during ischemia and heart attacks, the use of a CGRP receptor antagonist may potentially lead to severe side effects when used for cardiac patients. Indeed we[46] and others[47,48] demonstrated in the isolated rat Langendorff heart that CGRP has a cardioprotective role in preconditioning and olcegepant completely abolished the cardioprotection induced by preconditioning as well as by exogenous CGRP administration[46]. Additional, studies have shown that CGRP is regulated by multiple factors, such as transient ischemia, hyperthermia or autocoids, and the elevated level of CGRP during ischemia may therefore constitute a compensatory response[49-51].

Thus, there is a need to explore other receptor targets that may have less cardiac side effects such as highly specific calcium channel subtype blockers or targets at a molecular level eg. transcription factors like NFκB, which is believed to be involved in neurogenic inflammation and hence migraine[52].

12.5 SUMMARY

12.5.1 The role of 5-hydroxytryptamine (5-HT), adrenergic and CGRP receptor subtypes in migraine pathophysiology (Chapter 1)

Migraine affects a substantial fraction of the world population and is a major cause of disability in the work place. Migraine is a recurrent incapacitating neurovascular disorder characterized by attacks of debilitating pain associated with photophobia, phonophobia, nausea and vomiting. Though the patho-

physiology of migraine is still unclear, it is believed to be neurovascular in nature. Drugs used in the treatment of migraine affect vascular receptors. Earlier, α-adrenoceptor agonists (ergotamine, dihydroergotamine, isometheptene) were used. The last decade has witnessed the advent of sumatriptan and the second generation, all 5-$HT_{1B/1D}$ receptor agonists, which have a well-established efficacy in treating migraine. Currently, prophylactic treatments for migraine include 5-HT_2 receptor antagonists and β-adrenoceptor blockers. Despite such progress, in view of the complexity of the etiology of migraine, this disease still remains underdiagnosed and available therapies are underused. In this chapter, use of different current and prospective pharmacological targets for the migraine are reviewed, with special emphasis on each of the receptor subtypes of 5-HT, adrenoceptor and calcitonin gene-related peptide (CGRP) receptors.

12.5.2 Potential role of female sex hormones in the pathophysiology of migraine (Chapter 2)

Female sex steroids may contribute to the high prevalence of migraine in women, as well as changes in the frequency or severity of migraine that are in tandem with various reproductive milestones in women's life. While estrogen and progesterone do not seem to be involved in the pathogenesis of migraine *per se*, they may modulate several mediators and/or receptor systems *via* both genomic and non-genomic mechanisms. These actions may be perpetuated at the central nervous system, as well as at the peripheral (neuro)vascular level. For example, estrogen enhances neuronal excitability by elevating Ca^{2+} and decreasing Mg^{2+} concentrations; this may act in concurrence with other mechanisms triggering migraine. Further, estrogen is reported to enhance the synthesis and release of nitric oxide and neuropeptides, such as calcitonin gene-related peptide; this in turn reinforces vasodilatation and activates trigeminal sensory afferents with a subsequent stimulation of pain centres. In addition, female sex steroids may increase the function of receptors mediating vasodilatation, while responses of receptors inducing vasoconstriction are attenuated. The serotonergic, adrenergic and GABAergic systems are also modulated by sex steroids, albeit to a varying degree and with potentially contrasting effects on migraine outcome. Taken together, female sex steroids seem to be involved in an array of components implicated in migraine pathogenesis. Future studies will further delineate the extent and the clinical relevance of each of these mechanisms, and will thus expand the knowledge on the femininity of migraine.

12.5.3 Aims

The aims of the present investigation have been listed are: (i) to investigate whether the vasoconstriction/vasodilatation of the carotid circulation induced via 5-HT, adrenergic and CGRP receptors in pigs *in vivo* involves the meningeal artery; (ii) to characterize the 5-HT, adrenergic and CGRP receptors in porcine isolated meningeal arteries; (iii) to investigate the effect of female sex hormones on 5-HT, α-adrenergic, muscuranic and CGRP receptor responses in isolated blood vessels; (iv) to clone and characterize the porcine α_{2B}-adrenoceptor; (v) to assess whether there is a genetic basis for occurrence of chest symptoms after the use of sumatriptan.

12.5.4 Effects of current and prospective antimigraine drugs on the porcine isolated meningeal artery (Chapter 3)

In this chapter we studied the effects of current and prospective antimigraine drugs on porcine isolated meningeal artery. We investigated contraction to agonists at 5-HT receptors and α-adrenoceptors, as well as vasodilatation induced by α-CGRP, as these have been well described in the porcine carotid circulation *in vivo*, although the *locus* of this vasomotion remains to be investigated. Thus, this study was designed to investigate the effects of current and prospective antimigraine drugs on the isolated porcine meningeal artery, segments of meningeal artery were mounted in Mulvany myographs and concentration response curves to several compounds were constructed. Surprisingly, ergotamine, dihydroergotamine, isometheptene, clonidine and sumatriptan all failed to contract the porcine meningeal artery. In contrast, 5-HT and noradrenaline induced concentration-dependent contractions. The contractions

to 5-HT were antagonized by the 5-HT_{2A} receptor antagonist ketanserin, whilst those to noradrenaline were blocked by the α_1-adrenoceptor antagonist prazosin, the α_2-adrenoceptor antagonists rauwolscine and yohimbine as well as the $\alpha_{2C/2B}$-adrenoceptor antagonist OPC-28326. Furthermore, α-CGRP induced concentration-dependent relaxations that were antagonized by the $CGRP_1$ receptor antagonist olcegepant. Interestingly, but in agreement with their lack of contractile effects, forskolin-induced increases in cAMP remained unaffected by sumatriptan and ergotamine in the porcine meningeal artery. Finally, using RT-PCR, we could demonstrate the presence of mRNAs encoding for several 5-HT (5-$HT_{1B,}$ 5-HT_{1D}, 5-$HT_{1F,}$ 5-HT_{2A} and 5-HT_7) and adrenergic (α_{1A}, α_{1B}, $\alpha_{1D,}$ α_{2A}, α_{2B}, α_{2C}, β_1 and β_2) receptors, as well as that for a component of the CGRP receptor, the calcitonin receptor like receptor (CALCRL). These results show that the meningeal artery is not involved in the vasoconstriction of the carotid vascular bed elicited by antimigraine drugs in anaesthetized pigs. Moreover, although the mRNA of several 5-HT, adrenergic and CGRP receptors is located on these vessels, many of these receptors, in particular 5-HT_{1B} receptors as well as α_2- and β-adrenoceptors, are either not functional or their density is too low for producing a noticeable response.

12.5.5 A61603-induced contractions of the porcine meningeal artery are mediated by α_1- and α_2-adrenoceptors (Chapter 4)

It was recently shown that A61603 (N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]methane sulphonamide), a potent α_{1A}-adrenoceptor agonist, decreased carotid artery conductance in anaesthetized pigs by a novel non-adrenergic mechanism. In this study, we set out to pharmacologically characterize A61603-induced contractions of the porcine isolated meningeal artery. While the maximum contractile responses of the artery were similar, A61603 was more potent than noradrenaline or phenylephrine. Prazosin, as well as rauwolscine and yohimbine, antagonised the contractions to A61603. The 5-HT receptor antagonists, GR127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl) phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide) and ritanserin failed to affect the responses to A61603, but methiothepin, which, in addition, has a high affinity for α-adrenoceptors, proved an effective antagonist. The A61603-induced responses were suppressed by the cAMP stimulator forskolin, but not by the protein kinase C inhibitor chelerythrine. Our results suggest that the contraction of the porcine isolated meningeal artery by A61603 is mediated via a mixed population of α_1- (probably α_{1A}) and α_2-adrenoceptors and that the adenylate cyclase, but not the diacylglycerol, pathway seem to be involved.

12.5.6 Potential vascular α_1-adrenoceptor blocking properties of an array of 5-HT receptor ligands in the rat (Chapter 5)

This study set out to analyse the potential ability of some 5-HT receptor ligands widely used in cardiovascular experimental models to interact with vascular α_1-adrenoceptors in the pithed rat. These ligands included: methiothepin, methysergide and metergoline (5-HT_1/5-HT_2); WAY-100635, buspirone, ipsapirone and 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) (5-HT_{1A}); GR127935 (5-$HT_{1B/1D}$); ketanserin, ritanserin, spiperone and pizotifen (5-HT_2); granisetron and metoclopramide (5-HT_3); tropisetron (5-HT_3/5-HT_4); ergotamine (5-$HT_{1B/1D}$, 5-$ht_{5A/5B}$); clozapine (5-HT_6/5-HT_7); as well as LY215840 and mesulergine (5-HT_2/5-HT_7). For this purpose, the increases in diastolic blood pressure produced by the selective α_1-adrenoceptor agonist, phenylephrine, were analysed before and after the above antagonists or saline. The adrenoceptor antagonist properties of prazosin (α_1) and yohimbine (α_2) were also analysed for comparison. Thus, the phenylephrine-induced vasopressor responses were dose-dependently antagonised with the following apparent rank order of potency by:

prazosin≥methiothepin>ketanserin>clozapine≥lisuride>>buspirone; this potency correlates with the affinity of these compounds for α_1-adrenoceptor binding sites. In contrast, the other compounds were either devoid of any blocking effect on -or even potentiated (i.e. lisuride, methysergide, 8-OH-DPAT,

granisetron and GR127935)- the responses to phenylephrine. These results show that methiothepin, ketanserin, clozapine, lisuride and buspirone can block α_1-adrenoceptors in the rat systemic vasculature.

12.5.7 The role of CGRP in ischemic preconditioning in isolated rat hearts (Chapter 6)

Here we studied the role of CGRP in preconditioning (PC) in isolated perfused rat hearts, by measuring CGRP release during PC and mimicking this by exogenous CGRP infusion, either in the absence or presence of the CGRP antagonist olcegepant. As previously it is being described brief coronary artery occlusion (CAO) can protect the heart against damage during subsequent prolonged CAO; which is called as ischemic preconditioning (PC). CGRP increased left ventricular pressure (LVP) and coronary flow (CF) in a concentration dependent manner, which was effectively antagonized by olcegepant. Rat hearts were subjected to CAO and reperfusion, which was proceeded by: (1) sham pretreatment, (2) olcegepant infusion (3) PC by CAO and reperfusion, (4) as 3, but with olcegepant, (5) CGRP infusion and then washout, (6) as 5, but with olcegepant. Cardiac protection was assessed by reactive hyperaemia (RH), creatine kinase (CK) release, infarct size related to the area at risk (I/R%), and LVP recovery. PC increased CGRP release into the coronary effluent, and significantly protected the hearts by decreasing RH, reducing CK release, limiting infarct size, and improving LVP recovery. Exogenous CGRP induced preconditioning-like cardioprotection. Olcegepant completely abolished the cardioprotection induced by PC as well as by exogenous CGRP. Thus, since cardioprotection of PC-induced CGRP release can be mimicked by exogenous CGRP, and a CGRP antagonist can block both, our results indicate an important role for CGRP in ischemic preconditioning.

12.5.8 Rat carotid artery responses to α-adrenergic receptor agonists and 5-HT after ovariectomy and hormone replacement (Chapter 7)

This study was designed to compare the contractile responses to α-adrenoceptor agonists and 5-HT in the rat carotid artery after ovariectomy and subsequent hormone replacement with 17β-estradiol, progesterone or the combination of 17β-estradiol and progesterone. After one week of acclimatization, female Sprague-Dawley rats were either sham-operated or bilaterally ovariectomized. One week later, the ovariectomized rats were subcutaneously implanted with a pellet releasing over a 21-day period either placebo, 17β-estradiol, progesterone or the combination of the two hormones. Blood samples were collected to measure plasma noradrenaline and adrenaline. On day 25-28, the animals were sacrificed to isolate carotid artery and mount its segments in Mulvany myographs. Cumulative concentration response curves to α-adrenoceptor agonists and 5-HT were constructed in the absence or presence of suitable antagonists. The potency of noradrenaline in ovariectomized rats was significantly reduced in animals treated with progesterone as compared to those with placebo. In placebo-treated ovariectomized animals there was a noticeable response mediated by α_2-adrenoceptors, in contrast to that in sham-operated or ovariectomized rats treated with 17β-estradiol and progesterone, either alone or in combination. The plasma levels of noradrenaline and adrenaline were not significantly affected by either ovariectomy or the subsequent hormone replacement. The potency of 5-HT was significantly reduced in animals having circulating sex hormones as compared to that in placebo-treated ovariectomized animals. Our results gave us an idea that circulating progesterone and/or 17β-estradiol may reduce the contraction of the rat carotid artery in response to noradrenaline and 5-HT.

12.5.9 Effects of female sex hormones on responses to CGRP, acetylcholine and 5-HT in rat isolated arteries (Chapter 8)

This study sets out to investigate the effects of the female sex steroids, 17β-estradiol and progesterone (separately and in combination) on vasoactive responses to CGRP, acetylcholine and 5-HT in rat isolated mesenteric, caudal and basilar arteries. Female Sprague-Dawley rats were ovariectomized and 7 days later subcutaneously implanted with pellets releasing over a 21-day period 17β-estradiol, progesterone,

their combination or placebo. On Day 25-28, the animals were sacrificed, arteries isolated and mounted in Mulvany myographs and cumulative concentration response curves to CGRP, acetylcholine and 5-HT were constructed. The relaxant responses to CGRP were significantly potentiated in mesenteric and caudal arteries from rats treated with 17β-estradiol as compared to the placebo-treated rats. Acetylcholine-induced relaxations were potentiated in the caudal artery from rats treated with the combination of 17β-estradiol and progesterone, as compared to that from placebo-treated rats. The 5-HT-induced contractions in the three arteries were not significantly different in efficacy or potency. Our results show that 17β-estradiol potentiates CGRP-induced relaxations in the mesenteric and caudal arteries, whilst the combination treatment enhances acetylcholine-induced relaxations in the caudal artery.

12.5.10 Cloning of the porcine α_{2B}-adrenoceptor: Tissue distribution and pharmacological characterization (Chapter 9)

This study demonstrates the molecular cloning and characterization of the porcine α_{2B}-adrenoceptor. In addition, its tissue distribution was studied. Sequence analysis of the porcine cDNA clone (1341 bp) revealed an open reading frame of 446 amino acids encoding the α_{2B}-adrenoceptor, which showed 88% and 85% similarity with the human and guinea pig sequences, respectively. The porcine α_{2B}-adrenoceptor showed 7 transmembrane domains and differed from mouse and rat, both of which have a 5-amino acid extensions at the N-terminal. The expression of α_{2B}-adrenoceptor mRNA was demonstrated in renal, femoral, mesenteric, carotid, pulmonary and coronary arteries, external jugular and saphenous veins as well as in the brain cortex, cerebellum and trigeminal ganglion. When the receptor was transiently transfected in HEK293 cells, the agonist noradrenaline showed a pEC_{50} ≥7.67±0.4 and antagonist rauwolscine depicted a pK_b 9.76±0.6. Using dual luciferase assay in transfected HEK293 as well as COS-7 cells, we found significant ($P<0.05$) elevation of serum response element (SRE) and activator protein-1 (AP-1) upon stimulation with noradrenaline, while nuclear factor of κB cells (NFκB) and nuclear factor of activated T-cells (NFAT) were not activated.

12.5.11 The Phe-124-Cys and A-161T variants of the human 5-HT_{1B} receptor are not major determinants of the clinical response to sumatriptan (Chapter 10)

The 5-$HT_{1B/1D}$ agonist sumatriptan is highly effective in the treatment of migraine. However, some patients do not respond to sumatriptan or experience recurrence of the headache after initial relief. In addition, some patients report chest symptoms after the use of sumatriptan. We therefore, assessed whether two naturally occurring genetic variants of 5-HT_{1B} receptor (F124C changing a phenylalanine for a cysteine and polymorphism A/T at nucleotide position -161 in the 5′ regulatory region) play a role in the therapeutic response to sumatriptan. The 5-HT_{1B} receptor most likely mediates the therapeutic action and coronary side effects of sumatriptan, and both F124C and A-161T have relevant functional consequences on either the affinity of sumatriptan to bind to the 5-HT_{1B} receptor or on receptor expression level itself, respectively. Genomic DNA of a relatively small but very well-characterized set of migraine patients with consistently good response to sumatriptan (*n*=14), with no response (*n*=12), with recurrence of the headache (*n*=12), with chest symptoms (*n*=13), and patients without chest symptoms (*n*=27) was available for the genetic analyses and screened for the Phe-124-Cys variant and the A-161T polymorphism in the human 5-HT_{1B} receptor gene. F124C was not detected in any of the patients studied. In addition, we did not observe drastic changes in allele frequencies of the A-161T polymorphism that might hint to a causal relation with the therapeutic effect of sumatriptan. Thus, this study does not provide any evidence that the variants F124C and A-161T of the 5-HT_{1B} receptor gene are major determinants in the clinical response to sumatriptan.

12.6 SAMENVATTING

De rol van 5-hydroxytryptamine(5-HT), adrenerge en CGRP receptor subtypen in de pathofysiologie van migraine (Hoofdstuk 1)

Een aanzienlijk deel van de wereldbevolking lijdt aan migraine, wat een van de meest voorkomende oorzaken voor het ziekteverzuim op het werk is. Migraine is een terugkerende, belemmerende neurovasculaire ziekte, die gekarakteriseerd wordt door aanvallen met ernstige pijn die gepaard gaat met fotofobie, fonofobie, misselijkheid en braken. Hoewel de pathofysiologie van migraine nog steeds onduidelijk is, wordt aangenomen dat deze neurovasculair van aard is. Medicijnen die gebruikt worden voor de bestrijding van migraine werken op vasculaire receptoren. In het verleden werden hiervoor α-adrenoceptor agonisten (ergotamine, dihydroergotamine, isomethepteen) gebruikt. De laatste jaren zijn gekenmerkt door de opkomst van sumatriptan en de tweede generatie triptanen, allen 5-$HT_{1B/1D}$ receptor agonisten, welke een goed beschreven effectiviteit hebben in de behandeling van migraine. Op dit moment behelst de prophylactische behandeling van migraine 5-HT_2 receptor antagonisten en β-adrenoceptor blokkers. Ondanks deze vooruitgang wordt deze ziekte, mede gezien de complexiteit van de etiology van migraine, nog steeds onvoldoende gediagnostiseerd en worden de beschikbare therapieën niet voldoende gebruikt. In dit hoofdstuk wordt het gebruik van diverse huidige en toekomstige farmacologische targets voor migraine besproken, met bijzondere nadruk op elk subtype van de 5-HT, adrenoceptor en calcitonin gene-related peptide (CGRP) receptoren.

Potentiële rol van vrouwelijke geslachtshormonen in de pathofysiologie van migraine (Hoofdstuk 2)

Vrouwelijke geslachtshormonen kunnen bijdragen aan de hoge prevalentie van migraine bij vrouwen, evenals veranderingen in de frequentie of hevigheid van de migraine aanvallen die gepaard gaan met verschillende vruchtbaarheidsfasen in een vrouwenleven. Terwijl oestrogeen en progesteron niet noodzakelijk betrokken lijken te zijn bij de pathogenese van migraine, zouden ze verschillende mediatoren en/of receptor systemem kunnen beïnvloeden via zowel genomische als non-genomische mechanismen. Deze mechanismen kunnen zowel in het centraal zenuwstelsel als op perifeer (neuro)vasculair niveau plaatsvinden. Oestrogeen verhoogt bijvoorbeeld de neuronale excitabiliteit door een verhoogde Ca^{2+} en verlaagde Mg^{2+} concentratie; dit zou samen met andere tegelijkertijd optredende mechanismen een migraine aanval op gang kunnen brengen. Bovendien is van oestrogeen bekend dat het de synthese en afgifte van stikstofoxide en neuropeptiden zoals calcitonin gene-related peptide kan verhogen; dit versterkt vervolgens de vaatverwijding en activeert trigeminale sensorische afferenten, wat wordt gevolg door stimulatie van de pijncentra. Daarnaast zouden vrouwelijke geslachtshormonen de functie van receptoren die voor vaatverwijding zorgen kunnen verhogen, terwijl effecten van receptoren die vaatvernauwing merdieren verzwakt zijn. De serotonerge, adrenerge en GABAerge systemen worden ook gemoduleerd door geslachtshormonen, hoewel op verschillende niveaus en met potentieel tegenstrijdige effecten op het ontstaan van migraine. Alles bij elkaar genomen lijken vrouwelijke geslachtshormonen betrokken te zijn bij een aaneenschakeling van componenten in de pathogenese van migraine. Toekomstige studies zullen de omvang en de klinische relevantie van elk van deze mechanismen verder kenschetsen, en zo de kennis over de vrouwelijkheid van migraine vergroten.

Doelen

De doelen die zijn gesteld voor dit onderzoek zijn: (i) te onderzoeken of de vaatvernauwing/vaatverwijding van de carotide circulatie veroorzaakt door 5-HT, adrenerge en CGRP receptoren in varkens *in vivo* betrekking heeft op de meningeaal arterie; (ii) het karakteriseren van de 5-HT, adrenerge en CGRP receptoren in geïsoleerde meningeal arteriën van varkens; (iii) het onderzoeken van het effect van vrouwelijke geslachtshormonen op door 5-HT, α-adrenerge, muscarine en CGRP receptoren gemedieerde responsen in geïsoleerde bloedvaten; (iv) het kloneren en karakteriseren van de α_{2B}-adrenoceptor in varkens; (v)

vast te stellen of er een genetische basis is voor het vóórkomen van pijn op de borst na het gebruik van sumatriptan.

Effecten van huidige en toekomstige antimigraine medicatie op de geïsoleerde meningeal arterie van varkens (Hoofdstuk 3)

In dit hoofdstuk bestudeerden we de effecten van huidige en toekomstige antimigraine middelen op de geïsoleerde meningeal arterie van het varken. Wij onderzochten contractie in respons op agonisten op 5-HT en α-adrenerge receptoren, evenals dilatatie veroorzaakt door α-CGRP, omdat deze effecten goed beschreven zijn in de carotide circulatie in varkens *in vivo*, hoewel de *locus* van deze vasomotie tot op heden niet bekend is. Om de effecten van huidige en toekomstige antimigraine niddelen op de geïsoleerde meningeaal arterie in varkens te bestuderen zijn stukjes meningeaal arterie bevestigd in een Mulvany myograaf en zijn concentratie respons curves gemaakt om de respons op de verschillende stoffen te meten. Verassend genoeg waren ergotamine, dihydroergotamine, isomethepteen, clonidine en sumatriptan geen van allen in staat om de meningeaal arterie te doen contraheren. In contrast hiermee veroorzaakten 5-HT en noradrenaline wel concentratie-afhankelijke contracties. De contracties in respons op 5-HT werden geantagoneerd door de 5-HT_{2A} receptor antagonist ketanserine, terwijl de responsen op noradrenaline geblokkeerd werden door de α_1-adrenoceptor antagonist prazosine, de α_2-adrenoceptor antagonisten rauwolscine en yohimbine, evenals de $\alpha_{2C/2B}$-adrenoceptor antagonist OPC-28326. Bovendien veroorzaakte α-CGRP concentratie-afhankelijke relaxaties, wat werd geantagoneerd door de CGRP_1 receptor antagonist olcegepant. Interessant genoeg, maar in overeenkomst met hun gebrek aan contraherende effecten, bleven door forskoline geïnduceerde verhogingen van cAMP onveranderd in aanwezigheid van sumatriptan of ergotamine in de meningeaal arterie van het varken. Tot slot konden wij, gebruik makend van RT-PCR, de aanwezigheid van mRNA coderend voor veschillende 5-HT ($5\text{-HT}_{1B,}$ 5-HT_{1D}, $5\text{-HT}_{1F,}$ 5-HT_{2A} and 5-HT_7) and adrenerge (α_{1A}, α_{1B}, $\alpha_{1D,}$ α_{2A}, α_{2B}, α_{2C}, β_1 and β_2) receptoren aantonen, evenals mRNA dat een component van de CGRP receptor, de 'calcitonin receptor like receptor' (CALCRL) codeert. Deze resultaten tonen aan dat de meningeaal arterie niet betrokken is bij de vaatvernauwing van het carotide vaatbed die door antimigraine middelen wordt veroorzaakt in varkens onder anaesthesie. Hoewel mRNA van verscheidene 5-HT, adrenerge en CGRP receptoren op deze bloedvaten aanwezig is, zijn vele van deze receptoren, met name 5-HT_{1B} receptoren en α_2- en β-adrenoceptoren, niet functioneel of is hun dichtheid te laag om een zichtbare reactie teweeg te brengen.

Door A61603 geïnduceerde contracties van de meningeaal arterie van het varken worden gemedieerd door α_1- en α_2-adrenoceptoren (Hoofdstuk 4)

Het is recent aangetoond dat A61603 (N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]methane sulphonamide), een potente α_{1A}-adrenoceptor agonist, de conductantie van de arteria carotis in verdoofde varkens vermindert via een nieuw, non-adrenerg mechanisme. In deze studie hebben wij de door A61603 geïnduceerde contracties van de meningeaal arterie van het varken farmacologisch gekarakteriseerd. Terwijl de maximum contractiele responsen van het arterie hetzelfde waren, was A61603 potenter dan noradrenaline of phenylephrine. Prazosine, evenals rauwolscine en yohimbine, antagoneerde de contracties in respons op A61603. De 5-HT receptor antagonisten, GR127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl) phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) [1,1-biphenyl]-4-carboxamide) en ritanserine hadden geen invloed op de respons op A61603, maar methiothepine, dat bovendien een hoge affiniteit voor α-adrenoceptoren heeft, bleek een effectieve antagonist te zijn. De responsen op A61603 werden onderdrukt door de cAMP stimulator forskoline, maar niet door de proteine kinase C remmer chelerythrine. Onze resultaten suggereren dat de contractie van de geisoleerde meningeale arterie van varkens door A61603 tot stand komt door middel van een gemengde populatie van α_1- (waarschijnlijk α_{1A}) en α_2-adrenoceptoren en dat de adenylate cyclase, maar niet de diacylglycerol pathway hierbij is.

Potentiële vasculaire α_1-adrenoceptor remmende eigenschappen van een scala aan 5-HT receptor liganden in de rat (Hoofdstuk 5)

Deze studie had als doel het analyseren van het potentiële vermogen van enkele 5-HT receptor ligands die op grote schaal gebruikt worden in cardiovasculaire experimentele modellen om interactie aan te gaan met vasculaire α_1-adrenoceptoren in centraal gedenerveerde ratten. Deze liganden waren: methiothepine, methysergide en metergoline (5-HT_1/5-HT_2); WAY-100635, buspirone, ipsapirone en 8-hydroxy-2-(di-n-propylamino) tetraline (8-OH-DPAT) (5-HT_{1A}); GR127935 (5-$HT_{1B/1D}$); ketanserine, ritanserine, spiperon en pizotifen (5-HT_2); granisetron en metoclopramide (5-HT_3); tropisetron (5-HT_3/5-HT_4); ergotamine (5-$HT_{1B/1D}$, 5-$ht_{5A/5B}$); clozapine (5-HT_6/5-HT_7); evenals LY215840 en mesulergine (5-HT_2/5-HT_7). Toename van diastolische bloeddruk werd verkregen door de selectieve α_1-adrenoceptor agonist phenylephrine, wat werd geanalyseerd voor en na toevoeging van de voorgenoemde antagonisten of fysiologische zoutoplossing. De adrenoceptor antagonist eigenschappen van prazosine (α_1) en yohimbine (α_2) werden ook geanalyseerd ter vergelijking. De phenylephrine-geïnduceerde vasopressor responsen werden dosis-afhankelijk geantagoniseerd met de volgende schijnbare rankorde van potentie:

Prazosine ≥ methiothepine > ketanserine > clozapine ≥ lisuride >> buspiron; deze potentie komt overeen met de affiniteit van deze stoffen voor α_1-adrenoceptor bindingsplaatsen. In tegenstelling hiermee hadden de andere stoffen geen enkel remmend effect of versterkten ze zelfs (i.e. lisuride, methysergide, 8-OH-DPAT, granisetron en GR127935) de reacties op phenylephrine. Deze resultaten tonen aan dat methiothepine, ketanserine, clozapine, lisuride en buspirone α_1-adrenoceptoren kunnen blokkeren in de systemische vasculatuur van de rat.

De rol van CGRP in ischemische preconditionering in geïsoleerde rattenharten (Hoofdstuk 6)

Hier bestudeerden we de rol van CGRP in preconditionering (PC) in geïsoleerde gecatheteriseerde rattenharten, door de hoeveelheid vrijgekomen CGRP te meten tijdens de PC en dit na te bootsen door de infusie van exogeen CGRP, zowel in aan- als afwezigheid van de CGRP antagonist olcegepant. Eerder was beschreven dat korte coronaire arterie occlusion (CAO) het hart kan beschermen tegen schade tijdens volgende langdurige CAO, wat ischemische preconditioning (PC) wordt genoemd. CGRP verhoogde de linker ventriculaire druk (LVP) en de coronaire flow (CF) op een concentratie-afhankelijke manier, hetgeen effectief geantagoneerd werd door olcegepant. Rattenharten werden onderworpen aan CAO en reperfusie, wat werd voorafgegaan door: (1) sham voorbehandeling, (2) infusie van olcegepant (3) PC door CAO en reperfusie, (4) als 3, maar dan met olcegepant, (5) CGRP infusie gevolgd door uitwassen (6) als 5, maar dan met olcegepant. Cardiale bescherming werd bepaald door reactieve hyperaemie (RH), het vrijkomen van creatine kinase (CK), grootte van het infarct in relatie tot het risicogebied (I/R%), en LVP herstel. PC verhoogde de afgifte van CGRP in het coronaire effluent en beschermde de harten significant door de RH te verminderen, waardoor de afgifte van CK te verminderen, de grootte van het infart te beperken en het LVP herstel te verbeteren. Exogeen CGRP induceerde preconditionering-achtige bescherming van het hart. Olcegepant zorgde voor een volledige blokkade van de bescherming van het hart door zowel PC als exogeen CGRP. Aangezien de bescherming van het hart van PC-geïnduceerde CGRP afgifte kan worden nagebootst door exogeen CGRP, en een CGRP antagonist beide kan blokkeren, geven onze resultaten aan dat CGRP een belangrijke rol speelt bij de ischemische preconditionering.

Responsen van de a. carotis van de rat op α-adrenergic receptor agonisten en 5-HT na ovariectomy en hormoonsubstitutie (Hoofdstuk 7)

Deze studie is opgezet om de contractiele responsen op α-adrenerge receptor agonisten en 5-HT in de a. carotis van de rat na ovariectomie, gevolgd door hormoonvervanging met 17β-oestradiol, progesteron of de combinatie van 17β-oestradiol en progesteron, te vergelijken. Na een week acclimatisatie werden sham operaties of bilaterale ovariectomie uitgevoerd bij vrouwelijke Sprague-Dawley ratten. Een week later werd onderhuids een pellet geïmplanteerd in de geovariectomeerde ratten, dat over een

periode van 21 dagen placebo, 17β-oestradiol, progesteron of een combinatie van de twee hormonen afgaf. Er werden bloedmonsters afgenomen om de hoeveelheid noradrelanine en adrenaline te meten in het plasma. Op dag 25-28 werden de dieren geëuthanaseerd om de a. carotis te isoleren en stukjes in een Mulvany myograaf op te hangen. Er zijn cumulatieve concentratie response curves gemaakt van α-adrenerge receptor agonisten en 5-HT in de aan- of afwezigheid van hun respectievelijke antagonisten. De potentie van noradrenaline in geovariectomeerde ratten was significant minder in dieren die behandeld waren met progesteron in vergelijking met de dieren die met placebo behandeld waren. In de met placebo behandelde geovariectomeerde dieren was er een zichtbare reactie veroorzaakt door α_2-adrenoceptoren, in tegenstelling tot de ratten waarop sham operaties of ovariectomie waren uitgevoerd en die behandeld waren met 17β-oestradiol, progesteron of een combinatie van beide. De hoeveelheid noradrenaline en adrenaline in het plasma werd niet significant verandered door ovariectomie of de daaropvolgende hormoonsubstitutie. De potentie van 5-HT was significant verminderd in dieren waarin geslachtshormonen circuleerden in vergelijking met die in geovariectomeerde dieren die met placebo behandeld werden. Onze resultaten geven aan dat circulerend progesteron en/of 17β-oestradiol de contractie van de a. carotis in ratten in respons op noradrenaline en 5-HT and verminderen.

Effecten van vrouwelijke geslachtshormonen in respons op CGRP, acetylcholine en 5-HT in geïsoleerde bloedvaten van de rat (Hoofdstuk 8)

In deze studie onderzochten wij de effecten van de vrouwelijke geslachtshormonen 17β-oestradiol en progesteron (apart en samen) op vasoactieve responsen op CGRP, acetylcholine en 5-HT in de geïsoleerde a. mesenterica, a. caudalis en a. basilaris van de rat. Vrouwelijke Sprague-Dawley ratten werden geovariectomeerd en 7 dagen later werden er onderhuids pellets aangebracht die in een periode van 21 dagen 17β-oestradiol, progesteron, een combinatie van de twee hormonen, of placebo afgaven. Op dag 25-28 werden de dieren geëuthaniseerd, de bloedvaten geïsoleerd en opgehangen in een Mulvany myograaf. Vervolgens werden cumulatieve concentratie respons curves van CGRP, acetylcholine en 5-HT gemaakt. De relaxerende responsen op CGRP werden duidelijk gepotentieerd in de a. mesenterica en a. caudalis van ratten die behandeld waren met 17β-oestradiol in vergelijking met de ratten die met placebo behandeld waren. Door acetylcholine geïnduceerde relaxaties werden gepotentieerd in de a. caudalis van ratten die behandeld waren met een combinatie van 17β-oestradiol en progesteron vergeleken met de ratten die met placebo behandeld waren. De door 5-HT geïnduceerde contracties in de drie arteriën waren niet duidelijk verschillend in effectiviteit of potentie. Onze resultaten geven aan dat 17β-oestradiol de door CGRP geïnduceerde relaxaties in de a. mesenterica en a. caudalis potentieert, terwijl de behandeling met een combinatie van 17β-oestradiol en progesteron de door acetylcholine geïnduceerde relaxaties in de a. caudalis versterkt.

Het kloneren van de α_{2B}-adrenoceptor in varkens: Weefseldistributie en farmacologische karakterisering (Hoofdstuk 9)

Deze studie beschrijft de moleculaire klonering en karakterisatie van de α_{2B}-adrenoceptor in varkens. Ook werd de weefseldistributie bestudeerd. Sequentie analyse van de cDNA kloon (1341 bp) in varkens toonde een open 'reading frame' van 446 aminozuren die de α_{2B}-adrenoceptor codeerden, hetgeen een overeenkomst van 88% en 85% liet zien in vergelijking met de sequenties van respectievelijk mensen en cavia's. De α_{2B}-adrenoceptor in varkens toonde 7 transmembraan domeinen en verschilde met de muis en rat, welke beide een extensie van 5 aminozuren bij het N-terminale deel hebben. De expressie van α_{2B}-adrenoceptor mRNA was aantoonbaar in de a. renalis, a. femoralis, a. mesenterica, a. carotis, a. pulmonaris en de coronair arteriën, de v. jugularis externa en de v. saphena, evenals in hersenschors, het cerebellum en het trigeminale ganglion. In een transient transfectiesysteem gebaseerd op HEK293 cellen, toonde receptorbinding met de endogene ligand noradrenaline een pEC_{50} ≥7.67±0.4, en met de antagonist rauwolscine een pK_b van 9.76±0.6. Gebruik makend van de 'dual luciferase assay' in getrans-

fecteerde HEK293 en COS-7 cellen vonden we een significante (P≤0.05) verhoging van 'serum response element' (SRE) en activator eiwit-1 (AP-1) na stimulatie met noradrenaline, terwijl de nucleaire factor van κB cellen (NFκB) en de nucleaire factor van geactiveerde T-cellen (NFAT) niet geactiveerd werden.

De Phe-124-Cys en A-161T varianten van de humane 5-HT$_{1B}$ receptor zijn geen belangrijke determinanten van de klinische respons op sumatriptan (Hoofdstuk 10)

In dit hoofdstuk hebben we de associatie tussen de natuurlijk voorkomende enkele nucleotide vervangingen in de coderende en niet-coderende regio van de 5-HT$_{1B}$ receptor en de klinische respons op sumatriptan bestudeerd. De 5-HT$_{1B/1D}$ receptor agonist sumatriptan zeer effectief is in the behandeling van migraine. Sommige patiënten reageren echter niet op sumatriptan, of krijgen opnieuw last van hoofdpijn nadat de pijn in eerste instantie verminderde ('recurrence'). Bovendien geven sommige patiënten aan last te hebben van pijn in de borst na gebruik van sumatriptan. Wij onderzochten of twee genetische varianten (F124C dat een phenylalanine in een cysteine verandert en polymorphisme A/T op nucleotide positie -161 in de 5' regulerende regio) van de 5-HT$_{1B}$ receptor een belangrijke rol spelen in de therapeutische respons op sumatriptan. De 5-HT$_{1B}$ receptor medieert hoogstwaarschijnlijk het therapeutische effect en de coronaire bijwerkingen van sumatriptan, en zowel F124C and A-161T hebben relevante functionele consequenties op de bindingsaffiniteit van sumatriptan aan 5-HT$_{1B}$ receptor of op de receptorexpressie zelf, respectivelijk. Genomisch DNA van een relatief kleine, maar erg goed gekarakteriseerde groep migraine patiënten met consistent goede respons op sumatriptan (*n*=14), zonder respons (*n*=12), met terugkerende hoofdpijn (*n*=12), met pijn op de borst (*n*=13), en patiënten zonder pijn op de borst (*n*=27) was beschikbaar voor de genetische analyse en werd gescreend voor de Phe-124-Cys variant en het A-161T polymorphisme in het humane 5-HT$_{1B}$ receptor gen. F124C werd in geen van de geïncludeerde patiënten gedetecteerd. Verder vonden wij geen drastische veranderingen in allel frequentisch van het A-161T polymorphisme die zouden kunnen wijzen op een causale relatie met het therapeutische effect van sumatriptan. Concluderend hebben wij geen aanwijzingen gevonden dat de varianten F124C en A-161T van het 5-HT$_{1B}$ receptor gen belangrijke determinanten van de klinische respons op sumatriptan zijn.

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God has given 32 soldiers in front of the tongue, so as it should be opened only when required.

RESEARCH PAPERS

Full Papers

1. **S. Mehrotra**, S. Gupta, C.M. Villalón, P.R. Saxena, A.J.J.C. Bogers, A. MaassenVanDenBrink. Effects of current and prospective antimigraine drugs on the porcine isolated meningeal artery. (**Naunyn Schmiedberg's Archives of Pharmacology, In Press**).
2. **S. Mehrotra,** J. Zhang, A.W.R.H. Teunissen, P.R. Saxena, A. MaassenVanDenBrink, T.Walther, H.S. Sharma. Molecular cloning, tissue distribution and signaling characterization of the porcine α_{2B}-adrenoceptor. (**Submitted**).
3. ***S. Mehrotra**, *S. Gupta, C.M. Villalón, P.R. Saxena, A. MaassenVanDenBrink. Rat carotid artery responses to α-adrenergic receptor agonists and 5-HT after ovariectomy and hormone replacement (**Headache, In Press, *both authors contributed equally**).
4. **S. Mehrotra**, K.R.J. Vanmolkot, R.R. Frants, A.M.J.M. van den Maagdenberg, M.D. Ferrari, A. MaassenVanDenBrink. The Phe-124-Cys and A-161T variants of the human 5-HT_{1B} receptor are not major determinants of the clinical response to sumatriptan. (**Submitted**).
5. **S. Mehrotra**, S. Gupta, D. Centurión, C.M. Villalón, P.R. Saxena, A. MaassenVanDen Brink. A61603-induced contractions of the porcine meningeal artery are mediated by α_1- and α_2-adrenoceptors (**Submitted**).
6. **S. Mehrotra**, S. Gupta, C.M. Villalón, P.R. Saxena, D. Centurión, A. MaassenVanDenBrink. Current and prospective pharmacological targets in relation to antimigraine action. (**Invited review, Naunyn Schmiedberg's Archives of Pharmacology, In preparation)**
7. *S. Gupta, ***S. Mehrotra**, C.M. Villalón, P.R. Saxena, A. MaassenVanDenBrink. Effect of female sex steroids on CGRP, 5-HT and acetylcholine-induced responses in rat blood vessels. (**Headache, In Press, *both authors contributed equally**).
8. S. Gupta, **S. Mehrotra**, M. Perusquía, P.R. Saxena, C.M. Villalón, A. MaassenVanDenBrink. Potential role of female sex hormone in pathophysiology of migraine: review article. (**Pharmacology and Therapeutics, Invited review, In Press**).
9. S. Gupta, **S. Mehrotra**, C.M. Villalón, I.M. Garrelds, R. de Vries, J.P. VanKats, H.S. Sharma, P.R. Saxena, A. MaassenVanDenBrink. Characterisation of CGRP receptors in human and porcine isolated coronary arteries: evidence for CGRP receptor heterogeneity. (**European Journal Pharmacology, 530: 107-16, 2006**).
10. S. Gupta, **S.Mehrotra**, C.M. Villalón, P.R. Saxena, A. MaassenVanDenBrink. Characterization of CGRP receptor in human middle meningeal artery (**Life Sciences, 79: 265-71, 2006**).
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icin-induced canine external carotid vasodilatation via 5-HT_{1B} rather than 5-HT_{1D} receptors. (**British Journal of Pharmacology, 149: 82-91, 2006**).

Book chapters

1. **S. Mehrotra**, A. MaassenVanDenBrink, P.R. Saxena, H.S. Sharma, Molecular cloning and tissue distribution of porcine α_{2B}-adrenoceptor, In: **Headache and Migraine**, Editors: Sorbi, M.J., Couturier E.J.M.and Mérelle S.Y.M., A-D Druk B.V., Utrecht 2006, Pg 67-82
2. **S. Mehrotra**, S. Gupta, P.R. Saxena, A. MaassenVanDenBrink, The vascular effects of 5-HT receptors, adrenoceptors and CGRP-receptors subtypes in migraine pathophysiology-A three receptor story, In: **Headache and Migraine**, Editors: Sorbi, M.J., Couturier E.J.M.and Mérelle S.Y.M., A-D Druk B.V., Utrecht 2006 (**In press**).
3. S. Gupta, C.M. Villalón, **S. Mehrotra**, R. de Vries, I.M. Garrelds, P.R. Saxena, A. MaassenVanDenBrink. Potential of female sex-steroids in migraine pathophysiology. In: **Headache and Migraine**, Editors: Sorbi, M.J., Couturier E.J.M.and Mérelle S.Y.M., A-D Druk B.V., Utrecht 2006 (**In press**).

Abstracts

1. **S. Mehrotra**, S. Gupta, C.M. Villalón, P.R. Saxena, A. MaassenVanDenBrink. A61603-induced vasoconstrictions of the porcine isolated meningeal artery are mediated: possible involvement of novel receptor. Naunyn Schimedberg's Archives of Pharmacology, 2006; 373:95.
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ABBREVIATIONS

5-HT	5-hydoxytryptamine
AR	adrenoceptor
ANOVA	analysis of variance
ATP1A2	Na^+ / K^+-ATPase α_2-subunit gene
AU	arbitrary units
AVA	arteriovenous anastomoses
A-V SO_2	oxygen saturation difference the arterial and external jugular venous blood samples
BIBN4086BS	olcegepant
BK_{Ca}	Ca^{2+}-dependent K^+ channels for large conductance
bp	base pair
CACNA1A	$Ca_v2.1$ (P/Q-type) Ca^{2+} channel α_1-subunit gene
c-FOS	FBJ osteosarcoma oncogene
CGRP	calcitonin gene-related peptide
CLR	calcitonin receptor like receptor
CNS	central nervous system
CREB	cAMP response-element binding protein
[Cys(Acm)^{2,7}]-h-αCGRP	[acetimidomethyl-Cys^{2,7}]-h-αCGRP
[Cys(Et)^{2,7}]-h-αCGRP	[ethylamide-Cys^{2,7}]-h-αCGRP
GAPDH	glyceraldehyde-3-phosphate-dehydrogenase
cGMP	cyclic guanylylmonophosphate
CV	cerebral blood vessel
CRC	concentration response curve
DA	dural artery
EC_{50}	concentration required to elicit half the maximal response
E_{max}	maximum plateau response reached with an agonist
ESR1	estrogen receptor 1
ET-1	endothelin-1
FHM	familial hemiplegic migraine
GABA	γ-aminobutyric acid B
h	hours (time)
HRT	hormone replacement therapy
HELLP	haemolysis, elevated liver enzymes, low platelet counts
IBMX	isobutylmethylxanthine
IK_{Ca}	intermediate-conductance Ca^{2+}-activated K^+ channel
i.c.	intracarotid
i.p.	intraperitoneal
i.v.	intravenous
L-NAME	N^{ω}-nitro-L-arginine methyl ester HCl
µA	micro ampere
µM	micro molar
MBP	mean arterial blood pressure
min	minute
mM	milli molar
MRI	magnetic resonance imaging
NF-κB	nuclear factor-κB

NK_1	neurokinnin-1
nM	nano molar
NMDA	*N*-methyl-D-aspartic acid
NO	nitrix oxide
NOS	nitrix oxide synthase
NSAIDS	non-steroidal anti-inflammatory drugs
pA_2	the negative logarithm ($-^{10}$log) of the molar concentration of antagonist required to shift the concentration response curve of an agonist by two fold.
PCR	polymerase chain reaction
pEC_{50}	negative logarithm ($-^{10}$log) of EC_{50}
pK_b	antagonists potency, calculated by dose ratio (DR) of EC_{50} of agonists in presence of the antagonist by the EC_{50} of agonist in control situation and plotting $-^{10}$log (DR-1) in Schild plot while constraining the slope to unity.
pK_i	binding affinity constants
PR	progesterone receptor
PROGINS:	306-bp *Alu* insertion within the progesterone receptor gene
RAMP1	Receptor activity modifying protein
RCP	Receptor component protein
RDC	the orphan receptor (originally cloned from canine thyroid cDNA)
RT-PCR	reverse transcriptase polymerase chain reaction
s	second (time)
s.e.m.	standard error of mean
SCN1A	neuronal voltage-gated sodium channel gene
SK_{Ca}	small-conductance Ca^{2+}-activated K^+ channel
SNAP	S-nitroso N-penicillamine
TxA_2	thromboxane A_2

Translation of Sushruta Samshita (400 BC)

As the sun rises the intensity of pain increases and occupies eyes and eye brow regions. As sun is at high peak on midday so also pain attains maximum intensity (11)

After the sun passes noon, pain intensity also reduces, which becomes normalize after sunset. Sometimes cold and sometimes hot treatment provides relief. This may also be called as 'Bhaskaravarta' with symptoms of severe pain which worsen during maximum sunlight (12)