EFFECT OF ANTI-CGRP ANTIBODY GALCANEZUMAB ON NEUROPEPTIDE-MEDIATED TRIGEMINOVASCULAR FUNCTION IN RATS

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I. TABLE OF CONTENTS

II. PUBLICATIONS

1. Publication included in the PhD Thesis:

I. Friedrich, Nadine; Németh, Krisztina; Tanner, Martin; Rosta, Judit; Dobos, Ildikó; Oszlács, Orsolya; Jancsó, Gábor; Messlinger, Karl; Dux, Mária: Anti-CGRP antibody galcanezumab modifies the function of the trigeminovascular nocisensor complex in the rat.
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2. Publications not directly related to the PhD Thesis:

II. Benedicter, Nicola; Messlinger, Karl; Vogler, Birgit; Mackenzie, Kimberly D.; Stratton, Jennifer; **Friedrich, Nadine**; Dux, Mária: Semi-Automated Recording of Facial Sensitivity in Rat Demonstrates Antinociceptive Effects of the Anti-CGRP Antibody Fremanezumab.
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III. Rosta, Judit; Tóth, Máté; **Friedrich, Nadine**; Sántha, Péter; Jancsó, Gábor; Dux, Mária: Insulin sensitizes neural and vascular TRPV1 receptors in the trigeminovascular system.
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III. LIST OF ABBREVIATIONS

5-HT	serotonin
5-HT_{1B}	serotonin 1B receptor
5-HT_{1D}	serotonin 1D receptor
5-HT_{1F}	serotonin 1F receptor
AC	adenylate cyclase
ATP	adenosine triphosphate
BSA	bovine serum albumine
BK	bradykinin
C2	spinal cervical 2
C3	spinal cervical 3
cAMP	cyclic adenosine monophosphate
CGRP	calcitonin gene-related peptide
CLR	calcitonin receptor-like receptor
CNS	central nervous system
CREB	cAMP response element binding
CSD	cortical spreading depression
DAPI	4′,6′-diamidino-2-phenylindole hydrochloride
EIA	enzyme-linked immunoassay
Galcan	galcanezumab
H	histamine
HRP	Horseradish peroxidase
IB4	Griffonia simplicifolia isolectin B4
IgG	immunoglobulin G
MMA	middle meningeal artery
NK1	neurokinin 1
NK2	neurokinin 2

NK3	neurokinin 3
NKA	neurokinin A
NGF	nerve growth factor
PACAP	pituitary adenilate-cyclase activating polypeptide
PACAP27	pituitary adenylate cyclase-activating polypeptide 27
PACAP38	pituitary adenylate cyclase-activating polypeptide 38
PAR-2	proteinase-activated receptor-2
PBS	phosphate buffered saline
PGE2	prostaglandin E2
PKA	protein kinase A
RAMP	receptor activity modifying protein
RAMP1	receptor activity modifying protein 1
RAMP2	receptor activity modifying protein 2
RAMP3	receptor activity modifying protein 3
RCP	receptor component protein
SIF	synthetic interstitial fluid
SMA	smooth muscle actin
SP	substance P
SOM	somatostatin
TMB	3,3',5,5'-tetramethylbenzidine
TRPA1	transient receptor potential ankyrin 1
TRP	transient receptor potential
TRPV1	transient receptor potential vanilloid 1
VIP	vasoactive intestinal peptide
VV	venous vessel
vWF	von Willebrand factor

IV. INTRODUCTION

Migraine is a common primary headache disorder. Records of migraine-like headaches date back to ancient Mesopotamia. Hippocrates was the first to provide a scientific description of migraine. Many famous historical figures, such as Julius Caesar and Charles Darwin, suffered from migraine (Jones, 1999). Besides being a health problem, it is also a major social burden. In 2008, migraine affected more than 300 million people worldwide, in 2019, the number of people suffering from migraine was over one billion (Amiri et al., 2021; World Health Organization, 2008). Migraine is in the top five causes of years lived with disability. Migraine is more prevalent among women than men, the ratio is about three-to-one. In young women migraine is the first cause of years lived with disability and 13% of migraineurs have more then one attack per week (Buse et al., 2013; Russell et al., 2014; Steiner et al., 2020).

According to the third edition of The International Classification of Headache Disorders, there are two major types of migraine: migraine without aura and migraine with aura. Aura symptoms are variable. It can be a visual, a sensory, speech or motor disturbance. The aura symptoms are reversible, they last 5-60 min, usually followed by headache (Headache Classification Committee of the International Headache Society (IHS), 2018). As a visual aura symptom, scintillating scotoma primarily occurs; sensory symptoms often include paresthesia and numbness in the face or upper limbs, while expressive language dysfunction is observed as a linguistic symptom (Ferrari et al., 2022). The cause of aura can be cortical spreading depression (CSD), which is a fast, nearly complete depolarization in the cortical neurons followed by inhibition (Shibata & Tang, 2021). Migraine has characteristic symptoms: it is unilateral, pulsating, moderate or severe intensity headache, which is getting worse for routine physical activity that could be caused by mechanical hypersensitivity. It is often accompanied by nausea, photophobia or phonophobia. Migraine attacks last from 4 to 72 hours, and if there is 15 or more headache days per month and at least 8 headache attacks meet the criteria for migraine, the condition is called chronic migraine. Medication overuse headache seems to be a growing problem in the population suffering frequently from migraine attacks. It usually occurs when painkillers are taken frequently to relieve headaches (Headache Classification Committee of the International Headache Society (IHS), 2018).

Migraine is a complex neurovascular disorder. Its pathogenesis involves multiple components of both the peripheral and the central nervous system. Although the exact mechanism underlying the generation of a migraine attack is not completely understood, it is

widely accepted, that the headache pain is generated by the activation of the trigeminal nociceptive pathway (Bolay et al., 2015; Dux et al., 2020).

1. Meninges

Wilder Penfield was the first to highlight the connection between headaches and the meninges. During numerous intraoperative interventions, he examined patients' sensation of pain to noxious stimuli (mechanical, thermal or chemical), and concluded that by stimulating the dura mater encephali a headache-like sensation could be induced, however direct stimulation of the brain did not elicit pain in the patients. Independently of the type of stimulation the only sensation that was experienced by patients, was pain (Penfield & McNaughton, 1940). The meninges have several roles as protector of the brain and the spinal cord, supporter for arteries, veins and sinuses, and creator of subarachnoid space.

The meninges covering the brain have three layers: the outer dura mater encephali (pachymeninx), the arachnoid mater and pia mater, which together are called leptomeninges (Haines, 2018). The dura mater lines the inner surface of the skull and the spinal canal, and these two parts continue into each other through the foramen magnum. The dura mater encephali splits into two layers: periosteal and meningeal, but there is no significant border line between them. In the meningeal layer, there are several blood vessels and nerves, which are surrounded by elongated fibroblasts and collagen fibrils. These cells ensure the strength of the membrane. The dura mater is also responsible for the formation of the falx cerebri, falx cerebelli, the diaphragma sellae and the tentorium cerebelli. It follows the cranial nerves after they exit the skull, and forms a sheath around the trigeminal ganglion in the cavum trigeminale. In several places in the skull, these two layers separate and they enclose the venous sinuses (Szentágothai & Réthelyi, 2002).

2. Blood and nerve supply of the dura mater encephali

In the three cranial fossae, the arterial supply to the dura mater encephali is different. In the anterior cranial fossa, the main arteries are the anterior meningeal artery (a branch from the anterior ethmoidal artery) and the meningeal branches of the ophthalmic artery. The dura mater of the middle cranial fossa is supplied by the middle meningeal artery (originating from the maxillary branch of the external carotid artery). The posterior meningeal artery (a branch of the ascending pharyngeal artery) and meningeal branches from the occipital artery transport blood to the posterior cranial fossa (Haines, 2018).

Sensory and autonomic nerve fibers (sympathetic and parasympathetic) innervate the dura mater. It receives sensory branches from the ipsilateral trigeminal ganglion and the dorsal roots of C2 and C3 segments. The sympathetic innervation of the tissue originates from the superior cervical ganglia, and the parasympathetic fibers derive from the sphenopalatine and the otic ganglia (Bolay et al., 2015; Haines, 2018).

All three branches of the trigeminal nerve such as the ophthalmic, the maxillary and the mandibular nerve participate in the sensory innervation of the dura mater. The axons of the trigeminal nociceptors are either unmyelinated C- or thinly myelinated Aδ-fibers. The majority of these fibers run parallel to meningeal arteries and terminate as free nerve endings in the dura mater. The axons originate from the trigeminal ganglion, that lies within the cavum trigeminale in the middle cranial fossa, lined by the dura mater. The ganglion is the place where the peripheral branches of pseudounipolar neurons converge, and from where the central branches extend towards the central nervous system. Neither the dura mater encephali nor the trigeminal ganglia are protected by the blood-brain barrier. The central processes of trigeminal ganglion neurons forming the trigeminal nerve, enter the brainstem and terminate in the trigeminal brainstem nuclear complex, which consists of the principal sensory nucleus and the spinal trigeminal nucleus. The thick myelinated mechanosensitive afferents terminate in the principal sensory nucleus, whereas both myelinated and unmyelinated fibers descend in the spinal trigeminal tract projecting to the spinal trigeminal nucleus which is subdivided into three subnuclei: a subnucleus oralis, a subnucleus interpolaris, and a subnucleus caudalis. Based on clinical observations and animal studies it was recognized that the subnucleus caudalis is primarily responsible for processing nociceptive and thermoreceptive information from the face and head (Bolay et al., 2015; Szentágothai & Réthelyi, 2002).

3. Trigeminal nociceptors

Peripheral and central sensitiations of the trigeminal nociceptive pathway are considered as significant factors increasing migraine susceptibility of patients. According to clinical observations migraine sufferers have a lower pain threshold, which may develop due to sensitization of the trigeminal nociceptive primary sensory neurons (peripheral sensitization). Facial cutaneous allodynia that may accompany the headache attack may be the consequence of the sensitization of second-order neurons (central sensitization) (Bolay et al., 2015).

A significant population of trigeminal nociceptors are chemosensitive neurons expressing transduction channels of the transient receptor potential (TRP) family, as the

transient receptor potential vanilloid 1 (TRPV1) and the transient receptor potential ankyrin 1 (TRPA1) receptors. They may contain different neuropeptides. The alterations in the function of the nociceptive TRP ion channels may contribute to changes in neuropeptide release and through that to peripheral or central sensitization of the trigeminal nociceptive pathway (Dux et al., 2020). In human, the TRPV1 receptor is expressed in 16% of trigeminal neurons, mainly in small- and medium-sized neurons (Bolay et al., 2015). TRPV1 receptor is a non-selective cation channel. Noxious heat, acidic pH, various lipid metabolites, or even exogenous substances such as capsaicin or resiniferatoxin can activate the channel. Activation of the TRPV1 channel leads to an increase in intracellular sodium and calcium concentrations that augments nociceptive responses and release of neuropeptides from both the central and the peripheral terminals of the neurons (Bolay et al., 2015).

TRPA1 receptors are mainly colocalized with TRPV1 in the trigeminal ganglion neurons. The non-selective cation channel TRPA1 receptor can be activated by noxious temperature, but in this case noxious cold activates the receptor. Various endogenous and exogenous activators like acrolein, umbellulone of the „headache tree", and hydrogen peroxide are also agonists of the receptor. It is suggested that activation of TRPA1 channels of trigeminal chemosensitive neurons may contribute to inhaled environmental irritant-induced neuronal activation and headache (Bolay et al., 2015; Shibata & Tang, 2021). Similar to the TRPV1 receptor, the result of the TRPA1 receptor activation is inflow of sodium and calcium ions into the nociceptor that depolarizes the neuron and releases neuropeptides from the peripheral and central terminals of the nociceptor (Dux et al., 2020).

4. Neuropeptides of trigeminal neurons

Neuropeptides play a key role in the pathophysiology of migraine as they can be released parallel to the classical transmitter glutamate and they modulate the function of target cells in the central nervous system and at the periphery. These neuropeptides are synthesized in the cell body of neurons, following this, they undergo post-translational processing and are released from dense core vesicles by calcium-dependent exocytosis. Signal transduction occurs through cell surface receptors, the majority of the receptors are G-protein coupled receptors, thereby allowing for a slower and modulatory response to neuropeptides, which incidentally has comparatively high ligand affinity (Russo, 2017).

A. Calcitonin gene-related peptide

In the pathophysiology of migraine, calcitonin gene-related peptide (CGRP) is one of the most important neuropeptides. This 37-amino acid neuropeptide is encoded by the 11th chromosome. In human, two isoforms are known: α-CGRP and β-CGRP. The α subtype is mainly present in the central and peripheral nervous system, while the β-CGRP is found mainly in the enteric nerve terminals (Edvinsson et al., 2012).

The two isotypes of CGRP are transcribed from two different genes. α-CGRP is encoded by the CALC I gene, and the product of this gene is either calcitonin or CGRP, while β-CGRP is transcribed from CALC II gene. Between the two subtypes, the difference is three amino acids in human, but their biological activities are similar. The elimination of the released peptide is not fully understood, it may be metabolized in peripheral tissues via endopeptidases, but the possibility of reuptake into the neuron has also been suggested (Russell et al., 2014).

CGRP is found in various locations within the central and peripheral nervous system. In the trigeminal ganglion, about 50% of the neurons are CGRP-positive (Edvinsson et al., 2012; Tajti et al., 2015). The peripheral axons of these CGRP-positive nerve fibers are mainly located along the arteries from the circle of Willis and meningeal blood vessels (Edvinsson et al., 1987). Besides the neuronal presence, CGRP is found in non-neuronal structures, including endothelials cells, macrophages and adipocytes (Russell et al., 2014).

The receptor of CGRP is composed of three subunits. The calcitonin receptor-like receptor (CLR) has 7 transmembrane domains and it shows 56% homology with human calcitonin receptor. CLR is a G-protein-coupled receptor and belongs to the class B secretin-like family, which includes pituitary adenilate-cyclase activating polypeptide (PACAP), calcitonin, parathyreoid hormone and vasoactive intestinal polypeptide (VIP) receptors (Russell et al., 2014).

In order for the receptor to be transported from the endoplasmic reticulum to the plasma membrane, the heterodimerization of CLR and receptor activity modifying protein (RAMP) peptide is required. Three RAMP membrane proteins have been discovered: RAMP1, RAMP2 and RAMP3. Depending on which RAMP subtype binds to CLR, different receptor affinities are established. CLR and RAMP1 together form the classical CGRP receptor, CLR and RAMP2 primarily binds adrenomedullin, while CLR and RAMP3 form another adrenomedullin/amylin receptor, which has some affinity for CGRP (Russell et al., 2014).

In order for the CGRP receptor to function, a membrane-associated protein called receptor component protein (RCP) is required. RCP is necessary for the coupling of the receptor

to the G proteins, for proper cyclic adenosine monophosphate (cAMP) generation (Russo & Hay, 2023).

Upon CGRP receptor activation, adenylate cyclase is activated through $G\alpha_s$ signalling pathway, leading to an increased cAMP level. The cAMP contribute to activation of protein kinase A and the opening of ATP-sensitive potassium channels, which results in vasodilatation in the vascular smooth muscle. Additionally, CGRP can influence gene transcription by phosphorylating the cAMP response element binding (CREB) protein (Russell et al., 2014).

In the central nervous system, CGRP is primarily released during nociceptor activation and may contribute to the central sensitization by increasing glutamate transmission in the medulla and the dorsal horn of the spinal cord. The role of CGRP in the higher centers involved in nociceptive transmission is not fully understood, nonetheless, CGRP and its receptors are found in the thalamus, the anterior cingular cortex, and the insula too (Russo & Hay, 2023).

CGRP may play a role not only in central sensitization, but also in the peripheral sensitization. Presumably, through autocrine and paracrine pathways, CGRP may enhance its own synthesis and release, potentially sensitizing trigeminal ganglion neurons in a positive feed back loop, which can lead to pain exacerbation in migraine (Russo & Hay, 2023).

Clinical and experimental evidences indicate the role of CGRP in migraine pathophysiology. In migraine patients the plasma level of CGRP increased in the external jugular vein during migraine attacks (Goadsby et al., 1990). Furthermore, elevated levels of CGRP have been measured in tears and saliva during migraine attacks and in the plasma of chronic migraine patients between attacks (Russo & Hay, 2023). Another piece of evidence is that intravenous administration of CGRP in migrain patients triggers migraine-like headache (Lassen et al., 2002). Last but not least, one of the most significant proofs for the role of CGRP in migraine pathophysiology is the succesful use of anti-CGRP therapy in clinical practice (Russell et al., 2014).

B. Pituitary adenylate cyclase-activating polypeptide

There is increasing evidence that pituitary adenylate cyclase-activating polypeptide (PACAP) may also play a role in the pathophysiology of migraine. This neuropeptide is found in various locations in the nervous system and there are several areas where it is co-localized with CGRP, such as the sensory nerve fibers innervating meningeal blood vessels and the trigeminal ganglion neurons. In the rat trigeminal ganglion approximately 50% of CGRP expressing neurons also contain PACAP (Eftekhari et al., 2015). PACAP has two isoforms: PACAP-27 and PACAP-38. PACAP-38 isofom is dominating in the nervous system. It participates in the development and differentiation of the nervous system, acts as a neuromodulator, and can function as a neurotransmitter. PACAP has three receptors: PACAP1, which is completely selective for PACAP, VIP-PACAP1 and VIP-PACAP2, which bind equally to VIP, another member of the same neuropeptide family. PACAP receptors activate cAMP-dependent pathways via Gs protein activation.

The physiological effects of PACAP are similar to CGRP. PACAP has a potent vasodilator effect and induces dural mast cell degranulation in rats. Clinical trials have revealed that intravenous administration of PACAP induces headache in both control group and migraine patients, unlike CGRP, which only induces migraine-like headaches in migraine sufferers. It may be involved in the development of peripheral and central sensitization, could influence neurogenic inflammation, making it a potential target for therapeutic options (Edvinsson et al., 2018; Kuburas & Russo, 2023; Warfvinge & Edvinsson, 2020).

Regarding the origin of the peptide-containing meningeal nerve fibers there is a significant difference between PACAP and CGRP. CGRP is expressed exclusively by sensory neurons while PACAP may be released by both sensory and parasympathetic axons (Kuburas & Russo, 2023).

C. Shared functions of calcitonin gene-related peptide and pituitary adenylate cyclase-activating polypeptide in migraine pathophysiology

Both CGRP and PACAP are considered as mediators of migraine pathophysiology. Both peptides induce vasodilatation in the innervated tissue and they can cause migraine-like headaches when infused into people and migraine-like symptoms when injected into rodents. CGRP and PACAP are found in distinct, but overlapping areas of peripheral and central nervous system relevant to migraine (Kuburas & Russo, 2023). In rodents, under experimental conditions the two peptides share functions, including neurogenic inflammation of the

innervated tissue and they also accelerate nociceptive reactions. Despite the functional similarities they bind to different specific receptors and act by independent mechanisms possibly by distinct intracellular signalling pathways. Since multiple CGRP and PACAP receptors may contribute to migraine pathophysiology it is suggested that PACAP targeted therapy may provide a new option for migraine patients resistant to CGRP effect blocking agents. According to current knowledge PACAP may complement and augment the CGRP mediated sensory functions (Haanes & Edvinsson, 2019).

There is growing evidence suggesting that glutamate, the excitatory neurotransmitter of nociceptive primary sensory neurons in the trigeminal system, plays a role in the onset and maintenance of migraine pain by enhancing peptide release from nociceptors. Increased glutamate levels have been linked to both peripheral and central sensitization of the nociceptive pathway. The relationship between CGRP, PACAP and glutamate is complex, an interplay between neuropeptides and glutamate are suggested that may be involved in sensitization of the nociceptive pathway (Russo & Hay, 2023).

D. Substance P

Substance P (SP) is an 11 amino-acid neuropeptide, mainly expressed in the trigeminal sensory nerve fibers and along the dural vessels, similarly to CGRP. It is expressed by a minority of CGRP containing trigeminal neurons (Ma et al., 2001). Its effects are mediated through tachykinin receptors, which are 7-transmembrane G-protein-coupled receptors. There are three types: neurokinin 1 (NK1), neurokinin 2 (NK2), and neurokinin 3 (NK3), with SP primarily exerting its effects via the NK1 receptor. Upon receptor activation, intracellular calcium level and cAMP level increase. The SP-induced increased permeability of blood vessels mainly applies to postcapillary venules, thereby supporting neurogenic inflammation of the peripheral tissue. Additionally, SP plays a role in pain transmission. Its relationship with migraine is not entirely clear, but elevated SP levels have been measured in saliva during spontaneous migraine attacks without aura. Furthermore, increased SP levels have been observed during attack-free periods in both episodic and chronic migraine patients. However, during migraine attacks, plasma SP concentrations were not found to be elevated and clinically, NK receptor antagonists have shown no significant therapeutic effect in migraine patients (Dux et al., 2012; Harrison & Geppetti, 2001; Muñoz & Coveñas, 2014; Tajti et al., 2015; Viudez-Martínez et al., 2024).

E. Somatostatin

Somatostatin (SOM) is a neuropeptide existing in two active forms, consisting of 14 or 28 amino acids. The peptide has an analgesic effect in rodents and humans. It is widely distributed both in neural and non-neural tissues. Neurons, neuroendocrine, inflammatory and immune cells may release the peptide (Patel, 1999). It acts as neurotransmitter, paracrine/autocrine regulator or upon entering the systemic circulation it may exert systemic effects. In sensory nerves it inhibits nociceptive processes and release of other neuropeptides from sensory nerve endings (Szolcsányi et al., 1998). Five human SOM receptors have been cloned, that consist of 7 transmembrane domains with an N-terminal extracellular and an intracellular C-terminal domain. Somatostatin receptors are linked to G-proteins. They inhibit adenylate cyclase activity (Jakobs et al., 1983; Patel et al., 1995), reduce the conductance of voltage-dependent calcium channels (Patel et al., 1995; Schally, 1988) and activate potassium channels (Mihara et al., 1987).

In migraine attacks the pain suppressing role of SOM was suggested. SOM concentration was low interictally in cerebrospinal fluid, and further reduction was measured during headache attacks in migraineurs (Sarchielli et al., 2006; Vécsei et al., 1992).

5. The trigeminal nocisensor complex

Although the generation of migraine has been attributed to pathophysiological changes in the central nervous system, pain is induced by the activation of the trigeminal system. The prominent role of the trigeminovascular system in the pathophysiology of migraine pain is not only the activation of the trigeminal nociceptive pathway potentially leading to headache, but also the generation of neurogenic inflammation in the meningeal tissues (Dux et al., 2012).

Inside the skull, the dura mater encephali is the main pain sensitive structure (Ray & Wolff, 1940). In the sensory system neurogenic inflammation induced by the release of vasoactive neuropeptides is considered as a key component of the nociceptor sensitization. Peptides released by activated nociceptors increase blood flow and vascular permeability in the innervated tissue, and by degranulation of mast cells or activation of other meningeal cells further vasoactive and nociceptor sensitizing mediators can be released (Dux et al., 2012; Russell et al., 2014; Russo & Hay, 2023).

A few years ago the concept of the trigeminal nocisensor complex as a morphological substrate of meningeal nociceptor sensitization was formulated (Fig. 1) (Dux et al., 2012). The concept of the trigeminal nocisensor complex may provide an explanation for many

pathophysiological changes in the trigeminovascular system in migraine. The nocisensor complex consists of the trigeminal nociceptive primary afferent neurons with their peripheral and central terminals innervating the dura mater and providing the sensory information to second order neurons, respectively, the meningeal blood vessels and mast cells. According to current theories the combination of changes in the neural activity and vascular reactions may generate head pain during migraine attacks (Edvinsson et al., 2012).

Stimulation of dural nociceptors by noxious agents results in the transmission of nociceptive signals by the activation of peptidergic and non-peptidergic; Griffonia simplicifolia isolectin B4 (IB4) positive primary sensory neurons. Peptidergic nerves also release vasoactive peptides such as CGRP and SP, which generate a sterile neurogenic inflammation in the meningeal tissues; produce vasodilatation and plasma extravasation, respectively. Tissue mediators may excite or sensitize the TRPV1 and TRPA1 channels expressed by meningeal nociceptors. Mast cells may be activated by CGRP and SP released from the nociceptors and also by mast cell tryptase. A mutual activation of mast cells through the activation of the proteinase-activated receptor-2 by triptase was reported, which provides a possibility for a self triggering mechanism of mast cells resulting in an augmentation of the release of mast cell mediators such as histamine. Histamine released by mast cells may amplify vascular reactions and may also sensitize nociceptive afferents. Although the role of vasodilatation and increased blood flow to the dura mater is probably not the activation of nociceptors, vascular reactions may accelerate the removal of noxious substances and restoring homeostasis within the meningeal tissue (Dux et al., 2012).

Experimental results have shown that electrical stimulation or topical application of the TRPV1 receptor agonist capsaicin to the dura mater induces vasodilation due to CGRP released from trigeminal afferents, a response that could be inhibited by CGRP receptor antagonists (Dux et al., 2003; Kurosawa et al., 1995).

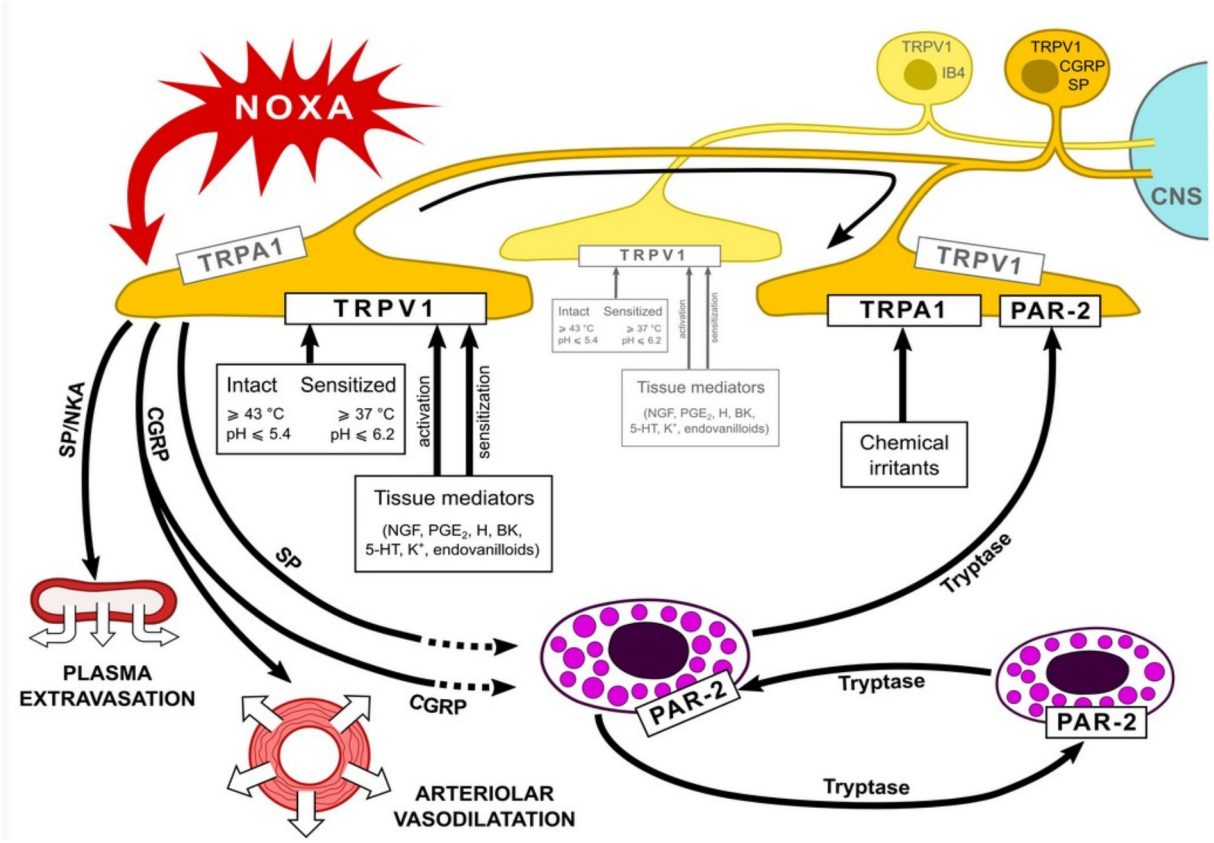

Fig. 1. Function of the trigeminovascular nocisensor complex (Dux et al., 2012)**.**
BK: bradykinin, CGRP: calcitonin gene-related peptide, 5-HT: serotonin, IB4: Griffonia simplicifolia isolectin B4, NKA: neurokinin A, PAR-2: proteinase-activated receptor-2, PGE2: prostaglandin E2, SP: substance P, TRPA1: transient receptor potential ankyrin 1, TRPV1: transient receptor potential vanilloid 1, NGF: nerve growth factor, H: histamine, CNS: central nervous system.

6. Calcitonin gene-related peptide-targeted therapy in migraine

Therapy of migraine headache usually combines preventive treatment with early intervention for acute attacks. Treatment of acute migraine episodes includes a number of different classes of medications, including analgesics such as aspirin, nonsteroidal antiinflammatory agents, barbiturates, antiemetics, ergot alkaloids. Most medications available for migraine prevention were originally developed for hypertension, depression or epilepsy, and were generally underused due to poor tolerability or insufficient efficacy (Deen et al., 2017).

Forty years ago, the discovery of the vasoactive neuropeptide CGRP and its role in migraine pathophysiology opened a new era in migraine treatment and prophylaxis (Amara et

al., 1982). Serotonin receptor agonists, reducing the release of CGRP from stimulated afferents and small-molecule CGRP receptor antagonists are effective in acute attack treatmant while anti-CGRP antibodies and anti-CGRP receptor antibody are used particularly for migraine prevention (Fig. 2.) (Edvinsson et al., 2012).

A. Triptans and ditans reducing the calcitonin gene-related peptide release from trigeminal afferents

Triptans target 5-HT_{1B} and 5-HT_{1D} serotonin receptors, and through receptor activation they reduce CGRP release. Nowadays, several triptans available on the pharmaceutical market, such as sumatriptan, almotriptan or eletriptan. These medications are selective partial agonists of 5-HT_{1B} and 5-HT_{1D} receptors, but also have binding affinity to 5-HT_{1F} receptor. The 5-HT receptors are found in both neuronal and vascular structures of the meningeal tissues. The 5-HT receptors are found in several areas in the brain, like in the trigeminal ganglion, the trigeminal nucleus caudalis and the ventroposteromedial nucleus of the thalamus. Activation of those receptors may reduce CGRP release, and as a central effect, it may influence the activity of the nociceptive pathway and the trigeminal response to nociceptive stimuli (Rubio-Beltrán et al., 2018).

Considering the vascular site of the effect, 5-HT_{1B} receptors are localized on smooth muscle and in the endothelium of intracranial blood vessels. Triptans induce vasoconstriction by the activation of this receptor. Because 5-HT_{1B} receptors are also expressed by other arterial blood vessels like the coronary arteries, triptans are contraindicated due to cardiovascular risks of the treatment (Benemei et al., 2017; Haanes & Edvinsson, 2019).

Ditans (lasmiditan) are newer agents that can be prescribed for acute migraine attacks in patients for whom triptans may be ineffective or contraindicated. They are selective agonists to the 5-HT_{1F} receptor and due to the receptor specificity, they lack the vasoconstrictor effect that is associated with triptans and are safer in patients with cardiac and vascular diseases (de Vries et al., 2020).

B. Small-molecule non-peptide antagonists of the calcitonin gene-related peptide receptor

Gepants are small-molecule non-peptide competitive antagonists at the classical CGRP receptor. An effect mediated by the inhibition of the amylin receptor (the second potent CGRP receptor) might also contribute to gepants' effect. Gepants do not cause vasoconstriction as opposed to triptans. They not just reduce headache pain, but also reduce photophobia, phonophobia and functional disability occuring during migraine attacks (Edvinsson, 2015; Edvinsson et al., 2018).

The first medicine which was effective in migraine and targeted the CGRP receptor was the intravenous olcegepant in 2004. It was effective in the acute treatment of migraine, but its further clinical development halted because of its hepatotoxicity (Olesen et al., 2004; Tepper, 2018). After molecular modifications, three new gepants appeared in the clinical practice: atogepant, rimegepant and ubrogepant. Ubrogepant and rimegepant are used for acute migraine therapy. Atogepant has promising result for prevention of migraine attacks (de Vries et al., 2020; Edvinsson et al., 2022).

C. Monoclonal antibodies targeting calcitonin gene-related peptide or its receptor

Nowadays four monoclonal antibodies are available for prevention of episodic and chronic migraine. Three of them, eptinezumab, fremanezumab and galcanezumab, are anti-CGRP antibodies, while erenumab is an anti-CGRP receptor antibody. Galcanezumab is not just useful for migraine treatment, but it is also effective in cluster headache attacks (Dodick et al., 2020)(Edvinsson, Haanes, et al. 2018). All these monoclonal antibodies are IgG isotypes, have a unique structure and affinity, and also a long serum half-life, so the patients can receive the antibody monthly or quarterly. It results good patient adherence and tolerability. The size of the anti-CGRP and anti-CGRP receptor antibodies are around 150 kilodalton, so they do not cross the blood-brain barrier and do not cause significant central nervous system side effects or toxicity. Because of the size and protein feature, the antibodies need to be administered parenterally (Dodick, 2019). Contrary to gepants, antibodies are eliminated via the reticuloendothelial system, so none of the antibodies have toxic metabolites, liver toxicity or hepatic drug interactions (Deen et al., 2017; Edvinsson et al., 2018; Kielbasa & Helton, 2019). All of the antibodies showed some adverse events during the trials, for example nausea, vomiting, fatigue, constipation, nasopharyngitis or injection site reactions. Another

disadvantage of the antibody therapy is the production of antidrug antibodies (Deen et al., 2017; Moriarty et al., 2019).

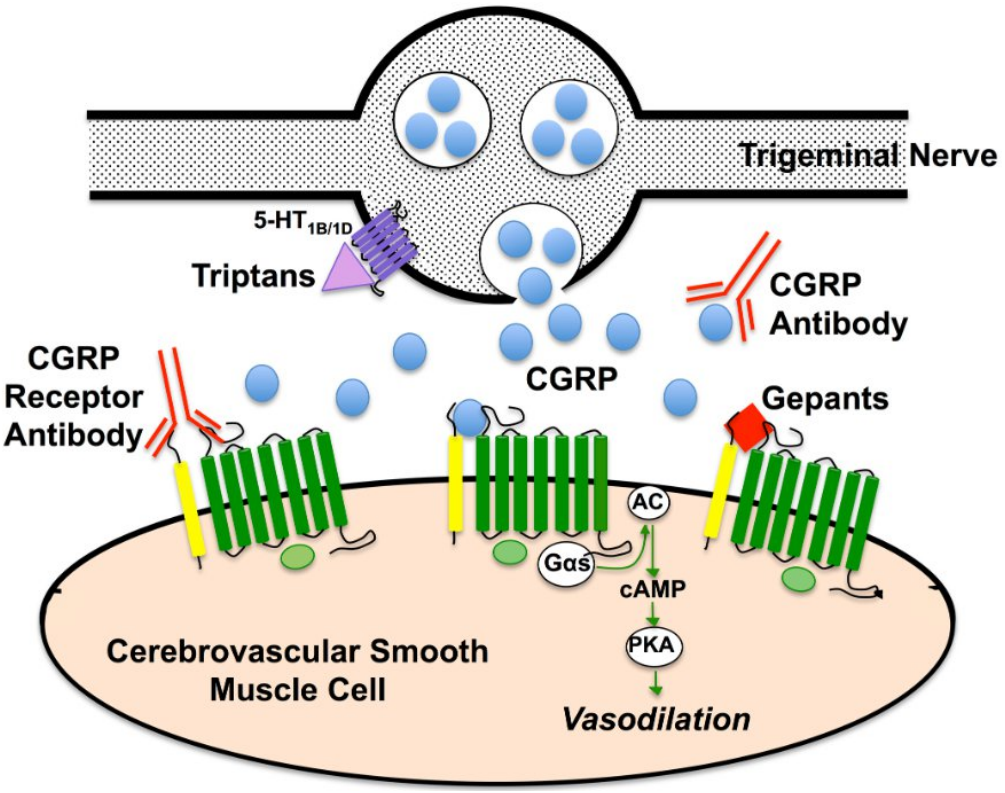

Fig. 2.: The targets for CGRP-related migraine therapies illustrated in a CGRP-containing trigeminal nerve varicosity that innervates a cerebrovascular smooth muscle cell (Edvinsson et al., 2018).

$5\text{-HT}_{1B/1D}$: serotonin 1B/1D receptor, CGRP: calcitonin gene-related peptide, AC: adenylate cyclase, cAMP: cyclic adenosine monophosphate, PKA: protein kinase A.

7. Animal models of migraine

Current knowledge about the pathophysiology of the complex disorder migraine is based mostly on animal models developed to study the trigeminal nociceptive pathway. Animal models of migraine focus on trigeminal sensory processing. Morphological and functional similarities between the human and rodent trigeminovascular system provide a basis for drawing conclusions about the pathophysiology of migraine. Data obtained from animal models led to advances in migraine therapy (Harriott et al., 2019). Different validated animal models relevant for headache exist, the researcher should consider the advantages and limitations of each model before selecting the most appropriate to answer a specific research question.

Our laboratory has a long tradition in migraine research. In ex vivo dura mater preparation of rats we can measure basal and stimulated peptide/mediator release from different cellular components of the dura mater. Using immunohistochemical methods in whole mount preparation of the dura mater and cryostate sections of trigeminal ganglia we can study the morphological changes affecting the trigeminovascular functions under pathophysiological conditions relevant to migraine (Schwenger et al., 2007).

V. THE AIM OF THE STUDY

The aim of our study was to define functional changes in the trigeminovascular nocisensor complex of rats that may affect the nociceptor function after treatment with the anti-CGRP antibody galcanezumab. To visualize the distribution of the anti-CGRP antibody in the dura mater and the trigeminal ganglion, we used fluorophore-labelled galcanezumab. To define the localization of the anti-CGRP antibody in the meningeal tissue and the trigeminal ganglion coexpression of the fluorophore-labelled galcanezumab with the neuropeptide CGRP, an endothelial marker von Willebrand factor, smooth muscle actin and histamine was studied. Changes in the release of CGRP, SP and SOM from meningeal afferents following stimulation of meningeal afferents with the TRPV1 receptor agonist capsaicin or the depolarizing agent KCl was measured in an ex vivo dura mater preparation after galcanezumab treatment. Provided that CGRP and SP are mainly colocalized in trigeminal neurons, stored in the same vesicles and co-released upon activation, we also asked if treatment with a CGRP-binding antibody galcanezumab changes the release of SP similar to that of CGRP. We also studied the effect of galcanezumab on meningeal mast cell function; in an ex vivo rat dura mater preparation we examined the effect of anti-CGRP antibody treatment on CGRP and compound 48/80-induced histamine release from meningeal mast cells.

VI. MATERIALS AND METHODS

1. Experimental animals

Adult male (weighing 270-320 g) and female (weighing 230-260 g) Wistar rats were used in all of our experiments. The animals were housed under a 12-h light/dark cycle, at a temperature of 22 ± 2 °C, in 50 – 70 % relaive humidity with free access to food and water. All experimental procedures were approved by the Ethical Committee for Animal Care of the University of Szeged (approval ID: XIV./1800/2021 and XIV./2368/2023) and carried out in accordance with the Directive 2010/63/EU of the European Parliament. All efforts were made to minimize animal suffering. The number of experimental animals was kept as low as possible.

2. Administration of the antibodies

Rats were anaesthetized with isoflurane at an increasing concentration up to 4 % (Aerrane, Baxter Hungary Kft, Hungary). One group of animals received 30 mg/kg of the anti-CGRP antibody galcanezumab (in 10 mg/ml solution) via subcutaneous injection into the shaved area at the neck and shoulder region. Galcanezumab was taken from the commercial injector containing 120 mg galcanezumab (Emgality, Eli Lilly Netherlands B.V., Utrecht, Nederland) and diluted with saline (0.9 % NaCl). The concentration of galcanezumab was chosen according to previously published data indicating morphological and functional changes in the rat trigeminal system following the administration of another anti-CGRP antibody fremanezumab at the same dose (Dux et al., 2022; Noseda et al., 2020; Vogler et al., 2023). Control rats received subcutaneous injection of the vehicle (0.9 % NaCl).

To visualize the presence and the distribution of galcanezumab in the dura mater and the trigeminal ganglion, a fluorophore-labelled antibody was used (labelling of antibodies with the fluorophore is described below). For comparison, another fluorophore-labelled antibody acting independently of the CGRP pathway was used. Bevacizumab (30 mg/kg in 10 mg/ml solution, Avastin, Roche, Switzerland), a humanized monoclonal antitumor antibody targeting the vascular endothelial growth factor, was administered subcutaneously in some experiments.

3. Labelling of antibodies with fluorophore

As a first step in the fluorescent modification of galcanezumab and bevacizumab, the original buffer (not defined by the manufacturer) was exchanged to 110 mM $NaHCO_3/Na_2CO_3$, pH 9.0 using desalting Zeba^{TM} spin column (7 K MWCO; # 89,890 Thermo Scientific USA) according to the manufacturer's protocol. Five equivalents of Cy3-active ester (succinimidyl ester, NHS-Cy3) was added to the IgG anibodies (galcanezumab: 120 mg/ml; 800 μM; bevacizumab: 25 mg/ml; 167 μM) in two portions, each time reacting at room temperature in the dark for 30 min. The excess reagents were removed, and buffer was exchanged with sterile and isotonic Salsol solution by using the above mentioned Zeba^{TM} spin column. Reaction yields, IgG concentration, and purity were checked by two types of capillary electrophoresis methods and intact protein mass spectrometry analysis (Török et al., 2021).

4. Immunohistochemical staining of the dura mater and the trigeminal ganglion

To visualize the exact localization of the antibody in the different cellular components of the dura mater and the trigeminal ganglion coexpression of the fluorophore-labelled antibodies with an endothelial marker von Willebrand factor, smooth muscle actin or histamine was studied with the indirect immunofluorescence method. Spatial relationship between structures containing the fluorophore-labelled antibody and CGRP-containing axons in the dura mater or neuronal cell bodies in the trigeminal ganglion expressing CGRP was also studied in histological preparations.

Rats injected with the fluorophore-labelled galcanezumab (Cy3-galcanezumab) or bevacizumab (Cy3-bevacizumab) 3 or 30 days prior to the experiment were deeply anaesthetized with thiopental sodium (200 mg/kg, intraperitoneally, Braun, Spain) and perfused transcardially with physiological saline followed by 4 % paraformaldehyde in phosphate buffer (pH 7.4). The animals were decapitated, skin and muscles were removed and the skull was divided into halves along the midline. The brain was removed, the parietal dura mater and the trigeminal ganglia were dissected and postfixed for 2 h in the same fixative. Then trigeminal ganglia were placed in 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 30 % sucrose at 4 °C for 24 h and cut into 16 μm thick longitudinal sections with a cryostat (Leica CM 1950, Switzerland).

Whole mount preparations of the dura mater and sections of trigeminal ganglia of rats treated with fluorophore-labelled antibody were processed for staining with the indirect immunofluorescence technique. Tissue samples were incubated with 5 % normal goat serum

containing 0.5 % Triton X-100 (Merck, Darmstadt, Germany) and 1 % bovine serum albumine (BSA) in PBS for 1 h at room temperature. Thereafter, they were rinsed in PBS and incubated for immunolabelling with appropriate primary antibodies raised against CGRP, smooth muscle actin, von Willebrand factor or histamine in PBS with 1 % BSA and 0.5 % Triton X-100 at room temperature overnight. For the specification of primary antibodies used, see Table 1. After another wash, the sections were incubated with the secondary antibodies at room temperature for 2 hours. IgG labelled with DyLight 488 or Alexa 488 was used as secondary antibody (both 1:500, Jackson Immunoresearch Laboratories, USA). Secondary antibodies were dissolved in PBS with 0.5 % Triton X-100 and 1 % BSA.

Some sections of trigeminal ganglia were mounted with Roti-Mount FluorCare DAPI (4′,6′-diamidino-2-phenylindole hydrochloride; Sigma Aldrich, Taufkirchen, Germany). Preparations of the dura mater and trigeminal ganglia were examined with a confocal fluorescence microscope (ZEISS LSM 700, Germany) using the appropriate filter settings of the confocal scanner.

Table 1. List of primary antibodies

Target	Host species	Manufacturer	Dilution
CGRP	mouse	Sigma-Aldrich, Germany	1:500
Von Willebrand factor	rabbit	abcam, Cambridge, UK	1:50
Smooth muscle actin	mouse	Sigma-Aldrich, Germany	1:1000
Histamine	rabbit	GeneTex, USA	1:100

5. Ex vivo measurement of calcitonin gene-related peptide, substance P and somatostatin release from meningeal afferents

Measurement of CGRP, SP and SOM release from the rat dura mater was performed in ex vivo dura mater preparation by the method originally developed by Ebersberger et al. (Ebersberger et al., 1999). Control male and female rats treated with galcanezumab or vehicle 7 days prior to the experiment were deeply anaesthetized with thiopental sodium (200 mg/kg, intraperitoneally) and decapitated. After removal of the skin and muscles, the skull was divided into halves along the sagittal suture and the cerebral hemispheres were removed. The hemiskull preparations were washed with carbogen-gassed synthetic interstitial fluid (SIF, containing in mM: NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 5, glucose 10 and Hepes 10, pH 7.4) at room

temperature for 30 min and then mounted in a humid chamber at 37 °C. The cranial fossa was filled with 300 µl of carbogen-gassed SIF solution. Samples of the superfusate were collected at periods of 10 min. Control samples were taken to determine basal peptide release in the presence of SIF, then the dura was stimulated for 10 min with the TRPV1 receptor agonist capsaicin at 100 nM concentration in case of CGRP and SP or with 60 mM KCl in case of SOM release. 200 µl of samples diluted with 50 µl of enzyme-linked immunoassay (EIA) buffer were placed into Eppendorf cups and immediately frozen at -70 °C for subsequent analysis.

6. Analysis of the calcitonin gene-related peptide content of samples

The EIA method was used for the measurement of CGRP content of the defrosted samples. For CGRP and SP measurements the same samples divided into halves (125 µl) were used. Samples were processed according to the instructions of the manufacturer (Bertin Pharma/SPIbio, France). The EIA is based on a double-antibody sandwich technique with capture and tracer antibodies binding the CGRP molecule; the tracer antibody is conjugated with acetylcholine esterase converting Ellman's reagent to a yellow substance. The absorbance of the reaction product indicating the CGRP content of the sample was measured photometrically at a wavelength of 405 nm, using a microplate reader (DYNEX MRX). According to the manufacturer the assay has 100 % reactivity to rat CGRP and <0.01 % cross-reactivity to other proteins of the calcitonin family. It detects both α-CGRP and β-CGRP with the same sensitivity. Peptide concentrations of the superfusates were measured in pg/ml, considering the added volume of EIA buffer. Changes in peptide release were expressed as percentage changes relative to the basal, unstimulated release.

7. Analysis of substance P content of samples

In the other half of the defrosted samples also used for CGRP measurement, the SP concentration was measured. The EIA method was used for the measurement of SP content. Samples were processed according to the instructions of the manufacturer of the EIA kit (MyBioSource, USA).

The kit is based on sandwich enzyme-linked immunosorbent assay technology. Capture antibody is pre-coated onto the wells. A biotin conjugated antibody is used as detection antibody. Horseradish peroxidase (HRP)-streptavidin conjugate was added and TMB substrate was used to visualize HRP enzymatic reaction. 3,3',5,5'-tetramethylbenzidine (TMB) was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic

stop solution. The density of yellow product was proportional to the SP content of the sample. The absorbance of the reaction product indicating the SP content of the sample was measured photometrically at a wavelength of 450 nm, using a microplate reader (DYNEX MRX). According to the manufacturer the assay has high sensitivity and excellent specificity for detection of rat SP. No significant cross-reactivity or interference between SP and other peptides was observed.

8. Analysis of somatostatin content of samples

The EIA method was used for the measurement of the SOM content of samples obtained before and after stimulation of the dura mater with 60 mM KCl. Samples were processed according to the instructions of the manufacturer (MyBioSource, USA).

This kit employs the double-antibody sandwich technique, similar to the kit detecting SP. The pre-coated antibody is an anti-rat SOM monoclonal antibody, while the detection antibody is a biotinylated polyclonal antibody. Samples, biotinylated antibodies and avidin-peroxidase conjugates are added to the wells. TMB substrate is used to form a blue product, that finally turns to yellow after addition of the stop solution. The color intensity indicating the SOM content of samples was measured photometrically at a wavelength of 450 nm, using a microplate reader (DYNEX MRX). According to the manufacturer no cross-reactivity with other peptides was observed.

9. Ex vivo measurement of histamine release from meningeal mast cells

Skull halves of control rats and animals treated with galcanezumab 7 days prior to the experiment were prepared as described above for the measurement of peptide release. Control samples were taken in the presence of SIF for 10 min to determine basal histamine release, then the dura mater was stimulated for 10 min by application of 300 µl of CGRP (Sigma-Aldrich, Germany) at 10 µM or 2.5 µg/ml compound 48/80 (Sigma-Aldrich, Germany). The concentrations of CGRP and compound 48/80 used in the experiments were found effective in releasing histamine in previous experiments of our laboratory (Schwenger et al., 2007). 100 µl of samples diluted with 25 µl of EIA buffer were placed into Eppendorf cups and immediately frozen at -70 °C for subsequent analysis.

10. Analysis of the histamine content of samples

The EIA method used for measurement of the histamine concentration of samples is based on the competition between unlabelled histamine of samples and acetylcholin esterase linked to histamine for limited specific anti-histamine antibody sites in wells (Bertin Pharma, France). Acetylcholine esterase converts Ellman's reagent to a yellow substance. The absorbance of the reaction product indicating the histamine content of the sample was measured photometrically at a wavelength of 405 nm, using a microplate reader (DYNEX MRX).

11. Statistics

Statistical analysis was performed using Statistica 13 software (StatSoft, USA). Following verification of the normal distribution of data, the Student's t-test and analysis of variance (factorial ANOVA or one-way ANOVA) extended by the unequal *N* honest significant difference (HSD) test were used as specified in the results. All values were expressed as mean ± standard error of the mean (SEM). A probability level of $p < 0.05$ was regarded as statistically significant.

VII. RESULTS

1. Localization of fluorophore-labelled antibodies in the dura mater

In whole mount preparations of the dura mater from animals treated with Cy3-galcanezumab 3 days prior to fixation, the fluorescence signal was detected mainly in the wall of small branches of the middle meningeal artery. The diameter of the labelled blood vessels was between 20-30 μm, which can be assigned to arterioles (Fig. 3A,C). Larger branches of the dural arteries and capillaries were free from Cy3-galcanezumab. A less prominent deposition of the fluorophore-labelled antibody could be seen in some larger venous blood vessels (Fig. 3C). Axons of CGRP-positive afferent neurons were detected in close vicinity to arterioles showing the fluorescence signal (Fig. 3A,C). In addition, some cells in the connective tissue of the dura mater distant from visible blood vessels showed Cy3-fluorescence (Fig. 3A,B). To see whether mast cells augmenting the neurogenic inflammation in the meningeal tissue by the release of their mediators also accumulate the fluorophore-labelled antibody, histamine immunofluorescence in the dura mater whole mount preparations was identified. Mast cells were mainly located in close proximity to blood vessels labelled with Cy3-galcanezumab, but no colocalization of mast cell histamine with Cy3-galcanezumab labelling was observed in the meningeal tissue (Fig. 3E).

Traces of Cy3-galcanezumab were still present in the dura mater 30 days after injection of the antibody (Fig. 3D).

In our experiments, Cy3-bevacizumab targeting the vascular endothelial growth factor was used as a control antibody. It does not target CGRP. Although the localization of Cy3-bevacizumab not targeting CGRP was similar to Cy3-galcanezumab in the blood vessels of the dura mater, deposition of the fluorophore-labelled bevacizumab was overall visibly less intense (Fig. 3F).

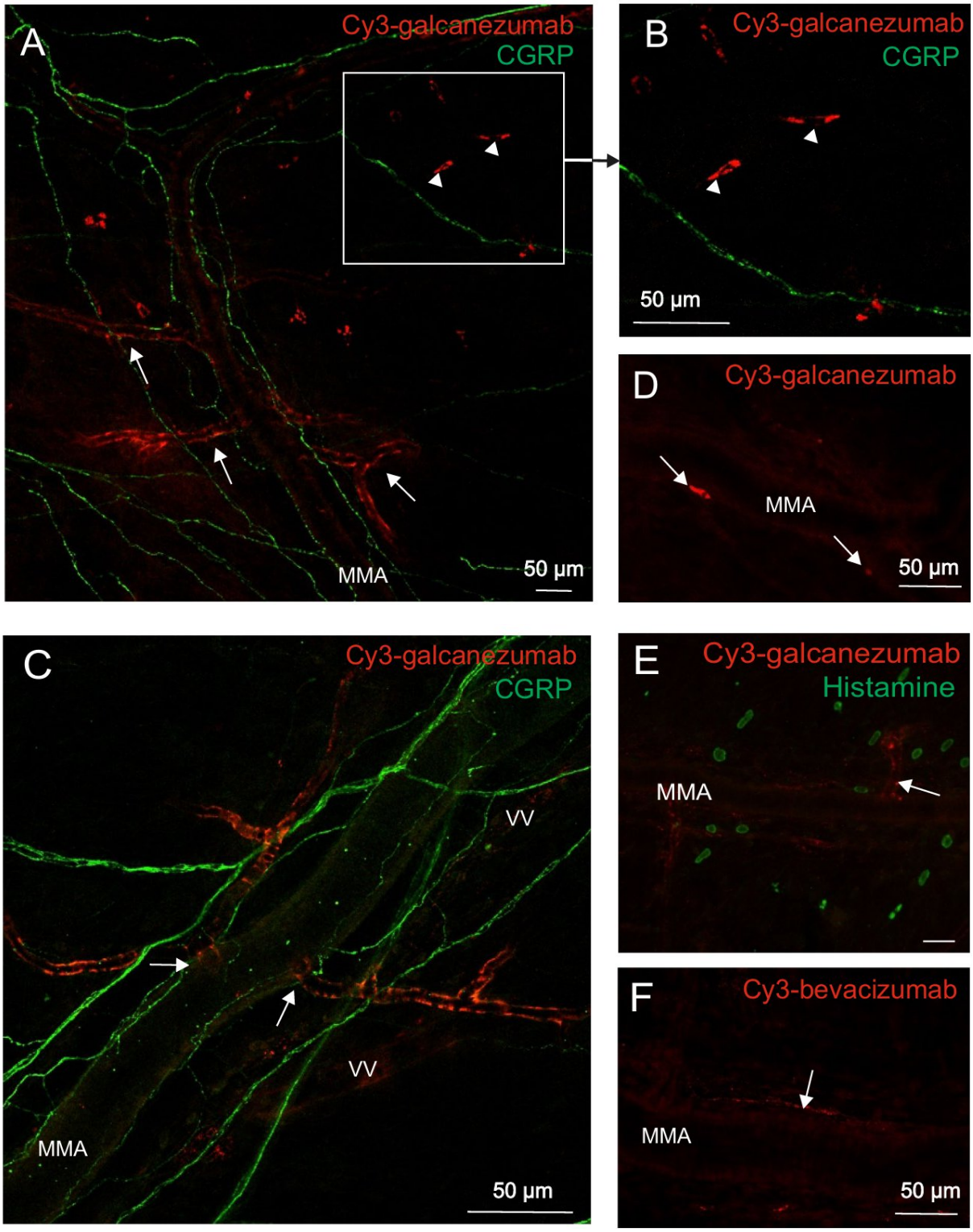

Fig. 3. Localization of Cy3-galcanezumab and Cy3-bevacizumab in the rat dura mater. Immunohistochemical localization of Cy3-galcanezumab (A-E) and Cy3-bevacizumab (F) in whole mount preparations of the rat dura mater 3 days (A-C, E–F) or 30 days (D) after injection of the antibodies. Arrows indicate blood vessels, arrowheads (A-B) cells with deposition of fluorophore-labelled antibody galcanezumab. MMA: middle meningeal artery, VV: venous vessel.

2. Localization of fluorophore-labelled antibodies in the trig 0eminal ganglion

Three days after treating the animals with Cy3-galcanezumab in sections of the trigeminal ganglia fluorescence signal was detected in the wall of blood vessels, similar to the dura mater. Labelled blood vessels were localized close to CGRP-immunopositive trigeminal neurons (Fig. 4A). The fluorescence signal was also detected around the soma of some trigeminal ganglion cells (Fig. 4A,F).

To clarify the exact localization of the fluorescence signal in the blood vessels of the trigeminal ganglia, immunohistochemistry identifying the endothelial marker von Willebrand factor and smooth muscle actin was carried out. Cy3-galcanezumab was not colocalized with the fluorescence marker for smooth muscle actin (Fig. 4D), but in almost all labelled blood vessels with the endothelial marker for von Willebrand factor (Fig. 4E). Capillaries positive for von Willebrand factor (marked by * in Fig. 4E) did not show Cy3-fluorescence for galcanezumab.

Traces of Cy3-galcanezumab were still present in the wall of some blood vessels in the trigeminal ganglion 30 days after injection of the antibody (Fig. 4B).

Localization of Cy3-bevacizumab in the blood vessels of the trigeminal ganglia was similar to that of Cy3-galcanezumab but deposition of the antibody around the soma of neurons in the ganglion was absent and the labelling was overall less intense (Fig. 4C).

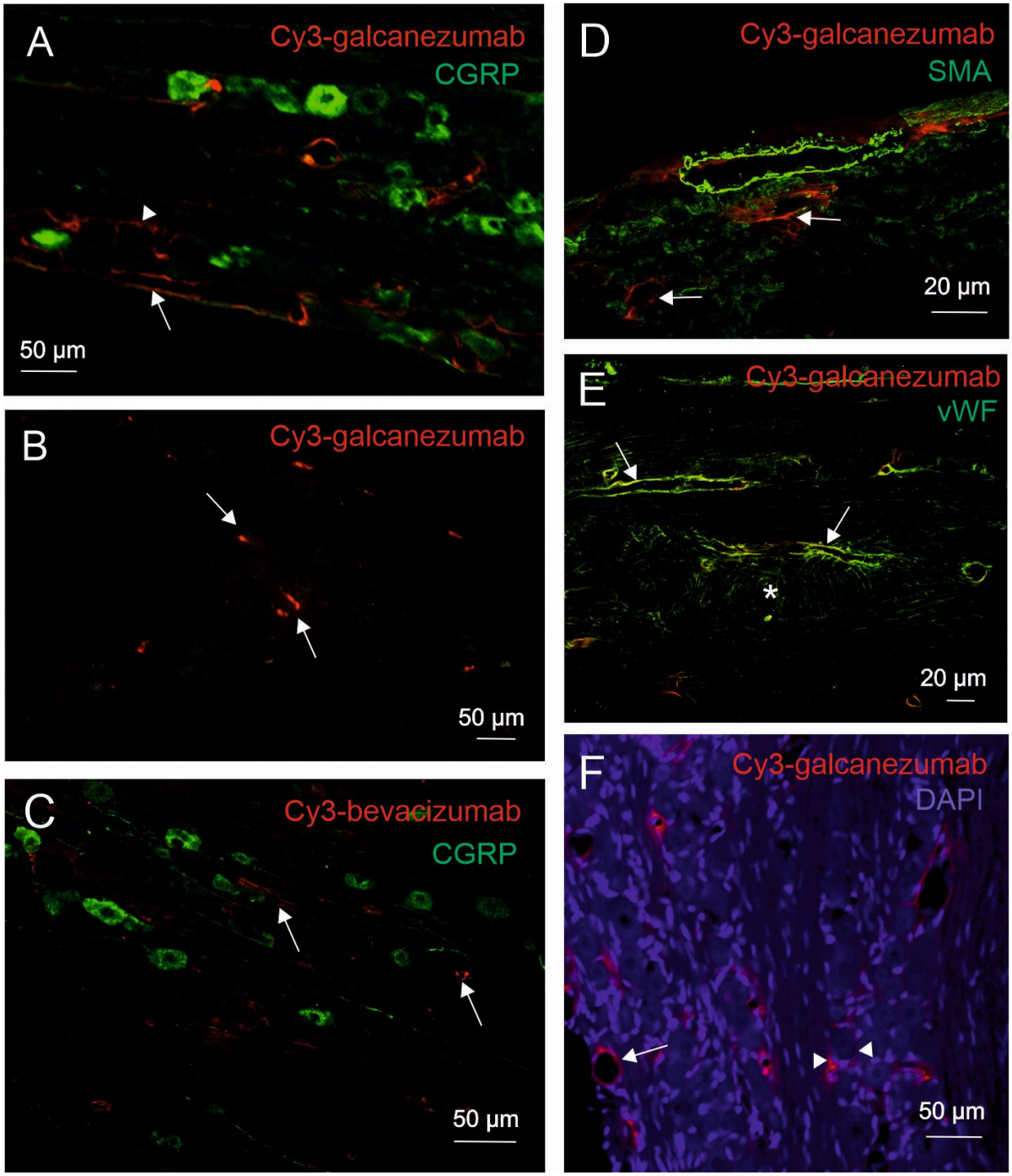

Fig. 4. Localization of Cy3-galcanezumab and Cy3-bevacizumab in the rat trigeminal ganglion.

Immunohistochemical localization of Cy3-galcanezumab (A-B, D-F) and Cy3-bevacizumab (C) in rat trigeminal ganglia 3 days (A, C-F) or 30 days (B) after injection of the antibodies. Arrows indicate blood vessels, arrowheads (A,F) ganglion cells with deposition of fluorophore-labelled galcanezumab. Around * in panel E, capillaries labelled with the anti-von Willebrand factor (vWF) antibody can be seen. SMA: smooth muscle actin.

3. Altered calcitonin gene-related peptide and substance P release from the dura mater after galcanezumab treatment

A. Basal release of calcitonin gene-related peptide and substance P

Using ex vivo hemisected rat skull preparations, we measured the concentrations of CGRP and SP released spontaneously by the meningeal afferents in the presence of SIF. In control animals, basal CGRP release was lower in males (19.32 ± 0.46 pg/ml, $n = 14$) than in females (23.48 ± 1.23, $n = 10$, t-test, $p < 0.005$), while SP concentrations measured in the same samples tended to be higher in males (6.17 ± 0.77 pg/ml) than in females (5.74 ± 1.24 pg/ml, t-test, $p = 0.76$).

B. Stimulated release of calcitonin gene-related peptide

In control animals treated with vehicle ($n = 24$), capsaicin stimulation increased the CGRP release to 231.02 ±12.78 % of the basal release. In galcanezumab-treated animals ($n = 32$), the stimulated increase in CGRP release was 184.50 ± 7.60 %, which was significantly different from the vehicle group (factorial ANOVA, $F_{1,52} = 4.60$, $p < 0.05$). There was no significant difference between the sexes ($F_{1,52} = 0.28$, $p = 0.595$) but post-hoc testing using the unequal *N* HSD test showed that the difference between the vehicle and the galcanezumab group was solely due to the female animals (Table 2, Fig.5A).

C. Stimulated release of substance P

The capsaicin-stimulated SP release was below the basal release in vehicle-treated animals (62.16 ± 5.56 %) but not different from the basal release in galcanezumab-treated animals (100.76 ± 9.0 %) of the unstimulated release, which was indicated as significant between the groups (factorial ANOVA, $F_{1,52} = 8.847$, $p < 0.005$). According to the unequal *N* HSD post-hoc test, the difference between the control and the galcanezumab group was solely due to the female animals (Table 2, Fig. 5B).

Table 2: Sex-specific releative changes in capsaicin-stimulated CGRP and substance P release

	Stimulated CGRP release				Stimulated substance P release			
Sex	Males		Females		Males		Females	
Treatment group	Vehicle (n = 14)	Galcan. (n = 6)	Vehicle (n = 10)	Galcan. (n = 26)	Vehicle (n = 14)	Galcan. (n = 10)	Vehicle (n = 6)	Galcan. (n = 26)
Mean (%)	212.19	233.89	257.39	173.10	72.30	95.99	47.96	101.86
SEM (%)	18.32	17.45	13.81	6.81	7.84	5.18	12.22	10.80
P_{HSD}	0.858		< 0.005		0.770		< 0.05	

P_{HSD} values are from unequal *N* honest significance post-hoc test following factorial ANOVA with factors treatment and sex. Galcan.: galcanezumab.

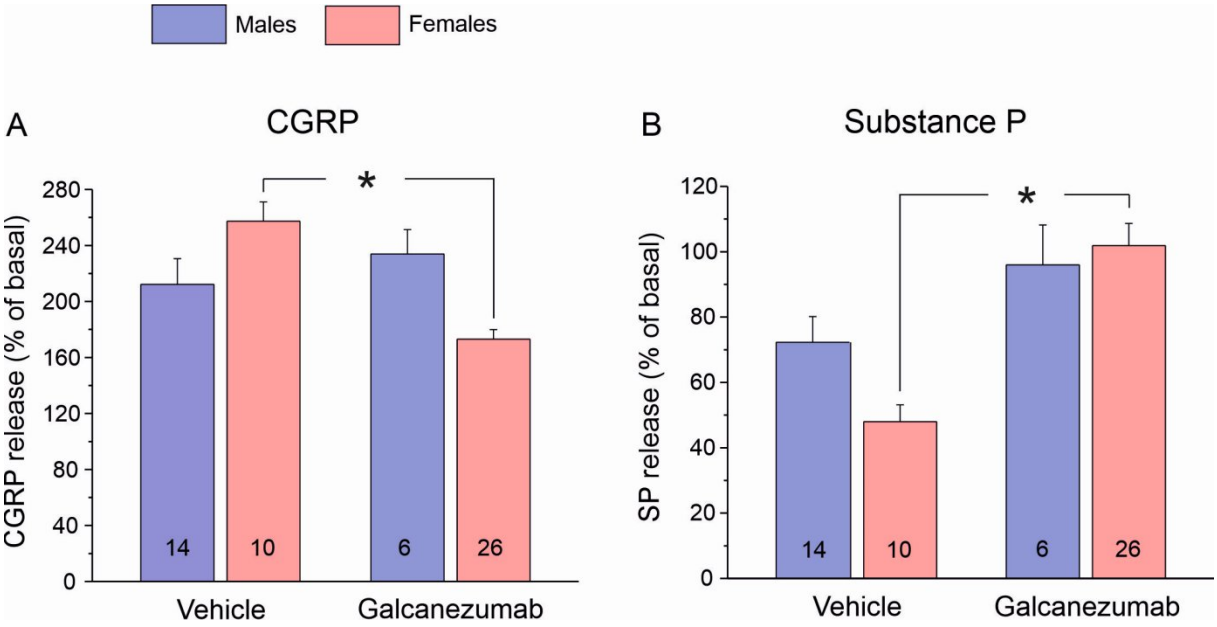

Fig. 5. Release of calcitonin gene-related peptide and substance P from meningeal afferents.

Relative changes in CGRP and SP release in vehicle-treated animals and in animals 7 days after treatment with galcanezumab. CGRP and SP content was measured in the same sample after stimulating the dura mater with capsaicin at 100 nM. The number of experiments is indicated in the bars. *: $p < 0.05$.

4. Basal and stimulated release of somatostatin from the dura mater

In other hemisected skull preparations we measured the SOM release from the dura mater stimulated with 60 mM KCl. Since we did not want to exclude the TRP receptor negative but SOM positive neurons from the measurement, in this series of experiments we stimulated the meningeal afferents with 60 mM KCl instead of capsaicin. In vehicle-treated control animals both basal and KCl stimulated SOM release tended to be higher in females. In males the basal release was 18.68 ± 0.03 pg/ml (n = 5), in females it was 19.16 ± 0,16 pg/ml (n = 6, t-test, $p <$ 0.05). In control animals stimulation with KCl induced no significant changes in SOM release (Table 3, Fig. 6). Seven days after galcanezumab treatment of the animals no significant change was observed in basal or stimulated SOM release. Factorial ANOVA showed no significant difference between the treatments ($F_{1,33}$ = 1.0, p = 0.332) and the sexes ($F_{1,33}$ = 0.0, p = 0.889).

Table 3: Sex-specific relative changes in KCl-stimulated somatostatin release

	Stimulated somatostatin release			
Sex	Males		Females	
Treatment group	Vehicle (n = 5)	Galcanezumab (n = 6)	Vehicle (n = 10)	Galcanezumab (n = 14)
Mean (%)	100.41	97.63	100.80	99.71
SEM (%)	0.29	0.68	0.61	0.17

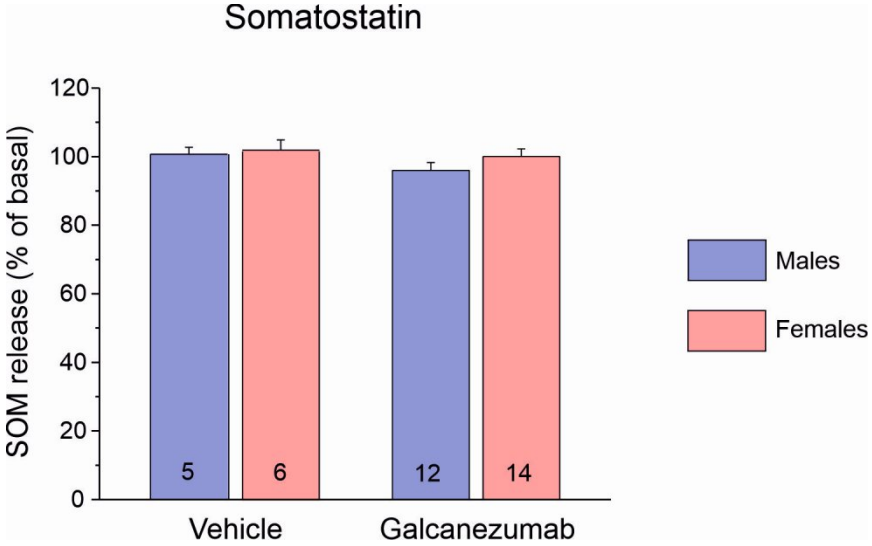

Fig. 6. Release of somatostatin from meningeal afferents.

Relative change in SOM release in vehicle-treated animals and in animals 7 days after treatment with galcanezumab. Release of SOM was induced with KCl at 60 mM. The number of experiments is indicated in the bars. *: $p < 0.05$.

5. Histamine releasing effect of different mast cell degranulating agents

In other hemisected skull preparations of male rats, we measured histamine release from the meningeal tissue. The basal histamine release was 31.39 ± 1.88 pg/ml in vehicle-treated (n = 13) and 36.71 ± 1.05 pg/ml in galcanezumab-treated animals (n = 25), which was significantly different (t-test, $p < 0.05$). The histamine-releasing effects of mast cell degranulating agents were differentially influenced by galcanezumab treatment. In vehicle-treated control animals, CGRP (10 µM) increased the histamine release to 134.9 ± 7.5% (n = 5) of the basal release, while 7 days after treatment with galcanezumab the increase was 110.5 ± 4.7% (n = 15), which was significantly different from the control (one-way ANOVA for treatment, extended by the unequal HSD test, $F_{1,18} = 7.03$, $p < 0.05$). We also tested the histamine-releasing effect of the widely used mast cell degranulating agent compound 48/80 at 2.5 µg/ml that was not different between vehicle (124.6 ± 6.2% of basal, n = 8) and galcanezumab-treated animals (126.4 ± 6.7% of basal, n = 10, one-way ANOVA, $F_{1,16} = 0.04$, p = 0.85; Fig.7).

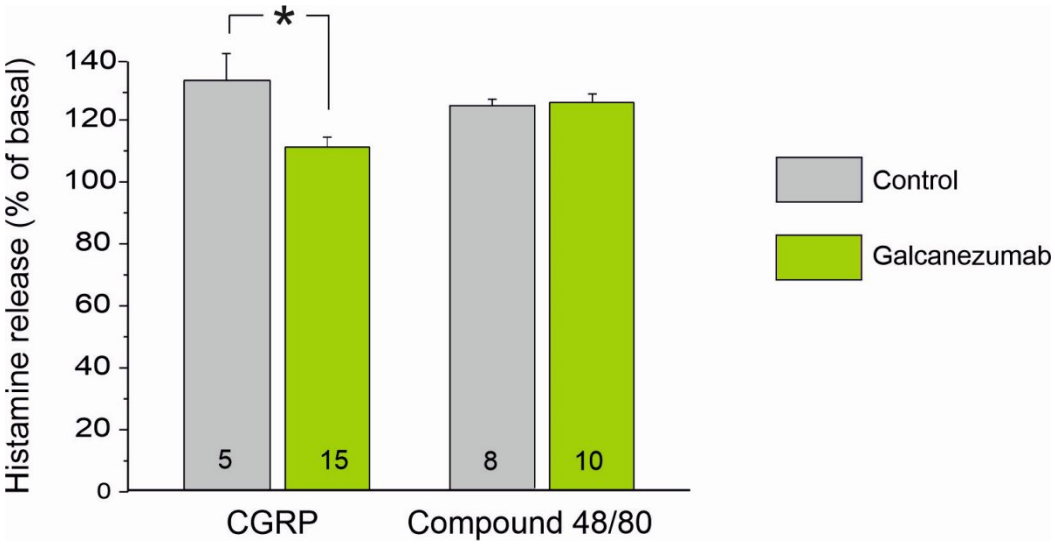

Fig. 7. Histamine release from meningeal mast cells.

Relative changes in histamine release induced by CGRP at 10 µM and 2.5 µg/ml compound 48/80 in control animals and rats treated with galcanezumab 7 days prior to the experiment. The number of experiments is indicated in the bars. *: p < 0.05.

VIII. DISCUSSION

Our study was initiated in an attempt to examine changes in the trigeminovascular system after the administration of the migraine preventing drug humanized monoclonal anti-CGRP antibody galcanezumab. Since the trigeminal nocisensor complex includes the peptidergic sensory neurons, the meningeal vascular elements and mast cells involved in the generation of the neurogenic inflammatory reaction of the dura mater and thereby playing significant role in the peripheral and central sensitization of the trigeminal nociceptive pathway, we measured functional changes in this system such as release of neuropeptides as well as histamine, regarded as relevant for headache generation in migraineurs.

To visualize the presence and the distribution of the antibody in the components of the nocisensor complex we used a Cy3 fluorophore-labelled anti-CGRP antibody. The impact of fluorophore labelling on protein function is critical, particularly regarding potential changes in protein conformation, dynamics, and interactions mediated by the fluorophore. Smaller fluorophores tend to be preferred for their lower likelihood of interfering with the native state and activity of proteins, making them a better choice in many experimental setups (Liu et al., 2018). Cy3 is recognized as one of the smaller fluorophores, particularly with respect to its practical applications in fluorescence studies (Liu & Lilley, 2017). Due to its fluorescent properties and ability to covalently bond with proteins, Cy3, a member of the cyanine dye family, is a versatile tool for labelling.

In our experiments rats were subcutaneously treated with the anti-CGRP antibody in a manner similar to the clinical use for the prevention of chronic migraine. Although the dose of galcanezumab used in our experiments was higher than the initial dose normally used for preventive migraine therapy, the slow elimination and the repeated administration of the antibody in humans may increase its concentration in trigeminal tissues (Detke et al., 2018; Monteith et al., 2017).

Galcanezumab, Cy3-galcanezumab and Cy3-bevacizumab treatments were well tolerated by the rats, they did not cause any visible change in their behaviour during the experiments.

1. Distribution of Cy3-galcanezumab in the dura mater and the trigeminal ganglion

Cy3-labelled galcanezumab was identified in the dura mater and the trigeminal ganglion already 3 days after the treatment of the animals. Cy3-galcanezumab could be detected in the trigeminovascular system for up to 30 days after the administration. In both tissues mainly blood vessels were labelled by the antibody. In whole mount preparations of the dura mater where the whole vascular system were visualized, we identified the labelled vessels as arterioles by measuring their diameter. Neither larger nor smaller diameter blood vessels contained the fluorophore-labelled antibody. Since profound perfusion of the animals with physiological saline and paraformaldehyde did not eliminate the laballing of these blood vessels, we wanted to determine which layer of the blood vessel wall binds the antibody. In sections of the trigeminal ganglia where the different layers of the labelled blood vessel wall could be distinguished, Cy3-galcanezumab was coexpressed to a great extent with the endothelial marker von Willebrand factor but not with smooth muscle actin. We assume that the strong affinity of some components of the luminal glycocalyx of endothelial cells in dural blood vessels to the circulating antibody may form a local depot that releases antibody in the dura mater and also in the trigeminal ganglion for at least 30 days after the injection (Hennigs et al., 2021).

The luminal surface of vascular endothelial cells is covered by membrane-bound, negatively charged proteoglycans, glycolipids and glycosaminoglycans, contributing to mechanotransduction, cell signaling and adhesion of blood components. Although the exact mechanism is unclear, the high affinity of the Cy3-galcanezumab to arterioles can be explained by the diverse gene expression of adhesion molecules along the vascular tree. We assume that the specific properties of endothelial cells are responsible for the selective accumulation of galcanezumab at specific segments of blood vessels (Chi et al., 2003).

In our experiments the fluorophore-labelled bevacizumab, a clinically approved anti-angiogenic antibody directed against vascular endothelial growth factor was used as control antibody. Since we do not know the exact mechanism of the interaction between the fluorophore-labelled anti-CGRP antibody and the vascular endothelium, we compared the distribution of the different fluorophore-labelled antibodies in the meningeal tissues. Although both Cy3-galcanezumab and Cy3-bevacizumab could be identified in blood vessels of the dura mater and the trigeminal ganglion, the intensity and the exact localization of labelling were different. Our finding makes it unlikely that the Cy3 fluorophore alone could be responsible for the accumulation and characteristic distribution of the antibody at the endothelial cells. However, it seems to be proved that the anti-CGRP antibody reaches high concentration for

weeks exactly at those places of the trigeminovascular system where functional changes can be expected.

In the dura mater and the trigeminal ganglion fluorophore-labelled blood vessels were in close proximity to CGRP-immunoreactive nerve fibers or cell bodies and in the dura mater also to mast cells, providing an antibody pool for the nocisensor complex for at least 30 days.

In an earlier study, the presence of the anti-CGRP antibody fremanezumab in the meningeal tissue of rats was visualized (Noseda et al., 2020). In this study, a very similar distribution of the fluorophore-labelled antibody was seen associated to the wall of meningeal blood vessels. Similar to our observations, they also detected the fluorescence signal around the soma of trigeminal neurons already 4 hours after the injection of the antibody. In our experiments we have not checked the presence of the antibody after such a short period but the robust labelling makes it likely that deposition of galcanezumab in the meningeal tissue starts much earlier after subcutaneos administration. Although the anti-CGRP antibody used (fremanezumab vs galcanezumab) and also the way of administration (intravenous vs subcutaneous) were different, the very similar distribution of the antibodies in the trigeminovascular system suggests a common fate of the two anti-CGRP antibodies in the trigeminovascular system.

Although the presence of the anti-CGRP antibody was robust both in the dura mater and the trigeminal ganglion already 3 days after the Cy3-galcanezumab injection, we performed the functional tests a few days later (7 days after the injection) to allow the development of possibly complete functional changes in the nocisensor complex.

2. Sex differences observed in calcitonin gene-related peptide and substance P release from the dura mater

Earlier observations indicated reduced basal and capsaicin-induced CGRP release from meningeal afferents 3 days after systemic treatment with the anti-CGRP antibody fremanezumab. Increases in meningeal blood flow induced by TRPA1 or TRPV1 receptor agonists were also reduced after the fremanezumab injection in an in vivo rat dura mater preparation (Dux et al., 2022).

CGRP, the central pathophysiological factor of migraine headache can be colocalized with other peptides in nociceptive neurons (Price & Flores, 2007). Based on earlier observations indicating that SP is present in a subpopulation of CGRP-containing sensory neurons, CGRP and SP are largely stored in the same vesicles and co-released upon activation of the afferents

(Gulbenkian et al., 1986; Plenderleith et al., 1990), we asked if treatment with an anti-CGRP antibody changes the SP release from meningeal afferents in the same way as it changes the CGRP release. To minimize the modifying effect of the inter-animal variability in peptide content we measured CGRP and SP concentrations in the same sample obtained from the ex vivo dura mater preparation. Corresponding to the earlier observations in our experiments systemic galcazenumab treatment reduced TRPV1 stimulation-induced CGRP release from meningeal afferents (Dux et al., 2022).

The anti-CGRP antibody fremanezumab reduces CGRP content of trigeminal neurons of rats (Vogler et al., 2023). Based on the similarities between the functional changes affecting CGRP release after fremanezumab and galcanezumab treatment we speculate that CGRP content of trigeminal neurons may be also reduced the same way after fremanezumab and galcanezumab treatment.

Our results revealed a sex difference in peptide release. Not only the basal but also the capsaicin-stimulated CGRP concentrations were higher in female rats. Migraine is more prevalent in the female population. The higher susceptibility of the female trigeminal system to release CGRP is reflected by our results. The higher sensitivity of trigeminal neurons towards factors modulating peptide release is indicated also by the stronger inhibition of CGRP release in female animals after galcanezumab treatment.

In control animals CGRP release was combined with a much lower SP release in the same sample corresponding to the lower number of SP containing neurons in the trigeminal system (Dux & Messlinger, 2025). In female animals an inverse relationship was observed between changes in CGRP and SP content of samples. Compared to male animals higher CGRP concentrations were combined with lower SP concentrations in vehiche-treated female rats after capsaicin application. While galcanezumab treatment decreased the capsaicin-induced CGRP release, SP content of the samples was increased in both sexes. The difference was significant in females.

While the clinical relevance of SP in migraine pathophysiology is not clear, we can not explain the function of increased SP production or release concomitant with a decreased CGRP production or release after galcanezumab treatment (Ashina et al., 2019). Anyway, our results raises the notion that less CGRP production in the neuron may set the protein producing machinery free that leads to an increased SP production or transport to the vesicles. According to our hypothesis galcanezumab treatment may affect not only the targeted CGRP production and function but other neuropeptides may be also affected by the treatment.

3. Somatostatin release from meningeal afferents

Immunohistochemical studies have demonstrated the presence of SOM within trigeminal neurons (Kai-Kai, 1989; Kummer & Heym, 1986; N. Lazarov & Chouchkov, 1990). SOM was identified in chemosensitive nociceptors, and in human trigeminal ganglion SOM was partially coexpressed with SP (Del Fiacco & Quartu, 1994). Activation of chemosensitive neurons releases not only the neuropeptides promoting activation of the nocisensor complex and sensitization of the trigeminal system but also SOM can be released from terminals of capsaicin-sensitive primary afferent neurons. SOM inhibits not only the acute inflammation and nociception in the innervated tissue, but may also exert a "sensocrine" function with systemic antiinflammatory and analgesic effects (Helyes et al., 2004).

Since we realized that galcanezumab treatment modifies not only the release of the targeted CGRP upon stimulation but concomitantly SP release is increased, we tested whether the migraine-preventing effect of the anti-CGRP antibodies is not only due to reduced expression and effectivity of CGRP but at least partially the result of an increased SOM release from the stimulated terminals. Thus it appeared possible that enhanced expression and release of SOM with its systemic pain inhibiting effect is also involved.

Capsaicin has been shown to induce SOM release in different tissues but only about 8% of trigeminal ganglion neurons express this neuropeptide (Kummer & Heym, 1986; Lazarov, 2002). Therefore, to ascertain a measurable effect in SOM release, we used KCl instead of capsaicin as a potent depolarizing stimulus activating not only the capsaicin sensitive neuron population. Our results indicated sex difference for basal SOM release in control animals with a higher release in females compared to males. However, basal as well as stimulated SOM concentrations were very similar in both vehicle and galcanezumab-treated animals suggesting that it is unlikely that an increased SOM release may contribute to a pain reducing effect of galcanezumab treatment.

4. Histamine release from rat dura mater

In the wholemount preparations of the dura mater we could not find any colocalization between Cy3-galcanezumab and immunohistochemically detected histamine indicating mast cells, but the close proximity of fluorophore-labelled blood vessels, CGRP immmunoreactive afferents and mast cells may provide a morphological basis for a mast cell function modifying effect of anti-CGRP antibody. Activation and consequent degranulation of mast cells releases different vasoactive and nociceptor sensitizing agents.

In our experiments histamine release was measured as an indicator of mast cell activation. We tested two different degranulating agents acting on different G-protein coupled mast cell receptors. CGRP acts on its canonical receptor while compound 48/80 acts on Mas-related G-protein coupled receptor member X2. The differential effects of mast cell degranulating agents on histamine release suggest that galcanezumab treatment modifies the mast cell-mediated amplifying of signals, but it does not alter the histamine content of mast cells.

In an earlier study treatment of the animals with another anti-CGRP antibody, fremanezumab, the fraction of trigeminal ganglion neurons immunoreactive to the CGRP receptor components CLR and RAMP1 was significantly lowered compared to the control (Vogler et al., 2023). Our present results indicate that changes in the CGRP receptor components induced by the antibody treatment may affect not only neurons but also other components of the trigeminal nocisensor complex such as mast cells expressing the receptor.

The role of histamine released by mast cells is not clear in migraine pathophysiology. Although histamine infusion generates pulsating headache in migraine patients, treatment with antihistamines seems to be uneffective in alleviating migraine attacks (Worm et al., 2019). After all, the contribution of activated mast cells in the vitious circle of nocisensor complex activation leading to sensitization of the nociceptive pathway cannot be ruled out since also other metabolites released by activated mast cells can be involved in nociceptor sensitization.

We do not know the reason for the difference between the basal histamine release in control (vehicle-treated) and galcanezumab-treated animals. The higher concentration of spontaneously released histamine measured in galcanezumab-treated rats may be due to changes in the number and/or function of mast cell receptors stimulated by tissue metabolites under basal conditions.

A limitation of our study was that only male animals were included in the histamine release experiments. After the publication of our data included in my PhD thesis additional experiments were done in our laboratory that provided information about histamine releasing effects of CGRP and compound 48/80 in female animals. Histamine releasing effect of CGRP was similar in both control and galcanezumab-treated male and female animals while compound 48/80-induced histamine release was more robust in female than in male but no difference between control vehicle-treated and galcanezumab-treated animals was observed (unpublished data from our laboratory). Also these additional results indicate sex differences in the activity and susceptibility of the components of the nocisensor complex to anti-CGRP antibody treatment.

5. Clinical relevance

An important question regarding the clinical relevance of our study is: where these anti-CGRP antibodies in the trigeminal nociceptive pathway act? The migraine-preventing effect of CGRP-targeting antibodies is considered mainly as a peripheral effect in the trigeminovascular system as penetration of the antibody through the blood brain barrier into the central nervous system is limited. No convincing evidence supports the assumption that migraine attacks would enhance the permeability of the blood brain barrier allowing the passage of chemical substances from the blood into the brain tissue (Lundblad et al., 2015). The trigeminal ganglion and the peripheral axons of the trigeminal nociceptors innervating the dura mater are not protected by the blood brain barrier and thus are likely targets of antibody treatment.

Experimental results suggest that the action of the anti-CGRP antibody is complex. It is not limited to the neutralization of released CGRP but it might also modify the production and/or release of other neuropeptides coexpressed with CGRP. Altered expression or function of CGRP receptors on trigeminal neurons and other components of the trigeminal nocisensor complex may prevent the sensitization of trigeminal neurons and the exaggeration of the initial nociceptive and vascular responses.

IX. SUMMARY

Migraine is a primary headache predominantly affecting women. Monoclonal antibodies binding CGRP are approved in the prophylaxis of migraine. The precise mechanism of their effect is yet unclear but they appear to protect trigeminal afferents from sensitization.

Our experiments detected a long-lasting multi-faceted change in the function of the trigeminovascular nocisensor complex after tretment of rats with the anti-CGRP antibody galcanezumab. Fluorophore-labelled galcanezumab was mainly accumulated in the arterioles of the dura mater and the trigeminal ganglion, in structures of the trigeminovascular system that are not protected by the blood brain barrier. Anti-CGRP antibody treatment modulated CGRP and SP release from activated nociceptors inversely. CGRP release was reduced while SP release increased upon activation of TRPV1 receptors of trigeminal nociceptors. SOM exerting systemic antinociceptive effect was not influenced by the antibody treatment.

Activating effects of CGRP on non-neural elements of the trigeminal nocisensor complex, notably the dural mast cells, seem to be mitigated by the antibody treatment. A shift in the equilibrium between pronociceptive and antinociceptive mediators released upon trigeminal activation may contribute to the beneficial effect of anti-CGRP antibody treatment in migraine.

Activity and susceptibility of the trigeminovascular system to anti-CGRP antibody treatment was higher in female animals.

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