NEUROGENIC VASCULAR RESPONSES MEDIATED BY CAPSAICIN-SENSITIVE NOCICEPTIVE AFFERENTS IN THE RAT DURA MATER ENCEPHALI: IMPLICATIONS FOR THE PATHOMECHANISM OF HEADACHES

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LIST OF ABBREVIATIONS

ACh	acetylcholine
CBF	cerebral blood flow
CGRP	calcitonin-gene related peptide
CSD	cortical spreading depression
DY	diaminido yellow
HA	histamine
IR	immunoreactive
MMA	middle meningeal artery
NO	nitric-oxide
NTG	nitroglycerine
NPY	neuropeptide Y
PAR	proteinase-activated receptor
PKA	protein kinase A
PKC	protein kinase C
SP	substance P
STN	spinal trigeminal nucleus
TG	trigeminal ganglion
TRPV1	transient receptor potential vanilloid type 1 receptor
VIP	vasoactive intestinal polypeptide

SUMMARY

The role of meningeal sensory nerves continues to be a major focus of current thinking on the origin of head pain, since pain-sensitivity of intracranial structures is primarily restricted to the blood vessels of the dura mater encephali. Headaches that accompany intracranial pathologies are thought to result from the activation of meningeal nociceptive sensory fibers. Anatomical studies revealed that meningeal nociceptive information is conveyed towards the central nervous system by trigeminal afferent nerves, which project to the spinal trigeminal nucleus. Our laboratory has previously demonstrated that topical application of capsaicin elicited a marked vasodilatory response in the rat dura mater, which was inhibited by capsaicin pretreatment and by a competitive capsaicin receptor antagonist, capsazepine. Pharmacological and neurochemical evidence indicated that this sensory neurogenic vasodilatory response is mediated by the release of calcitonin gene-related peptide (CGRP) from capsaicin-sensitive afferent nerves.

In the present study, we demonstrated that a significant population of the nerve fibers of the rat dura mater displays transient receptor potential vanilloid type-1 receptor (TRPV1) immunoreactivity, suggesting that they are nociceptive in function. Further, we explored the possible functional role of meningeal capsaicin-sensitive sensory nerves in headache syndromes associated with certain pathological states through the study of capsaicin-induced dural vasodilation, CGRP release and morphology of meningeal nerves using laser Doppler flowmetry, measurement of peptide levels and immunohistochemistry.

The pathologies examined in these studies included diabetic neuropathy and activation of proteinase-activated receptor-2 (PAR-2). Previous studies demonstrated that PAR-2, which mediates proinflammatory reactions, is localized on and modulate the function of cutaneous capsaicin-sensitive afferent nerves. Since cutaneous and meningeal capsaicin-sensitive afferents share similar functional properties, the present study examined the possible role of PAR-2 in neurogenically mediated vascular responses of the rat dura mater. Our findings indicated that activation of PAR-2 induced vasodilation of meningeal arteries through the release of CGRP and nitric oxide (NO) from the sensory nerves and the vascular endothelium, respectively. Moreover, activation of PAR-2 was observed to sensitize the capsaicin-mediated meningeal sensory neurogenic vasodilatory response possibly via interaction with signal

transduction pathways involved in the activation of TRPV1 receptors. Inflammation-induced sensitization of meningeal nociceptors appears to be a major contributor of the development of head pain. It is suggested that activation and modulation by PAR-2 of neurogenic sensory vascular responses and meningeal C-fiber nociceptor function may bear of significance as regards the pathomechanism of headaches.

Neuropathic alterations associated with diabetes affect cephalic pain mechanisms and vascular responses mediated by sensory nerves. Therefore, we studied the function of dural nociceptors in rats with streptozotocin-induced experimental diabetes mellitus. The results indicated impairments of meningeal vasodilatory mechanisms involving peptidergic capsaicin-sensitive nociceptors, whereas endothelium-dependent vascular responses seemed to be preserved. It has been concluded that limited removal of inflammatory mediators and/or tissue metabolites may contribute to the enhanced incidence of headaches in diabetics.

In conclusion, the present study furnished further firm experimental support for the notion that capsaicin-sensitive trigeminal afferents play a prominent role in meningeal nociceptor function and vascular reactions and contribute significantly to the pathomechanisms of headaches.

INTRODUCTION

1. Meningeal nociception

1.1. Innervation of the dura mater encephali

1.1.1. The dura mater encephali

The brain and the spinal cord are enveloped by three membranes (meninges): the pia mater, the arachnoid mater and the most external of the meninges, the dura mater. The dura mater is a thick sheet of dense fibrous connective tissue separated from the arachnoid by the very narrow subdural space. The cerebral and the spinal dura mater are continuous with each other at the foramen magnum (238). The dura mater encephali is composed of two layers, an

inner (meningeal) and an outer (endosteal), but these are united except where they separate to enclose the venous sinuses draining blood from the brain.

The principal blood supply of the dura mater encephali comes through the middle meningeal artery (MMA), a branch of the maxillary artery. There are also meningeal branches of the ophthalmic, anterior ethmoidal and internal carotid arteries and the accessory meningeal artery. All these arteries lie, like the venous sinuses, between the two layers of the dura mater (166).

Early neurosurgical studies have indicated that the human dura mater is sensitive to painful stimuli along the middle meningeal arteries and dural sinuses at the sites where cerebral veins enter these sinuses (74;193;198). Based on his intraoperative observations, the neurosurgeon Penfield pointed to the significance of the pain-sensitive intracranial structures in relation to the mechanism of headache (192). In contrast, the arteries within the brain tissue are quite insensitive to ordinary mechanical stimulation, only the largest cerebral arteries composing the circle of Willis seem to be sensitive to mechanical stimuli (193). Therefore, the sensory innervation of the meningeal vasculature continues to be a major focus of the study of meningeal nociception and pathophysiology of headaches.

1.1.2. Anatomy of the meningeal innervation

The first description of the innervation of the dura mater was done by Arnold in 1851, who described “nervi tentorii” as branches of the trigeminal nerve (9). Later, a high number of morphological observations have established that the dura mater encephali is richly innervated by nerve fibers (72;136;193). Meningeal nerves originate mainly from two sources: the ipsilateral trigeminal (Gasserian) ganglion (TG) and some of the upper cervical ganglia (C1-3) give rise to sensory fibers, while sympathetic fibers take their origin predominantly from the ipsilateral superior cervical ganglion. In addition to the sensory and the sympathetic nerves, a less prominent parasympathetic innervation originating from the ipsilateral otic and sphenopalatine ganglia was also described.

All the three divisions of the TG- ophthalmic, maxillary and mandibular- participate in the innervation of the dura mater (Fig.1). Most of the anterior part of the dura mater is innervated by the tentorial nerve (“nervus tentorius”), a branch of the ophthalmic division,

while in the medial fossa the spinosal nerve ("nervus spinosus"), the meningeal branch of the mandibular division and the medial meningeal nerve ("nervus meningeus medius"), a ramus of the maxillary division innervate the dura mater. The posterior part of the dura mater is mostly supplied by ascending meningeal branches of the cervical nerves.

Anatomical neuronal tract tracing and denervation studies have revealed a similar pattern of the innervation and blood supply of the meninges in man and animals. These experiments have demonstrated the existence of the trigeminal (151;165;211), sympathetic (129;227) and parasympathetic innervation in the rat, cat, monkey and the guinea pig dura mater (6;68;130;227). Hence, the dura mater of these laboratory animals may serve as useful models to investigate the mechanisms of meningeal nociceptive functions and headache in humans.

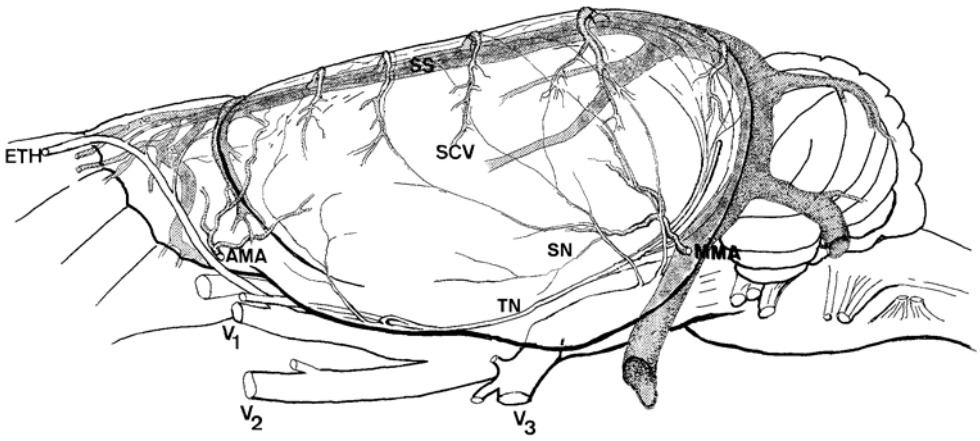


Fig. 1 Topography of innervation and blood supply of the dura mater encephali of the rat. Ophthalmic nerve (V3), maxillary nerve (V2), mandibular nerve (V3), ethmoidal nerve (ETH), anterior meningeal artery (AMA), middle meningeal artery (MMA), sagittal sinus (SS), superior cerebral veins (SCV), tentorial nerve (TN), spinosal nerve (SN) (7).

Electron microscopic investigations have revealed that the dura mater is richly innervated by myelinated and unmyelinated axons, which terminate in two locations: around the meningeal vessels or far from blood vessels in the connective tissue (7). Quantitative anatomical data showed that the majority of meningeal axons are unmyelinated and a large proportion of these are autonomic, only ~20% of the unmyelinated axons are sensory (7;77). Further, morphometric analysis of meningeal nerves disclosed myelinated axons with

relatively large diameter (>5 μm) that could be classified as A-beta fibers (216). Consistent with electron microscopical observations, electrophysiological studies confirmed that fast A-, slow A- and C- fiber populations exist in the dura mater encephali (147;148;205;215).

1.1.3. Neurochemical features of the meningeal nerves

The majority of unmyelinated nerve fibers innervating the rat dura mater terminate as free nerve endings (7). However, there are no morphological criteria for discriminating sensory nerve endings in the dura mater from sympathetic and parasympathetic nerve fibers. According to the less characteristic morphological features of free nerve terminals, the expression pattern of neurotransmitters and neuropeptides adds more knowledge to their functional properties.

Immunohistochemical investigations revealed that dural nerve fibers exhibit considerable neurochemical diversity and contain a variety of neuropeptides or neurotransmitters. Meningeal nerve fibers immunoreactive (IR) to antibodies for substance P (SP), CGRP and neurokinin A are thought to belong to the sensory system, while nerve fibers immunopositive for neuropeptide Y (NPY) are probably sympathetic, and those positive for vasoactive intestinal polypeptide (VIP) are probably of parasympathetic origin.

- Immunohistological studies detected that nerve fibers showing SP- and CGRP-like immunopositivity are widely distributed over the whole dura mater and form dense network around the large meningeal arteries and less dense network in dural connective tissue (221). In the dura mater of the rat, CGRP-like IR nerve fibers (~20% of axons), most of which have small diameter, are more abundant than SP-positive nerve fibers (<10% of axons) in the dura mater encephali (128). According to detailed morphological studies, most of these peptidergic nerve fibers cross the connective tissue and terminate as free nerve endings far away from meningeal blood vessels (167).

Autonomic nerves are much more intimately associated with walls of the meningeal arteries:

- NPY-positive fibers, which are extremely abundant in all areas of the dura mater encephali of the rat, show a vessel-dependent course along the meningeal arteries (128;232). Removal of the superior cervical ganglion, as well as chemical sympathectomy, markedly reduced the number of visible NPY fibers in the dura mater

(64;169). These results confirmed that NPY-containing meningeal nerve fibers derived from the sympathetic system.

- Previous histochemical studies detected acetylcholinesterase-containing nerve fibers in close relation with the main meningeal blood vessels and in the meningeal tissue (6;68). Chemical sympathectomy did not seem to interfere with the pattern of the dural cholinergic innervation indicating its parasympathetic origin. According to immunohistochemical data, the overall density of VIP-IR parasympathetic nerve fibers is markedly less than that for CGRP, SP, or NPY (128).

In 1998, Edvinsson and colleagues confirmed these observations also in humans (67). They provided immunohistological evidence for the presence of SP-, CGRP-, neuropeptide K-, peptide histidin-methionoine-, VIP-, and numerous NPY-containing nerve fibers in the human dura mater.

Taken together, these immunohistochemical findings disclosed a rich innervation of the dura mater by peptidergic nerves, the majority of which are closely associated with blood vessels, indicating that they are vasomotor in nature. These studies corroborated and extended previous investigations suggesting that the rich innervation of the dura mater may bear of importance for the pathogenesis of headaches.

1.1.4. Meningeal nociceptors

Electronmicroscopic observations described meningeal innervation as consisting of mostly unmyelinated C-fibers and thin myelinated axons (>1.5–3 μm in diameter) (7;75). In addition, the dura mater contains a substantial number of large myelinated fibers (>5 μm in diameter) (7;77;216). Meningeal thin A- and C-fibers are suspected to fulfill nociceptor functions, while dural large A-fiber afferents have been suggested to be responsible for mechanosensitivity, as in other tissues. However, the fact that pain is the only sensation that can be evoked by stimulation of the intracranial meninges, regardless of the nature of the stimuli (198) determines a substantial role of polymodal nociceptive primary afferents in the dura mater encephali.

Besides innervating blood vessels, many nerve fibers run freely within the connective tissue of the dura mater (7;77). Some of these axons terminate in close contact to collagenous fibers as Ruffini-like receptors in rat dura mater encephali (7). Considering their location and ultrastructure, Ruffini-like terminals have been discussed as slowly adapting mechanoreceptors which detect intracranial tensile forces (77). However, the majority of afferents supplying the dura mater are unencapsulated „free nerve endings". The terminals associated with the wall of the blood vessels have been proposed to monitor the chemical composition of the blood, while other C-fiber terminals have been suspected to fulfill nociceptor functions. However, the possible physiological significance of afferent terminals located in the connective tissue has remained unresolved. According to Strassman, these nerve endings which terminate in nonvascular regions of the dura mater might represent silent receptors that may become responsive under pathological conditions, such as inflammation (147;215). This hypothesis is based on the experimental findings of meningeal nociceptive sensitization.

1.1.4.1.Sensitization of meningeal nociceptive afferents

Electrophysiological studies identified a population of neurons in the spinal trigeminal nucleus (STN) that respond to electrical stimulation of the dura mater (52;108;213). STN neurons sensitive to dural stimulation have been classified as nociceptive-specific neurons (activated only by intense pressure), wide dynamic range neurons (activated by noxious pressure and also by innocuous stimuli) or low threshold mechanoreceptive cells (activated only by innocuous stimuli) (54). The results of correlative morphometric and immunohistochemical studies supported the existence of trigeminal nociceptive neurons, which innervate the dura mater and project to the STN. Trigeminovascular primary afferent neurons are small (165;211) and contain SP- and/or CGRP (184;221), similar to nociceptive sensory ganglion neurons innervating other tissues (94;100;133).

Further electrophysiological studies have provided further information on the properties of meningeal afferents through the study of the nature of stimuli, which activate meningeal afferents. STN neurons increase their activity in response to topical applications of inflammatory mediators such as bradykinin, serotonin or acidic solutions onto the dura mater.

The application of these agents resulted in increased activity of central trigeminal neurons, which were also sensitive to mechanical stimuli (53;246;247). These findings provided evidence that meningeal afferents may be activated by noxious chemical stimuli through polymodal nociceptive primary meningeal afferents (24).

Some types of headaches share certain clinical features such as allodynia– pain induced by innoxious stimuli– that suggest an exaggerated intracranial mechanosensitivity (21;31). Sensation of nociceptors associated with severe headaches has been postulated to result from mechanical or chemical stimulation of pain-sensitive intracranial structures. Indeed, topical application of inflammatory and algesic agents onto the surface of the dura mater enhanced the sensitivity of most mechanosensitive STN neurons to subsequent mechanical stimulation (30;215). Besides, neurons characterized initially as mechanically insensitive units developed long-lasting mechanosensitivity after exposure of the dural surface to a mixture of inflammatory agents (146). These findings indicated that sensitization may lead to intracranial hypersensitivity to dural mechanical stimulation either due to a decrease in threshold and/or an increase in the magnitude of suprathreshold responses (146).

Experimental data revealed that the mechanical threshold of the dural afferents to stimulation with von Frey hairs were mostly above physiological levels of intracranial pressure, which is generally below 2.5 kPa (134) suggesting that the meningeal sensory nerves become activated only under pathophysiological conditions (146;148). However, the mechanical threshold of dural C-fibers tested with von Frey monofilaments was low (215) as compared to cutaneous nociceptors (28). Therefore, elevations of intracranial pressure which might occur during pathological conditions (>10 kPa reached in experimental meningitis) (99), may result in the recruitment of a large proportion of mechanosensitive afferents (148). Consequently, abnormal intracranial pressure associated with inflammation-induced neuronal hyperexcitability may lead to an intensification of pain (214;215). This hypothesis is also strenghtened by clinical observations showing that normally innocuous stimuli produce an increase in intracranial pressure or altered haemodynamics which can evoke dramatically increased head pain during headache attacks (21). Moreover, meningeal arteries and veins with their extensive capillary plexus, apart from their nutritive function, are proposed to take part in the regulation of the intracranial pressure (140).

1.2. Mechanism of primary headaches

1.2.1. The trigeminovascular system

The cranial blood vessels are largely innervated by fibers of the Vth (trigeminal) cranial nerve. This system is known as the trigeminovascular system (163). Immunohistochemical data revealed that CGRP- and SP-containing primary sensory neurons comprise large populations within the TG, which is the main source of the afferent fibers supplying the dura mater encephali (144;196). In line with these data, chemical stimulation of the dura mater encephali can evoke marked releases of CGRP and SP from meningeal sensory nerves (81). These findings support the view that CGRP and SP are important transmitter agents in the trigeminovascular system. Further, both CGRP and SP have potent vasodilator effects on meningeal blood vessels (240). In addition, topical administration of SP has been shown to produce marked plasma extravasation in the dura mater of the rat and the guinea pig (160;185).

Antidromic electrical stimulation of trigeminal afferent fibers results in an increase in blood flow of both meningeal and cerebral blood vessels, which is mediated by CGRP (84;168). Similarly, electrical stimulation of the large dural venous sinuses leads to large increases in cerebral blood flow (CBF) (89). In turn, stimulation of the cerebral cortex was followed by a transient increase in CBF and induced a long-lasting increase in the blood flow of the meningeal arteries (23). This phenomenon is associated with activation of central trigeminal neurons located in the ipsilateral STN. Mapping of the activated trigeminal neurons in the STN indicated that stimulation of the cerebral cortex may result in the activation of meningeal axons and central trigeminal projections probably associated with nociception (23). In support of this suggestion, retrograde tracing studies revealed that a significant population of TG neurons that innervate the large cerebral arteries has collateral projections to the MMA, as well as the surrounding dura mater (183). Taken together, these observations suggest that activation of trigeminovascular afferents can evoke a series of meningeal, cortical and brainstem events, which are consistent with the development of headaches.

1.2.2. The neuronal versus vascular hypothesis of primary headaches

Headache as one of the most common medical problems has become one of the best classified and defined pathology of neurologic disorders. Primary headaches, such as migraine, tension-type headache, or cluster headache can develop without being associated with a clearly defined disease process.

- *Migraine* is characterized by recurrent, pulsating head pain that is throbbing and frequently unilateral. It can be divided into two major subtypes: migraine without aura that is a clinical syndrome characterized by headache with specific features and associated syndromes, and migraine with aura, which is characterized also by visual, sensory, or motor symptoms (aura): nausea, vomiting, sensitivity to light, sound, or movement (1;29;85;187) that sometimes precede or accompany the headache.
- *Tension-type headache* is characterized by moderate constant, nonpulsatile pain that is bilateral in distribution, pressing or tightening in quality, and unaccompanied by major systemic disturbances or neurological signs (88).
- *Cluster headache* is characterized by recurrent short-lasting attacks of unilateral periorbital steady pain accompanied by ipsilateral autonomic signs (lacrimation, nasal congestion, ptosis, miosis, lid edema, redness of the eye) (88).

At the present time, the lack of information on the primary cause of headache presents a significant gap in our understanding of the aetiology of headache disorders. To explain the origin of head pain, two hypotheses - the vascular versus the neuronal - have been put forward.

The vascular hypothesis of headache is based on the observation that changes in the diameter of intracranial blood vessels or gross changes in cortical perfusion triggers pain (172). Clinical data indicate blood flow increases in large intracranial arteries during headache attacks (49;186). It has been concluded that activation of perivascular meningeal sensory nerves results from streching produced by vasodilation during migraine attacks (90;242).

The following clinical observations support the hypothesis:

- Sensitization of central trigeminal neurons occurs after dilation of meningeal blood vessels (47).
- The potent vasodilator NO donor, nitroglycerine (NTG) can provoke headache in both healthy subjects and migraineurs (113;206;223).
- Variation in vessel diameter have been shown to be strictly associated with headache attack as assessed by functional brain imaging (15;249).
- The most effective antimigraine drugs, such as ergotamine, dihydroergotamine and the triptans have been shown to be potent constrictors of large intracranial vessels (76;121;239).

However, more recent data show that NTG, CGRP and the clinically effective antimigraine drugs, the triptans, originally known as vasoactive substances, have additional actions at several levels of the central nervous system (46;138;139).

The neurogenic theory implies that migraine is a primary neurological disturbance with secondary vasomotor changes (20). It was originally based on the experimentally evoked phenomenon of cortical spreading depression (CSD) of Leao (143). CSD manifests as a slowly propagating wave of intense neuronal burst activity, followed by depression of spontaneous and evoked activity (143). Since a similar change in neuronal activity can be observed during migraine aura, CSD has been suspected to be the underlying pathophysiological mechanism of migraine with aura. Besides changes in neuronal activity, alterations in cortical blood flow also occur during CSD (142). These changes in CBF are similar to those observed in cortical perfusion during migraine attacks as assessed by functional brain imaging. Further, the velocitiy of CSD spreading equals to the temporal characteristics of visual symptoms associated with migraine aura. Moreover, functional imaging studies confirmed that the visual aura is generated by CSD within the occipital lobe (33;141). Although CSD may explain the pathogenesis of the aura phase of migraine, the possible mechanisms whereby CSD may cause activation of the trigeminal system and consequently headache, are not well understood. The connection between CSD and the initiation of headache can be explained by chemical messengers, which are released during CSD. These substances may induce perivascular inflammation and consequent activation or sensitization of perivascular nociceptors (159). Besides, released substances may activate the nociceptors directly and cause headache. Consideringly, the phenomenon of CSD suggests

that vascular responses are apparently secondary to local changes in the neuronal activity (142).

The neurogenic theory is supported by:

- observation that many triggers provoke attacks with such a short latency that only some kind of neural mechanisms can explain the triggering (21)
- advances in functional brain imaging, which suggest that vascular changes are not the primary cause for head pain (163). Human neuroimaging studies during migraine attacks confirmed the activation of a trigeminal brainstem area that may serve as a specific migraine generator (3;13).

To place emphasis on the interaction between nerves and vessels, a more integrated *„neurovascular theory"* has been suggested involving both vascular and neural components. A current concept on the pathogenesis of primary headaches holds the integrated neurovascular theory as activation of both vascular and neural components can produce activation of the trigeminovascular afferent system producing head pain.

1.2.3. Animal models in the study of the pathomechanisms of headache

The activation of trigeminal afferent nerves and vasodilation of intracranial blood vessels are the principal features of headache disorders. Thus, modeling these symptoms in laboratory animals may serve as a useful approach in our understanding of primary headache syndromes.

- In vitro, contraction or relaxation can be measured on isolated segments of intracranial blood vessels. This model is well suited to pharmacological and physiological investigations and may be applied for human preparations, too (93;224).
- In vivo, through a thin cranial window , the diameter of cranial, dural and pial arteries may be directly measured with intravital microscopy (92). Similarly, blood flow studies using laser Doppler flowmetry directly record changes in perfusion of intracranial blood vessels as a sign of trigeminovascular activation (60;137). These models are suitable for studying the effect of stimulation of the trigeminovascular system on either the cortical or the meningeal blood flow (86;240).

- The study of c-fos gene expression, which is a salient sign of neuronal activation, provides a reliable method for mapping of functional trigeminovascular pathways. Moreover, it can be used as a marker for nociceptor activation (175). Following electrical, chemical and mechanical stimulation of the meningeal arteries, increases in c-fos expression can be detected at distinct levels of the central nervous system (17;108;126).
- CSD has been suggested to represent a correlate of the migraine aura (143). Experimentally evoked CSD has been shown to induce trigeminal neuronal activation, meningeal plasma extravasation and changes in intracranial blood flow (62;234).
- Genetic factors, which may be involved in the pathophysiology of some types of migraines, have recently been determined. Hence, transgenic mouse migraine models have become available. These molecular models are based on a P/Q-type Ca^{2+}current shift resulting from a mutant gene, which encodes a voltage-gated Ca^{2+} channel subunit (228).

Since an increased level of CGRP was observed in the jugular venous blood during headache attacks (66;87), measuring the level of CGRP proved to be a useful tool for studying the activation of the trigeminovascular system (149). The role of meningeal CGRP-containing sensory nerves in the pathomechanism of headaches is also supported by the findings that a non-peptide CGRP blocker, BIBN4096BS, an effective antimigraine drug, which does not cross the blood brain barrier, is very effective in preventing both CGRP-induced and electrically-evoked vasodilation of meningeal vessels (195).

2. Capsaicin-sensitive nociceptive C-fibers in the dura mater encephali

2.1. Characterization of capsaicin-sensitive nociceptive primary sensory neurons

In his pioneering studies N. Jancsó demonstrated that capsaicin, the pungent ingredient of chili peppers produced inflammation via a mechanism which is dependent on the presence of intact sensory nerves (118;120). It has also been described that rats can be desensitized against chemically induced pain and inflammation with repeated incremental doses of capsaicin (119). N. Jancsó pointed out that capsaicin-sensitive sensory nerves possess a dual function: on the one hand, they transmit noxious impulses elicited by chemical irritants

towards the central nervous system (afferent, nociceptive function) and, on the other hand, in their stimulated state they elicit local vascular reactions through the release of a "neurohumor" from their terminals (efferent, local regulatory function) (118;120). The discovery of the selective neurotoxic action of capsaicin by G. Jancsó has permitted the direct morphological identification and the neurochemical characterization of capsaicin-sensitive primary sensory neurons (115;116). Morphologically, capsaicin/chemo-sensitive primary afferent neurons correspond to small sensory ganglion cells of dorsal root and cranial nerve ganglia, which bear mostly unmyelinated, C-fiber axons. The peripheral terminals of these neurons innervate most tissues of the body. Capsaicin-sensitive nociceptive neurons/axons can be reliably visualized in different tissues by making use of the neurotoxic action of capsaicin at the electron microscopic level (59;131;203).

Using the neonatally capsaicin-pretreated rat as a model of nociceptive C-fiber deficient animal, many new facets of the functions of capsaicin-sensitive neurons have been revealed. Importantly, functional studies on rats treated neonatally with capsaicin showed that capsaicin-sensitive primary afferent neurons are also involved in the transmission of nociceptive stimuli evoked by noxious heat (104;114;174). Neurochemical and immunohistochemical studies revealed that capsaicin-sensitive primary afferent neurons contain multiple neuropeptides, such as SP, neurokinin A, somatostatin, cholecystokinin and CGRP (79;100;102;145;177;209). Capsaicin-sensitive primary afferent neurons participate, *inter alia*, in the mediation of nociceptive impulses elicited by irritant chemicals and noxious heat, neurogenic inflammatory processes, involving neurogenic sensory vascular responses and plasma extravasation, smooth muscle contractile responses, glandular secretion and immune reactions (82;102;114;117;155;173). There is ample evidence that the sensory-efferent, local regulatory functions of capsaicin-sensitive afferents are mediated by neuropeptides released from the axonal endings stimulated either orthodromically by e.g. chemical irritants or antidromically by electrical stimulation or through axon reflexes (83;157;158).

The relative selectivity of capsaicin with regard to the morphological characteristics of target neurons and nocifensive behavior suggested the presence of a specific receptor associated with a specific population of primary afferents which might be involved in the transmission of pain (35). In 1997, Caterina and his colleagues have succeeded in cloning the

„capsaicin receptor" as a transducer of painful thermal stimuli (37). The cloned capsaicin-receptor named as the TRPV1 receptor is structurally related to members of the transient receptor potential family and serves as a calcium-permeable non-specific cation channel. TRPV1 is preferentially expressed by a population of primary sensory neurons and-besides exogeneous agents, such as capsaicin- is gated by noxious heat, protons and endogenous ligands such as anandamide, N-oleoyldopamine and lipoxygenase products of arachidonic acid (111;225;253). TRPV1 knockout mice showed impaired detection of painful heat and chemically induced thermal hyperalgesia confirming that TRPV1 is essential for selective modalities of pain sensation and for tissue injury-induced thermal hyperalgesia (36;51).

2.2. Capsaicin-sensitive nerves in the dura mater encephali

Experimental results of Moskowitz and colleagues showed that administration of capsaicin-induced plasma extravasation in the dura mater encephali of the rat and guinea pig (160). Further experimental data showed that the majority of STN neurons innervating the dura mater and nociceptive-specific thalamic neurons with receptive fields around the MMA respond to application of capsaicin onto the dural surface (205;215;247). Therefore, capsaicin-sensitive C-fiber afferents are thought to constitute a significant nociceptive input from the meninges. This suggestion is supported by clinical observations demonstrating the phenomenon of capsaicin-induced experimental head pain (163;164).

Using an experimental neuroanatomical technique making use of the selective neurotoxic action of capsaicin (131), capsaicin-sensitive afferent axons have been directly identified in the rat dura mater under the electron microscope (59). In the same study, a marked vasodilatory action of capsaicin has been demonstrated in the dura mater further disclosing the functional significance of capsaicin-sensitive afferent nerves in this vascular bed. Since in other tissues the capsaicin-sensitive afferent nerves are nociceptors, these nerves are likely candidates for sensory fibers which transmit nociceptive information also from the dura mater (214).

2.3. Capsaicin-sensitive nociceptive C-fibers under pathological conditions

2.3.1. Neurogenic inflammation and pain

Capsaicin-sensitive nociceptive sensory afferents possess a dual function: first, they are involved in the transmission of nociceptive information and second, they participate in the initiation of local regulatory responses, such as increases in blood flow and vascular permeability through the axonal release of vasoactive neuropeptides. These phenomena are known as *neurogenic inflammation,* a term introduced by N. Jancsó (118). Jancsó also demonstrated that pretreatment with capsaicin suppressed not only the sensation of pain, but prevented the neurogenic inflammatory response as well (119). The findings disclosed that the integrity of capsaicin-sensitive afferents is of pivotal importance for the development of neurogenic inflammation.

The changes in vascular permeability and other vascular phenomena of inflammation, such as vasodilation were considered to be mediated by endogenous vasoactive substances, released upon noxious stimulation (237). It has been reported that the potent vasoactive peptides such as SP and CGRP are released from peripheral sensory nerve endings in response to antidromic nerve stimulation, and are involved in inflammatory processes and pain sensation (11;14;194). It has also been demonstrated that chemical irritants which stimulate sensory nerve endings induce arteriolar vasodilation and plasma extravasation via local release of sensory neuropeptides (19).

Hence, by inference, the sensory neuropeptides CGRP and SP have been considered to be the major contributors of capsaicin-induced inflammatory reactions. This suggestion has been supported by experimental findings which demonstrated that capsaicin activates the TRPV1 receptor of sensory nerve terminals, triggering the release of SP and CGRP (18;235). Consideringly, release of CGRP from sensory nerves is regarded as a reliable approach to characterize the nociceptive function of peptidergic afferent nerves (61;135).

2.3.1.1.Involvement of capsaicin-sensitive sensory nerves in meningeal neurogenic inflammation

The dura mater is richly innervated with peptidergic nerves including paravascular axons and contains an abundant population of mast cells (77;136). The sensory nerves of the

dura mater are of trigeminal origin and participate in pathophysiological phenomena. Indeed, in the rodent dura mater, stimulation of the Gasserian ganglion resulted in marked dilation of meningeal arteries, increase in vascular permeability and degranulation of dural mast cells, i.e. characteristic signs of neurogenic inflammation (58;241).

It has been proposed that activation of trigeminal sensory nerves by capsaicin could initiate neurogenic inflammatory processes in the dura mater encephali. This hypothesis was based mainly on previous findings of N. Jancsó showing that stimulation of trigeminal afferents by capsaicin elicited plasma leakage in extracranial tissues through capsaicin-sensitive afferents (119). The findings of Moskowitz and others showing dural plasma extravasation following intravenous capsaicin provided direct evidence for the involvement of capsaicin-sensitive nerves in meningeal inflammatory processes (160).

It is generally recognized that vasoactive neuropeptides contained in unmyelinated C-fibers can be released from perivascular sensory axons by antidromic stimulation of sensory nerves and mediate vasodilation and plasma extravasation in the periphery (11;14;194). Several lines of independent experimental and clinical observations supported the notion that neuropeptides are intimately involved in the mechanisms of meningeal vascular reactions.

- Activation of the trigeminovascular system resulted in the release of SP and CGRP from meningeal afferents (61;81;248).
- Histological studies provided evidence for a role of SP in the mechanisms of electrically evoked meningeal plasma extravasation (45;160;185).
- CGRP was shown to be the major peptide producing increases in meningeal blood flow (137;168).
- Both CGRP and SP evoke release of histamine (HA) from mast cells which may promote plasma extravasation and consequent inflammation in the rat dura mater (189).
- Bacterial meningitis causes depletion of SP and CGRP from meningeal afferents (99)
- Clinical observations showed elevated serum levels of CGRP during headache attacks (66;87).

- In the light of these findings it has been suggested that CGRP and SP serve as mediators of pain and inflammation associated with cluster- and migraine headaches (65) through the activation of peptidergic capsaicin-sensitive trigeminal afferents.

2.3.2. Interaction of the TRPV1 receptor with the proteinase-activated receptor-2

2.3.2.1. The proteinase-activated receptor family

In inflammed tissues, proteolytic enzymes have been assumed to be involved in "pain-production" (16). However, the mechanisms, by which these proteases may influence inflammatory processes remained unidentified. Lollar and Owen suggested that anchored cell surface factors might serve as targets of tissue proteases such as thrombin (153). In 1991, two groups independently isolated a new member of the G-protein-coupled seven transmembrane domain receptor family, the proteinase-activated receptor (PAR); a new signaling mechanism involving a cleavage of the amino-terminal extension of the thrombin receptor was also unraveled (197;233). Further studies revealed additional members of the proteinase-activated receptors and these newly defined molecules were named after the order of finding: four types of PARs have been identified (PAR-1-4) (112;123;181;245). The unique way of receptor activation gives the link between members of PAR family. Vu and colleagues showed this novel mechanism in which thrombin cleaves its receptor's amino-terminal extension to create a new receptor amino terminus that functions as a tethered ligand and activates the receptor initiating a distinct intracellular signaling mechanism (233). Subsequent findings confirmed this mechanism as a shared identical signaling way for all members of the PAR family (56;150;204).

Under experimental conditions, besides proteolytical cleavage of the receptors, PARs can be activated by attached synthetic agonist peptides mimicking the new amino terminus created by cleavage (233). The activation, which is mediated by soluble peptides, showed reversibility, strong excitability and more specificity against irreversible cleavage by the physiological activation. Activation of PARs induces a multiple intracellular cascade mechanism- including activation of protein kinase C (PKC), phosphoinositide-specific phospholipase C (PLC) (25;95;190), inositol triphosphate formation (26) or inhibition of

adenylate cyclase (25)- and consequently affect various cellular processes via calcium mobilization and enzyme phosphorylation.

PARs are expressed by human platelets, fibroblasts, monocytes, smooth muscle cells, endothelial cells and neurons. They are presented in a high variety of tissues (101;112;229;245;251). Besides thrombin, which is the most potent agonist of PAR-1,-3,-4 but not PAR-2, several proteases are also capable of activating PARs: coagulation factors, pancreatic and extrapancreatic trypsin, mast cell proteases, leukocyte proteases, cell-surface proteases and non-mammalian proteases (188).

Both PARs and serine proteases, which are capable of activating PARs are widely expressed in the nervous system (57;207;236). Consequently, these serine proteases have been proposed to mediate neuronal processes at least partly via PARs both in the peripheral and the central nervous system (55;179;220). PARs have been considered to mediate cell proliferation and migration, apoptosis and other pathophysiological processes. However, most neuronal signaling mechanisms, which might be consequent to PARs activation, are so far uncleared in details. There are investigations, which demonstrated a contribution of PARs to pro-inflammatory mechanisms in the periphery (40;80;176), although protective effects of PARs have also been revealed by some authors (34;41;231).

2.3.2.2.Involvement of PAR-2 in neurogenic inflammation

PAR-1, PAR-3 and PAR-4 serve as potential thrombin receptors, while PAR-2 can not be activated by thrombin, but another serine protease: trypsin. PAR-2 was also cloned in human and rat as a naturally occurring, widely expressed trypsin receptor in a wide variety of tissues (22;48;180). PAR-2 is highly expressed in the gastrointestinal tract on enterocytes, where the released luminal trypsin is capable of activating the receptor (132). However, PAR-2 is also expressed in tissues that are not exposed to trypsin, including the nervous system and the skin, where PAR-2 may serve as targets for other proteases distinct from trypsin. Considering that mast cells are present in the close vicinity of peripheral nerves, mast cell proteases have been postulated to activate neuronal PAR-2. The mast cell-derived tryptase has been proved to be a potent activator of PAR-2 expressed by human endothelial cells, keratinocytes and isolated myocytes (42;170).

PAR-2 expression is apparently upregulated by pathological states including inflammatory reactions, neurodegenerative disorders or abnormal cell proliferation (2;171;178;182). Inflammatory/immune cells such as mast cells and neutrophils serve as source of PAR-2 activating proteases, suggesting involvement of PAR-2 in inflammatory reactions. Trypsin and mast cell tryptase have proinflammatory effects in different tissues by promoting NO-dependent vasodilation (69;200;202) and plasma extravasation (96) presumably through activation of PAR-2.

PAR-2 is expressed on a subset of primary afferent neurons, which contain CGRP and SP (212) suggesting that PAR-2 may participate in neurogenic inflammatory processes. It has been shown that activation of PAR-2 induced vasodilation and increase in vascular permeability by a neurogenic mechanism via activation of sensory nerves and subsequent release of neuropeptides (neurogenic inflammation) (43;219). It has been hypothesized that PAR-2 may play a role in the sensitization of afferent nerve endings and contribute to the pathogenesis of pain under inflammatory conditions. PAR-2 has been shown to be coexpressed with TRPV1 in dorsal root ganglion neurons (50) suggesting a functional interaction between the two receptors. In support of this, Dai reported that activation of PAR-2 reduced temperature threshold for TRPV1 activation below the body temperature (50). Moreover, PAR-2 agonists induce thermal hyperalgesia, which may result from an enhancement of TRPV1 receptor activity through PAR-2-triggered intracellular signals (5). Therefore, PAR-2 activation might trigger the sensation of pain through sensitization of the TRPV1 receptor (5). Although, PAR-2 has been shown to coexist with TRPV1 in trigeminal ganglion neurons (127;191), there had been virtually no studies demonstrating a direct effect of PAR-2 activation on capsaicin-sensitive nerves with regard the meninges.

2.3.3. Diabetic neuropathy

The metabolic disease, diabetes mellitus affects nearly every organ including the nervous system. Impairments of peripheral sensory nerves results in sensory abnormalities, which may lead to the development of diabetic neuropathy. Painful diabetic neuropathy is associated with one or more kinds of stimulus-evoked pain including sensory loss, increased responsiveness to noxious stimuli (*hyperalgesia*), hyperresponsiveness to normally

innocuous tactile stimuli (*allodynia*), and persistent pain (44;244). Importantly, it has been known for a long time that diabetic patients suffer more frequently from headaches than non-diabetics, regardless the type of diabetes (161;199;210).

In diabetic patients, peripheral nerve biopsies showed morphological evidence of degeneration and demyelination of nerve fibers of all diameters (8;152). The underlying mechanism of the progressive degeneration of nerve fibers in diabetic neuropathy is still uncleared. However, degenerative processes of either myelinated or unmyelinated afferent fibers may be accounted for by the irreversible impairment of sensory functions, which accompany diabetes. Consistent with anatomical data, hyperosmolarity of tissue fluids, as one of the most common sign of diabetes mellitus, has an effect on the conduction velocity of both myelinated A- and unmyelinated C-fibers (162). Dysfunction of large diameter A-fibers may be responsible for certain symptoms of diabetic neuropathy, e.g. tactile allodynia (122), while hyperreactivity of damaged small diameter C-fibers, as assessed as an increase in C-fiber firing frequency in experimental diabetes, may result in diabetic neuropathic pain (27;38). However, the exact mechanism of persistent pain in diabetic patients is unidentified yet. Various bioassays provided direct evidence for decreased expressions of some sensory neuron-specific transmitters such as SP and CGRP in experimental diabetes (226;252). In addition, decreased cell-specific expression of the TRPV1 receptor (105;106) and reduction in the number of TRPV1 expressing peripheral nerve fibers were observed in diabetic skin (70). These findings suggest a dysfunction of capsaicin-sensitive nociceptive C-fibers in diabetic neuropathy (105;106). Available experimental evidence indicates that the decreased expression of TRPV1, as well as the structural and functional alterations of peripheral sensory fibers associated with diabetes may result from a deficiency of neurotrophic factors supplying peptidergic nociceptors (73;97).

3. The aim of the study

The experiments were initiated in an attempt to reveal the possible physiological and pathophysiological significance of capsaicin-sensitive trigeminovascular sensory nerves, which innervate the rat dura mater encephali.

We aimed to reveal the possible contribution of capsaicin-sensitive afferent nerves to neurogenic vascular reactions elicited through the activation of PARs, in particular PAR-2. In addition, we examined possible functional interactions between TRPV1 and PAR-2 receptors and sought for the possible morphological correlates of such interactions between these receptors.

To explore the effect of pathologies affecting the sensory nerves, we studied neurogenic vascular responses in the dura mater of rats with streptozotocin-induced experimental diabetes mellitus. Diabetes mellitus has been shown to affect sensory nerve functions in many organs but the effects on meningeal sensory neurogenic vascular reactions were not investigated yet.

MATERIALS AND METHODS

1. Measurement of capsaicin-sensitive neurogenic vascular reactions with laser Doppler flowmetry

1.1. Experimental animals and surgery

All experimental procedures were approved by the Ethical Committee for Animal Care of the University of Szeged. All efforts were made to minimize the number of animals used and their suffering. Adult male Wistar rats weighing 300-350 g were used in all experiments. Number of animals in each series of experiments was between 4-12.

Capsaicin-desensitization

In some experiments one group of animals was given subcutaneous (s.c.) injections of capsaicin (Sigma-Aldrich Chemie Gmbh, Germany) on three consecutive days at doses of 10, 20 and 100 mg/kg. Intact animals which received the solvent for capsaicin (6% ethanol and 8% Tween 80 in saline) served as controls.

Experimental diabetes mellitus

- Diabetes mellitus was induced by a single intravenous injection of streptozotocin (65 mg/kg, i.v., Sigma-Aldrich Chemie Gmbh, Germany). Rats treated with the solvent for streptozotocin (citrate-phosphate buffer, 0.1 M, pH 4) served as controls.

- One group of diabetic animals received daily injections of insulin (Lantus 1 IU/100 g body weight, s. c., Aventis Pharma Deutschland GmbH, Germany) from the third day after streptozotocin treatment.

The blood glucose levels of control, diabetic and insulin-treated diabetic animals used for the experiments were 102 ± 18, 384.5 ± 46 and 154 ± 38.9 mg/dl, respectively.

Two, 4 or 6 weeks after streptozotocin treatment and four days after completion of treatment with capsaicin, the animals were anaesthetized with an initial intraperitoneal (i.p.) dose of thiopentone (150 mg/kg, Thiopental, Biochemie GmbH, Austria). Additional doses of thiopentone (25 mg/kg, i.p.) served to hold the anaesthesia at a level at which noxious stimuli failed to elicit motor reflexes or changes in the systemic blood pressure. Systemic blood pressure was recorded with a pressure transducer connected to a catheter inserted into the right femoral artery. The body temperature of the animals was monitored with a thermoprobe inserted into the rectum and was held at 37-37.5 °C with a heating pad. The animals were tracheotomized and breathed spontaneously (59;137). For the measurement of meningeal blood flow, a cranial window was prepared according to Kurosawa (137). The head of the animal was fixed in a stereotaxic frame, the scalp was incised in the midline and the parietal bone was exposed on one side. A cranial window measuring 4x6 mm was drilled into the parietal bone to expose the underlying dura mater. To avoid thermal lesions, the bone was cooled with saline. At the end of the experiments, the animals were killed with an overdose of thiopentone (250 mg/kg, i.p.).

1.2. Drug administration

The cranial window was filled with synthetic interstitial fluid (SIF) containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM glucose and 10 mM Hepes (147;215). All drugs but capsaicin and capsazepine were dissolved in SIF and diluted immediately before use. A stock solution of capsaicin (32 mM) and capsazepine (1mM) was prepared with 6% ethanol and 8% Tween 80 in saline, and was further diluted with SIF to the required concentration. All drugs were purchased from Sigma-Aldrich Chemie Gmbh, Germany.

- Drug administration: 40 µl of the solution containing the drug was applied topically onto the surface of the dura mater. The solution was removed after 3 or 5 min in different series of experiments and the dura mater was washed repeatedly with SIF to allow the blood flow to return to the basal level. In some experiments, the effects of repeated applications were tested: the same concentrations of capsaicin or PAR-2 agonist peptide were applied three times, separated by wash-out periods.
- Pretreatment of the dura mater: receptor antagonist or NO synthase inhibitor was preapplied to the dura mater for 5 or 15 min- according to the results of preliminary experiments- in a concentration of 10^{-4} or 10^{-5} M with regard to the efficiency of the drugs.

Capsaicin-sensitive meningeal vascular responses:

- Capsaicin was tested at increasing concentrations (10^{-8}, 5×10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M). In the same animal, two to three different concentrations of capsaicin were tested.
- To determine the mechanisms involved in the capsaicin-induced changes in blood flow, the TRPV1 receptor antagonist capsazepine (10^{-5} M), the CGRP receptor antagonist $CGRP_{8-37}$ (10^{-5} M) or the nonspecific cation channel blocker, ruthenium red (RR, 10^{-5} M) was preapplied before the application of capsaicin (10^{-7} and 10^{-5} M) for 5 min.
- To test the effect of ablation of capsaicin-sensitive nerves on capsaicin-induced vascular reactions, the effect of capsaicin (10^{-7} and 10^{-5} M) was tested in capsaicin-desensitized animals.

Changes in capsaicin-sensitive meningeal vascular responses in experimental diabetes mellitus:

- Capsaicin (10^{-7} and 10^{-5} M)-induced meningeal blood flow changes were compared in control, diabetic and insulin-treated diabetic animals.

The contribution of PAR-2 activation to capsaicin-induced changes in blood flow:

- Trypsin (5×10^{-7}-10^{-5} M), as a naturally occurring activator of the PAR-2, and SLIGRL-NH_2 (10^{-6}-10^{-4} M), a selective PAR-2 agonist peptide were tested in control and capsaicin-desensitized animals.

- To study the involvement of PAR-2 activation on capsaicin-induced meningeal vasodilation, the effect of capsaicin (10^{-8} M) was tested before and after SLIGRL-NH_2 (10^{-6} M) application.
- To determine the mechanisms involved in the PAR-2 agonist peptide-induced changes in blood flow, $CGRP_{8-37}$ (10^{-5} M) and the NO synthase inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 10^{-4} M) were pre-applied to the dura mater before the administration of SLIGRL-NH_2 (10^{-6} M) for 5 and 15 min, respectively.

To investigate meningeal vascular reactivity, the vasodilatory effects of HA (10^{-5} M), CGRP (10^{-5} M) and acetylcholine (ACh, 10^{-4} M) were also studied in the experiments investigating the effects of PAR-2 activation and experimental diabetes mellitus on capsaicin-induced meningeal blood flow changes. The doses of HA, CGRP and ACh were chosen to produce responses that were similar in magnitude. All drugs were purchased from Sigma-Aldrich Chemie Gmbh, Germany.

1.3. Measurement of dural blood flow and evaluation of data

Measuring of blood flow is based on the phenomenon of Doppler-shift. Laser light generated by the optics enter the tissue and become scattered and absorbed by moving red blood cells and static tissue structures. The basic unit of measurement is the perfusion unit (PU), which depends on the actual number and velocity of cells flowing through a given volume of tissue during a given time period. The dural blood flow was measured with 1 mm diameter needle-type probes of a laser Doppler flowmeter (Perimed, Sweden). The probes were placed over branches of the medial meningeal artery lying distant from visible cortical blood vessels. Under these experimental conditions, the flow signal recorded from the cortical blood vessels is minimized (137). Meningeal blood flow, systemic blood pressure and body temperature were recorded simultaneously and stored on a computer through use of the Perisoft program (Perimed, Sweden). The mean blood flow measured during a 5-min period prior to drug application was regarded as the basal flow. Changes induced in the blood flow by the application of drugs were expressed as percentage changes relative to the basal flow (mean ± S.E.M.) calculated for the 3 or 5-min application period or separately for each minute

of application (n=4-8 for each value). Statistical analysis of the experimental data of blood flow measurements was performed by means of ANOVA followed by the Tukey test. A probability level of $P < 0.05$ was regarded as a statistically significant difference between groups.

2. Demonstration of capsaicin-sensitive meningeal afferents and trigeminal ganglion neurons by immunohistochemistry

2.1. The preparation of whole mount specimens of the rat dura mater

Control and diabetic rats were anaesthetized, then perfused transcardially with 150 ml saline and subsequently with 500 ml 4% paraformaldehyde in phosphate buffer (pH 7.4) and then were decapitated. After removal of the skin and muscles, the skull was divided into halves along midline and the cerebral hemispheres were removed. The parietal dura mater was cut out and postfixed in the same fixative for 1 hour. Preparations were then processed for TRPV1 immunohistochemistry and for TRPV1-CGRP or TRPV1-PAR-2 double immunostaining.

2.2. The preparation of tissue sections from the Gasserian ganglion

Control rats were anaesthetized, perfused and decapitated as described above. After removal of the skin and muscles, the skull was divided into halves along midline and the cerebral hemispheres were removed. The bilateral Gasserian ganglion were removed, postfixed in the same fixative for 1 hour and then cryoprotected in a phosphate-buffered 30% sucrose solution overnight at 4°C. The tissue was serially sectioned at 40 μm thickness, mounted on slides and processed for TRPV1 and CGRP double immunostaining.

2.3. Immunohistochemical and cresyl-violet staining procedures

After brief rinsing in phosphate buffered saline (PBS), dural preparations and trigeminal sections were incubated with primary antibodies: a rabbit anti-TRPV1 antibody (Alomone

Labs.,1:1000) and a mouse anti-CGRP (Abcam, USA,1:500)/ or a goat anti-PAR-2 antibody (Santa Cruz Bio, 1:200) overnight at 4°C. Then dural preparations and trigeminal sections were rinsed 3 times in PBS and incubated with secondary antibodies: donkey anti-rabbit antibody labelled with FITC, and rabbit anti-mouse antibody labelled with CY3 / or rabbit anti-goat antibody labelled with CY3 for 2 hours (Jackson, 1:500). After a final rinsing in PBS, preparations and tissue sections were mounted on slides and coverslipped (Vectashield, Vector Labs., USA).

To identificate the size distribution of trigeminal neurons some trigeminal sections were stained with 0.1% cresyl violet and were then dehydrated and cleared in increasing concentrations of alcohol and xylene.

2.4. Analysis of immunohistochemical data

Images were captured via video link to a Leica DMLB fluorescent microscope equipped with a Retiga 2000R (QImaging, Surrey, BC, Canada) digital camera and scanned by the computer. Excitation light of 635 nm and 455 nm wavelength, were used to view red-emitting CGRP/PAR-2- and green emitting TRPV1-positivity, respectively. Image-Pro Plus image analysis program (Media Cybernetics) was used to quantify IR nerve fibers and to determine size and number of trigeminal neurons (n=4). The total number and size of TG neurons, and the number and size of TRPV1-IR cells were counted by using serial horizontal sections of the TG. The TRPV1-IR nerve fiber density was estimated by counting the number of intercepts with superimposed counting frames containing cycloid curves (110). Nerve fiber intercepts were counted in dural areas of at least 10 mm^2. Nerve fiber density was expressed as a number of intercepts of single axons and nerve fiber bundles per mm^2 ± S.E.M.

Statistical analysis of the experimental data was performed with Student's t-test. A probability level of $P < 0.05$ was regarded as a statistically significant difference between groups.

3. Demonstration by retrograde labeling of capsaicin-sensitive trigeminal primary afferent neurons which project to the parietal dura mater and the middle meningeal artery

3.1. Surgery

The experiments were carried out in 4 male Wistar rats, ranging in weight from 300 to 350 g. The animals were anaesthetized with an initial dose of chloral-hydrate (350 mg/kg, i.p., Sigma-Aldrich Chemie Gmbh, Germany). Additional doses of chloral-hydrate (35 mg/kg) served to hold the anaesthesia during surgery. Surgical procedure was the same as described earlier. The opened cranial window allowed to identify the MMA.

3.2. Retrograde labeling technique

In each rat, 0.1 μl of a 3% suspension of the fluorescent dye diaminido yellow (DY) in distilled water was applied onto the surface of the dura mater using a micropipette. The cranial window was then covered with gelaspon and an outermost layer of parafilm to avoid dye-penetration to neighbouring tissues. The cut muscle and skin were sutured, and then the animals were allowed to recover.

3.3. Immunohistochemical procedure, data analysis

3 days later, the animals were re-anaesthetized, perfused and decapitated as described above. Both ipsi- and contralateral trigeminal ganglia were removed, postfixed, cryoprotected, sectioned, mounted on slides and processed for TRPV1 and CGRP double immunohistochemistry as described above. An ultraviolet filter providing excitation light of 360 nm wavelength was used to examine the blue-emitting DY-positive TG cells, while excitation light of 635 nm and 455 nm wavelength, were used to view red-emitting CGRP- and green emitting TRPV1-positive neurons, respectively.

4. Measurement of CGRP release from the rat dura mater by enzyme-linked immunoassay

- To determine whether capsaicin-induced CGRP release is altered in experimental diabetic neuropathy, capsaicin-induced CGRP release was compared in control, diabetic and insulin-treated diabetic rats.
- To determine whether capsaicin-induced CGRP release is influenced by activation of PAR-2, the PAR-2 agonist SLIGRL-NH_2-induced CGRP release was compared in control vs. capsaicin-desensitized rats.

4.1. Rat CGRP enzyme-linked immunoassay and evaluation of data

The release of CGRP from the dural afferent nerves was measured by the method of Ebersberger (61). Control, diabetic, insulin-treated diabetic and capsaicin-desensitized rats were deeply anaesthetized with thiopentone (150 mg/kg, i.p.) and decapitated. After removal of the skin and muscles, the skull was divided into halves along the midline and the cerebral hemispheres were removed. The skull preparations were washed with SIF at room temperature for 30 min and then mounted in a humid chamber at 37 °C. The cut lines of the skull halves were covered with vaselin, and the cranial fossae were filled with 400 µl of SIF solution. Three consecutive samples of the superfusate were collected at periods of 5 min by carefully removing the content of the skull halves with a pipette. The second sample was collected after incubation in the presence of capsaicin (10^{-5} M, in control, diabetic and insulin-treated diabetic rats; n=5 in each group) or SLIGRL-NH_2 (10^{-4} M, in control and capsaicin-desensitized rats; n=6 in both groups). All samples were frozen at -80 °C for later analysis. The CGRP contents of the samples were measured by enzyme-linked immunoassay (ELISA) (SPIbio, France). The absorbance of the reaction product representing the CGRP content of the sample was determined photometrically, using a microplate reader (DYNEX MRX). The minimum detection limit of the assay was 2 pg/ml CGRP. CGRP content was measured in pg/ml as mean ± S.E.M. Changes induced in CGRP release were expressed as percentage changes relative to the basal release (mean ± S.E.M.) calculated for the 5-min application period.

Statistical analysis of the experimental data was carried out with ANOVA followed by the Tukey test. A probability level of $P < 0.05$ was regarded as a statistically significant difference between groups.

RESULTS

1. The capsaicin-sensitive afferent innervation of the dura mater encephali of the rat

1.1. Capsaicin-sensitive sensory neurogenic meningeal vascular responses

Administration of capsaicin at concentrations of 5×10^{-8} and 10^{-7} M, but not 10^{-8} M, produced a significant increase in blood flow (13.5 ± 8.1%, 18.5 ± 5.1% and 1.8 ± 4.5%, respectively). The highest increase in dural blood flow was observed during the second and third minutes of the application period and the blood flow returned to the basal level in 4 ± 1.2 min after removing capsaicin 10^{-7} M (Fig. 2 a). In contrast, topical application of capsaicin at 10^{-6} or 10^{-5} M elicited an immediate decrease in blood flow (21.9 ± 6.4% and 27.9 ± 7.1% decreases, respectively, Fig. 2 b). Following the administration of capsaicin at 10^{-6} and 10^{-5} M, the blood flow returned to the control level after 5 ± 0.6 and 12 ± 0.8 min, respectively.

The effects of capsaicin were reproducible at all concentrations: no significant decreases could be observed in the blood flow-increasing or -decreasing effects of three consecutive applications of the same capsaicin concentration. Topical application of the vehicle for capsaicin did not induce significant changes in blood flow (3 ± 1.5% increase). The mean arterial blood pressure of the rats did not exhibit significant changes after the application of capsaicin (control: 93 ± 5 mmHg vs after capsaicin: 91 ± 3 mmHg).

To obtain direct pharmacological evidence of the involvement of TRPV1 receptor in capsaicin-evoked meningeal vasodilation, capsazepine, a specific TRPV1 antagonist, was used. Topical application of capsazepine (10^{-5} M) failed to affect the basal blood flow (2.7 ± 1.8% increase). After pre-application of capsazepine, a significant inhibition of the capsaicin-induced vasodilation was observed: the original vasodilatory effect of 10^{-7} M capsaicin turned into a moderate vasoconstriction (5.9 ± 2.1% decrease in blood flow, Fig. 3).

To study the involvement of CGRP in capsaicin-evoked meningeal vasodilation, $CGRP_{8-37}$, a specific CGRP receptor antagonist was preapplied before capsaicin (10^{-7} M). $CGRP_{8-37}$ (10^{-5} M) did not induce significant changes in dural blood flow (1.9 ± 0.8% increase). However, preapplication of $CGRP_{8-37}$ resulted in a significant inhibition of the

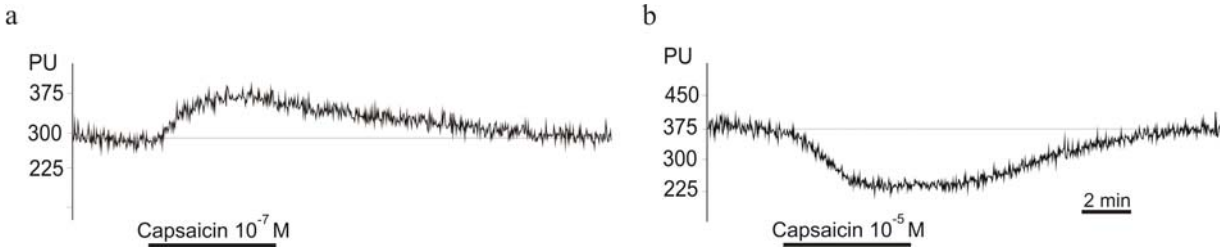


Fig. 2. Effect of topical application of capsaicin on meningeal blood flow. Original recordings indicating the vasodilatory and vasoconstrictor effects of capsaicin at concentrations of 10^{-7} M (a) and 10^{-5} M (b), respectively.

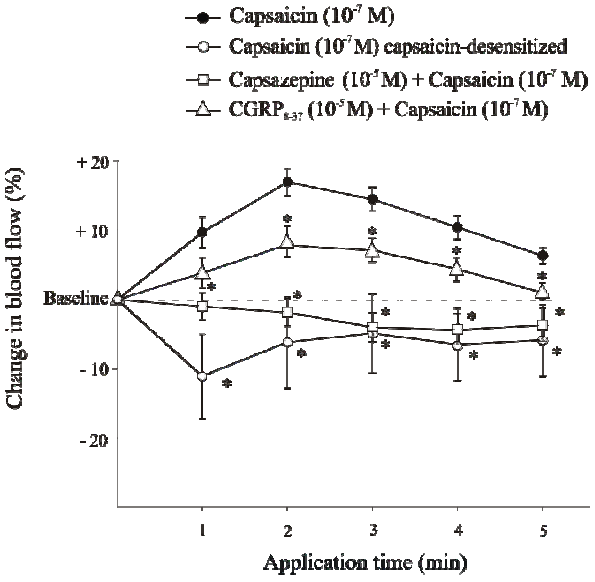


Fig. 3. Effects of the TRPV1-receptor antagonist capsazepine, capsaicin-desensitization and the CGRP-receptor antagonist $CGRP_{8-37}$ on capsaicin (10^{-7} M)-induced vasodilation in the dura mater encephali of the rat (*: $P < 0.05$; n=6 control, n=5 desensitized).

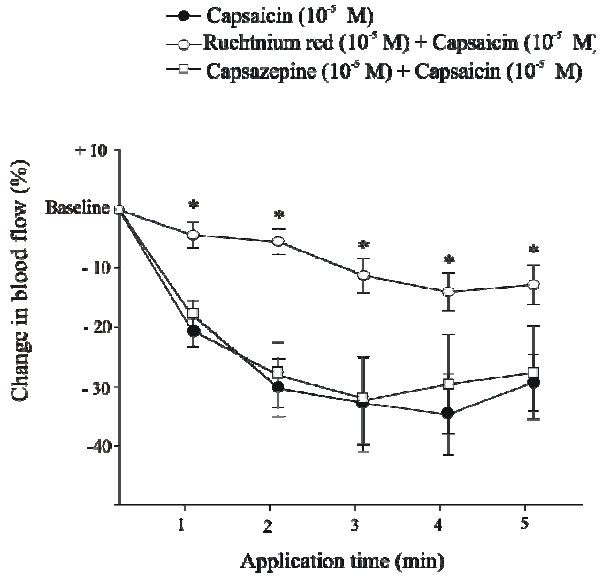


Fig. 4. Effect of the the non-specific cation channel blocker ruthenium red and capsazepine on capsaicin (10^{-5} M)-induced vasoconstriction in the dura mater encephali of the rat (*: $P < 0.05$; n=4).

capsaicin-induced vasodilation: 13.8 ± 2.1% vs 3.9 ± 4.3% (averaged data during the 5-min application period) (Fig. 3).

To determine the contribution of TRPV1 to the vasoconstriction caused by capsaicin at 10^{-5} M, capsazepine and RR, the non-specific cation channel blocker were preapplied. Although capsazepine-pretreatment (10^{-5} M) had no effect on capsaicin-induced

vasoconstriction (decreases in blood flow: 28.5 ± 7.7% vs 26.9 ± 6.4%, Fig. 4), preapplication of RR (10^{-5} M) significantly decreased the vasoconstrictor effect of 10^{-5} M capsaicin (decreases in blood flow: 27.3 ± 3.7% vs 9.1 ± 3.6%, Fig. 4). Neither capsazepine, nor RR had significant effects on the basal blood flow (increase by 2.7 ± 1.8% and decrease by 1.8 ± 2.1%, respectively).

In capsaicin-desensitized animals, the vasodilatory effect of capsaicin applied at 10^{-7} M was significantly inhibited (Fig. 3) while the vasodilation produced by the dural application of HA (10^{-5} M) was unaffected in all the capsaicin-desensitized animals.

The topical application of HA, CGRP and ACh induced significant increases in blood flow by 12.8 ± 3.4%, 14.6 ± 2.8% and 11.5 ± 0.7%, respectively, indicating that endothelium-dependent vascular reactions were intact. The systemic blood pressure was not influenced by these agents.

1.2. TRPV1- immunoreactive nerve fibers in the dura mater encephali of the rat

Immunofluorescence staining with an antiserum against TRPV1 showed many TRPV1-IR nerves running in small nerve bundles or as single axons in the dura mater encephali of the rat (Fig. 5). Topographically, these nerves were localized either in association with dural blood vessels (51.16 ± 6.13 intercepts of single axons/mm^2), or they were observed in „avascular" regions of the meningeal tissue areas (39.44 ± 8.57 intercepts of single axons/mm^2) at a distance from larger blood vessels, where they appeared to form loose nerve plexuses. The use of double immunofluorescence staining confirmed the existence of CGRP-containing TRPV1 immunopositive meningeal nerve fibers (Fig. 6) and revealed that these fibers are present in significant number in rat dura mater.

1.3. TRPV1-immunoreactive neurons in the rat Gasserian ganglion

Double immunostaining revealed that CGRP was localized in the majority of the TRPV1-IR neurons in the trigeminal ganglion of the rat supporting our findings about the colocalization of CGRP and TRPV1-immunoreactivity in dural nerve fibers (Fig. 7).

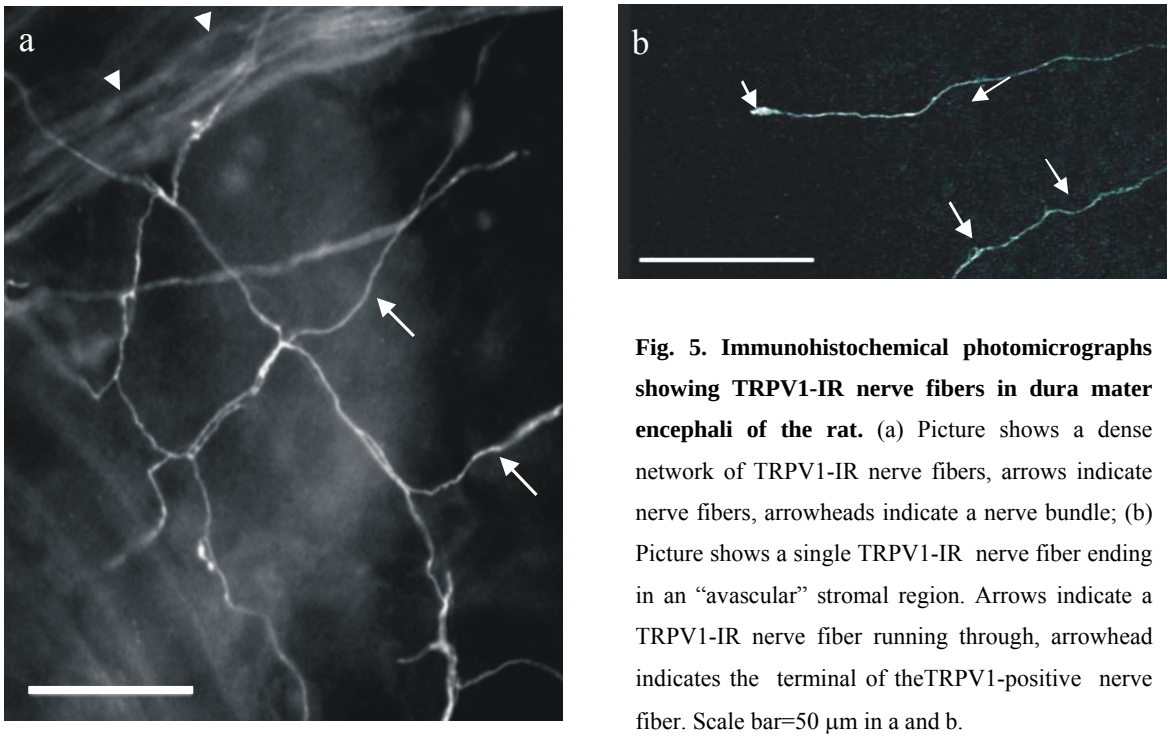
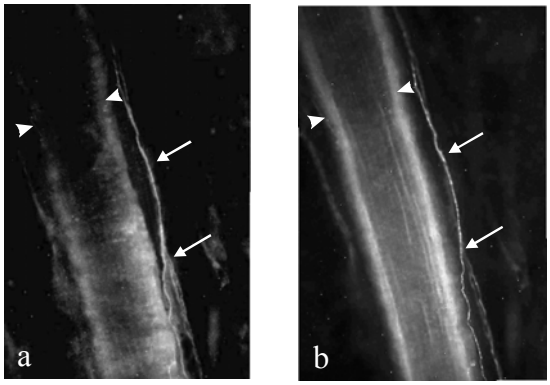


Fig. 5. Immunohistochemical photomicrographs showing TRPV1-IR nerve fibers in dura mater encephali of the rat. (a) Picture shows a dense network of TRPV1-IR nerve fibers, arrows indicate nerve fibers, arrowheads indicate a nerve bundle; (b) Picture shows a single TRPV1-IR nerve fiber ending in an "avascular" stromal region. Arrows indicate a TRPV1-IR nerve fiber running through, arrowhead indicates the terminal of theTRPV1-positive nerve fiber. Scale bar=50 μm in a and b.

Fig. 6. Double immunostaining for CGRP (a) and TRPV1 (b) in the dura mater encephali of the rat. Figures show a single nerve fiber (arrows) along an arteriolar branch (arrowheads) of the MMA, which show positivity for both TRPV1 and CGRP antibody. The pictures indicate the presence of CGRP-cotaining TRPV1-positive nerve fibers in the dura mater encephali of the rat. Scale bar=50 μm in b and applies for both photomicrograph.



The majority of the nerve cell bodies were concentrated at the periphery of the ganglion, leaving a broad central zone rich in nerve fibers and interspersed with isolated columns made up of perikarya. Immunofluorescence staining revealed that 17.9 ± 2.11% of the trigeminal cells showed positivity for the TRPV1 antibody. The topographical distribution of the TRPV1-IR neurons was similar to the whole cell population. Morphometric analysis of trigeminal ganglion neurons showed that TRPV1 was localized mostly in small-sized ($\leq 600\ \mu m^2$) neurons (Fig. 8).

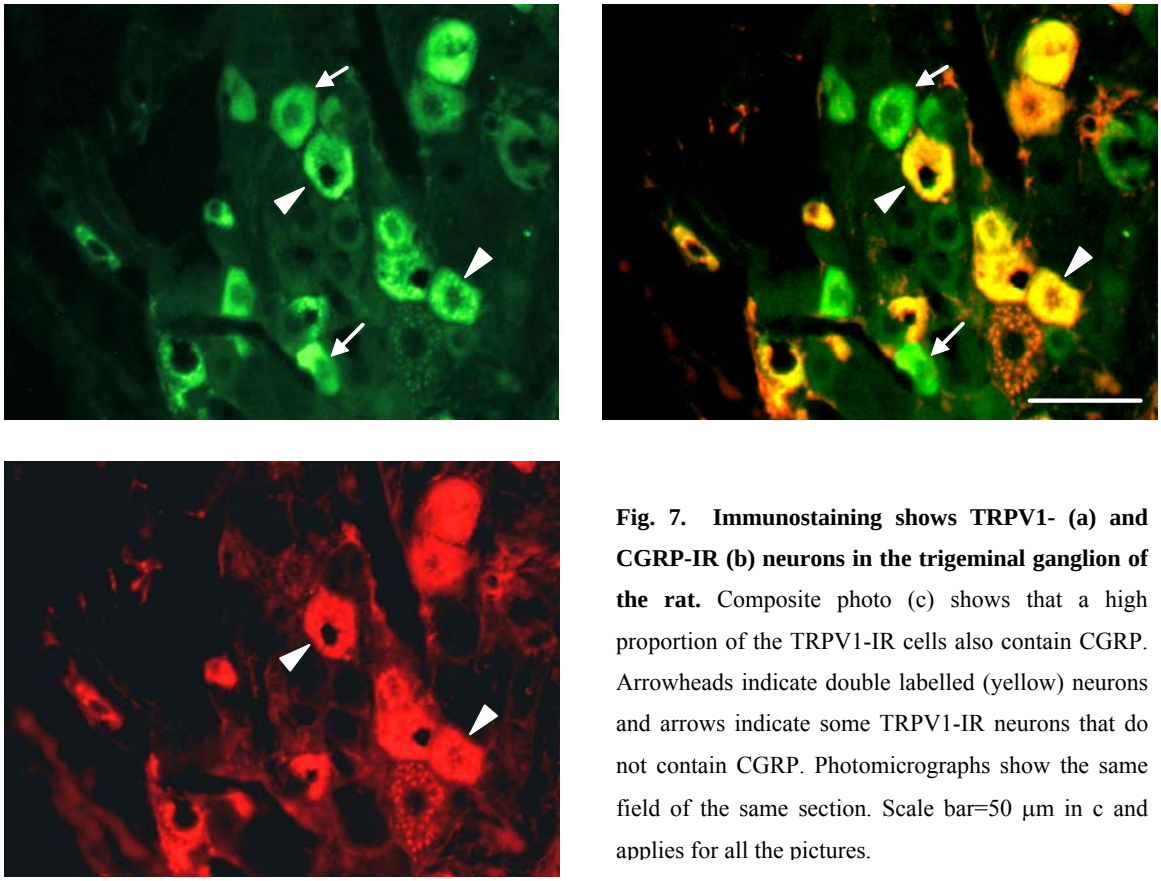


Fig. 7. Immunostaining shows TRPV1- (a) and CGRP-IR (b) neurons in the trigeminal ganglion of the rat. Composite photo (c) shows that a high proportion of the TRPV1-IR cells also contain CGRP. Arrowheads indicate double labelled (yellow) neurons and arrows indicate some TRPV1-IR neurons that do not contain CGRP. Photomicrographs show the same field of the same section. Scale bar=50 μm in c and applies for all the pictures.

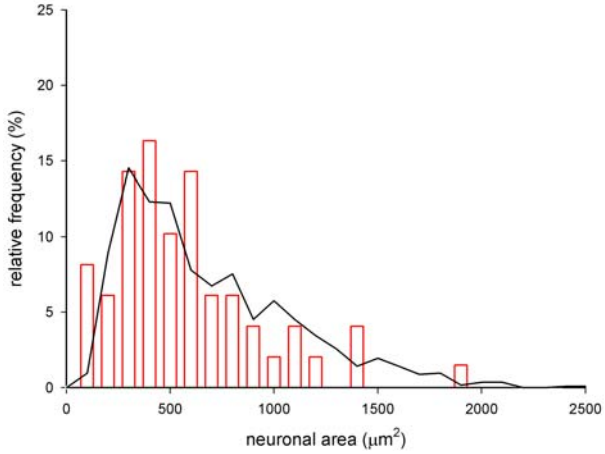


Fig. 8. A histogram showing the cell size spectrum of the Gasserian ganglion of the rat. Red bars indicate TRPV1-IR cells. The overlaid line graph shows the frequency distribution of cell diameters in the entire trigeminal population. The data were obtained from 1127 trigeminal neurons. TRPV1-immunopositivity was found mostly in small- and medium-sized neurons.

1.4. Demonstration of capsaicin-sensitive Gasserian ganglion cells which project to the parietal dura mater and the middle meningeal artery

Using a retrograde labeling technique, an average of 36 ± 4.3 cells per rat in the ipsilateral ganglion was found to project to the parietal dura mater. Immunofluorescence staining with the antiserum to TRPV1 revealed that some of the retrogradely labelled cells show positivity for the TRPV1 antibody (19 of 36 cells). Furthermore, the use of double immunofluorescence staining detected colocalization of CGRP immunoreactivity with TRPV1 immunoreactivity in some of the DY-labelled cells (Fig. 9).

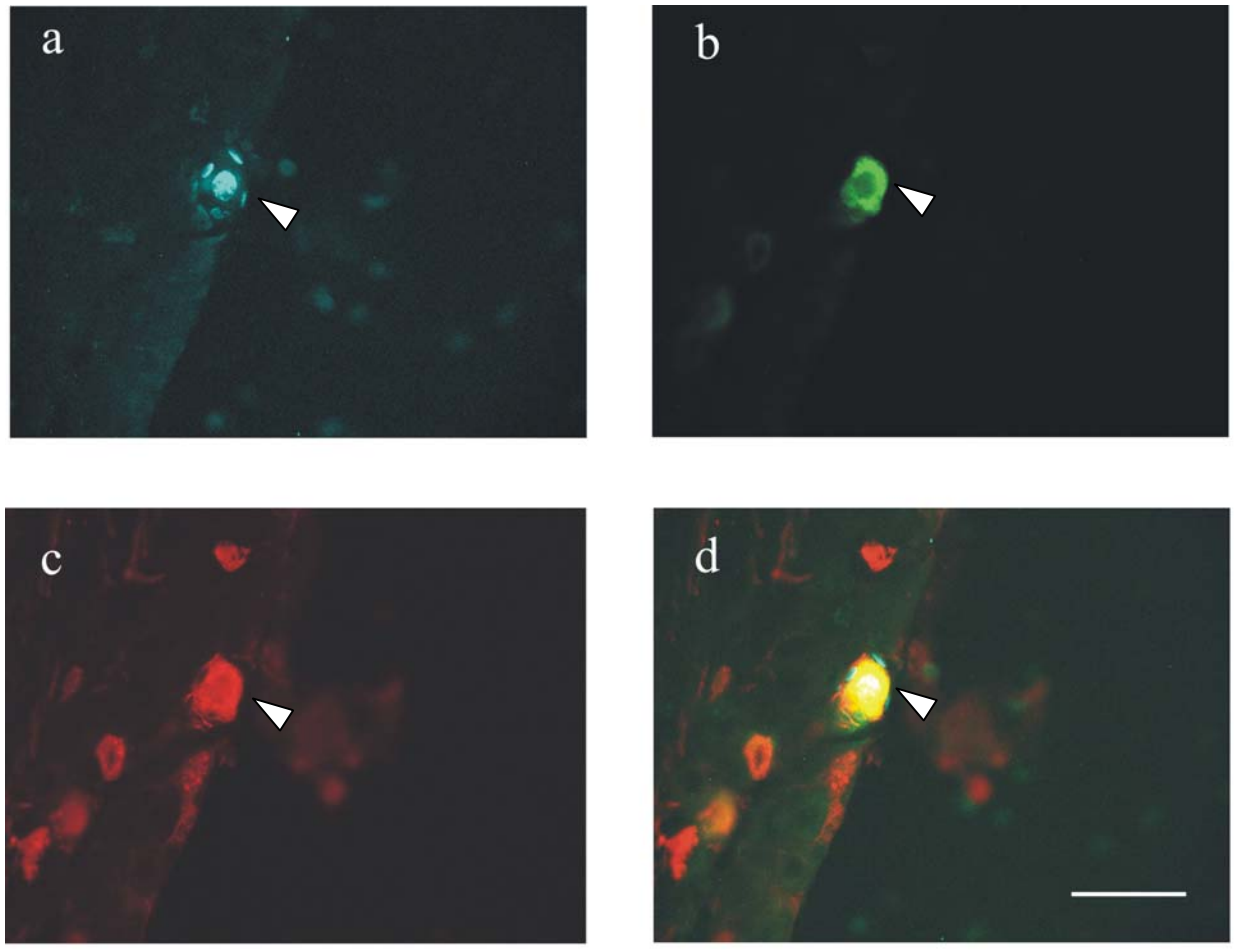


Fig. 9. TRPV1 (b) and CGRP (c) immunostaining in trigeminal ganglion cells retrogradely labelled with DY (a) transported from the MMA. Merged picture (d) shows colocalization of CGRP and TRPV1 with DY retrogradely transported from the MMA. (Arrowhead indicates the labeled neuron) The photomicrographs show the same field of the same section. Scale bar=50 μm in d and applies for all the pictures.

2. Involvement of TRPV1 in vascular responses elicited by the activation of PAR-2

2.1. PAR-2-mediated vascular responses in the dura mater encephali of the rat

Administration of trypsin (5×10^{-7}-10^{-5} M) and the PAR-2 agonist peptide SLIGRL-NH_2 (10^{-6}-10^{-4} M) induced dose-dependent increases in the blood flow of the meningeal blood vessels in control rats (Fig. 10). Since trypsin is a naturally occurring, but not a selective activator of the PAR-2, the selective agonist peptide SLIGRL-NH_2 was used in our further experiments. The effect of SLIGRL-NH_2 was reproducible: three consecutive applications of SLIGRL-NH_2 at 10^{-5} M produced similar increases in meningeal blood flow: 16 ± 1.8, 16 ± 2.8 and 14 ± 2.4%, respectively. During the 10-min wash-out periods, the meningeal blood flow returned to the original baseline level. The mean arterial blood pressure was not affected by SLIGRL-NH_2 (105 ± 12 mmHg and 102 ± 9 mmHg before and after drug application).

The SLIGRL-NH_2-induced meningeal vasodilatory reactions were significantly inhibited after local pretreatment with the CGRP receptor antagonist $CGRP_{8-37}$ (Fig. 11) or the NO synthase inhibitor L-NAME. Prior application of $CGRP_{8-37}$ and L-NAME reduced the increase in blood flow to SLIGRL-NH_2 from 17.1 ± 2.1% to 7.7 ± 2.6% and from 16.7 ± 1.8% to 6.2 ± 1.2%, respectively. Topical application of L-NAME, but not $CGRP_{8-37}$, reduced the basal meningeal blood flow. At the end of the 15-min L-NAME application period, the basal meningeal blood flow had decreased to 82.4 ± 5.6% of the initial value. The mean arterial blood pressure of the animals was not significantly affected by the local administration of $CGRP_{8-37}$ or L-NAME.

In order to find out whether capsaicin-sensitive afferents contribute to the mechanism of the vasodilatory effect of PAR-2 activation, the effect of SLIGRL-NH_2 was also studied in capsaicin-desensitized rats. The vasodilatory effect of SLIGRL-NH_2 (10^{-5} M) was significantly reduced, the increases in blood flow in the control and the capsaicin-desensitized animals were 16.19 ± 0.9% and 3.4 ± 1.2%, respectively. The vasodilatory effect of 10^{-5} M histamine was unaffected by capsaicin-desensitization (control: 15.3 ± 1.3%, desensitized:

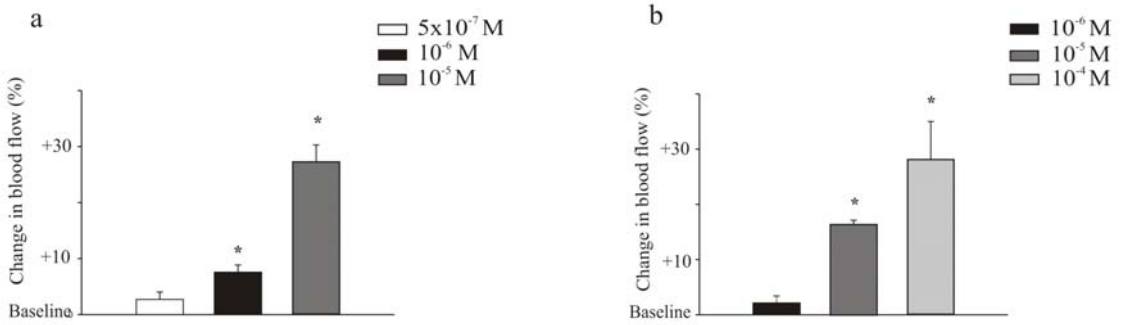


Fig. 10. Effects of topical application of trypsin (a) and SLIGRL-NH_2 (b) on meningeal blood flow. Changes in blood flow are calculated as mean percentage changes ± S.E.M. for 5 min of the application (*: Significantly different from the control basal blood flow (Baseline); $P < 0.05$; n=5-6).

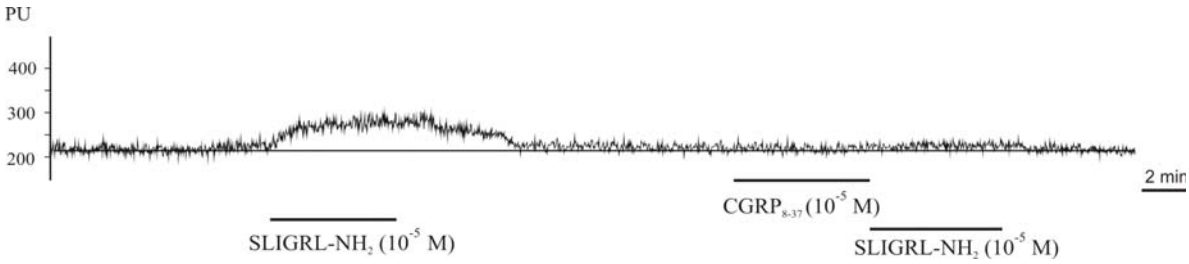


Fig. 11. Original recording indicating the effect of CGRP receptor antagonist $CGRP_{8-37}$ on SLIGLR-NH_2-induced meningeal blood flow changes.

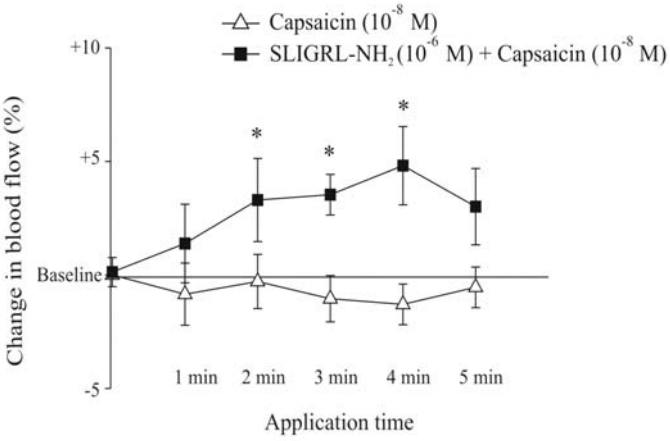


Fig. 12. Effect of preapplication of SLIGRL-NH2 (10^{-6} M) on capsaicin (10^{-8} M)-induced meningeal blood flow changes. *: The difference between the two groups is statistically significant (P<0.05; n=8).

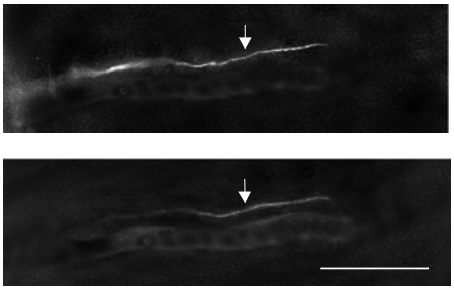


Fig. 13. Immunostaining for TRPV1-(a) and PAR-2 (b) indicates coexpression of TRPV1 with PAR-2 in dura mater encephali of the rat. Picture indicates a single nerve fiber (arrows) showing immunopositivity for both TRPV1 and PAR-2 antibody. Photomicrographs show the same section. Scale bar=50 μm in b and applies for a also.

18.3 ± 1.8%). The mean arterial blood pressure was not affected by application of the above agents.

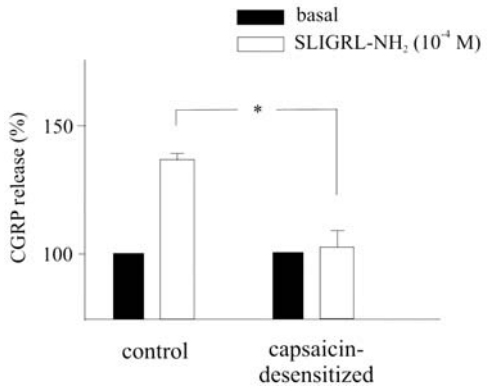
2.2. Capsaicin-sensitive vascular responses modulated by PAR-2 activation

In the control rats, neither capsaicin nor SLIGRL-NH_2 administered at 10^{-8} and 10^{-6} M, respectively, had a significant effect on the basal meningeal blood flow. However, when SLIGRL-NH_2 (10^{-6} M) was administered prior to the application of capsaicin (10^{-8} M), a marked increase of 8.3 ± 2.1% in meningeal blood flow was recorded for the 5-min application period (Fig. 12.).

2.3. Detection of PAR-2-immunopositive capsaicin-sensitive nerve fibers in the rat dura mater

The use of double immunofluorescence staining with the antiserum to TRPV1 and PAR-2 revealed the colocalization of TRPV1 with PAR-2 in some of the TRPV1-immunopositive meningeal nerve fibers of the dura mater encephali of the rat (Fig. 13).

2.4. Capsaicin-sensitive meningeal nerves significantly contribute to PAR-2-induced CGRP release



The basal release of CGRP from the dura mater preparations of the control and the capsaicin-desensitized rats was 16.1 ± 1.6 and 13.3 ± 0.6 pg/ml, respectively. Although the basal release of CGRP was lower in the capsaicin-desensitized animals than in the controls the difference was not significant.

Fig. 14. Effect of capsaicin-desensitization on SLIGRL-NH_2-induced CGRP release in the dura mater. Bars represent changes induced in CGRP release by application of SLIGRL-NH_2 at 10^{-4} M in control dura mater preparations and after systemic capsaicin-desensitization (*: $P < 0.05$; n=6).

SLIGRL-NH_2 (10^{-4} M) induced a significant increase of 33.6 ± 13.3% in the release of CGRP from the dura mater preparation of the control rats. The PAR-2 agonist peptide-induced increase in the release of CGRP was significantly diminished in the capsaicin-desensitized rats as compared with the controls, it amounted to 2.76 ± 5.4% increase of the basal release. The CGRP content of the samples obtained after the washing-out of SLIGRL-NH_2 was similar to the basal release values both in control and capsaicin-pretreated rats (Fig. 14).

3. Effect of streptozotocin-induced diabetes mellitus on the structure and function of capsaicin-sensitive meningeal afferent nerves

3.1. Loss of capsaicin-sensitive neurogenic vasodilation in diabetic rats

In the diabetic rats, capsaicin-induced vasodilation was abolished 6, but not 2 or 4 weeks after the induction of diabetes (Fig. 15). The blood flow-increasing effect of capsaicin at 10^{-7} M 2 and 4 weeks after the induction of diabetes was 9.4 ± 2.8 and 12 ± 1.8%, respectively. Six weeks after the injection of streptozotocin, the usual vasodilatory effect of capsaicin administered at 10^{-7} M had become a slight vasoconstriction (5.9 ± 2.1 % decrease in blood flow). Since the capsaicin-induced increases in blood flow were similar 2, 4 or 6 weeks after

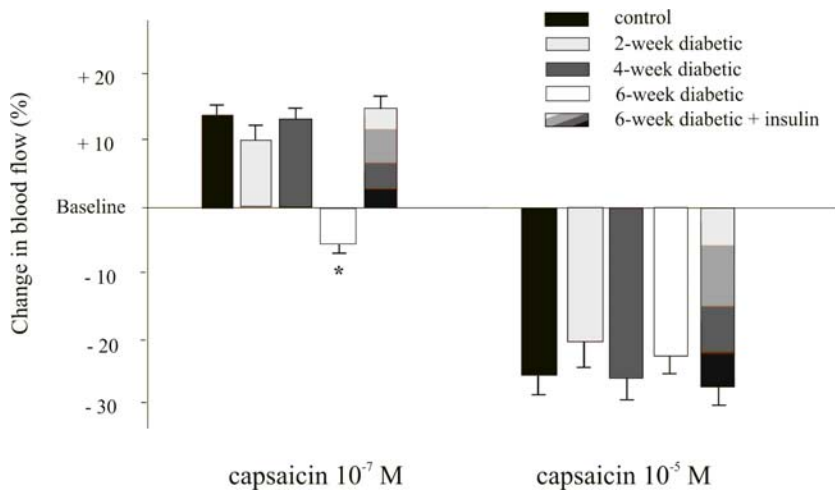


Fig..15. Effects of topical applications of capsaicin at different concentrations on meningeal blood flow in control, diabetic and insulin-treated diabetic rats. Changes in blood flow are calculated as mean percentage changes ± S.E.M. for 5 min of the application (*: $P < 0.05$; n=5-8).

the injection of the vehicle, the data were pooled and a single control group was formed. Administration of insulin restored the vasodilatory effect of capsaicin at 10^{-7} M (15 ± 2.9% increase).

No significant differences were found among the control, the diabetic and the insulin-treated diabetic rats as regards the vasoconstrictor effect of capsaicin at 10^{-5} M (Fig. 15). 2, 4 and 6 weeks after the induction of diabetes the blood flow was decreased by 20.9 ± 4.7, 25.8 ± 5.4 and 23.5 ± 5.4%, respectively. In insulin-treated diabetic rats, capsaicin at a concentration of 10^{-5} M elicited a decrease in meningeal blood flow by 28.6 ± 4.2%.

The vasodilatory effects of CGRP, HA and ACh were also studied in the animals treated with streptozotocin. No significant differences were found between the control and the 6-week diabetic animals as regards the vasodilatory effects of HA, CGRP and ACh (Table 1).

Table 1. Vasodilatory effect of HA, CGRP and ACh in control, diabetic and insulin-treated diabetic rats.

	HA	*CGRP*	*ACh*
Control	12.8± 3.4%	14.6±2.8%	11.5±0.7%
Diabetic	11±1.6%	19±3%	15±1.8%
Insulin-treated diabetic	17.2 ± 2.8%	22.1±2.8%	10±1.5%

Values are calculated as mean percentage ± S.E.M. for 3 min of the application period, the results obtained from three control, three diabetic and three insulin-treated diabetic rats.

3.2. Diminution of TRPV1-immunoreactive nerve fibers in diabetic rats

In 6-week diabetic rats, decreases in the number of both perivascular and stromal TRPV1-IR nerves were observed. Quantitative analysis of the distribution of TRPV1-IR nerve fibers disclosed a statistically significant reduction in nerve fiber density of diabetic rats. Significant decreases in the density of nerves associated with both blood vessels and the largely „avascular" stromal regions of the dura mater were observed (Table 2).

Table 2. Density of TRPV1-immunopositive nerve fibers of the dura mater of control and 6-week diabetic rats.

	Perivascular region		„Avascular" region	
	Single axons	Nerve bundles	Single axons	Nerve bundles
Control	51.16 ± 6.13	68.63± 6.31	39.44± 8.57	9.50± 4.58
Diabetic	19.76 ±. 2,28*	53.88± 7.41*	11.80± 3.86*	4.14± 3.76*

Values demonstrate the number of intercepts of single axons and nerve bundles in 1 mm^2 of the dura mater. Each value is expressed as mean ± S.E.M. and represents the results obtained from three control and three diabetic rats (*:Significantly different from the control, P<0,05).

3.3. Reduced release of CGRP from capsaicin-sensitive meningeal nerves in diabetic rats

The basal release of CGRP from the dura mater preparations of the control, the diabetic and the insulin-treated diabetic rats was 18.2 ± 0.8, 19.1 ± 0.7 and 18.1 ± 0.55 pg/ml, respectively (5 min samples). Capsaicin (10^{-5} M) induced a marked release of CGRP from the dura mater preparations of control rats, amounting to 61.4 ± 3.8 pg/ml. The capsaicin-induced release of CGRP was significantly diminished in the diabetic rats as compared with the controls (69.04 ± 7.69% of the control) (10 min samples). Insulin treatment of diabetic animals restored the effect of capsaicin in increasing the release of CGRP (93.3 ± 4.9% of the control). The CGRP contents of the samples obtained after the washing-out of capsaicin were similar to the basal release values (22.28 ± 0.47, 19.8 ± 0.35 and 21.5 ± 1.43 pg/ml in the control, the diabetic and the insulin-treated diabetic rats, Fig. 16; 15 min samples).

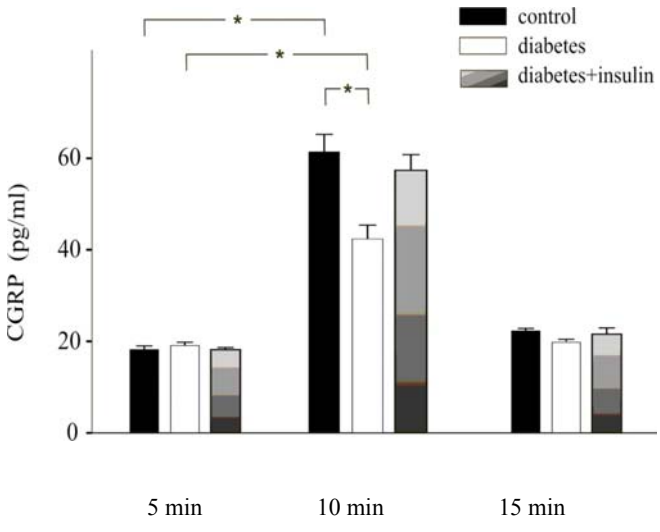


Fig. 16. Capsaicin-induced in vitro CGRP release from the dura mater of hemisected skulls of control, diabetic and insulin-treated diabetic rats (*: P < 0,05; n=5).

DISCUSSION

1. Morphology and function of capsaicin-sensitive afferent nerves in the rat dura mater

In the present study, we investigated a vascular phenomenon of meningeal arteries, which is mediated by capsaicin sensitive afferent nerves that innervate the dura mater encephali of the rat. Dux have previously observed that chemical stimulation of the meningeal sensory fibers by capsaicin at low concentrations elicited a moderate, but significant, vasodilatory response, which was mediated by the release of CGRP from capsaicin-sensitive afferent nerves (59). Capsaicin-sensitive vasodilator mechanisms have already been described in other organs, such as the skin (118;145), the gingival mucosa (71), and the gastrointestinal system (103;201). The release of vasoactive peptides from sensory nerves comprises the crucial mechanism of the local regulatory, sensory-efferent function of capsaicin-sensitive sensory neurons (102).

Laser Doppler flowmetry revealed that the capsaicin-induced meningeal vasodilation was markedly inhibited either by local administration of the specific competitive TRPV1 receptor antagonist, capsazepine or by a prior application of a CGRP antagonist, $CGRP_{8-37}$ (59). These findings, also supported by the present work, strongly suggested that capsaicin-induced neurogenic vasodilation is elicited through the activation of the capsaicin receptor, TRPV1 and it is mediated by the consequent release of CGRP from sensory nerves of the dura mater encephali of the rat.

Capsaicin at high concentrations, however, was observed to induce a robust meningeal vasoconstriction, which was not affected by either ablation of capsaicin sensitive nerves or capsazepine pretreatment (59). This vasoconstrictor response was markedly decreased by the preapplication of ruthenium red, an inorganic dye that blocks nonselective cation channels. This is in accordance with the results of Griffith, who suggested an operation through separate receptor-coupled ion channel complexes at high and low concentrations of capsaicin (91). Since the vasoconstrictor effect of high-dose capsaicin seems to be non-neural, potential targets of capsaicin may be located on the smooth muscle of meningeal blood vessels. Although Kark and colleagues recently reported opposite functional roles of neuronal and smooth muscle located TRPV1 in an in vitro preparation (125), the possibility that the biphasic

effects of capsaicin is based on two vanilloid receptor subtypes presented in the dural vasculature requires further study.

Previous studies using an experimental neuroanatomical approach demonstrated the existence of capsaicin-sensitive sensory nerves in the rat dura mater (59). The present study confirmed and extended these findings by showing a rich network of meningeal sensory nerves using TRPV1 immunohistochemistry. TRPV1 immunopositive nerve fibers were detected around the blood vessels as well as in the stromal region of the dura mater encephali of the rat. Double immunostaining techniques revealed the presence of the powerful vasodilator neuropeptide CGRP in the majority of the paravascular and stromal TRPV1-IR meningeal nerve fibers. These data are in accordance with formerly published results of Shimizu, who demonstrated CGRP immunoreactivity in 70% of the TRPV1-IR nerve fibers in the rat dura mater (208).

Retrograde tracing combined with immunohistochemistry served to identify the origin of nerve fibers innervating the meningeal area subjected to laser Doppler flowmetry. Using specific antibodies against TRPV1, we detected TRPV1 immunoreactivity in about 16% of primary sensory neurons in the rat TG. In accord with previous findings (12;109), morphometrical analysis of trigeminal neurons detected TRPV1-immunopositivity mostly in small neurons. Double labeling immunohistochemistry using antibodies to TRPV1 and CGRP showed colocalization of CGRP in the majority of TRPV1-IR trigeminal neurons. Using a retrograde labeling technique combined with double immunostaining for TRPV1 and CGRP, we provided evidence for the existence of CGRP-containing TRPV1-immunopositive primary sensory neurons with peripheral endings around the MMA. These results are in line with recent data showing colocalization of CGRP and TRPV1 in trigeminal neurons innervating the MMA (208). In the periphery, the capsaicin-sensitive afferent nerves expressing the TRPV1 receptor are thought to be nociceptors (37). Hence, by inference, capsaicin-sensitive afferent nerves are likely candidates for sensory fibers which transmit nociceptive information from the dura mater encephali (214). The release of the vasoactive peptide CGRP from sensory nerves is regarded as a reliable approach to characterize the nociceptive function of peptidergic afferent nerves (61). Most of the sensory nerve fibers innervating the dura mater encephali contain CGRP (128;167), which has been shown to play a crucial role in the mechanisms of meningeal vasodilation and blood flow regulation (59;137). Considering that

the release of CGRP is involved in meningeal neurogenic vasodilation associated with severe types of headache (65;87), activation of TRPV1 located on CGRP-containing nociceptive afferent nerves is suggested to contribute to the pathomechanism of head pain. Our immunohistological results, however, showed TRPV1 expressing nerve fibers not only perivascularly, but also in the stromal region of rat dura mater. The functional relevance of these endings in the avascular regions of the dura mater has not been revealed, yet (7).

2. Interaction of TRPV1 and PAR-2 in the mediation of vascular reactions of the rat dura mater

The present experiments have revealed a new meningeal neurovascular mechanism, which is mediated by the activation of PAR-2. The results indicated that PAR-2 activation induces a CGRP- and NO-dependent vasodilation in the rat dura mater involving meningeal capsaicin-sensitive afferent nerves. Also, the results suggested that activation of PAR-2 may modulate the meningeal nociceptor function by initiating neuronal signalling mechanisms leading to sensitization of the TRPV1 receptor expressed on capsaicin-sensitive afferent nerves.

The increase in meningeal blood flow evoked by activation of PAR-2 was significantly reduced either by ablation of capsaicin-sensitive afferents by systemic capsaicin treatment or administration of the specific CGRP receptor antagonist $CGRP_{8-37}$. In support of these findings, the PAR-2 agonist-induced release of CGRP was significantly decreased in capsaicin-desensitized animals. Consequently, the release of CGRP from capsaicin-sensitive sensory nerves may play a significant role in the mechanism of PAR-2-induced vasodilation. Our immunohistochemical studies disclosed that a large population of rat trigeminal sensory neurons, as well as meningeal afferents, express the TRPV1, and many of these neurons/nerves also contain CGRP. Double immunostaining revealed that approximately 80% of mostly small TRPV1-IR neurons also express PAR-2 (50;191). In line with these data, we provided immunohistological evidence for the colocalization of PAR-2 with TRPV1 in meningeal afferents using double labeling immunohistochemistry.

Furthermore, the present study suggests a functional interaction between the capsaicin-receptor TRPV1 and the PAR-2 by showing that prior activation of PAR-2 resulted

in a significant vasodilatory response produced by otherwise ineffective concentrations of capsaicin. In the skin, it has been already demonstrated that activation of PAR-2 resulted in a TRPV1 mediated thermal sensitization indicating a functional association between PAR-2 and TRPV1 at a behavioral level (230). Previous investigations demonstrated a sensitization of TRPV1 following activation of certain G-protein-coupled receptors by e.g. nerve growth factor or bradykinin in a PKC dependent manner (39;154). The PAR-2-mediated thermal hyperalgesia might result from an enhancement of capsaicin receptor activity through phosphorylation of the TRPV1 by PKC and/or protein kinase A (PKA) (4;50). The signalling pathway for the PAR-2 involves the activation of PLC resulting in the formation of inositol triphosphate and diacylglycerol, followed by calcium mobilization and the activation of PKC (156). A recent observation revealed that diacylglycerol may serve as an endogenous ligand for the rat TRPV1 (243). Therefore, activation of the PAR-2 may contribute to increased nociceptor excitability resulting in an enhancement of the TRPV1-mediated neuropeptide release from primary sensory neurons (107).

The possibility that similar mechanisms are involved in the sensitization of both cutaneous and meningeal nociceptors requires further study, although a sensitizing effect of the PKA second messenger cascade have been already revealed on rat dural mechanonociceptors (147). Inflammation of the dura mater may further contribute to the sensitization of nociceptors by the elevated levels of activating proteases released by inflammatory cells and by the upregulated expression of the PAR-2 in the inflamed tissue (182). Given the suggestion that meningeal sensory nerves are activated only under abnormal, potentially harmful conditions (148), the PAR-2 may contribute significantly to the pathomechanism of headaches via meningeal nociceptor sensitization.

The present findings revealed an additional component of the PAR-2 induced meningeal vasodilation, which involves NO. Inhibition of NO synthase with L-NAME resulted in a reduced response to PAR-2 activation. These findings are in line with earlier observations concerning the role of NO in meningeal vascular reactions (217). In neurogenic inflammatory processes, complex interactions of CGRP and NO have been observed: NO plays a modulatory role in the release of the sensory vasodilator CGRP (124;218), and, in turn, CGRP-induced vasodilation is partly mediated by endothelium-derived NO. Furthermore, inflammatory role of PAR-2 has become more emphatic through studies demonstrating that

PAR-2 is also expressed on mast cells and its activation can enhance their degranulation and mediator release (170;250).

3. Involvement of capsaicin-sensitive afferent nerves in meningeal vascular responses under pathological conditions: the effect of diabetes mellitus

In the present experiments, we have observed an attenuation of the TRPV1 receptor-mediated meningeal sensory vasodilation, which can be elicited by the topical application of capsaicin, 6 weeks after the induction of diabetes. In the streptozotocin-treated animals the vasodilatory effect of capsaicin administered in low concentration was abolished as assessed by laser Doppler flowmetry. Insulin treatment was effective in preventing the reduction of capsaicin-induced meningeal vasodilation suggesting that impairment of sensory neurogenic vasodilation was not a consequence of the toxic effect of the administration of streptozotocin.

The present study revealed a significant reduction in the number of TRPV1-positive meningeal nerves that may be responsible for the impairment of sensory neurogenic vasodilation in diabetic rats. The dysfunction of the vascular endothelium, which is generally manifested in the later stages of diabetes mellitus may be another factor contributing to the disturbances of the regulation of blood flow. However, a diminished sensitivity of the arterial smooth muscle or an impaired endothelial function is unlikely to contribute to the reduction of neurogenic sensory vasodilation under the conditions of the present experiments, since the vasodilatory effects of CGRP, HA and ACh were unaltered after streptozotocin treatment.

The capsaicin-induced release of CGRP was demonstrated in an in vitro preparation of the dura mater by using a sensitive enzyme-linked immunoassay. Measurement of the peptide release revealed an attenuated capsaicin-induced release of CGRP from preparations of the dura mater of diabetic rats. In accord with our findings, a decreased level of CGRP was observed in the rat trigeminal ganglion 5 weeks after the induction of diabetes (226). These observations support the suggestion that the changes in the vasodilatory responses in streptozotocin-induced diabetes are related to changes in the neuronal peptide level (32).

Our results indicate an impairment of an important sensory nerve dependent vasomotor mechanism of the dura mater in experimental diabetes. It is noteworthy, that similar reductions in capsaicin-induced CGRP-mediated cutaneous vasodilator responses have been

demonstrated in diabetic patients (10). In the diabetic rat skin, a reduction in neurogenic plasma extravasation and a decrease in peptide levels have also been demonstrated (78). Although vasodilation may not be significantly implicated in the generation of pain in meningeal pain-sensitive structures, an increase in blood flow may have a beneficial effect by removing, for example, inflammatory mediators and tissue metabolites inducing or aggravating headache attacks (75). Indeed, capsaicin-sensitive meningeal nociceptors may fulfill a unique biological function: they are involved in the detection of noxious stimuli, and they contribute to the activation of protective, antinociceptive mechanisms by promoting the removal of pain-producing agents from the site of the injury. In diabetes, this putative homeostatic effect of sensory nerve-mediated vasodilation is largely abolished, and the clearance of tissue metabolites, transmitters and inflammatory mediators implicated in the generation of cranial pain is impeded. An insufficient vasodilator function of meningeal sensory nerves may result in disturbances of tissue homeostasis, resulting ultimately in the further activation and/or sensitization of meningeal nociceptors. A dysfunction of these particular meningeal peptidergic nerves may be of relevance as concerns the increased incidence and severity of migraine headache in diabetic patients (210).

In the present study, we characterized the functional properties of meningeal capsaicin-sensitive afferents in the dura mater encephali and determined their physiological/pathophysiological relevance to headaches. Similarly to other nociceptive systems, the capsaicin-sensitive sensory nerves participate in the transmission of nociceptive information and initiation of local vascular responses via the release of vasoactive neuropeptides in rat dura mater. Our findings confirmed previous results of Dux which demonstrated that these particular peptidergic sensory nerves mediating capsaicin-induced neurogenic vasodilation comprise a novel class of meningeal sensory vasomotor fibers (59). The present study has extended these findings by showing that this particular class of afferent nerves is affected by diabetic pathology and contributes to vascular and possibly nociceptor dysfunctions which may affect the tissue integrity and meningeal sensation. The findings additionally have provided the first experimental evidence for the participation of PAR-2 in capsaicin-induced, TRPV1-mediated sensory neurogenic vasodilatory responses of the dura mater encephali of the rat. The results further suggest that the activation of PAR-2 may

contribute significantly to meningeal nociceptor sensitization and in turn to the pathomechanism of headaches. Clinical efficiency of certain small-molecule CGRP receptor antagonists in the treatment of migraine (63;98) suggest a significant role for the capsaicin-sensitive sensory C-fibers in intracranial nociception. Clinical trials support the significance of meningeal capsaicin-sensitive afferents as good targets of antinociceptive drugs by proving the efficacy of capsaicin and a small molecule TRPV1 antagonist in patients with migraine or cluster headache (78;222).

We believe that our results promote the understanding of the pathomechanism of headaches by providing new insights into the role of capsaicin-sensitive nociceptive meningeal afferents in the pathophysiology of headaches.

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APPENDIX

PAPER I.

PAPER II.

PAPER III.

PAPER IV.