Meningeal trigeminovascular reactions mediated by TRPV1 and TRPA1 receptors: effect of adriamycin treatment

PhD Thesis

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CB	cannabinoid
cGMP	cyclic guanosin monophosphate
CGRP	calcitonin gene-related peptide
CLR	calcitonin receptor-like receptor
DNA	deoxyribonucleic acid
EIA	enzyme-linked immunoassay
5HT	5-hydroxytryptamine
MMA	middle meningeal artery
mRNA	messenger ribonucleic acid
NADA	N-arachidonoyl-dopamine
NK1	neurokinin 1
NKA	neurokinine A
PACAP	pituitary adenylate cyclase-activating polypeptide
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PU	perfusion unit
RAMP1	receptor activity-modifying protein 1
RCP	receptor component protein
RNA	ribonucleic acid
SIF	synthetic interstitial fluid
SP	substance P
TRP	transient receptor potential
TRPA	transient receptor potential ankyrin
TRPA1	transient receptor potential ankyrin 1
TRPC	transient receptor potential canonical
TRPM	transient receptor potential melastatin

TRPML	transient receptor potential mucolipin
TRPP	transient receptor potential polycystin
TRPV	transient receptor potential vanilloid
TRPV1	transient receptor potential vanilloid 1
V	venous vessel

SUMMARY

A significant population of trigeminal primary afferents are chemosensitive nociceptors which express the transient receptor potential vanilloid 1 (TRPV1) and the transient receptor potential ankyrin 1 (TRPA1) receptors. Many of these afferents are peptidergic and contain calcitonin gene-related peptide (CGRP), substance P (SP) or neurokinine A (NKA). CGRP participates in the central transmission of nociceptive impulses, increases tissue perfusion and may also sensitize the nociceptive pathway. Since somatosensory and visceral chemosensitive nerves share common characteristics, functional alterations observed in the meningeal trigeminovascular system may provide useful information on possible impairments in nociceptive functions of other organs following metabolic, hormonal or toxic changes affecting the integrity of the whole organism.

The present experiments were initiated in an attempt to study the possible activation of meningeal TRPV1 receptors by exogenous and endogenous agonists and to reveal the effect of systemic adriamycin treatment on TRPV1 receptor-mediated sensory effector/local regulatory responses.

Meningeal blood flow was measured in a rat open cranial window preparation with laser Doppler flowmetry. Release of CGRP evoked by endovanilloids was measured with enzyme-linked immunoassay (EIA) in an *ex vivo* dura mater preparation.

In control and adriamycin-treated animals the dura mater was repeatedly stimulated by topical applications of capsaicin, a TRPV1 receptor agonist, or acrolein, a TRPA1 receptor agonist. The blood flow increasing effects of CGRP, histamine, acetylcholine and forskolin were also measured. Capsaicin- and acrolein-induced neural CGRP release was compared in control and adriamycin-treated rats. TRPV1 content of trigeminal ganglia and TRPV1-, CGRP- and CGRP receptor component-immunoreactivity were examined in dura mater samples obtained from control and adriamycin-treated rats.

Topical application of NADA induced a significant dose-dependent increase in meningeal blood flow that was markedly inhibited by pretreatments with the TRPV1 antagonist capsazepine, the CGRP antagonist $CGRP_{8-37}$, or by prior systemic capsaicin desensitization. In *ex vivo* dura mater preparations NADA evoked a significant increase in CGRP release. Cannabinoid CB1 receptors of CGRP releasing nerve fibers apparently counteracted the TRPV1 agonistic effect of anandamide in a dose-dependent manner, a result

which is confirmed by the facilitating effect of CB1 receptor inhibition on CGRP release and its reversing effect on the blood flow.

In adriamycin-treated animals the vasodilator effects of capsaicin, acrolein and CGRP were significantly reduced while histamine-, acetylcholine- and forskolin-induced vasodilatation were unaffected. Measurements of CGRP release revealed altered dynamics upon repeated stimulations of TRPV1 and TRPA1 receptors. In whole-mount dura mater preparations immunohistochemistry revealed altered CGRP receptor component protein (RCP)-immunoreactivity in adriamycin-treated animals, while CGRP receptor activity modifying protein (RAMP1)-, TRPV1- and CGRP-immunostaining were left apparently unaltered. Adriamycin treatment slightly reduced the TRPV1 protein content of trigeminal ganglia.

The present findings demonstrate that endovanilloid compounds are potential activators of meningeal TRPV1 receptors under physiological conditions. Endovanilloids may activate the trigeminovascular nocisensor complex and that may play a significant role in the pathophysiology of headaches. Systemic adriamycin treatment resulted in alterations of the functions of the trigeminovascular system leading to reduced meningeal sensory neurogenic vasodilatation. This may affect local regulatory and protective mechanisms of chemosensitive afferents leading to alterations in tissue integrity.

INTRODUCTION

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. It may be accompanied by autonomic, emotional and behavioral reactions (Merskey and Bogduk, 1994). In contrast to the relatively more objective nature of other senses, pain is highly subjective. Acute nociceptive pain associated with tissue injury has distinct warning and protective functions. Acute nociceptive pain can elicit protective reflexes (e.g. withdrawal of a damaged limb). A close correlation between stimulus intensity and pain perception can be observed; the pain is indicative of real or potential tissue damage. In chronic pain conditions an abnormal function of pain processing can be observed (Bennett, 1999; Petersel et al., 2011). Primary headaches such as migraine or cluster headache belong to chronic pain conditions that are stand-alone illnesses generally considered as consequences of pathophysiological changes affecting the nociceptive mechanisms of the head and neck regions (Levy, 2010; Niazi et al., 2013; de Tommaso and Sciruicchio, 2016).

Nociceptors innervate the skin, joints, internal surfaces of the body such as the meninges or the periosteum and also internal organs. Intense mechanical-, thermal- and chemical stimuli detected by peripheral nociceptor endings are encoded and conveyed to the central nervous system. Intra- and extracranial structures are innervated by peripheral axons of the trigeminal nerve (fifth cranial nerve), while other parts of the body are supplied by primary sensory neurons of dorsal root ganglia. Translation of nociception into pain perception can be modified by peripheral or central mechanisms sensitizing the nociceptive pathway. Changes in the activity of descending pain controlling pathway may also influence the pain perception and may contribute to pain conditions such as headaches (Coppola et al., 2013; Mainero et al., 2011).

Intracranial nociception

Clinical and experimental observations provide evidence for an essential contribution of peripheral, intracranial nociceptive processes to the generation of headaches (Edvinsson, 2017; Goadsby et al., 2017; Olesen et al., 2009). A large body of evidence supports the hypothesis that headaches, including primary headaches such as migraine, are of trigeminovascular origin (Hoffmann et al., 2017; Messlinger, 2009). They are induced or

influenced by nociceptors innervating the cranial meninges, particularly the dura mater encephali and large intracerebral blood vessels. Intraoperative studies by Ray and Wolff demonstrated that headache-like pain, but not other sensations can be evoked by electrical, mechanical, thermal or chemical stimulation of dural blood vessels and sinuses or large intracerebral arteries (Ray, B.S. and Wolff, H.G, 1940). Collaterals of meningeal nerve fibers project through the skull forming functional connections between extra- and intracranial tissues. Presence of such collaterals offers explanation how noxious stimulation of pericranial tissues can directly influence meningeal nociception associated with headache generation (Kosaras et al., 2009; Schueler et al., 2013).

The dura mater encephali

The dura mater encephali is the outer membrane of the meninges covering the central nervous system. It has two layers; an outer periosteal layer rich in blood vessels, nerves and large collagenous bundles with vascular and fibrous projections into the bone and an inner meningeal layer composed of mesothelial cells. Venous sinuses lie between the two layers of the dura mater encephali. The meningeal layer builds septums between the two hemispheres of the cerebrum (falx cerebri) and the cerebellum (falx cerebelli), it separates the cerebellum and brainstem from the occipital lobes of the cerebrum (tentorium cerebelli) and the pituitary gland (diaphragma sellae) from the cranial cavity (Szentágothai and Réthelyi, 2002).

The main artery of the dura mater is the middle meningeal artery (originating from the maxillary branch of the external carotid artery). It enters the middle cranial fossa through the foramen spinosum and divides into two terminal branches (anterior and posterior). The anterior cranial fossa is supplied with blood also through the anterior meningeal artery (a branch from the anterior ethmoidal artery) and the meningeal branches of the ophthalmic artery. The posterior meningeal artery (a branch of the ascending pharyngeal artery) and meningeal branches from the occipital artery transport blood to the posterior cranial fossa (Szentágothai and Réthelyi, 2002).

Innervation of the dura mater encephali

The meningeal innervation has been extensively studied in rodents but there is general agreement that the findings conform in principle with the human meningeal system. Electron microscopic examinations revealed thin myelinated Aδ and unmyelinated C nerve fibers in

the cranial dura mater (Andres et al., 1987; Messlinger et al., 1993). Besides the sensory trigeminal fibers originating in the ipsilateral trigeminal ganglion (O'Connor and van der Kooy, 1986), a dense network of sympathetic fibers mainly from the superior cervical ganglion (Keller et al., 1989) and a sparse innervation by parasympathetic fibers originating from the sphenopalatine and otic ganglia has been described (Bleys et al., 1996). Sensory and autonomic nerve fibers form dense plexuses around dural arterial blood vessels, but single axons can be observed also in the avascular regions of the meningeal tissue, i.e. in areas distant from larger blood vessels.

The sensory innervation of the dura mater is served by all three branches of the trigeminal nerve; the ophthalmic (V1), the maxillary (V2) and the mandibular (V3) divisions (McNaughton, M., 1938). The central terminals of meningeal nociceptors enter the brain stem at the pontine level and synapse in the caudal part of the trigeminal nucleus. The occipital region of the dura mater is innervated by the first and second spinal ganglia (Keller et al., 1985) that project to the cervical dorsal horn. Ultrastructural analyses of trigeminal afferents revealed that the majority of meningeal Aδ and C fibers terminate as free nerve endings. Encapsulated Ruffini-like receptors and lamellated nerve terminals have additionally been described in higher vertebrates including man, particularly at sites where cerebral veins enter the sagittal sinus (Andres et al., 1987).

The trigeminal nociceptive pathway

The majority of the neurons in caudal part of the spinal trigeminal nucleus and the cervical dorsal horn send axons to contralateral thalamic nuclei that relay ascending somatosensory information to the primary somatic sensory cortex. The major thalamic nuclei processing the nociceptive information are the ventroposteromedial nucleus and the posterior nuclear complex. Tracing studies revealed that neurons in the caudal part of the trigeminal nucleus also project to other brainstem- and diencephalic structures such as the brainstem reticular formation, the nucleus of the solitary tract, the superior salivatory nucleus, the periaqueductal grey matter, the inferior colliculus, the parabrachial nuclei, the hypothalamus and the cerebellum (Bernard et al., 1989; Guy et al., 2005; Malick et al., 2000; Mantle-St John and Tracey, 1987).

Perception of headache is a complex function of the cerebral cortex and involves distinct parts of the brain that process sensory discriminative, affective-emotional and

cognitive aspects of nociception. Clinical studies utilizing modern imaging techniques indicated that the activation of a cortical network involving the primary and secondary somatosensory cortices, the insular cortex, the anterior cingulate cortex and the frontal cortex is associated with nociceptive experience (Gauriau and Bernard, 2004; Hadjipavlou et al., 2006; Noseda et al., 2010).

The transmission of trigeminal nociceptive information to second order neurons is controlled by the inhibitory pathway descending from the periaqueductal gray matter and from the rostral ventromedial medulla. The periaqueductal grey matter located around the Sylvius aqueduct in the mesencephalon is the key structure in descending pain modulation with its powerful inhibitory properties on pain perception. Inputs from higher cerebral cortical structures, hypothalamus and amygdala converge on the periaqueductal grey matter and the rostral ventromedial medulla to exert pain-suppressive effect in the trigeminal nucleus (Aimone and Gebhart, 1988; Gebhart, 2004; Lakos and Basbaum, 1988; Yaksh, 1985).

Chemosensitive primary sensory neurons in the trigeminal system

Chemosensitive primary sensory neurons represent a unique population of trigeminal ganglion neurons. They are small or medium-sized pseudounipolar neurons with thinly myelinated Aδ- or unmyelinated C-fibers (Jancsó and Király, 1981; Jancsó et al., 1977). They express different members of the transient receptor potential (TRP) receptor family (Benemei et al., 2015; Jancsó and Király, 1981; Jancsó et al., 1977; Lehmann et al., 2016). TRP receptors are nonselective cation channels (Julius, 2013). In general, TRP channels act as molecular sensors of multiple stimuli; changes in pH, chemical agents, temperature and osmolarity. The TRP ion channel family is composed of six subfamilies, classified as canonical (TRPC), vanilloid (TRPV), ankyrin (TRPA), melastatin (TRPM), polycystin (TRPP), and mucolipin (TRPML) (Nilius and Owsianik, 2011; Wu et al., 2010). The ability to respond to different stimuli has focused attention on the TRP channels in many different physiological and pathophysiological processes.

Functions of the transient receptor potential vanilloid 1 (TRPV1) receptor

The transient receptor potential vanilloid 1 (TRPV1) channel is the best-characterised TRP channel to date. The functional channel is a tetramer formed by four subunits. A receptor

subunite has six transmembrane domains with a short pore-forming hydrophobic loop localized between the fifth and sixth transmembrane segments. In the peripheral nervous system TRPV1 is preferentially expressed in small to medium sized nociceptor neurons of the trigeminal- and dorsal root ganglia (Huang et al., 2012; Hwang et al., 2005).

The receptor has been identified in peripheral terminals of nociceptors innervating the skin (Tsukagoshi et al., 2006), meningeal tissue (Dux et al., 2003), urinary bladder (Liu et al., 2014), respiratory tract (De Logu et al., 2016; Zhao et al., 2016), cochlea (Vass et al., 2004) and the heart (Freichel et al., 2017). Presence of TRPV1 receptors on structures of the central nervous system such as hypothalamus, hippocampus and substantia nigra has been also documented (Cristino et al., 2006). Expression of the receptor is not restricted to neuronal tissues; non-neuronal tissues expressing TRPV1 are vascular smooth muscle (Sand et al., 2015), keratinocytes of the epidermis (Caterina and Pang, 2016), urothelium and smooth muscle of the urinary bladder (Lazzeri et al., 2004), polymorphonuclear granulocytes (Köse and Nazıroğlu, 2015) and macrophages (Ninomiya et al., 2017).

The TRPV1 receptor can be activated by high temperature (>43°C), acidic pH, and a wide range of both exogenous and endogenous compounds. Its main exogenous ligand is capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), agent in red pepper. Another naturally occuring agonist of the receptor is resiniferatoxin, a diterpene related to phorbol esters (Szallasi and Blumberg, 1989). Resiniferatoxin shares a vanillyl group with capsaicin and it is a particularly strong irritant that was isolated from the latex of the Moroccan cactus-like plant *Euphorbia resinifera* (Appendino and Szallasi, 1997; Hergenhahn et al., 1984). Piperine and zingerone, two pungent tasting compounds found in black pepper and ginger, respectively, have also been shown to activate TRPV1 receptors (Liu and Simon, 1996). Reactive oxygen species may modulate TRPV1 function by modifying different cysteine residues participating in disulfide bonds (Taylor-Clark, 2016).

Different metabolites of membrane lipids have been recently characterised as endogenous activators of the TRPV1 receptor (Di Marzo et al., 2002). Eicosanoids that are products of lipoxygenase and endovanilloids acting also as endocannabinoids activate TRPV1 channels (Zygmunt et al., 1999). Arachidonylethanolamide (anandamide) is probably the most widely studied endogenous ligand that acts on both cannabinoid (CB) and TRPV1 receptors. Anandamide and N-arachidonoyl-dopamine (NADA) have been previously identified in dorsal root ganglion neurons as potential endogenous activators of TRPV1 under

physiological or pathophysiological conditions (Dinis et al., 2004; Khasabova et al., 2013; van der Stelt et al., 2005).

Similar to many other channel proteins, TRPV1 contains multiple phosphorylation sites in its amino acid sequence for protein kinase C (PKC) (Bhave et al., 2003; Premkumar et al., 2004), protein kinase A (PKA) (De Petrocellis et al., 2001; Rathee et al., 2002) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Zhang et al., 2011). The presence of multiple phosphorylation sites in TRPV1 implies possible regulatory actions by these kinases. Activation or sensitization of TRPV1 can be achieved by inflammatory agents such as bradykinin, serotonin, histamine, or prostaglandins, which stimulate TRPV1 either by protein kinase C-dependent pathways (Premkumar and Ahern, 2000; Vellani et al., 2001), by releasing the channel from phosphatidylinositol 4,5-bisphosphate (PIP2)-dependent inhibition (Prescott and Julius, 2003), by a protein kinase A-mediated recovery from inactivation (Bhave et al., 2003) or by formation of 12-hydroperoxyeicosatetraenoic acid (Shin et al., 2002).

Capsaicin produces burning pain in humans when brought into contact with the skin or mucosa, but its topical application to the skin has been found useful in the treatment of some pain states (Nagy et al., 2004; Sawynok, 2003; Szallasi and Blumberg, 1999). Experimental evidence has been presented that capsaicin causes persistent functional desensitization of polymodal primary nociceptors after repeated or prolonged application (Carpenter and Lynn, 1981; Jancsó et al., 1967). This desensitization was suggested to occur due to functional and morphological alterations of sensory neurons (Jancsó et al., 1977, 1985, 1967; Király et al., 1991; Mohapatra and Nau, 2003; Szallasi and Blumberg, 1992). PIP2 is a quantitatively minor membrane phospholipid that is a positive cofactor for TRPV1, acting via direct interaction with the receptor. Depletion of PIP2 by calcium-induced activation of phospholipase C limits TRPV1 activity leading to capsaicin-induced desensitization of the channel.

Calcium influx via TRPV1 activation may trigger the degeneration of chemosensitive neurons, that leads to desensitization of the channel protein complex itself and, in extreme cases, to the degeneration of TRPV1-expressing sensory neurons due to calcium overload resulting in lysosomal breakdown and activation of proteases (Jancsó et al., 1978, 1984; Király et al., 1991; Olah et al., 2001).

Function of the transient receptor potential ankyrin 1 (TRPA1) receptor

TRPA1 is the sole member of the TRPA subfamily in mammals. The TRPA1 channel is characterised by multiple N-terminal ankyrin repeats. It is expressed in a subset of TRPV1 receptor expressing chemosensitive primary sensory neurons (Salas et al., 2009). Recent studies found evidence that TRPA1 is involved in sensory neural responses to mustard oil (allyl isothiocyanate), allicin, cinnamaldehyde and gingerol (Nieto-Posadas et al., 2011). Acrolein that is a component of tobacco smoke and other inhaled environmental irritants are known triggers of migraine headache attacks in susceptible individuals (Benemei et al., 2014; Silva-Néto et al., 2014). They may induce also coughing, apnea and lacrimation through the activation of chemosensitive nociceptors. Moreover, TRPA1 may serve as a sensor for noxious cold temperature (Pan et al., 2017). Other studies identified TRPA1 as a candidate for the auditory hair cell transduction channel (Corey et al., 2004). Recent observations indicate that two gaseous signaling molecules produced also under physiological conditions in different tissues, hydrogen sulphide and nitric oxide may interact and generate nitroxyl, the redox sibling of nitric oxide. Nitroxyl activates the sensory chemoreceptor channel TRPA1 via formation of amino-terminal disulphide bonds, which results in sustained calcium influx and activation of the nociceptor. Similar to the TRPV1 receptor, reactive oxigen species produced in oxidative stress may activate/sensitize the TRPA1 receptor leading to disulphide formation and altered kinetic of channel opening (Dux et al., 2016; Eberhardt et al., 2014).

Peptidergic population of trigeminal chemosensitive neurons

A significant population of TRPV1 and TRPA1 receptor expressing trigeminal nociceptors are peptidergic. Retrograde tracing experiments indicated that nearly 80 % of dural afferents expressing TRPV1 receptors also exhibited calcitonin gene-related peptide-(CGRP) immunoreactivity (Shimizu et al., 2007). Meningeal nerve fibers immunoreactive for calcitonin CGRP, substance P (SP) or neurokinine A (NKA) are considered to be afferents of the trigeminal sensory system. Few nerve fibers immunopositive for pituitary adenylate cyclase-activating polypeptide (PACAP) were also found in rat dura mater (Edvinsson et al., 2018; Jansen-Olesen and Hougaard Pedersen, 2018). Although PACAP can be found also in parasympathetic nerve fibers innervating the dura mater, its colocalization with CGRP in some nerve fibers indicates that at least some of those fibers have sensory function (Edvinsson et al., 2001). The majority of the CGRP-immunoreactive fibers are distributed to

branches of the anterior and middle meningeal arteries and to the superior sagittal and transverse sinuses (Keller and Marfurt, 1991; Messlinger et al., 1993). SP-like immunoreactivity was found coexpressed with CGRP in a small proportion of thin unmyelinated nerve fibers. However, the CGRP-immunoreactive nerve fibers outnumber the SP-positive ones.

Chemosensitive meningeal afferents containing neuropeptides possess a dual function. Peptides contained in primary sensory neurons serve not only as transmitters at the central synapses of nociceptive afferents (Duggan et al., 1987; Hökfelt et al., 1980; Lawson et al., 1993) but they also play a role in the mechanisms of the neurogenic inflammatory response of the innervated tissue (Fusco et al., 2003; Jancsó et al., 1967; Maggi and Meli, 1988). Besides transmitting the nociceptive information to the central nervous system, stimulation of nociceptive afferents leads also to neuropeptide release from their peripheral terminals. This unique efferent function of nociceptors induces neurovascular reactions; neurogenic vasodilatation induced by CGRP release, and neurogenic plasma extravasation mediated by SP.

Different mediators originating from different sources have been shown to modulate the release of sensory neuropeptides from peripheral endings of chemosensitive neurons. For many of these mediators the presence of specific receptors on cell bodies or terminals of sensory neurons has been documented. Histamine acting on H_1 receptors and serotonin/5-hydroxytryptamine (5HT) on 5-HT_3 receptors are considered to excite sensory nerves and cause neuropeptide release directly (Dux et al., 2002; Fischer et al., 2017). Purinergic P2Y receptors and P2X receptor channels activated by adenosine triphosphate (ATP) are also expressed in trigeminal afferents, partly colocalized with TRPV1 receptors (Ichikawa and Sugimoto, 2004; Ruan and Burnstock, 2003). Experimental results indicate that ATP acting on P2Y receptors enhances the proton-induced CGRP release from the isolated rat dura mater (Zimmermann et al., 2002).

A large variety of transmitters and mediators has been shown to negatively influence the neuropeptide release from peripheral endings of primary sensory neurons. Prejunctional suppression of transmitter release can be mediated by the G-protein coupled 5-HT_{1D} and 5-HT_{1F} receptors localised on trigeminal nerve fibers (Amrutkar et al., 2012; Buzzi and Moskowitz, 1991). A wide variety of experimental results have shown that 5-HT_1 receptor agonists inhibit the neurogenic inflammation in the dura mater encephali of experimental

animals. Clinical observations prove that the 5-$HT_{1B/1D}$ receptor agonists (“triptans”) used in migraine therapy selectively suppress neurotransmission from meningeal sensory nerve fibers (Burstein and Jakubowski, 2004; Macone and Perloff, 2017).

Stimulation of cannabinoid (CB) receptor 1 of chemosensitive neurons decreasing intracellular cyclic adenosine monophosphate (cAMP) production also reduces the transmitter release from nerve terminals. In the trigeminal system cannabinoid CB1 receptor immunoreactive neurons were found mainly in the maxillary and mandibular divisions of the trigeminal nerve (Price et al., 2003). Earlier observations indicate, that activation of trigeminal CB1 receptors inhibited dural vasodilatation brought about by electrical stimulation of the dura mater (Akerman et al., 2004), and the release of CGRP induced by thermal stimulation in an *in vitro* dura mater preparation (Fischer and Messlinger, 2007). Activation of CB1 receptors may have a particular role in the regulation of CGRP release from TRPV1 expressing neurons, since both receptors can be activated by the same endogenous lipid metabolites anandamide and NADA acting on both TRPV1 and CB1 receptors, although with different efficacies (Price et al., 2004).

Possible role of the trigeminal nocisensor complex in meningeal nociception

The elements of the trigeminal nocisensor complex include the trigeminovascular chemosensitive primary afferent neurons, the meningeal blood vessels and the dural mast cells (Dux et al., 2012). The elements of this complex are both anatomically and functionally interconnected and may be regarded as an important entity in pathophysiological processes of meningeal nociception. Activation and sensitization of meningeal nociceptors by inflammatory agents is an important peripheral mechanism in the initiation of a migraine attack (Goadsby, 2007). Some of these agents may act on their specific receptors expressed on nociceptors, whereas others may activate the TRPV1 and TRPA1 ion channels leading to the release of neuropeptides from nerve terminals. Dural mast cells localized in the close vicinity of blood vessels and also of afferent nerves have high sensitivity for sensory neuropeptides. CGRP and SP may activate meningeal mast cells resulting in the release of mast cell constituents and mediators, such as histamine and the proteolytic enzyme, tryptase (Johnson and Erdös, 1973; Li et al., 2012). Besides the direct vasodilatory effect of histamine, released mast cell constituents may further activate meningeal chemosensitive nociceptors resulting in additional neuropeptide release and central activation.

Consequences of the activation of dural chemosensitive nocisensors may be regarded as components of a positive feedback regulation, which may exaggerate the initial nociceptive and vascular responses. Considering the decreased dural CGRP levels after prolonged electrical stimulation of trigeminal afferents (Knyihár-Csillik et al., 1998; Samsam et al., 2001), it is possible that depletion of the peptide and the consequently supressed activation level of the trigeminovascular nocisensor complex may be related to the cessation of head pain. In addition, neurogenic sensory vasodilatation may have also beneficial effects by removing tissue metabolites inducing or aggravating headache attacks (Dux et al., 2003; Marics et al., 2017a, 2017b) (Fig. 1)**.**

The significance of neurogenic plasma extravasation in migraine pathogenesis is controversial (Williamson and Hargreaves, 2001). Although neurokinin 1 (NK1) receptor antagonists effectively inhibit plasma extravasation in experimental animals, clinical studies suggested that a migraine attack can neither be alleviated nor prevented by neurokinin receptor antagonists. Hence, neurogenic plasma extravasation was suggested to be of minor significance as a pathogenetic factor of migraine (Diener and RPR100893 Study Group, 2003). In contrast, neurogenic sensory vasodilatation mediated by CGRP appears to play an important role in the pathomechanism of migraine. Although the findings are somewhat controversial, its release into the jugular blood has been demonstrated during migraine attacks in humans (Goadsby et al., 1990; Messlinger, 2018; Ramachandran, 2018).

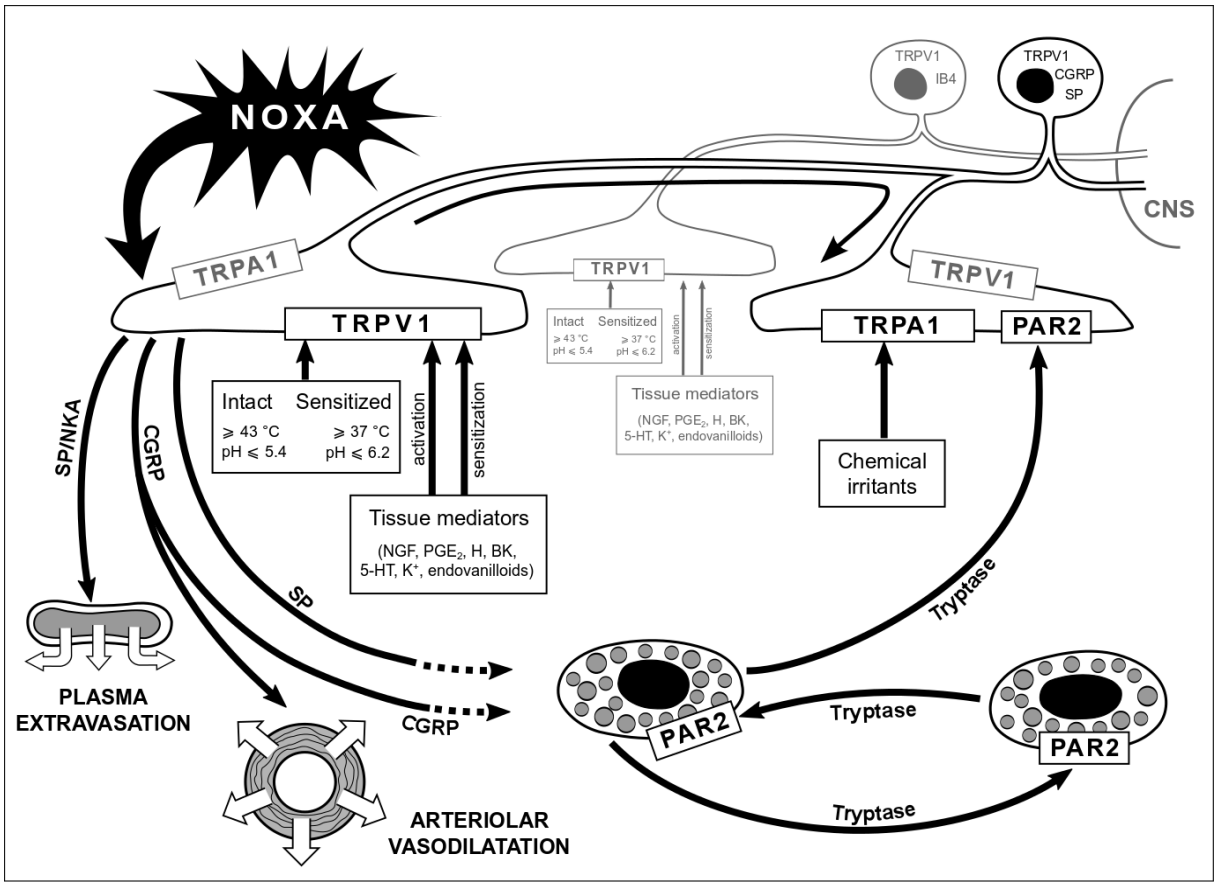

Figure 1. The trigeminal nocisensor complex. TRPA1: transient receptor potential ankyrin 1, TRPV1: transient receptor potential vanilloid 1, SP/NKA: substance P/neurokinine A, CGRP: calcitonin gene-related peptide, PAR2: proteinase-activated receptor-2, NGF: nerve growth factor, PGE2: prostaglandin E2, H: histamine, BK: bradykinine, 5-HT: 5-hydroxytryptamine, CNS: central nervous system, IB4: Griffonia simplicifolia isolectin B4 (Dux et al., 2012).

CGRP and the CGRP receptor

CGRP is a sensory neuropeptide consisting of 37 amino acids. It has two forms; αCGRP and βCGRP which are derived from two separate genes and differ by only three amino acids in humans (Wimalawansa et al., 1990). αCGRP is found in nerves throughout the central and peripheral nervous systems (Maggi, 1995; Rosenfeld et al., 1983), innervating the vasculature (Dux et al., 2003; Keller and Marfurt, 1991; Messlinger et al., 2011), whereas βCGRP is found mainly in the enteric nervous system (Mulderry et al., 1985).

CGRP is regarded as one of the key mediators in both nociceptive transmission and meningeal arterial vasodilatation that are critical pathophysiological components of headaches. Current migraine therapies are based on reducing CGRP effects by either

inhibiting its release from meningeal nociceptors (triptans acting on presynaptic 5-$HT_{1B/1D}$ receptors) or blocking the CGRP receptor (non-peptide CGRP receptor antagonists "gepants") (Karsan and Goadsby, 2015). Administration of humanized monoclonal antibodies targeting CGRP or its receptor appears also a promising new strategy in the therapy of migraine (Bigal et al., 2015; Dux, M. and Messlinger, K., 2015). Antibodies proved to be superior over placebo in reducing the frequency of migraine days in frequent or chronic migraine. Their beneficial effect is a clear proof of the principle that blocking the CGRP system is therapeutic in migraine.

The CGRP receptor consists of a large peptide with seven transmembrane domains, the calcitonin receptor-like receptor (CLR) complemented by a single transmembrane domain, the receptor activity-modifying protein 1 (RAMP1). RAMP1 is responsible for the specific binding of CGRP. The functional CGRP receptor complex contains also a third intracellular protein, the receptor component protein (RCP), which couples the receptor to the intracellular signal pathway through stimulatory G-protein and adenylyl cyclase (Evans et al., 2000; Flühmann et al., 1995; Messlinger, 2018; Muff et al., 1998).

CLR and RAMP1 are present on smooth muscle of dural arterial blood vessels, as well as on mononuclear and Schwann cells (Lennerz et al., 2008). Also some thicker CGRP-negative A-fibers of rodent and human dura may express CLR and RAMP1 receptor components (Eftekhari et al., 2013). In the trigeminal ganglion CGRP receptor immunoreactivity has been found in neurons and satellite glial cells (Eftekhari and Edvinsson, 2010; Lennerz et al., 2008).

CGRP is the currently known most potent vasodilator with a relatively long-lasting effect. Its potency is tenfold higher than that of the most potent prostaglandins (Brain et al., 1985). Several pathways are thought to be involved in CGRP-dependent vasodilatation, including endothelium-dependent and endothelium-independent pathways (Brain and Grant, 2004). The most commonly observed pathway is the endothelium-independent pathway. CGRP binding to its receptors on smooth muscle activates adenylate cyclase to trigger cAMP production. CGRP-induced vasodilatation in human intracranial arteries is mediated by cAMP production (Edvinsson et al., 1998). The subsequent activation of protein kinase A may lead to the phosphorylation and opening of ATP-sensitive potassium channels, resulting in hyperpolarisation and consequent relaxation of smooth muscle cells. Glibenclamide, the ATP-sensitive potassium channel blocker, effectively blocks the CGRP-induced vasorelaxation

(Nelson et al., 1990). In experimental animals, increase in the diameter of dural arteries induced by CGRP was blocked by systemic infusion of glibenclamid, although *in vitro* measurements could not confirm this finding (Gozalov et al., 2008). Other potassium channels have also been suggested to be involved, such as large-conductance calcium-activated potassium channels in pial arteries (Hong et al., 1996).

Although in endothelial cells of human cerebral arteries the presence of messenger RNA (mRNA) for all components of the CGRP receptor have been demonstrated, since removal of the endothelium induced no functional difference in CGRP-induced vasorelaxation, the endothelial receptors might be less sensitive than the smooth muscle cell receptors (Jansen-Olesen et al., 2003). Activation of endothelial CGRP receptors leads to production of NO that diffuses into the smooth muscle cells and activates guanylate cyclase leading to cyclic guanosin monophosphate (cGMP) production and vasorelaxation (Gray and Marshall, 1992). CGRP-induced increase in meningeal blood flow was suppresed by inhibition of endothelial nitric oxide synthase (Akerman et al., 2002). In experimental animals, vasodilatation evoked by electrical stimulation of the dura mater has been shown to be mainly mediated by CGRP released from dural afferent nerves. Systemic or topical administration of NO-synthase inhibitors reduced the electrically evoked increases in meningeal blood flow (Messlinger et al., 2000) (Fig. 2).

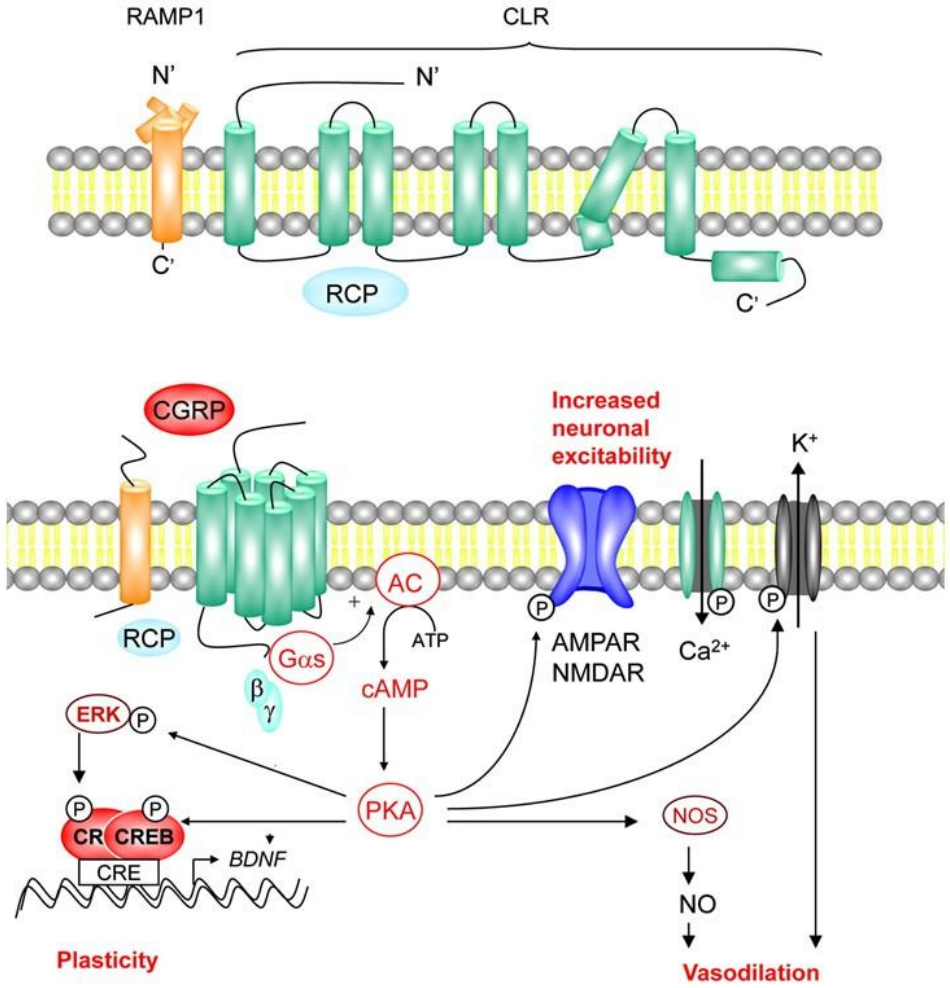

Figure 2. The CGRP receptor and its intracellular signaling. RAMP1: receptor activity-modifying protein 1, CLR: calcitonin receptor-like receptor, RCP: receptor component protein, CGRP: calcitonin gene-related peptide, AC: adenylate cyclase, cAMP: cyclic adenosine monophosphate, ATP: adenosine triphosphate, PKA: protein kinase A, CREB: cAMP response element-binding protein, CRE: cAMP response element, BDNF: brain-derived neurotrophic factor, Gαs: G-protein α subunit, AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, NMDAR: N-methyl-D-aspartate receptor, NOS: nitric oxide synthase, NO: nitric oxide (Benarroch, 2011).

Adriamycin-induced neurotoxicity

Anthracyclines comprise an important class of chemotherapeutic agents used in the treatment of various malignancies (Carvalho et al., 2009; Kalyanaraman et al., 2002). Adriamycin is one of the most commonly used antracycline derivative in both adult and pediatric oncology (Fig. 3). Its antineoplastic effect is based on various mechanisms. Adriamycin inhibits both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis

by intercalating DNA base pairs leading to breaks in the helical strands (Buja et al., 1974; Kellogg et al., 1998; Meriwether and Bachur, 1972; Pigram et al., 1972). Adriamycin also inhibits DNA-dependent enzymes such as topoisomerase II (Pommier et al., 2010; Tewey et al., 1984). Oxidative damage to DNA induced by free radicals have been identified as the main citotoxic effect of adriamycin in different types of lymphoma, in cases of breast- (Pilco-Ferreto and Calaf, 2016) and lung cancer (Farhane et al., 2017). Increase in intracellular calcium concentration via impairment of membrane- and intracellular transporters may be an additional mechanism of antitumor effect (Keyes et al., 1987; Kohnoe et al., 1992).

Besides its beneficial effects, adriamycin may also have serious side effects altering cardiac functions. Electrocardiographic abnormalities are non-dose-related and reversibel changes suppressed by the termination of the adriamycin treatment. The dose-dependent, usually irreversibel congestive dilatative cardiomyopathy can be a fatal consequence of adriamycin treatment (Carvalho et al., 2014; Ferreira et al., 2008; Renu et al., 2018).

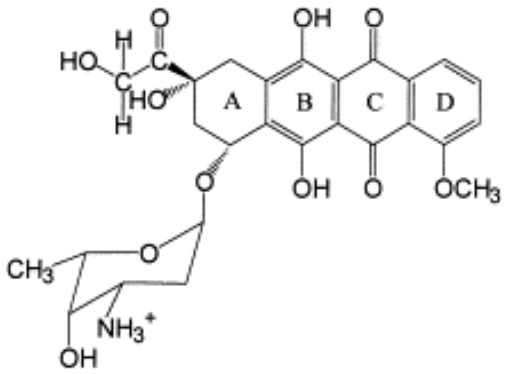

Figure 3. The chemical structure of adriamycin

Clinical observations and experimental results indicate that adriamycin exerts also a neurotoxic effect. Primary sensory neurons (Bigotte and Olsson, 1982; Kondo et al., 1987; Minow and Gottlieb, 1975), motoneurons (Liu et al., 1996; Yamamoto et al., 1984) and sympathetic efferents (Jeon et al., 2000) can be affected. Recent studies demonstrated marked structural, neurochemical and functional impairments of primary sensory neurons in animal models of adriamycin toxicity (El-Agamy et al., 2017; Kosoko et al., 2017). Neurotoxic propensity of adriamycin manifests as deleterious actions on chemosensitive sensory neurons which express transient receptor potential nociceptive ion channels (Boros et al., 2016). Recent observations in our laboratory indicated profound adriamycin-induced changes in the density of intraepidermal chemosensitive afferent axons, whereas the distribution and density

of subepidermal nerve fibers were apparently unaffected (Boros et al., 2016). Adriamycin can reach the sensory ganglion cells in different ways; it may be taken up directly from the blood due to the high vascular permeability in the dorsal root ganglia (Klosen et al., 1993) or it can be transported to the perikaryon by retrograde axonal transport (Bigotte and Olsson, 1982; Kondo et al., 1987). Adriamycin is not able to pass the blood-brain barrier but it may enter the choroid plexus and the circumventricular organs from where axonal transport may deliver it to different areas in the central nervous system (Bigotte and Olsson, 1984; Koda and Van der Kooy, 1983).

Aims of the study

Recent investigations into the mechanisms of headaches indicate that CGRP plays a central role in trigeminovascular functions including both the afferent transmission of nociceptive signals of meningeal origin and the initiation of vascular changes in the dura mater. Activation by specific agonists of nociceptive ion channels TRPV1 and TRPA1 expressed in CGRP-containing primary sensory neurons elicit meningeal vasodilatation. Previous findings demonstrated that the TRPV1 receptor-activation-induced meningeal vasodilatory response is a sensitive functional marker of the integrity of meningeal afferent nerves. Therefore, the aim of the present experiments was to examine the possible contribution of endogenous vanilloid compounds (endovanilloids) to meningeal TRPV1 receptor mediated vascular reactions and CGRP release. Further, we also initiated experiments to study the role of CB1 receptor activation in reactions induced by topically applied endogenous vanilloid/cannabinoid compounds, anandamide and NADA, which have been previously identified in dorsal root ganglion neurons. A further aim of this study was to explore the effects of adriamycin, a toxic anticancer agent on changes in meningeal blood flow elicited by specific agonists of the TRPV1 and TRPA1 receptors. We also examined changes in TRPV1 protein content of trigeminal ganglia, the expression and distribution of the TRPV1 receptor, and the CGRP and vascular CGRP receptor components using immunohistochemistry in the dura mater after systemic adriamycin treatment.

Since somatosensory and visceral chemosensitive nerves share many common characteristics, functional alterations observed in the meningeal trigeminovascular system may provide useful information on possible impairments in nociceptive functions of other organs following metabolic, hormonal or toxic changes affecting the integrity of the whole organism.

MATERIALS AND METHODS

Experimental animals

The experiments were approved by the Ethical Committee for Animal Care of the University of Szeged. Study procedures were carried out in accordance with the Directive 2010/63/EU of the European Parliament. All efforts were made to minimize the number of animals used and their suffering. Animals were raised and maintained under standard laboratory conditions.

Control, capsaicin-desensitized and adriamycin-treated adult male Wistar rats weighing 270-350 g were used. The number of animals in different experimental groups was between 6-17. Capsaicin desensitization of animals was induced by subcutaneous injections of capsaicin on three consecutive days at increasing doses of 10, 20 and 100 mg/kg (Ferdinandy et al., 1997). One group of animals received a cumulative dose of 15 mg/kg of adriamycin (Doxorubicin, Pharmacia Italia, Italy) by intravenous injection of 2.5 mg/kg of the drug three times a week for 2 weeks (Katona et al., 2004; Tong et al., 1991). Rats given the solvent for capsaicin (6% ethanol and 8% Tween 80 in saline) or vehicle for adriamycin (saline) served as controls, respectively. All experiments were performed 2-7 days after the termination of the treatment of the animals.

In vivo measurement of meningeal blood flow

Rats were anaesthetized with an initial dose of thiopental sodium (100-120 mg/kg, i.p. Thiopental, Biochemie GmbH, Austria or Insera Arzneimittel GmbH, Germany). Additional doses of thiopental sodium (25 mg/kg i.p.) were administered throughout the experiment to avoid changes in systemic blood pressure or nociceptive reactions to noxious stimuli. Systemic blood pressure was recorded with a pressure transducer via a cannula inserted into the 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 (Dux et al., 2003; Kurosawa et al., 1995).

A cranial window for the measurement of dural blood flow was prepared according to Kurosawa (Kurosawa et al., 1995). 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 4 x 6 mm was drilled into the parietal bone to expose the underlying dura

mater and to allow identification of the branches of the middle meningeal artery (Fig. 4). To avoid thermal lesions, the bone was cooled with saline. The cranial window was carefully filled with a modified synthetic interstitial fluid (SIF) containing (in mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 5 $CaCl_2$, 10 glucose and 10 Hepes, pH = 7.4 (Levy and Strassman, 2002; Strassman et al., 1996). Dural blood flow was measured with the needle-type probes of a laser Doppler flowmeter (Perimed, Sweden). The probes were placed over branches of the middle meningeal artery lying distant from visible cortical blood vessels (Fig. 4). Under these experimental conditions, the flow signal recorded from the cortical blood vessels is minimized (Kurosawa et al., 1995). Blood flow was recorded at a sampling rate of 1 Hz and was expressed in perfusion units (PU). Meningeal blood flow, systemic blood pressure and body temperature were recorded simultaneously. Data were stored and processed with the Perisoft program (Perimed, Sweden). The mean blood flow measured during a 3 or 5-min period prior to drug application was regarded as the basal flow in different experiments. Changes induced in blood flow by the application of drugs were expressed as percentage changes relative to the basal flow (mean ± SEM) calculated for the 3-min application period. The effects of TRPV1-, CGRP- and CB1 receptor antagonists on the endogenous vanilloid-induced blood flow changes were determined by comparing the changes in blood flow in response to stimulation before and after the application of the respective antagonist(s).

At the end of the experiments, the animals were killed with an overdose of thiopental sodium (250 mg/kg i.p.).

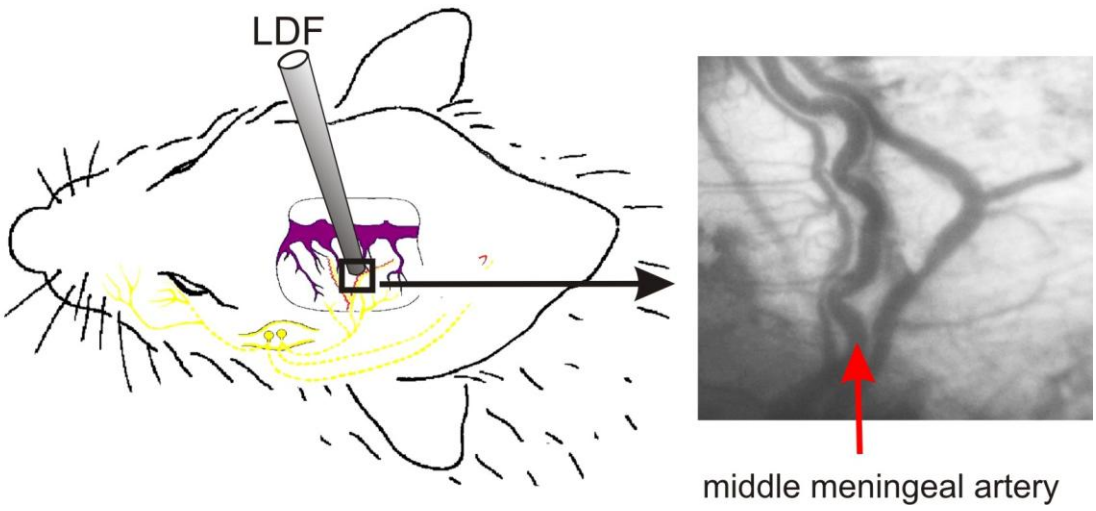

Figure 4. Cranial window preparation for blood flow measurement in rat. LDF: laser Doppler flow probe

Topical epidural application of drugs

Effects of endovanilloids on meningeal blood flow: the role of chemosensitive afferents

Stock solutions of capsaicin, capsazepine, anandamide, NADA and the CB1 receptor antagonist AM 251 were prepared. Capsaicin (32 mM) and capsazepine (1 mM) were dissolved in saline containing 6% ethanol and 8% Tween 80. Stock solutions of anandamide (14 mM), NADA (11 mM) and AM 251 (10 mM) were prepared with ethanol. Before the experiment drugs were further diluted with SIF to their final concentration. Substances were applied topically onto the exposed dura mater in 40 µl volume.

The blood flow increasing effect of capsaicin (100 nM) placed onto the exposed surface of the dura mater was measured, then anandamide or NADA were applied at increasing concentrations (anandamide 100 nM, 1 µM and 10 µM, NADA 10 nM, 100 nM and 1 µM). To minimize the desensitizing effect of repeated vanilloid applications, in this series of experiments drugs were applied for 3 min.

To determine the contribution of TRPV1 receptor and the role of CGRP release in the endovanilloid-induced changes in meningeal blood flow, the TRPV1 receptor antagonist capsazepine (10 µM) or the CGRP receptor antagonist $CGRP_{8-37}$ (100 µM) were applied for 5 min prior to NADA (100 nM). Meningeal blood flow changes induced by the topical applications of capsaicin (100 nM) and NADA (100 nM) were determined in capsaicin-desensitized animals too. After completion of the measurement of the vanilloid-induced blood flow changes, blood flow increasing effect of histamine at 10 µM was also measured in control and capsaicin-desensitized rats.

To study the role of CB1 receptor activation on endovanilloid-induced meningeal vasodilatation, anandamide at 10 µM was administered before and after the application of CB1 receptor antagonist AM 251 (100 µM) for 5 min. In some experiments the vasodilatory effect of anandamide (10 µM) was measured also after blocking both CB1- and CGRP receptors with pretreatment of the dura mater with AM 251 (100 µM) and $CGRP_{8-37}$ (100 µM).

Effects of adriamycin on meningeal blood flow responses

A stock solution of capsaicin (32 mM) was prepared with the aid of 6 % ethanol and 8 % Tween 80 in saline and was further diluted with SIF. Stock solution of forskolin (10 mM) was prepared with ethanol. Acrolein, CGRP, histamine and acetylcholine were dissolved in

SIF and diluted immediately before use.

To study the effect of adriamycin treatment on meningeal vascular responses, the dura mater was stimulated with repeated applications of capsaicin (100 nM), acrolein (300 μM) and CGRP (10 μM) for 3 min. During the washout periods between consecutive drug applications the blood flow recovered to basal values. The effects of single histamine (10 μM), acetylcholine (100 μM) and forskolin (10 μM) applications were also tested in control and adriamycin-treated animals.

All drugs used in *in vivo* blood flow measurements but anandamide, NADA and AM 251 (Tocris Bioscience, United Kingdom) were purchased from Sigma-Aldrich Chemie Gmbh, Hungary.

Measurement of CGRP release in *ex vivo* preparations

The release of CGRP from dural afferents was measured by the method of Ebersberger (Ebersberger et al., 1999). Control and adriamycin-treated rats were deeply anesthetized with thiopental sodium (150-200 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 without touching the dura mater encephali. The skull preparations were washed with carbogen-gassed SIF at room temperature for 30 min and then mounted in a humid chamber at 37 °C. The cranial fossae were filled with 300 μl of SIF solution. Samples of the superfusate were collected at periods of 5 min by carefully removing the content of the skull halves with a pipette.

Control samples were taken to determine basal CGRP release. In some experiments the second sample was collected after incubation in the presence of NADA at 100 nM and the third sample after capsaicin at 100 nM. In another series of experiments the effect of CB1 receptor antagonist pretreatment was studied on the anandamide- (10 μM) induced CGRP release; in these preparations after measuring the basal CGRP release, anandamide (10 μM) was applied twice for 5 min. CB1 receptor antagonist AM 251 was applied at 100 μM prior to the second anandamide application.

In other experiments CGRP releasing effect of repeated applications of capsaicin at 100 nM or acrolein at 300 μM was studied in control and adriamycin-treated animals. Capsaicin and acrolein were applied three times separated by washout periods with SIF. CGRP releasing effect of KCl (60 mM) application was also measured and compared to basal

CGRP release. 100 µl of samples diluted with 25 µl of enzyme-linked immunoassay (EIA) buffer were placed into Eppendorf cups and immediately frozen at -70 ^{o}C for later analysis. The CGRP contents of the samples were measured with an EIA kit (Bertin Pharma, SPIbio, France). The absorbance of the reaction product representing the CGRP content of the sample was determined photometrically, using a microplate reader (DYNEX MRX, USA). The CGRP concentrations of the superfusates were expressed in pg/ml. The minimum detection limit of the assay was 2 pg/ml CGRP. Changes induced in CGRP release by capsaicin, endovanilloids, acrolein and KCl were expressed as percentage changes relative to the basal release. Changes in anandamide-induced CGRP release were compared before and after CB1antagonist pretreatment.

Measurement of the TRPV1 protein content in the trigeminal ganglion

Control and adriamycin-treated animals were deeply anesthetized with thiopental sodium (200 mg/kg, i.p.). The animals were decapitated, the skin and muscles were removed and the skull was divided into halves along the midline. Trigeminal ganglia were cut out and homogenized in phosphate buffered saline. The samples were stored overnight at -20 °C. Two freeze-thaw cycles were performed before centrifuging the homogenates at 4 °C for 5 min at 5 *g*. The supernatants were removed and frozen at -70 °C for subsequent analysis. Concentration of TRPV1 protein in tissue samples was determined by EIA method (Aviva Systems Biology, USA) and expressed in pg/mg tissue.

Immunohistochemistry

Control and adriamycin-treated rats not used for *in vivo* blood flow recordings or *ex vivo* CGRP release experiments previously, were anesthetized deeply with thiopental sodium (200 mg/kg, i.p.) and perfused transcardially with physiological saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The skin and muscles of the skull were removed and the skull was divided into halves along the sagittal suture. After removing the brain, samples of the dura mater containing branches of the middle meningeal artery were cut out, postfixed for 2 h in the same fixative and processed for staining with the indirect immunofluorescence technique using a rabbit polyclonal antiserum raised against the TRPV1 receptor (1:500, Alomone Laboratories, Israel) in combination with a monoclonal mouse anti-CGRP antibody (1:500, Sigma-Aldrich, Germany). IgGs labeled with Cy3 and DyLight 488

were used as secondary antibodies (both 1:500, Jackson Immunoresearch Laboratories, USA). CGRP receptor components RCP and RAMP1 were visualized using mouse antiserum against RCP in combination with goat anti-RAMP1 (both 1:50, Santa Cruz Biotechnology, USA) primary and corresponding secondary antibodies labeled with Alexa 555 and Alexa 488 (both 1:500, Molecular Probes, USA). Whole mount preparations of the dura mater were examined under a confocal fluorescence microscope (ZEISS LSM 700, Germany).

Statistical analysis of the data

All values were expressed as means ± SEM. Statistical analysis of the data was performed using Statistica 12 or 13 (StatSoft, Tulsa, USA). In all groups normality was tested by the Shapiro-Wilk test. According to the distribution of data the Student’s t-test or the Wilcoxon test was used. ANOVA with repeated measurements and Fisher's least significant difference test were used to analyze the consecutive measurements of CGRP levels and blood flow increases induced by repeated applications of capsaicin, acrolein and CGRP. One-way ANOVA followed by the Bonferroni test was used for the statistical analysis of blood flow increasing effect of endovanilloids. A probability level of $p < 0.05$ was regarded as statistically significant.

RESULTS

Effects of endovanilloids on the trigeminovascular system

Endovanilloid-induced changes in meningeal blood flow

In line with previous findings (Dux et al., 2003), topical application of capsaicin at a concentration of 100 nM produced a significant increase in meningeal blood flow. In control animals, the blood flow increasing effect of capsaicin (100 nM) amounted to 16.4 ± 3.4 % (n = 7). Topical application of NADA significantly and dose-dependently increased meningeal blood flow which amounted to 7.4 ± 2 % (n = 10) and 24 ± 4.7 % (n = 11) at concentrations of 10 and 100 nM, respectively. However, NADA applied at a concentration of 1 μM decreased meningeal blood flow by 7.7 ± 4.3 % (n = 6).

In contrast, the other endovanilloid anandamide tested in our *in vivo* experimental model induced only slight changes in meningeal blood flow. At concentrations of 100 nM and 1 μM anandamide increased blood flow by 3.4 ± 1.5 (n = 8) and 2.5 ± 1% (n = 10), respectively, whereas at the highest concentration (10 μM), it decreased meningeal blood flow by 2.1 ± 0.8 % (n = 10).

In accord with previous observations, systemic capsaicin desensitization of experimental animals completely abolished the blood flow increasing effect of capsaicin at a concentration of 100 nM. Application of capsaicin at 100 nM onto the exposed dura mater failed to influence meningeal blood flow; it was 99.8 ± 1 % of the basal flow (n = 10). Correspondingly, the increase in meningeal blood flow induced by NADA (10 nM) was also reduced to 0.9 ± 1.2 % of the basal blood flow (n = 13) in capsaicin desensitized animals (Fig. 5).

To obtain pharmacological evidence for the involvement of TRPV1 receptor activation and consequent CGRP release in endovanilloid-induced meningeal vasodilatation, the specific antagonist of the TRPV1 receptor capsazepine (10 μM) or the CGRP receptor antagonist $CGRP_{8-37}$ (100 μM) were applied topically prior to NADA (100 nM). Application of capsazepine and $CGRP_{8-37}$ failed to influence basal blood flow, but significantly inhibited the vasodilatory effect of NADA (Fig. 5). Following the administration of capsazepine the blood flow increasing effect of NADA (100 nM) was only 2.23 ± 3.3 % (n = 8). After pretreatment of the dura mater with the CGRP receptor antagonist $CGRP_{8-37}$, application of NADA slightly decreased meningeal blood flow by 4.82 ± 1.42 % (n = 11).

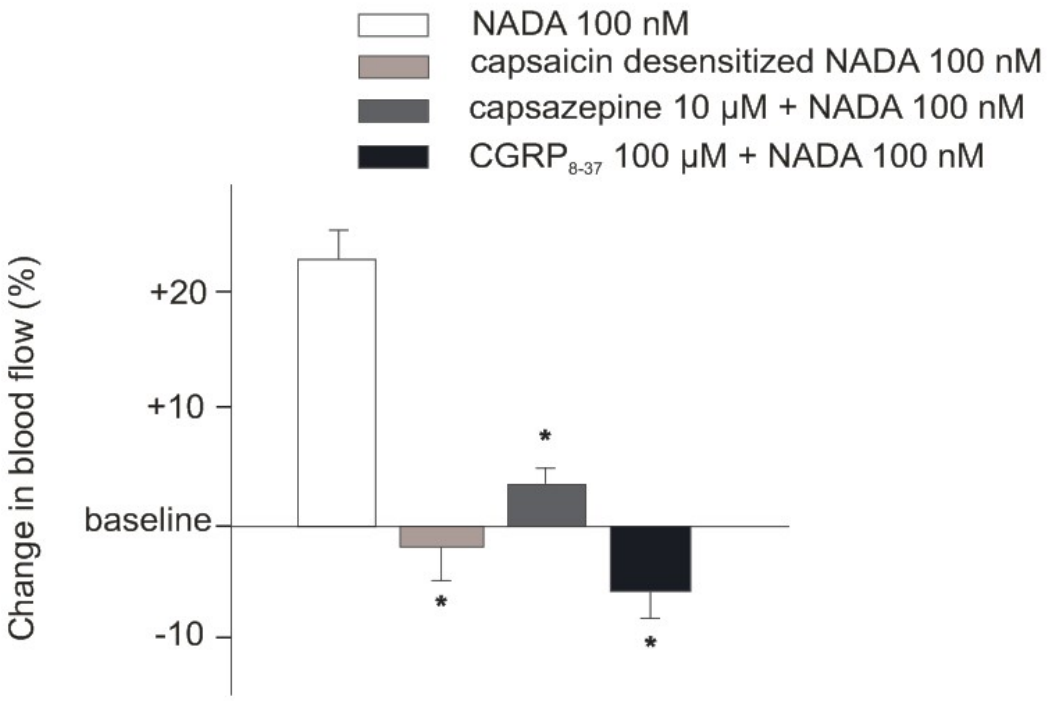

Figure 5. Effect of systemic capsaicin desensitization and preapplication of capsazepine or $CGRP_{8-37}$ on NADA-induced changes in meningeal blood flow. *: statistically different from the corresponding control values.

Application of the CB1 receptor antagonist AM 251 at a concentration of 100 µM did not influence basal meningeal blood flow. Although anandamide at 10 µM decreased meningeal blood flow in control rats by 2.1 ± 0.8 %, this effect turned into an increase by 4.1 ± 0.6 % following the administration of AM 251 (n = 10, Fig. 6A). This vasodilatory effect of anandamide was abolished by additional blockage of CGRP receptors with $CGRP_{8-37}$ (100 µM). Anandamide (10 µM) applied after simultaneous blockage of CB1 and CGRP receptors reduced meningeal blood flow by 1.1 ± 1.8 % (n = 6, Fig. 6B).

Systemic blood pressure of control and capsaicin-desensitized animals was in the same range (110 ± 22.4 and 97 ± 17.3 mmHg, respectively). Systemic blood pressure of the animals was not influenced by topical applications of endovanilloids or capsaicin. Desensitization of the animals with capsaicin did not influence vasodilator capacity of blood vessels, since topical application of histamine resulted in similar increases in meningeal blood flow in control and desensitized rats, it was 22.3 ± 0.7 (n = 10) and 19.3 ± 0.5% (n = 7), respectively.

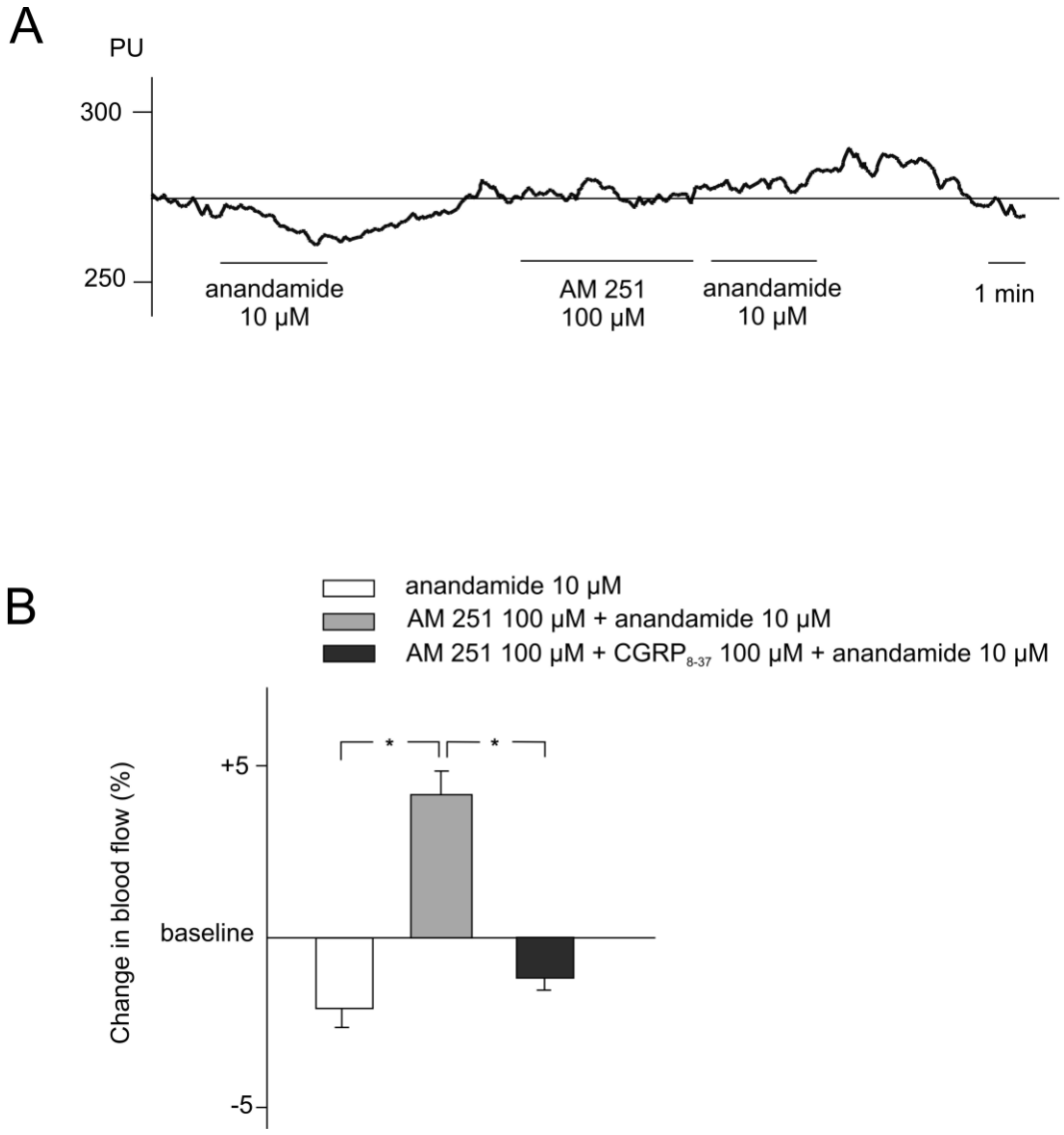

Figure 6. Original recording (A) and statistical evaluation (B) of blood flow changes induced by anandamide (10 µM) before and after the application of AM 251 (100 µM) or AM 251 (100 µM) and $CGRP_{8-37}$ (100 µM). *: statistically different from the effect of anandamide after AM 251 pretreatment.

CGRP releasing effect of endovanilloids measured in an ex vivo dura mater preparation

In control dura mater preparations, basal release of CGRP was 22.6 ± 5 pg/ml. NADA at 100 nM produced a marked increase in the release of CGRP which amounted to 140.3 ± 16.2 % of the basal release (n = 11, Fig. 7A). In this series of experiments the capsaicin-induced CGRP release measured following a challenge with NADA was 328.8 ± 63.6 % of the basal value (n = 11). Under these experimental conditions, applications of capsaicin were

used to control the functional integrity of the dura mater preparations.

In the other series of experiments, anandamide (10 µM) increased the release of CGRP to 122.2 ± 9.6 % (n = 10). After blocking the CB1 receptors with AM 251 (100 µM) an increase of 170.4 ± 23.7 % (n = 10) was measured. The changes in anandamide-induced neuropeptide release measured after blocking the CB1 receptors were significantly different both from the baseline release and the anandamide-induced CGRP release (Fig. 7B).

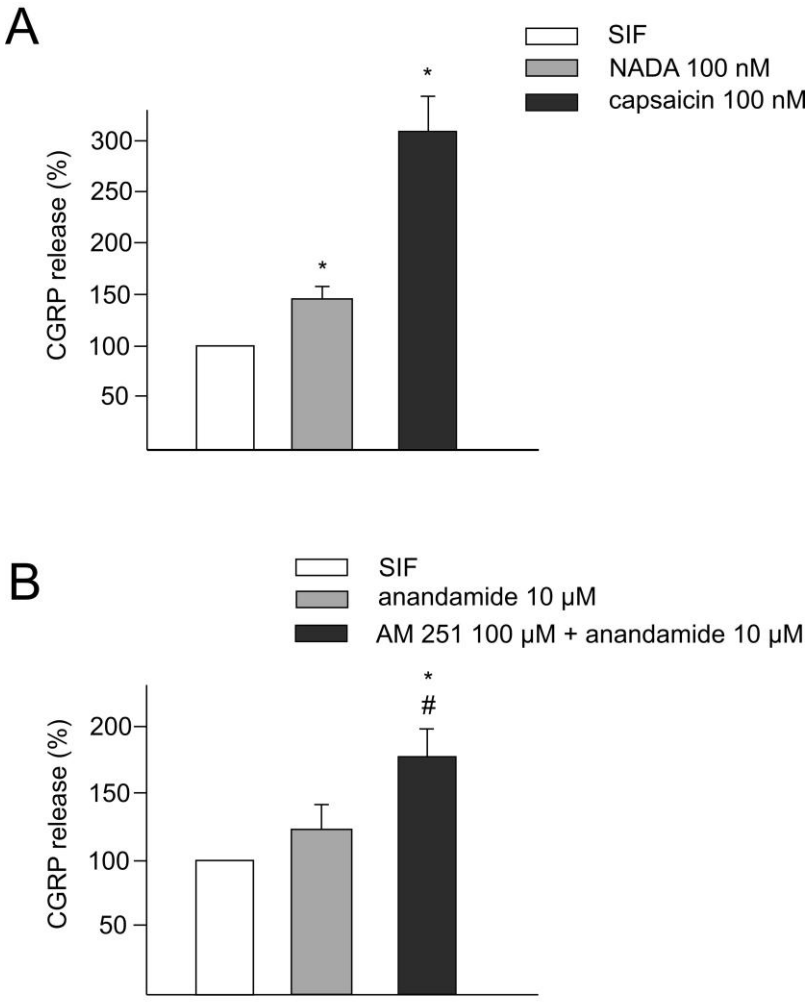

Figure 7. CGRP concentrations released by meningeal afferents after stimulation with NADA (100 nM) and capsaicin (100 nM) applications (A) and after anandamide (10 µM) application with and without prior AM 251 (100 µM) treatment (B). *: statistically different from the CGRP releasing effect of SIF, #: statistically different from the CGRP releasing effect of anandamide.

Effects of adriamycin treatment on TRPV1 and TRPA1 receptor function in the dura mater

Effect of adriamycin treatment on TRPV1 and TRPA1 receptor-mediated vasodilatation

The basal blood flow values measured in meningeal arteries were in the same range in control and adriamycin-treated animals. It was between 200-500 PU in different experiments. In control animals topical application of capsaicin at a concentration of 100 nM significantly increased meningeal blood flow by 28.6 ± 7.9 % (n = 8). Capsaicin-induced increases in meningeal blood flow were reproducible, the second and third applications of capsaicin separated by washout periods increased blood flow by 30.3 ± 4 and 21.5 ± 3.4 %, respectively (n = 8). In adriamycin-treated rats, the blood flow increasing effect of the same capsaicin concentration was attenuated during the first application (11 ± 3 %, n = 14) and almost completely abolished upon the further applications (2.6 ± 1.8 and 1 ± 0.6 %, n = 14, Fig. 8A).

Topical application of acrolein at 300 μM had similar effects on meningeal blood flow. In control animals three consecutive applications increased meningeal blood flow by 14.4 ± 2.4, 13.3 ± 3.7 and 8.9 ± 2.5%, respectively (n = 9). Acrolein-induced increases in blood flow were significantly reduced in adriamycin-treated animals in response to three consecutive applications and amounted to 4.1 ± 1.1, 2.6 ± 1.3 and 1.4 ± 1.8%, respectively (n = 14, Fig. 8B).

The vasodilatory effects of repeated applications of CGRP were also reduced in adriamycin-treated animals, but in this case no tendency of further reduction could be observed upon repeated stimulations. Increases in blood flow induced by CGRP at 10 μM amounted to 20.3 ± 5.3, 21.8 ± 3.9 and 24.7 ± 5.3 % (n = 15) in control and 9.8 ± 1.6, 10 ± 1.9 and 8.6 ± 1.8% (n = 17, Fig. 8C) in adriamycin-treated animals.

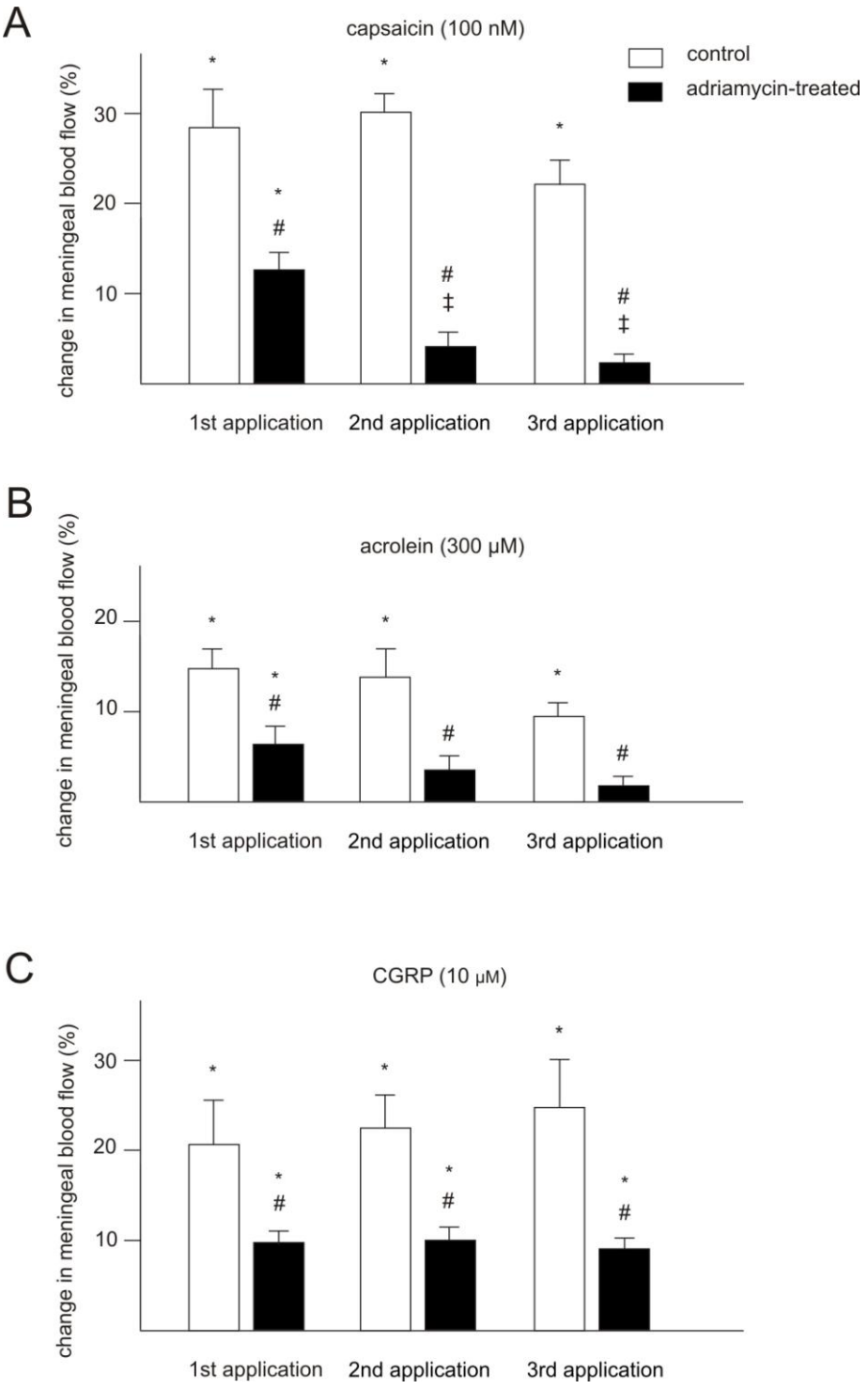

Figure 8. Effect of repeated applications of capsaicin (100 nM , A), acrolein (300 µM, B) and CGRP (10 µM, C) on meningeal blood flow. *: statistically different from the basal flow, #: statistically different from the control, ‡: statistically different from the effect of the first application.

In control and adriamycin-treated animals no significant differences in the meningeal blood flow increasing effects of single topical applications of histamine (21.3 ± 3.9, n = 10 vs. 22.3 ± 4.6 %, n = 16), acetylcholine (15.8 ± 3.7, n = 12 vs. 16.9 ± 4.5 % n = 14) or forskolin (22 ± 8, n = 10 vs. 22.9 ± 9.8 %, n = 10) could be observed (Fig. 9).

Systemic blood pressure of adriamycin-treated animals (84 ± 10 mmHg) was slightly less than values measured in control (98 ± 12 mmHg). Drugs administered topically to the dura mater did not influence systemic blood pressure.

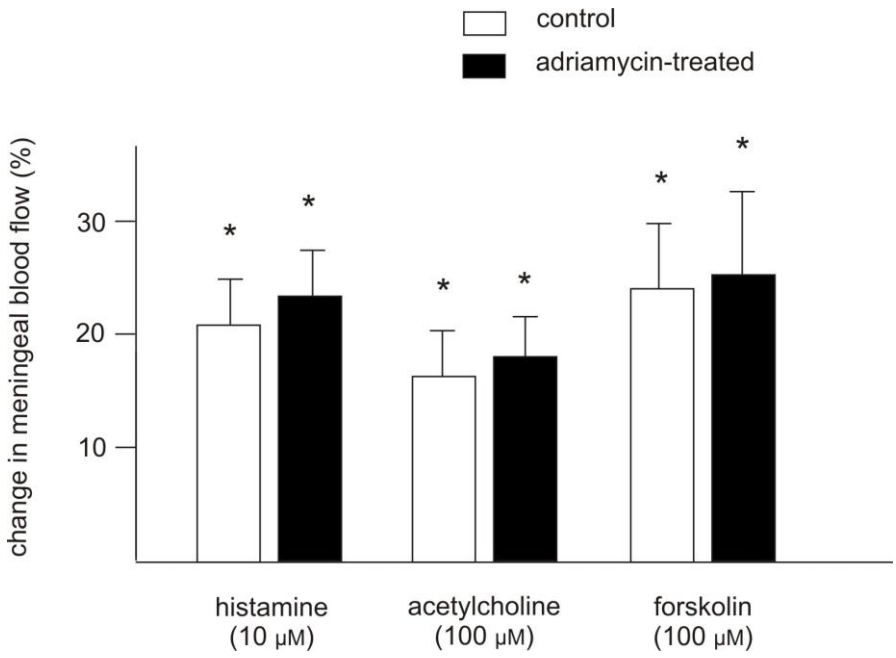

Figure 9. Effects of dural applications of histamine (10 µM), acetylcholine (100 µM) and forskolin (100 µM) on meningeal blood flow. *: statistically different from the basal flow.

Effect of adriamycin treatment on the release of CGRP from meningeal afferents induced by the activation of TRPV1 or TRPA1 receptors and depolarization

A slight but not significant difference in the basal release of CGRP was measured between *ex vivo* dura mater preparations of control and adriamycin-treated animals. It was 13 ± 2.7 pg/ml in control and 20 ± 1.9 pg/ml in adriamycin-treated rats (p = 0.054). In control rats, three consecutive applications of capsaicin (100 nM) induced significant increases in the release of CGRP (294.2 ± 51.6, 229.5 ± 56.7 and 251.4 ± 101.4 %, n = 7). In contrast, in adriamycin-treated animals, the first capsaicin application produced a significant increase in CGRP release that was even higher than the response to the first application of capsaicin in

control rats. In adriamycin-treated animals; it was 564.1 ± 71.2 % (n = 9) of the basal release. Further administrations of capsaicin failed to augment the basal release; the second and third applications amounted to 117.1 ± 19.9 and 80.1 ± 12.5 % (Fig. 10A).

In control rats, TRPA1 receptor activation by acrolein at 300 μM increased CGRP release to 277.2 ± 25.9, 361.9 ± 50.6 and 385.6 ± 83.3 % (n = 6) of the basal level at the three consecutive applications. In adriamycin-treated animals, the CGRP-releasing effect of the first acrolein application was comparable to that seen in control animals (273.9 ± 56.2 % of the basal release, n = 6), but the second and third administrations of acrolein induced only moderate increases in CGRP release (162.5 ± 40.3 and 189.1 ± 56.8 % of basal release, respectively, Fig.10B). KCl at 60 mM depolarised meningeal afferents that increased CGRP release in both control and adriamycin-treated animals to 141.2 ± 24 % (n = 7) and 189.3 ± 29 % (n = 9), respectively. The difference between control and adriamycin-treated groups was statistically not significant (p = 0.241).

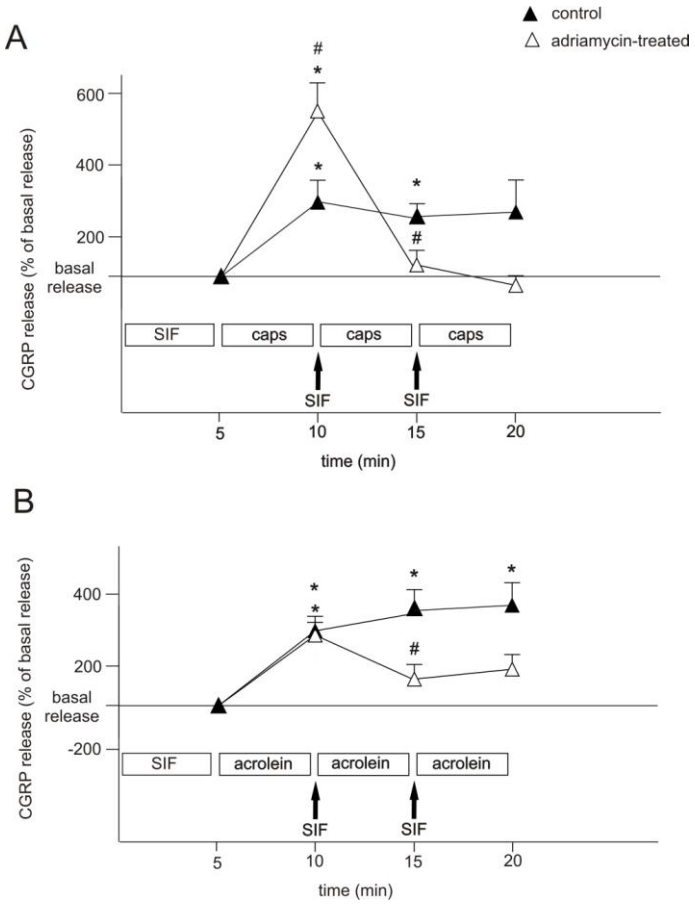

Figure 10. Capsaicin- (100 nM, A) and acrolein- (300 μM, B) induced release of CGRP from meningeal afferents. *: statistically different from the basal release, #: statistically different from the control.

Effect of adriamycin treatment on TRPV1 protein expression in the trigeminal ganglion

TRPV1 protein content of trigeminal ganglia obtained from control rats amounted to 6.25 ± 2.7 pg/mg (n = 10). Adriamycin treatment reduced TRPV1 content to 4 ± 0.5 pg/mg (n = 11), although this difference did not reach statistical significance (p = 0.147).

TRPV1-, CGRP- and CGRP receptor component-immunoreactivity in the dura mater after adriamycin treatment

In whole mount preparations TRPV1- and CGRP-immunoreactive nerve fibers were distributed over the whole parietal dura mater. TRPV1- and CGRP-immunoreactive nerve fibers were observed in both nerve bundles running parallel with branches of the middle meningeal artery and as single axons in regions distant from larger blood vessels. CGRP and TRPV1 were colocalized in most of these nerve fibers. No obvious difference in the density and distribution of TRPV1- and CGRP-immunoreactive afferents was seen in dura mater preparations of control and adriamycin-treated animals (Fig.11).

In dura mater preparations of control animals the CGRP receptor component proteins RCP and RAMP1 were present in the wall of meningeal arteries and veins. In dura samples of adriamycin-treated animals RAMP1-immunoreactivity was identifed, but no RCP-immunoreactive structures could be observed in the wall of meningeal blood vessels (Fig.12).

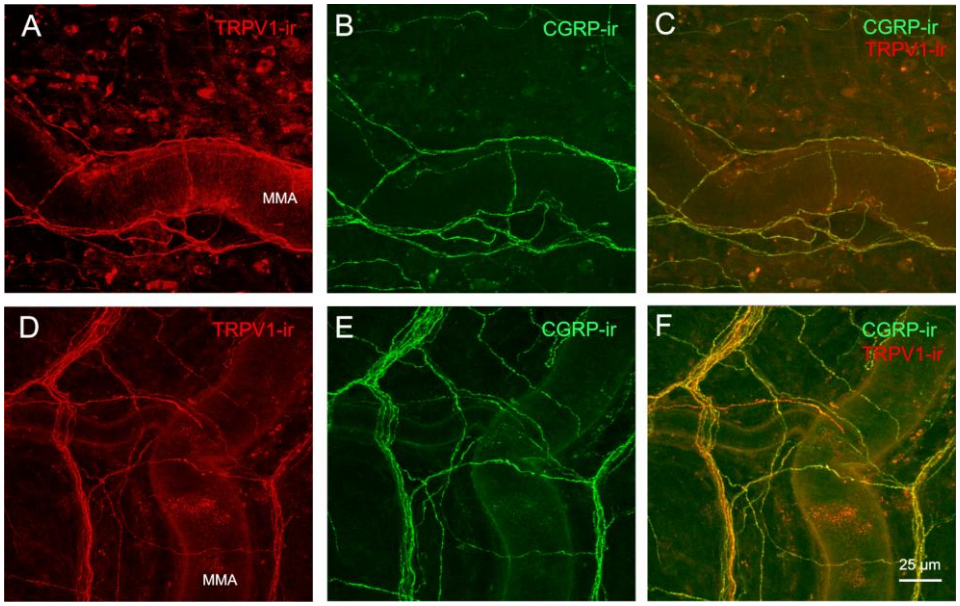

Figure 11. Photomicrographs showing the distribution of TRPV1- and CGRP-immunoreactivity in the dura mater of control (A, B, C) and adriamycin-treated (D, E, F) rats. Scale bar in F applies for all photomicrographs. MMA: branch of the middle meningeal artery.

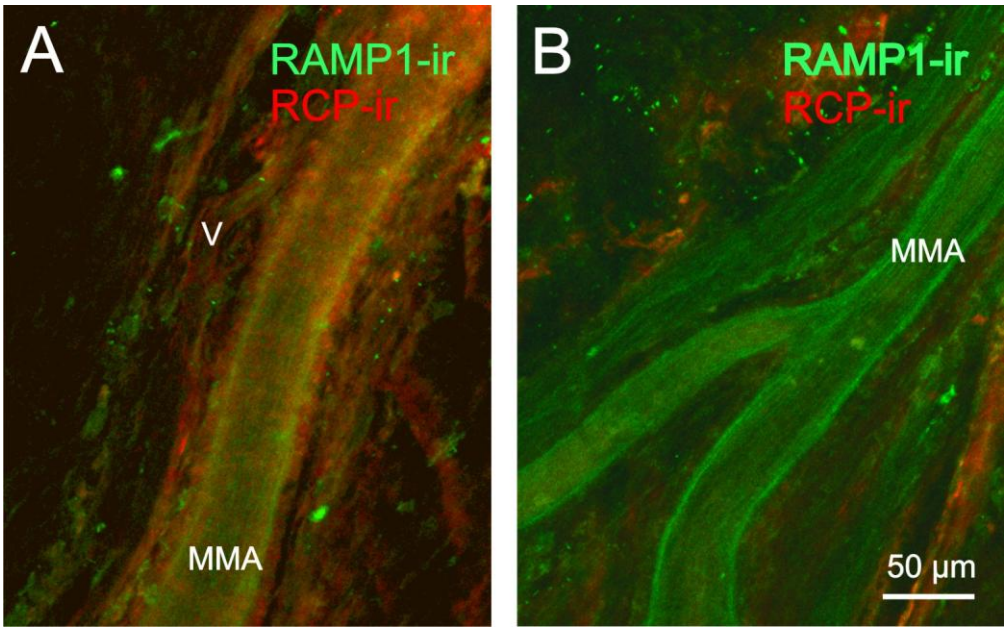

Figure 12. Photomicrographs showing the distribution of CGRP receptor component RCP- and RAMP1-immunoreactivity in the dura mater of control (A) and adriamycin-treated (B) rats. Scale bar in B applies also for A. MMA: branch of the middle meningeal artery, V: venous vessel.

DISCUSSION

The present findings, by showing that endovanilloids produce meningeal vasodilatory responses and release of CGRP, support and corroborate earlier findings which demonstrated that capsaicin, the archetypical TRPV1 agonist is a potent activator of the trigeminovascular nocisensor complex of the dura mater. Our observations also disclosed that trigeminovascular nociceptive functions are profoundly affected by adriamycin, a widely used antitumor agent, at doses producing cardiomyopathy in experimental models (Tong et al., 1991) in part due to damage to sensory nerve function (Boros et al., 2016; Katona et al., 2004).

The rat dura mater preparation is a well-established animal model of meningeal nociception. It is suitable for obtaining information about the pathophysiological processes of human headaches (Akerman et al., 2003; Dux et al., 2003; Holland et al., 2005; Messlinger et al., 2011). Since somatosensory and visceral chemosensitive nerves expressing TRPV1 and TRPA1 receptors share many common functional characteristics, functional changes in the trigeminovascular system may provide useful information about the impairments affecting different organs when systemic changes (e.g. following adriamycin treatment) occur. The sensory efferent/local regulatory functions of activated nociceptive trigeminovascular afferents can be reliably characterised by measuring the increase in meningeal blood flow and the release of CGRP from dural afferent terminals (Dux et al., 2017; Ebersberger et al., 1999; Marics et al., 2017b).

Endovanilloids may activate the trigeminovascular system

Endovanilloids are endogenous membrane lipid metabolites that can be synthesized in sensory ganglion neurons or they can be taken up by the neurons from the surrounding tissue (Dinis et al., 2004; Ligresti et al., 2004). Earlier results indicate that the endovanilloid/endocannabinoid anandamide binds to the same binding site of the TRPV1 receptor as the exogenous agonist capsaicin (Ross et al., 2001). Further, anandamide may activate the TRPV1 receptor under experimental and pathophysiological conditions leading to sensitization of nociceptive primary afferent neurons (Singh Tahim et al., 2005; Sousa-Valente et al., 2014; Tognetto et al., 2001). Although tissue content of anandamide measured under physiological conditions is moderate (Pacher et al., 2006), its level may increase through neurogenic inflammatory processes mediated by the components of the trigeminal nocisensor

complex, i.e. meningeal peptidergic nociceptive afferents and dural mast cells (Dux et al., 2012). The cellular concentration of endovanilloids may be elevated either by increased activity of the synthetizing enzymes and/or by increased endovanilloid transport across the cell membrane (McVey et al., 2003).

Anandamide and NADA act on both TRPV1 and CB1 receptors of nociceptors with different efficacies. In contrast to TRPV1 receptor activation, CB1 receptor inhibits the release of CGRP from trigeminal nerve fibers (Ahluwalia et al., 2003). The presence of CB1 receptors on trigeminal nerve fibers and ganglion cells was confirmed earlier by immunhistochemical studies (Ahluwalia et al., 2003; Hermann et al., 2003).

The present findings indicate that the vascular actions of anandamide and NADA are different in the trigeminovascular system. While anandamide induced only slight, if any, increase, NADA produced a marked increase in meningeal blood flow. This difference may be explained by the different activities of these agents on TRPV1 and CB1 receptors (Starowicz et al., 2007).

Systemic pretreatment of the animals with capsaicin produces functional impairment of chemosensitive nociceptors that is characterised, inter alia, by depletion of sensory neuropeptides CGRP and SP. Desensitization of the animals with capsaicin significantly inhibited NADA-induced increases in meningeal blood flow. In contrast, histamine-induced vasodilatation mediated by a direct action on endothelial and smooth muscle receptors was not affected by capsaicin-desensitization (Dux et al., 2002, 2007). Pretreatment of the dura mater with capsazepine, a specific antagonist of the TRPV1 receptor and blockage of vascular CGRP receptors by $CGRP_{8-37}$ inhibited the NADA-induced vasodilatation. Our results indicate that endogenous vanilloids may activate trigeminal afferents expressing the TRPV1 nociceptor ion channel and induce the release of CGRP from their terminals.

Similar to the concentration-dependent effects of the exogenous vanilloid capsaicin on mesenteric, renal and meningeal blood flow (Dux et al., 2003; Gardiner et al., 2002; Rózsa et al., 1984; Tamaki et al., 2012), NADA and also anandamide reduced meningeal blood flow at higher concentrations. The mechanism of capsaicin-induced vasoconstriction is not clear. It is generally supposed to be a direct vascular action of capsaicin (Duckles, 1986; Pórszász et al., 2002; Toda et al., 1972), although a TRPV1 receptor mediated effect can not be excluded (Kark et al., 2008).

In our *in vivo* experimental model anandamide evoked only moderate vasodilatation

compared to NADA. The relatively strong effect of anandamide on CB1 receptors may counteract the effect of TRPV1 activation reducing the amount of CGRP released by the stimulated nerve terminals. Indeed, blockage of CB1 receptors with AM251 potentiated the anandamide-induced vasodilatation. In the *ex vivo* dura mater preparation more than threefold increase in the amount of released CGRP was observed by blocking the presynaptic CB1 receptors. In our *in vivo* meningeal blood flow model additional blockage of CGRP receptors abolished the enhanced anandamide-induced meningeal vasodilatation, indicating the role of CGRP release. Although *in vivo* anandamide failed to significantly increase meningeal blood flow, *ex vivo* a slight but not significant increase in meningeal CGRP-release was measured after the application of anandamide at the same concentration (10 μM). The putative blood flow increasing effect of the moderate amount of CGRP released by anandamide is probably counterbalanced by its direct vasoconstrictor effect.

Functional impairments of the trigeminal nocisensor complex after systemic adriamycin treatment

Our results revealed a marked impairment of neurogenic sensory vasodilatation in adriamycin-treated rats. Diminished blood flow responses were even more obvious after repeated stimulation of the nociceptors. Activation of the nociceptive ion channels TRPV1 and TRPA1 expressed in chemosensitive dural afferents resulted in altered pattern of CGRP release. In control rats specific agonists of TRPV1 and TRPA1 receptors capsaicin and acrolein, respectively, elicited reproducible releases of CGRP of largely similar magnitudes. In dura mater preparations obtained from rats treated with adriamycin, a strikingly different pattern of CGRP release appeared upon repetitive stimulation: CGRP release was impaired only after the second and third stimulation period with capsaicin or acrolein. In adriamycin-treated dural specimens there was even an increase in initial CGRP release after capsaicin application compared to control rats. This was an unexpected finding, since the capsaicin-induced meningeal vasodilator response, which is mainly mediated by CGRP, was markedly attenuated in adriamycin-treated animals in all three capsaicin applications.

The initially increased CGRP release may be the consequence of a disturbed calcium homeostasis in adriamycin-treated animals. Calcium is essential in the normal function of sensory nerve endings that ensures the release of neuropeptides (Akerman et al., 2003). A high intracellular calcium level can be induced by adriamycin by influencing different

transporters which are involved in the calcium homeostasis of the cell. Increased intracellular calcium concentration can be also a sign of toxic cell damage/death (Jancsó et al., 1984) elicited by other mechanisms affected by adriamycin treatment. These processes may contribute to the rapid depletion of the CGRP from meningeal afferents upon the first stimulation (Keyes et al., 1987; Kohnoe et al., 1992).

Previous observations indicated an impaired cellular calcium homeostasis after adriamycin treatment. Disturbances of calcium homeostasis have been reported in skeletal- (Ertunc et al., 2009), cardiac- (Sag et al., 2011) and smooth muscle cells (Shen et al., 2009) where different channels and transport proteins were likely candidates of the adriamycin effect. Administration of adriamycin increased calcium influx also in HeLa cells (Dasdia et al., 1979). In our experimental model calcium influx was induced by opening the TRPV1 or TRPA1 channels by their respective agonists. A harmful effect of adriamycin on these receptors may result increased calcium influx into sensory nerves inducing increased peptide release. Subsequent decreases in CGRP release even beyond control levels may result from a reduced peptide content of sensory nerves due to the excessive first response upon capsaicin administration. The desensitisation of the TRPV1 receptor is mediated also by calcium-dependent proteins through dephosphorylation of the TRPV1 receptor protein (Docherty et al., 1996; Koplas et al., 1997; Mohapatra and Nau, 2003). The elevated intracellular calcium level produced by adriamycin may initiate this process. Adriamycin treatment had a similar effect on CGRP release induced by the activation of TRPA1 receptors, although in this case the amount of CGRP released upon the first acrolein application did not exceed the amount released from the control samples. The comparatively moderate release of CGRP upon stimulation with acrolein, as compared to the capsaicin-induced release, may be related to the fact that TRPA1 is expressed only by a subpopulation of TRPV1 positive nociceptors and adriamycin may affect differently TRPA1-containing neurons.

The discrepancy between the quantity of CGRP released by the first stimulation of chemosensitive nociceptors with capsaicin or acrolein and the intensity of the vasodilatory response elicited by CGRP release has drawn our attention to possible alterations in vascular functions that may lead to impaired vasodilatation upon CGRP release in adriamycin-treated animals. Since earlier observations indicated apoptotic and necrotic changes in vascular smooth muscle after chronic exposure to adriamycin (Murata et al., 2001), we tested the effects of different vasodilator agents applied directly onto the dura mater. Blood flow

increasing effects of histamine, acetylcholine and, in particular, exogenous CGRP were measured. Blood flow increases induced by these substances are independent of the functional condition of meningeal nerves, since they act directly on receptors localized on endothelial and/or smooth muscle cells of dural blood vessels (Brunt et al., 2015; Dux et al., 2002; Holzer, 1998). Histamine- and acetylcholine-induced vasodilatations were unaltered in adriamycin-treated animals suggesting that damage to vascular smooth muscle is unlikely to be responsible for the decreased vasodilatation to CGRP released by stimulation with capsaicin or acrolein. Conversely, in adriamycin-treated animals the blood flow increasing effect of CGRP was reduced in all three applications to about half of that measured in control.
In our experiments we tested the integrity of intracellular mechanisms involved in CGRP-induced vasodilatation. In adriamycin-treated rats they seemed to be preserved; forskolin, activating adenylyl cyclase and increasing intracellular levels of cAMP (Kanse et al., 1991) elicited similar increases in blood flow in both groups of animals. Immunohistochemistry revealed a loss of CGRP receptor component RCP-staining in arterial and venous dural blood vessels of adriamycin-treated animals. RCP component of the receptor complex is considered to enhance receptor coupling to the G-protein signaling machinery. Its expression seems to correlate with CGRP efficacy *in vivo*, suggesting its crucial role in the regulation of CGRP signaling (Dickerson, 2013). RCP protein staining was undetectable in dura samples of adriamycin-treated animals indicating a significant change in protein structure resulting in a loss of immunohistochemical staining and an impairment of CGRP binding of the receptor complex.

Our functional results indicate an altered TRPV1 and TRPA1 receptor function in meningeal afferents. Decreased TRPV1 protein content of the trigeminal ganglia of adriamycin-treated animals clearly signalised the impairment of chemosensitive nociceptors. Decreased TRPV1 content of the trigeminal ganglia was not reflected by the peripheral axons of trigeminal neurons innervating the dura mater. Whole mount preparations of control and adriamycin-treated animals displayed similar density and distribution of TRPV1- and CGRP-immunoreactive nerves. Despite the known limitations of immunohistochemistry to detect moderate changes in protein content, our findings indicate that reduction of capsaicin-induced neurogenic sensory vasodilatation was brought about in the absence of apparent changes in the distribution and localization of TRPV1- and CGRP-immunoreactive dural afferents. Although we did not study the immunohistochemical distribution and localization of TRPA1

receptor in the dura mater, we assume that they might be similarly unaltered after adriamycin treatment, since the TRPA1 receptor is colocalised with TRPV1 on chemosensitive neurons and earlier observations in our laboratory indicated that pathophysiological conditions altering the functions of chemosensitive afferents may have the same effect on both receptors (Marics et al., 2017a, 2017b).

Our data indicate multiple but selective impairments of receptor functions in the trigeminovascular system after adriamycin treatment. A similar selective impairment of vascular contractility after adriamycin treatment has been reported earlier. In isolated arteries adriamycin treatment downregulated the expression of α1A adrenoceptors but left the expression of endothelin A receptors unaltered (Murata et al., 2001). Anthracycline-induced toxicity is believed to be related to the generation of reactive oxygen species. Indeed, production of superoxide radicals contributed to reduced expression of the α1A receptor of blood vessels. Also receptors of chemosensitive afferents can be targets of reactive oxygen species. Both TRPV1- and TRPA1 receptors can be modified by these mediators altering the gating properties of the ion channels (Nishio et al., 2013; Ruan et al., 2014).

Earlier observations in our laboratory indicated not only functional, but also morphological changes in peripheral nociceptor endings following adriamycin administration. In the skin, loss of intraepidermal sensory nerve endings but not subepidermal axons could be observed after adriamycin treatment (Boros et al., 2016). Although our results obtained from dura mater preparations do not indicate loss of TRPV1- or CGRP-immunoreactivity in general, we cannot exclude the possibility of a similar destruction of the most terminal compartments of sensory axons that, however, are hardly detected in preparations of the dura mater due to inherent differences in the organization of cutaneous and dural innervation.

CONCLUSIONS

This study demonstrates that endovanilloids similar to exogenous vanilloid compounds are effective in activating the trigeminovascular nociceptive complex resulting in the release of CGRP and a consequent increase in meningeal blood flow. Chemosensitive afferents expressing the TRPV1 and TRPA1 receptors may contribute significantly not only to the vascular reactions but also to the nociceptive mechanisms of the dura mater possibly associated with the pathomechanisms of headaches. Endovanilloids may be implicated in the sustained activation of the trigeminal sensory system leading to peripheral and/or central sensitization of the nociceptive pathway and, eventually head pain.

Chemotherapy with adriamycin impairs meningeal nociceptive mechanisms involving TRPV1- and TRPA1-dependent activation of peptidergic trigeminovascular afferents that results in diminished sensory neurogenic vasodilatation of dural blood vessels. Alterations in neuronal CGRP release and changes in the RCP receptor complex protein expression may underlie the changes observed in meningeal vascular responses. Adriamycin-induced impairments of vascular functions may affect sensory nerve-mediated local tissue reactions and protective mechanisms, such as neurogenic inflammation, operative in the meninges.

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