Final report

TRP-dependent mechanisms in meningeal nociception: relevance to human pathophysiology

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The aim of our proposal was to explore the role of chemosensitive primary sensory neurons expressing the transient receptor potential vanilloid 1 (TRPV1) and the transient receptor potential ankyrin 1 (TRPA1) receptors in intracranial pain conditions such as the primary headache migraine and subarachnoid hemorrhage. The experimental findings obtained in established animal models of human disorders are as follows:

Altered metabolic conditions modify the function of meningeal nociceptors

Obese persons have increased risk of developing migraine and may suffer from more frequent and severe headache attacks. Clinical and experimental observations suggest multiple links between the pathophysiology of obesity and the primary headache migraine (Chai et al., 2014), among which the role of calcitonin gene-related peptide (CGRP) released from trigeminal afferents might be of particular relevance. Endogenous metabolites activating TRPV1 or TRPA1 receptors of chemosensitive nociceptors in the rat dura mater may release the vasodilator neuropeptide CGRP from dural afferents leading to increased meningeal blood flow and activation of the nociceptive pathway (Dux et al., 2003; Goadsby and Edvinsson, 1993).

We examined the TRPV1 and TRPA1 receptor functions in obese rats maintained on a long-term high-fat, high-sucrose diet. Our results revealed an enhanced basal and acrolein- (TRPA1 agonist) or capsaicin-induced (TRPV1 agonist) CGRP release from meningeal afferents of obese insulin-resistant rats and an attenuated CGRP release to KCl. Obesity was also associated with an augmented vasodilatation in meningeal arteries measured with laser Doppler flowmetry after dural application of acrolein or capsaicin, a reduction in TRPA1 protein expression in the trigeminal ganglia and elevations in circulating proinflammatory cytokines IL-1β and IL-6 in addition to increased fasting blood glucose and insulin concentrations. Only minor morphological changes of dural TRPV1- and CGRP-immunoreactive afferent nerves were observed in obese animals.

Chronic migraine has a well documented association with insulin resistance and metabolic syndrome (Bhoi et al., 2012; Rainero et al., 2005). Clinical data suggest that sucrose-induced hypoglycemia is a precipitating factor of migraine attacks. In the frame of the present project we examined the effect of insulin on TRPV1 receptor-dependent meningeal nociceptor functions in rats. In *ex vivo* dura mater preparations the application of insulin resulted in a dose-dependent increase in CGRP release from trigeminal afferents. Pretreatment of the dura mater with insulin increased also the capsaicin-induced CGRP release. Pretreatment with an insulin receptor antagonist (BMS-754807) or a TRPV1 receptor antagonist capsazepine decreased the insulin-triggered CGRP release. The results of our *in vivo* experiments confirmed that insulin enhanced the meningeal blood flow increasing effect of capsaicin. Insulin, acting on trigeminal chemosensitive neurons, may activate intracellular processes leading to opening of the TRPV1 channel, which, in turn results in calcium inflow and consequent peptide release. Immunohistochemical double staining indicated the colocalization of insulin receptor and TRPV1 receptor in a significant population of the trigeminal ganglion neurons.

Trigeminal nociceptor function can be influenced by drugs used in human therapy

Adriamycin is one of the most commonly used antracycline derivative for its favorable antineoplastic activity. Besides its deleterious effects on cardiac muscle, adriamycin exerts neurotoxic effects on primary sensory neurons in experimental animals and also in man (Bigotte and Olsson, 1982; Minow and Gottlieb, 1975). In the frame of the present project we studied the effect of systemic treatment with adriamycin. In our *in vivo* open cranial window preparation we measured a significantly reduced vasodilatory effect of a TRPV1 receptor agonist capsaicin and a TRPA1 receptor agonist acrolein indicating impaired function of the trigeminovascular system. Vasodilatory effects of histamine- and acetylcholine (acting directly on endothelial and smooth muscle receptors) were unaltered in adriamycin-treated animals while the blood flow increasing effect of CGRP was reduced to about half of that measured in control animals. We also tested the integrity of intracellular mechanisms involved in CGRP-induced vasodilatation. In adriamycin-treated rats they seemed to be preserved; forskolin, activating adenylyl cyclase elicited similar increases in blood flow in both groups of animals. Measurements of CGRP release in an *ex vivo* dura mater preparation revealed an altered dynamic 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 unaltered.

The sympathomimetic drug phenylephrine is clinically used at high doses as a mydriaticum and for the treatment of nasal congestion. Among its adverse side effects a transient burning sensation and the occurrence of headaches are striking. To clarify the pain enhancing effect of phenylephrine we studied the effect of the α1-adrenoceptor agonist phenylephrine on trigeminovascular reactions. Phenylephrine dose-dependently released CGRP from trigeminal afferents and from isolated trigeminal ganglia. The phenylephrine-evoked release was blocked by the TRPV1 antagonist BCTC and did not occur in trigeminal ganglia of TRPV1-deficient mice. Phenylephrine caused calcium transients in cultured trigeminal ganglion neurons responding to the TRPV1 agonist capsaicin and in HEK293T cells expressing human TRPV1. Local application of phenylephrine at micromolar concentrations to the exposed rat dura mater reduced meningeal blood flow, whereas concentrations above 10 mM increased meningeal blood flow. The flow increase was abolished by preapplication of the CGRP receptor antagonist $CGRP_{8-37}$ or the TRPV1 antagonist BCTC but not the TRPA1 antagonist HC030031. According to our results we propose that activation of TRPV1 receptors may underlie the human pathophysiology leading to cranial pain upon application of high dose phenylephrine.

Understanding the mode of the therapeutic effect of CGRP-inactivating migraine therapies

CGRP has been attributed a key role in primary headaches. Increases in plasma CGRP levels have been found in acute phases of migraine and also interictally in chronic migraine (Cernuda-Morollón et al., 2013; Fanciullacci et al., 1995). Inhibition of CGRP receptors is now applied in migraine therapy and inactivation of CGRP or CGRP receptors by newly developed humanized monoclonal antibodies is promising in reducing the frequency of headache attacks in chronic migraine. To understand the site and mode of the therapeutic effect of CGRP-inactivating migraine therapies, we studied vasodilatation mediated by CGRP released from peripheral and central terminals of trigeminal ganglion neurons upon chemical (KCl) stimulation. Activation of meningeal afferents increased meningeal and medullary blood flow

that was accompanied by increased CGRP concentrations in jugular plasma and cerebrospinal fluid. It also induced significant CGRP release in the hemisected rat skull preparation. Injection of lidocaine into the trigeminal ganglion reduced increases in medullary blood flow and CGRP concentration in the cerebrospinal fluid upon meningeal KCl application. Our results indicate that the medullary blood flow response is most likely mediated by CGRP released from activated central terminals of trigeminal afferents. CGRP released from perivascular afferents innervating dural arteries is preferably drained into dural venous vessels eventually appearing in the blood of the jugular vein. In contrast, CGRP released from trigeminal afferents innervating pial arteries of the cerebral cortex and the medulla oblongata most likely diffuses into the cerebrospinal fluid of the subarachnoidal space or the cisterna magna, respectively, since cortical blood vessels are equipped with a blood-brain barrier.

Increased CGRP levels have been observed in different extracellular fluid compartments during primary headaches such as migraine but it is not entirely clear how CGRP is drained from the meninges. We have used an *in vivo* preparation of the rat to examine after which time and at which concentration CGRP applied onto the exposed parietal dura mater appears in the jugular venous blood and the cerebrospinal fluid collected from the cisterna magna. Recordings of meningeal (dural) and cortical (pial) blood flow were used to monitor the vasodilatory effect of CGRP. We also developed a new *ex vivo* dura mater preparation to examine how much of a defined CGRP concentration applied to the arachnoidal side penetrates the dura. CGRP levels in the jugular plasma *in vivo* were slightly elevated compared to baseline values 5-20 min after dural application of CGRP (10 μM), in the cerebrospinal fluid a significant three-fold increase was seen after 35 min. Meningeal but not cortical blood flow showed significant increases. CGRP at 1 nM only partly penetrated the dura mater. We conclude that only a small fraction of CGRP applied onto the dura mater reaches the jugular blood and, in a delayed manner, also the cerebrospinal fluid. The dura mater may constitute a barrier for CGRP and limits diffusion into the cerebrospinal fluid of the subarachnoidal space, where the CGRP concentration is too low to cause vasodilatation.

In recent years humanized monoclonal antibodies against the sensory neuropeptide CGRP and CGRP receptor have been developed and clinical studies proved their efficacy in the prevention of migraine attacks (Mitsikostas and Reuter, 2017). Despite the beneficial effect of the antibody therapy the mechanism and the site of action of CGRP/CGRP receptor inhibition are still unclear although these questions may be relevant for the long-term efficacy and safety of these treatments. To clarify the tissue distribution of the monoclonal anti-CGRP antibodies recently introduced in the prevention of migraine, we developed a CY3-labelled fluorescent CGRP antibody Galcanezumab (Emgality, Eli Lilly). Rats were treated with subcutaneous injection of the CY3-labelled antibody. In our morphological experiments accumulation of the Cy3-labelled Galcanezumab was observed in arterioles of the dura mater and the trigeminal ganglion. Since mast cells of the dura mater have strong functional connection with meningeal afferents and blood vessels and they are involved in the neurogenic inflammatory reaction, we tested whether CGRP antibodies influence the function of meningeal mast cells. Histamine released by meningeal mast cells of rats treated with subcutaneous injections of the anti-CGRP antibody Galcanezumab was measured after stimulation with CGRP, trypsin acting on proteinase activated receptor 2 and compound 48/80. Galcanezumab treatment reduced the amount of histamine released by CGRP and trypsin while compound 48/80-induced histamine release was not affected by the treatment. Galcanezumab treatment also reduced the CGRP content of trigeminal ganglia compared to control. Reduced neuropeptide content of trigeminal ganglion neurons and the limited contribution of mast cells in neurogenic inflammatory reaction of the meningeal tissue may modify the activity of the trigeminal nociceptive pathway and may be beneficial in migraine prevention.

In rats subcutaneous injection of the anti-CGRP monoclonal antibody fremanezumab (Teva Pharmaceuticals, Redwood City, CA, USA) was followed by reduced basal and capsaicin-evoked CGRP release from day 3 up to 30 days. The difference was enhanced after the administration of migraine triggering glycerol trinitrate application. The samples from the female rats showed a higher CGRP release compared to that of the males. The increases in meningeal blood flow induced by TRPA1 receptor agonist acrolein and TRPV1 receptor agonist capsaicin were reduced 13–20 days after the fremanezumab injection, and the direct vasoconstrictor effect of high capsaicin (10 μM) was intensified. Our results indicate that the antibody may not only prevent the released CGRP from binding to its receptor but may also influence the CGRP release stimulated by noxious agents relevant for the generation of migraine pain.

Modulation of TRESK potassium channel activity modifies nociceptor sensitivity

In 2010 a frameshift mutation of two-pore domain potassium channel (TRESK) leading to a complete loss of TRESK function has been reported in migraine patients with aura (Lafrenière et al., 2010). Since function of TRESK may modify the trigeminal nociception we have started experiments to study the effects of cloxyquin, a specific activator and A2764, an inhibitor of TRESK channel on trigeminal nociception. In *ex vivo* mouse preparation pretreatment of the dura mater with cloxyquin reduced, while A2764 potentiated the CGRP releasing effect of capsaicin acting on trigeminal TRPV1 receptors. Results of *in vivo* laser Doppler blood flow measurements supported the role of TRESK channels in the sensitivity and CGRP releasing effect of the nociceptive cation channel TRPV1. Increases in meningeal blood flow induced by topical capsaicin application on the exposed dura mater were reduced by activation and increased by blocking of the TRESK channels. Our results indicate that pharmacological modulation of TRESK activity can be a suitable way to modify nociceptor sensitivity.

Experimental subarachnoid hemorrhage modifies trigeminal nociceptor function

Subarachnoid hemorrhage is characterized by pain, sustained cerebral vasoconstriction and ischemia, the pathomechanism of which is still rather unclear. Cerebral vasospasm is a potentially incapacitating or lethal complication in patients with aneurysmal subarachnoid hemorrhage. The development of effective preventive and therapeutic interventions has been largely hindered by the fact that the underlying pathogenetic mechanisms of cerebral vasospasm remain poorly understood. An imbalance between vasoconstrictor and vasodilator substances, inflammatory processes, disturbance of neuronal mechanisms regulating vascular tone are all potential pathogenetic factors (Kolias et al., 2009).

In our experiments an established model of experimental subarachnoid hemorrhage was used. Rats were injected with 150-200 μl own blood into the cisterna magna. Three days later the animals were sacrificed, the dura mater as a whole-mount preparation and cryostat sections of the trigeminal ganglia were processed for indirect immunohistochemical staining with the anti-CGRP antibody. Using stereological methods, we observed decreased density of CGRP-immunoreactive afferents in the dura mater and decreased number of CGRP-immunoreactive neurons in the trigeminal ganglion.

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