

Neuropeptide Effects in the Trigeminal System: Pathophysiology and Clinical Relevance in Migraine

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The neuropeptides substance P, calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide (VIP) have been considered as important mediators in migraine and other primary headaches. CGRP and VIP have been found at increased concentrations in jugular venous plasma during attacks of migraine or cluster headache, and CGRP receptor antagonists have recently been shown to be effective in migraine therapy. Substance P and CGRP are produced from a subset of trigeminal afferents, whereas VIP derives from parasympathetic efferents. Release of these neuropeptides in the meninges can cause arterial vasodilatation, mast cell degranulation and plasma extravasation in animal experiments, but only CGRP seems to be relevant in migraine. Animal models have confirmed the important role of CGRP in meningeal nociception. The activity of spinal trigeminal neurons is a sensitive integrative measure of trigeminal activity and is partly under the control of CGRP, most likely via central mechanisms. CGRP released from central terminals of trigeminal afferents in the spinal trigeminal nucleus seems to facilitate nociceptive transmission via presynaptic mechanisms. The central effect of CGRP is substantiated by suppression of nociceptive c-fos activation and neuronal activity in the spinal trigeminal nucleus following CGRP receptor inhibition. These proposed functions are supported by the localization of CGRP receptor components in the rat cranial dura mater, trigeminal ganglion and spinal trigeminal nucleus. The currently available data indicate multiple sites of CGRP action in trigeminal nociception and the pathogenesis of migraine; however, central CGRP receptors are likely to be the essential targets in the treatment of migraine using CGRP receptor antagonists. (Keio J Med 60 (3) : 82–89, September 2011)

Keywords: CGRP, headache, dura mater, trigeminal ganglion, trigeminal nucleus

Introduction

It has long been known that neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) are expressed by afferent neurons which have been ascribed a nociceptive function in various tissues.¹ They have been found to be released in the innervated tissues upon noxious stimulation and to exert efferent, or so-called effector, functions such as arterial vasodilatation and plasma extravasation from postcapillary venules, a

process termed neurogenic inflammation². The idea that neurogenic inflammation of meningeal tissues underlies the generation of migraine pain, in particular, has been put forward by distinguished groups that have performed a considerable number of animal experiments.³

The basic idea used to be that neurogenic inflammation, once it occurs in the cranial dura mater, may release or produce noxious substances. These lead to increasing activation of nociceptive primary afferents with the consequence of activation and sensitization of central

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trigeminal neurons that are involved in headache generation. This theory was substantiated by the effect of specific 5-HT_{1B/D} agonists, the triptans, which inhibit neurogenic inflammation on one hand and on the other hand are effective drugs for migraine therapy.⁴ However, blockade of the receptor for substance P, the NK1 receptor, though very effective in blocking plasma extravasation in the cranial dura mater in animal experiments,⁵ was ineffective in the therapy of migraine pain.⁶ What has survived of the neurogenic inflammation theory is the importance of CGRP as a key mediator in migraine generation.⁷ Here we propose a new interpretation of the neurogenic events following neuropeptide release in the meninges and how these events may interplay with new therapeutic options that arise as a consequence of neuropeptide functions. We also discuss the locations of the most important receptor sites for CGRP in the trigeminovascular system, and where they may have relevance as targets for an effective migraine therapy.

Role for Neuropeptides in the Trigemino-vascular System

Substance P, neurokinin A and CGRP are released by primary afferent neurons in the peripheral nervous system (i.e., the cranial meninges), while other neuropeptides such as neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP) are typically produced by postganglionic sympathetic and parasympathetic efferents, respectively.^{8,9} The latter two neuropeptides will not be discussed further because they do not seem to play a significant role in migraine; however, increased jugular venous blood levels of VIP have been found in those suffering attacks of cluster headache.¹⁰ Based on the findings of historical intraoperative studies of the Wolff group from 1930 to 1950, i.e., that noxious stimulation of cerebral arteries and blood vessels of the dura mater, but not the pia mater, produce headache-like sensations,¹¹ the cranial dura mater of animals became the preferred tool for basic research on headache generation. With the implicit understanding that the human trigeminovascular system is very similar to that of other mammals, major parts of our knowledge about meningeal pathophysiology and headache generation are now derived from animal experiments. However, histochemical and pharmacological studies on isolated human intracerebral arterial blood vessels have been performed,¹² but functional data from the intact human dura mater is virtually lacking because of technical and ethical considerations.

The distribution of neuropeptides has been extensively studied by immunohistochemistry in the cranial dura mater of mammals.^{8,13} Ramifications of the middle meningeal artery as well as the meningeal sinuses are densely innervated by CGRP-immunoreactive nerve fibers, whereas innervation with substance P-immunoreactive fibers is comparably sparse.¹⁴ Although terminals of im-

munopositive nerve fibers are found everywhere in the dura, it is noticeable that single CGRP-immunopositive nerve fibers run tightly along arterial and precapillary vessels, suggesting a vascular function. Vigorous stimulation of the rat dura *in vivo* or in preparations *in vitro* (using trains of electrical stimulation or capsaicin) changes the appearance of immunopositive nerve fibers and results in thickened varicosities with a peculiar “beads-on-a-string” appearance.^{15,16} This may indicate redistribution of CGRP. In an established *in vitro* preparation of the hemisected rat skull combined with enzyme-linked immunoassays, electrical and noxious chemical stimuli caused marked release of CGRP into the superfusate, whereas the release of substance P remained at baseline.^{15,17} These release studies are relevant in terms of pathological changes in migraine for the following reasons. During migraine attacks, increased jugular venous plasma levels of CGRP, but not substance P, have been reported¹⁸; this increase probably indicates massive activation of CGRP release from trigeminal afferents. Furthermore, the site of CGRP release in migraine patients is not clear; however, sites other than peripheral trigeminal fibers have to be considered.

The inflammatory response to neuropeptides has been studied based on principal functional components in animal experiments. For example, the main function of substance P has been described as plasma extravasation, whereas CGRP has a pronounced role in arterial vasodilatation and increase in blood flow, as outlined above. CGRP has the most potent vasodilator effect at intracranial arteries,¹⁹ and the vasodilatory mechanism of CGRP (but not substance P) is linked to an intracellular increase in cyclic adenosine monophosphate (cAMP), which can be fully antagonized by CGRP₈₋₃₇, the classical competitive receptor antagonist.²⁰ CGRP-dependent vasodilatory effects in the rodent dura can be observed through a cranial window by video microscopy²¹ or by using blood flow as a readout via laser Doppler flow recordings.²² For example, administrations of the CGRP receptor antagonists CGRP₈₋₃₇ or olcegepant (BIBN4096BS, Boehringer Ingelheim) were able to inhibit increases in meningeal blood flow induced by periodic electrical stimulation of the dura in rats. Interestingly, inhibition was observed when CGRP inhibitors were applied topically or administered systemically.^{22,23} These striking actions of the inhibitors raise questions about the localization of the CGRP receptors.

Role for CGRP Receptors in the Cranial Meninges

The CGRP receptor expressed by vascular smooth muscle cells and other tissues has a rather complicated structure. A large peptide with seven transmembrane domains, named the calcitonin receptor-like receptor (CLR or CRLR), is supplemented by a small single transmembrane peptide, named the receptor activity modifying

protein 1 (RAMP1), to form the ligand binding site that is specific for CGRP.²⁴ Exchange of RAMP1 with other RAMP molecules changes the receptor specificity, e.g., the adrenomedullin receptor is formed with RAMP2. In addition, a small intracellular protein component, called receptor component protein (RCP), is necessary for receptor protein trafficking and links the receptor to the intracellular signal-transduction machinery mainly through G_s proteins and adenylate cyclase. As a consequence, only the combination of these three components (CLR, RAMP1, RCP) reliably constitutes the functional CGRP receptor. Given the documented role of cAMP in sensitization, the hypothesis that CGRP may cause sensitization in meningeal afferents has been addressed via recordings from trigeminal ganglion neurons in rat nociceptors.²⁵ While cAMP analogues induced sensitization to mechanical stimuli, topical application of CGRP caused no change in activity or mechanical sensitivity of meningeal afferents, although CGRP did induce increased blood flow.²⁶ The results were surprising at the time of publication; however, our studies elucidating the CGRP receptor distribution within the dura disentangled these puzzling findings.

The distribution of CGRP receptors in the rat cranial dura has been extensively studied by our group.²⁷ Multiple-color immunofluorescence examinations demonstrated co-localization of CLR and RAMP1 immunoreactivity in meningeal nerves, indicating functional CGRP receptors; however, CLR was neither co-localized with protein gene product 9.5 (a pan-neuronal axonal marker) nor with CGRP. Instead, CLR was co-localized with glial fibrillary acidic protein (GFAP) as a marker for glia, indicating that CGRP receptor components may be expressed by Schwann cells.²⁷ While the functional significance of CGRP receptors on Schwann cells is currently unexplored, findings of CGRP receptors on arterial blood vessels are in line with CGRP functions (**Fig. 1A**). In addition, we detected CGRP receptors in large, round to elongated mononuclear cells in juxtaposition to meningeal vessels (**Fig. 1B**). The morphology and immunophenotype of these cells were compatible with mast cells, which have been found to accompany dural arteries in considerable numbers.²⁸ *In vivo* treatment of rat dura with mast cell degranulator compound 48/80 causes loss of immunoreactivity for histamine in mast cells, indicating degranulation. The treatment also caused delayed and prolonged activation of meningeal afferents.²⁹ Although the exact mediators and mechanisms responsible for the mast cell activation are unknown, these results indicate mast cell factors and possibly histamine as key contributors. We have observed that histamine is able to excite a subpopulation of primary meningeal afferents in a rat hemisected skull preparation.³⁰ Interestingly, histamine was not only released by superfusion with compound 48/80 but also to a minor extent by CGRP in the rat hemisected skull preparation.³⁰ Thus, localization of CGRP

receptor components on mast cells may indicate that CGRP can act as a mild mast cell degranulator.²⁷ Together, the dural CGRP receptor distribution is in line with existing functional data and suggests targets important in our understanding of the pharmacologic effector sites of CGRP inhibitors. This forms the starting point in our understanding of the exact interplay of substructures in meningeal nociception and the relation to CGRP signaling and headache generation.

Possible Role for CGRP Receptors in the Trigeminal Ganglion

In the rat trigeminal ganglion, immunoreactivity of the CGRP receptor components CLR and RAMP1 was seen in partly overlapping populations of mostly small-diameter neurons.²⁷ Subpopulations of CGRP-immunoreactive neurons were also immunopositive for either CLR or RAMP1, but the co-existence of CGRP with CLR and RAMP1 was extremely rare (**Fig. 1C, D**). Taken together, this pattern suggests the existence of separate CGRP-releasing neurons on one hand and CGRP-receptive neurons on the other hand. Whether CGRP can directly activate primary afferent neurons is disputed by calcium imaging in which CGRP increases intracellular calcium in a subpopulation of cultivated rat dorsal root ganglion neurons.³¹ In cultivated rat trigeminal ganglion neurons, treatment with CGRP has been found to induce CGRP promoter activity and to increase CGRP release.³² In a separated culture of rat trigeminal ganglion glia cells, the expression of inducible nitric oxide synthase and the production of nitric oxide has been found to be dose-dependently increased by CGRP.³³ Immunoreactivity for both CGRP receptor components has indeed been localized on satellite glia cells in the rat trigeminal ganglion.³³

Thus, signaling between trigeminal ganglion neurons using CGRP as a mediator seems possible, although functional data should be handled with care because of the limitations imposed by cell culture.

Possible Role for CGRP Receptors in the Trigeminal Brainstem

Recordings from neurons that receive afferent input from the cranial dura in the subnucleus caudalis of the spinal trigeminal nucleus (SpV) are excellently suited to assess the activation state of the rat meningeal nociceptive system and to test conditions that may be relevant for headache generation. We employed this model to examine the effect of the CGRP receptor antagonist olcegepant (BIBN4096BS) on the spontaneous and heat-evoked activity of neurons with receptive fields in the exposed parietal dura.³⁴ Olcegepant decreased both spontaneous and evoked activity in a dose-dependent manner when applied intravenously (**Fig. 2A-C**). However, topical application to the dura was ineffective. These findings in-

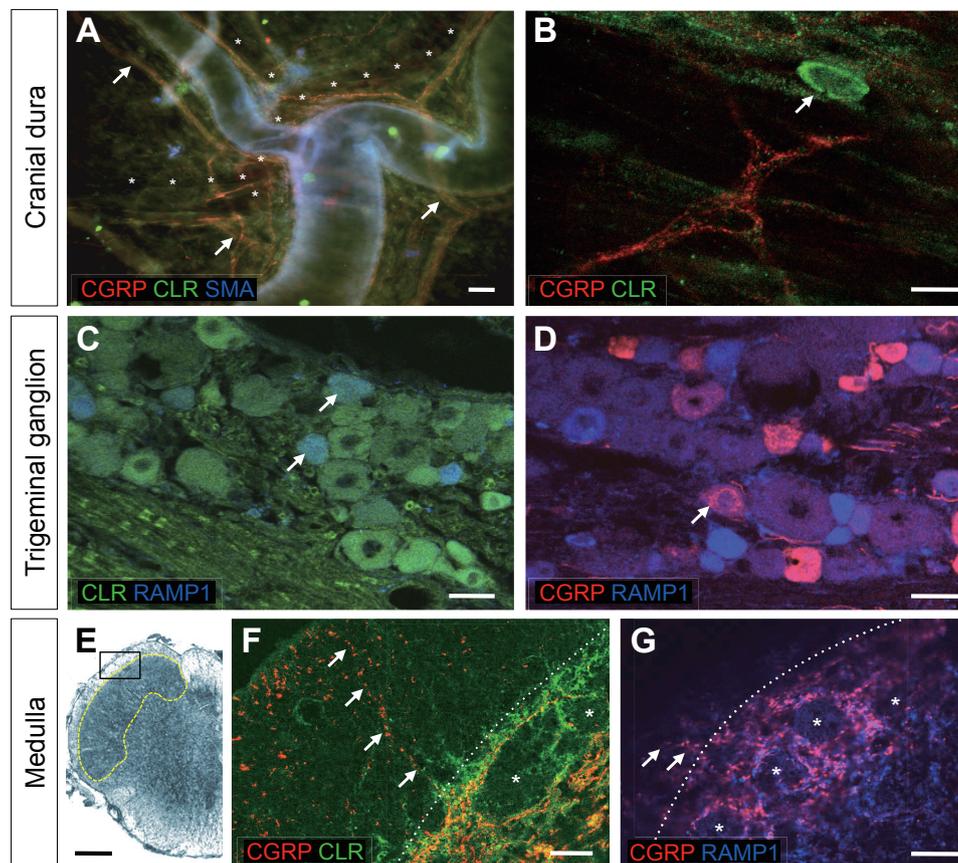


Fig. 1 Distribution of CGRP receptor components CLR and RAMP1 in the cranial dura, trigeminal ganglion and medulla oblongata. (A) Triple immunolabeling of CGRP (red), CLR (green) and smooth muscle marker SMA (blue). CLR immunoreactivity is associated with arterial vessels (turquoise) but not with veins (asterisks). CGRP fibers (arrows) frequently run close to CLR-positive arteries and mononuclear cells with no co-localization. (B) Larger mononuclear cells (arrow) exhibited granular cytoplasmic CLR staining shown here in close proximity to a CGRP-positive branching fiber. (C) CLR (green) and RAMP1 (blue) staining in the trigeminal ganglion showed homogeneous cytoplasmic staining; co-localization results in the mixed color turquoise (arrows). (D) CGRP (red) and RAMP1 (blue) staining in the trigeminal ganglion showed granular to homogeneous staining with nuclear sparing in many small- and medium-sized neuronal cell bodies. Co-localization results in the mixed color magenta (arrow). (E) Dark-field photomicrograph of an unstained horizontal section through the medulla oblongata. The spinal trigeminal nucleus (SpV) is outlined in yellow and the images F and G were taken from the posterior-lateral zone (black square). (F) CLR (green) and CGRP (red) immunolabeling in the SpV demonstrates irregular networks arranged around unstained regions (asterisks, “glomerular structures”). Laminae I and II demonstrate three staining patterns: CLR only (green), CGRP only (red) and CLR+CGRP (yellow). Note the incoming primary afferent CGRP-positive fibers (arrows). (G) Double labeling of RAMP1 (blue) with CGRP (red) in the SpV also shows irregular networks around unstained regions (asterisks, “glomerular structures”). Laminae I and II demonstrate three staining patterns: RAMP1 only (blue), CGRP only (red) and CGRP+RAMP1 (magenta). Note incoming primary afferent fibers (arrows). Scale bars: 50 μ m in A, C and D; 10 μ m in B, F and G; 1 mm in E.

indicate that the site of CGRP receptor inhibition may be located centrally to the primary afferents innervating the dura mater. As a complementary method, *c-fos* activation confirmed our extracellular recordings.³⁵ Specifically, a massive noxious stimulus in the form of a systemic infusion of capsaicin caused a marked increase in the number of *c-fos*-immunoreactive neurons in the superficial layers of SpV. The number of *c-fos*-activated neurons was significantly reduced when olcegepant was infused prior

to the capsaicin infusion. Together, these experimental results argue that CGRP is an important nociceptive neuromodulator in the trigeminal brainstem.

Accordingly, we observed CLR and RAMP1 immunoreactivity in the spinal trigeminal tract and in the trigeminal nucleus of the spinal medulla (Fig. 1E). We noted the highest density in superficial laminae,²⁷ and the distribution of CGRP receptors components (CLR and RAMP1) overlapped with that of CGRP (Fig. 1F, G). Interestingly,

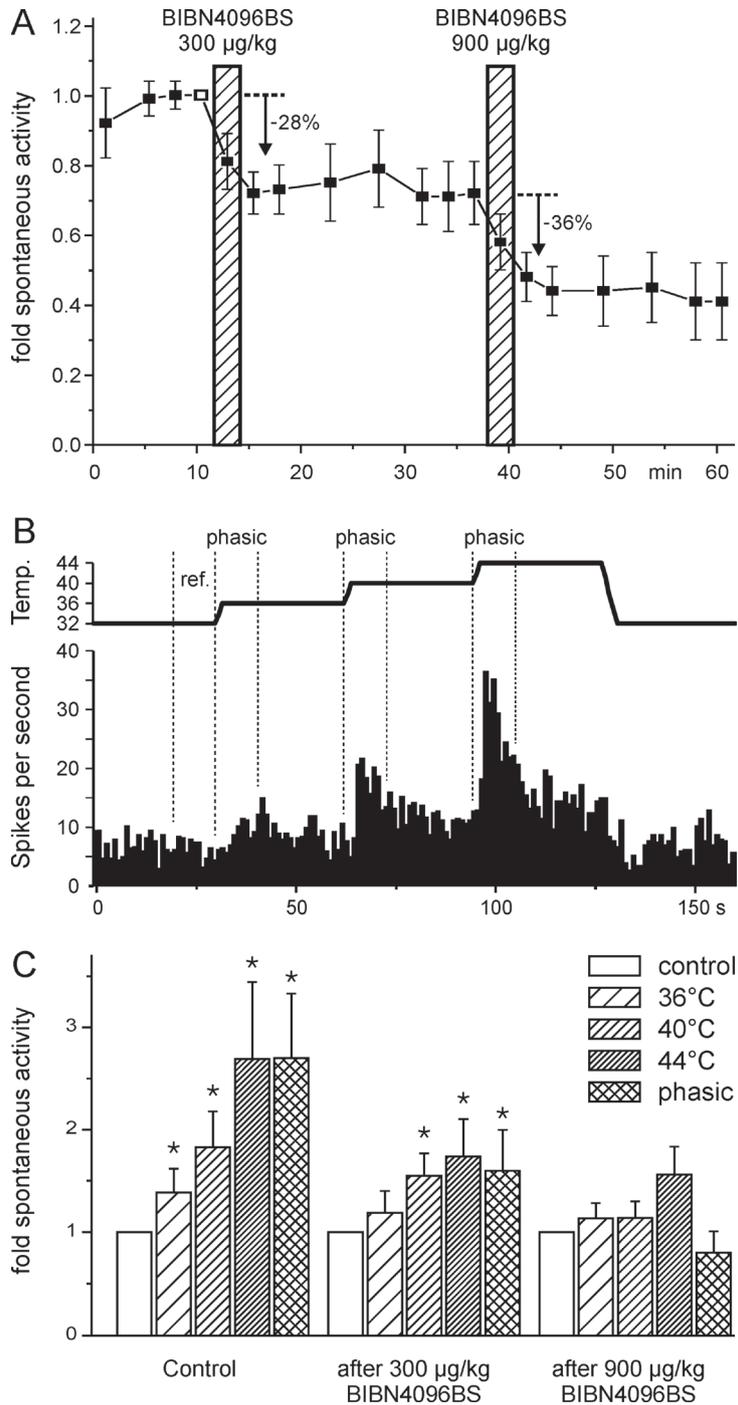


Fig. 2 Effects of i.v. BIBN4096BS (olcegepant) on the activity of spinal trigeminal nucleus neurons with dural afferent input. (A) Normalized activity of 17 separate extracellular single-unit recordings on a real-time scale. Each point represents the mean activity during intervals of 150 s, normalized to the 150-s period before first drug application (open square). Hatched bars indicate injection of olcegepant (BIBN4096BS). Note the longer time between some 150-s intervals that were used for thermal testing. (B) Representative spinal trigeminal neuron with phasic and tonic coding of dural heat stimulation. The activity is an average of 4 subsequent heat step stimulation trials. The temperature of the dura was stepwise increased as indicated, from 32 °C to 44 °C, each step lasting for 30 s. Stepwise temperature increase caused a phasic response in neuronal activity (mean of first 10-s periods of temperature steps versus reference activity). (C) Normalized neuronal activity of 13 neurons in the spinal trigeminal nucleus. Average and phasic responses to thermal stimulation protocols were reduced after administration of BIBN4096BS (olcegepant) compared to the control period. After a cumulative dose of 1.2 mg/kg BIBN4096BS, no significant activation by heat stimuli was observed. * $p < 0.05$ versus control period.

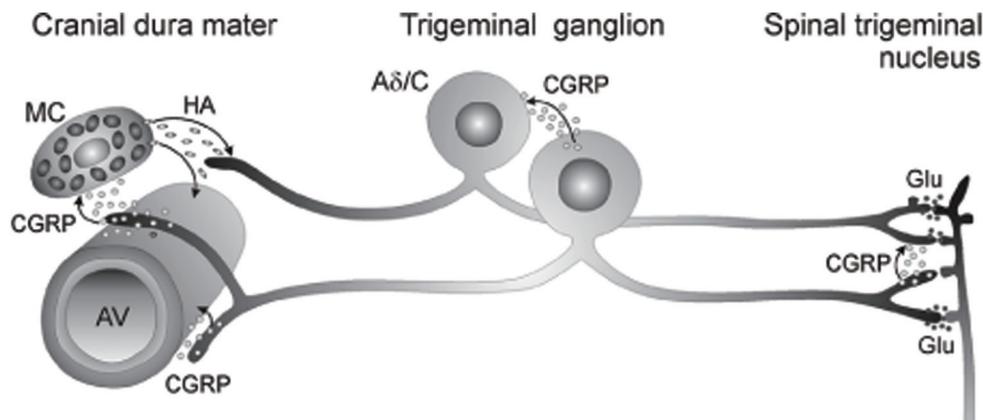


Fig. 3 Schematic representation of calcitonin gene-related peptide (CGRP) signaling in the cranial dura mater encephali, the trigeminal ganglion and the spinal trigeminal nucleus.

In the dura, CGRP causes dilatation of arterial vessels (AV) and secretion of substances such as histamine (HA) from mast cells (MC). HA amplifies vasodilatation and can activate specific primary afferents. In the trigeminal ganglion, CGRP may signal to other trigeminal ganglion neurons (A δ /C) that express CGRP receptors. In the trigeminal nucleus, CGRP facilitates nociceptive transmission presumably via a presynaptic mechanism by increasing neurotransmitter (e.g., glutamate, Glu) release from adjacent primary afferent terminals. Possible contributions of Schwann cells and satellite cells to CGRP signaling are not depicted.

there was no co-localization with the glial cell marker GFAP. CGRP receptor immunoreactivity was exclusively seen in structures resembling nerve fibers but never in cell bodies. The functional interpretation of this data is that CGRP-releasing terminals of primary afferents synapse at CGRP receptor-expressing axons, which most likely include the terminals of primary trigeminal afferents. Therefore we postulate that CGRP actions within the trigeminal nucleus follow a presynaptic mechanism whereby distinct terminals of primary afferents control the neurotransmitter release in other primary afferents (**Fig. 3**). The advantage of such a presynaptic mechanism would be that distinct afferent inputs could be selectively activated under the control of CGRP receptors (or inhibited in the case of CGRP receptor antagonists). CGRP receptor inhibition in the C1/2 segments of the cervical dorsal horn brought about by iontophoretic application of CGRP receptor antagonists reduces spontaneous and glutamate-induced neuronal activity in neurons with facial receptive fields.³⁶ In rat brain stem slices, the excitability of neurons in the spinal trigeminal nucleus (SpV) is increased by CGRP superfusion and prevented by inhibition of CGRP receptors using CGRP₈₋₃₇.³⁷ Therefore, the evidence is convincing that CGRP is an activating neuro-modulator in the spinal trigeminal nucleus.

Where Is the Effective Site of Action of CGRP Receptor Antagonists?

Given the multiple receptor sites in the trigeminovascular system – arterial vessels, mononuclear cells and

Schwann cells in the dura; neurons and satellite glial cells in the trigeminal ganglion; and central/afferent projections in the trigeminal nucleus – the question arises as to where CGRP receptor antagonists most effectively inhibit trigeminal activity (**Fig. 3**). The answer is of considerable importance, since CGRP receptor antagonists such as olcegepant and telcagepant have been shown to be effective in migraine therapy.^{38,39} Since CGRP does not activate meningeal afferents²⁶ and local application of CGRP antagonist onto the cranial dura is not effective in reducing spinal trigeminal activity,³⁴ a peripheral site of action, at least for acute effects of CGRP receptor inhibition, is not very likely. In contrast, all experiments are consistent with a central action of CGRP receptor antagonists. However, the trigeminal ganglion itself has not been excluded as an important receptor site and may function as a central component in the postulated mechanism. In recent studies, our group has addressed this question using functional animal experiments in which phosphorylation of extracellular receptor kinase (pERK) is a very quick step in the nociceptive cascade of primary afferents. We assessed pERK in both trigeminal ganglia after unilateral injection of capsaicin into the facial skin.³⁵ As expected, significantly more neurons in the trigeminal ganglion on the injected side showed the pERK signal compared to the control side. Intravenous infusion of CGRP receptor inhibitor olcegepant before the capsaicin injection showed no difference in pERK in the ganglia compared to controls without olcegepant. Most recently, we succeeded in injecting olcegepant vs. vehicle through the infraorbital channel directly into the

rat trigeminal ganglion while monitoring ongoing activity in neurons that receive input from meningeal afferents where we evoked short mechanical stimuli to the exposed dura (unpublished data). The injection of olcegepant into the ganglion did not significantly change ongoing or mechanically evoked activity compared to vehicle injections. Therefore we suggest that there is reasonable evidence to exclude the trigeminal ganglion as an important site of acute actions of CGRP receptor antagonist.

Conclusion

Systemic administration of CGRP receptor antagonist reduced ongoing and heat-evoked activity of spinal trigeminal neurons with meningeal afferent input. Likewise, pretreatment with CGRP receptor antagonist reduced the increase in c-fos activation of neurons in the spinal trigeminal nucleus induced by capsaicin infusion. In contrast, pretreatment with CGRP receptor antagonist did not reduce the increase in ERK phosphorylation in the trigeminal ganglion induced by facial capsaicin injection. Direct injection of CGRP receptor antagonist into the trigeminal ganglion did not reduce ongoing and mechanically evoked activity of spinal trigeminal neurons with meningeal afferent input. Therefore, CGRP receptor antagonists may act centrally rather than peripherally and promptly inhibit incoming activity from the trigeminal system involved in meningeal nociception. Similarly, we hypothesize that the action of CGRP receptor antagonists such as olcegepant in migraine therapy may, at least in part, depend on central mechanisms.

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