Research Report

Localization of CGRP, CGRP receptor, PACAP and glutamate in trigeminal ganglion. Relation to the blood–brain barrier

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A B S T R A C T

Calcitonin gene-related peptide (CGRP) receptor antagonists have demonstrated anti-migraine efficacy. One remaining question is where do these blockers act? We hypothesized that the trigeminal ganglion could be one possible site. We examined the binding sites of a CGRP receptor antagonist (MK-3207) and related this to the expression of CGRP and its receptor in rhesus trigeminal ganglion. Pituitary adenylate cyclase-activating polypeptide (PACAP) and glutamate were examined and related to the CGRP system. Furthermore, we examined if the trigeminal ganglion is protected by the blood–brain barrier (BBB). Autoradiography was performed with [3H]MK-3207 to demonstrate receptor binding sites in rhesus trigeminal ganglion (TG). Immunofluorescence was used to correlate binding and the presence of CGRP and its receptor components, calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1), and the distribution of PACAP and glutamate in rhesus and rat TG. Evans blue was used to examine large molecule penetration into the rat TG. High receptor binding densities were found in rhesus TG. Immunofluorescence revealed expression of CGRP, CLR and RAMP1 in trigeminal cells. CGRP positive neurons expressed PACAP but not glutamate. Some neurons expressing CLR and RAMP1 co-localized with glutamate. Evans blue revealed that the TG is not protected by BBB. This study demonstrates CGRP receptor binding sites and expression of the CGRP receptor in rhesus and rat TG. The expression pattern of PACAP and glutamate

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Abbreviations: BBB, blood–brain barrier; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; RAMP1, receptor activity-modifying protein 1; PACAP, pituitary adenylate cyclase-activating polypeptide; PBS, phosphate buffered-saline; BSA, bovine serum albumin; PFA, paraformaldehyde; Htx–eosin, hematoxylin–eosin; PBST, phosphate buffered-saline (PBS) containing 0.25% Triton X-100

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suggests a possible interaction between the glutamatergic and CGRP system. In rat the TG is outside the BBB, suggesting that molecules do not need to be CNS-penetrant to block these receptors.

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1. Introduction

Migraine is today recognized as a neurovascular disorder which originates in the brain, involving the hypothalamus and thalamus, as well as certain brainstem regions (Goadsby, 2012). The acute migraine attack is often preceded by prodromal symptoms, which suggest the central nervous system (CNS) as a likely point of origin (Charles, 2013). The pain during a migraine attack is associated with the release of the peptide calcitonin gene-related peptide (CGRP) which has a key role in migraine pathophysiology (Goadsby et al., 1988; Edvinsson and Goadsby, 1990; Ho et al., 2010). Clinical studies have demonstrated increased levels of CGRP putatively from the trigeminal system that can be found in serum, cerebrospinal fluid, and saliva (Goadsby et al., 1990; Goadsby and Edvinsson, 1993; Bellamy et al., 2006; Cernuda-Morollon et al., 2013). In addition, systemic infusion of CGRP can trigger a “migraine-like headache” in patients (Hansen et al., 2010). It is hypothesized that CGRP acts at second order neurons in the trigeminal nucleus caudalis (TNC) and at the C1–2 level of the spinal cord, to transmit pain signals to thalamus and higher cortical pain regions (Goadsby, 2007; Levy et al. 2005). The trigeminovascular system is involved in the regulation of the cranial vasculature and is a key element in the transmission of pain. The trigeminal ganglion contains neurons that peripherally innervate the intracranial vasculature and dura mater. The trigeminal ganglion also centrally projects to the brainstem, in related extensions down to the spinal cord and to parts of the CNS where nociceptive information is processed to higher cortical regions (Liu et al., 2009; Goadsby, 2012). Stimulation of the trigeminal ganglion resulted in release of CGRP and elevation of CGRP in the external jugular vein (Goadsby et al., 1988; Limmroth et al., 2001). Neural activity in the trigeminal nociceptive system in migraine patients has been demonstrated using imaging techniques (Borsook et al., 2006). The wide distribution of CGRP receptors in the trigeminal system is consistent with a role in migraine pathophysiology (Lennerz et al., 2008; Eftekhari and Edvinsson, 2010; Eftekhari et al., 2010; Bhatt et al., 2014).

The most important evidence for the role of CGRP in migraine pain came from the development of CGRP receptor antagonists (Olesen et al., 2004; Ho et al., 2008a, 2008b) which act by blocking the action of CGRP on the CGRP-receptor complex. The CGRP receptor is a G protein-coupled receptor of the B-type consisting of calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1), both necessary to yield a functional CGRP receptor (Walker and Hay, 2013). It has been suggested that elevated neuronal RAMP1 could

![Fig. 1 - In vitro autoradiography with [3H]MK-3207, a CGRP receptor antagonist, in the rhesus monkey. The two upper images to the left show [3H]MK-3207 high binding density within the trigeminal ganglion (total binding, yellow and red signal), and also its root (middle panel, red signal). Binding is also found in areas with collagen. To the right, the same sections were stained with Htx-eosin. Since scanning of the autoradiography and the HTX-staining were performed in different scanners with different resolution, some discrepancies may occur. In the lower panel, self-block on the adjacent slide with no binding using unlabeled MK-3207 (non-displaceable binding) is demonstrated.](image-url)
potentially sensitize the trigeminal ganglia of individuals to CGRP actions (Zhang et al., 2007). However, little is currently known about the regulation of RAMP1 levels in migraine.

Recent findings have identified the blockade of the CGRP receptor as a mechanism to reduce migraine pain (Salvatore and Kane, 2011; Moore and Salvatore, 2012). Clinical studies using CGRP receptor antagonists have demonstrated clinical efficacy comparable to that of triptans in the treatment of acute migraine attacks (Edvinsson and Linde, 2010; Ho et al., 2010; Salvatore and Kane, 2011). Therefore, it is of great interest to define where the CGRP receptor is expressed and on which possible sites drugs blocking CGRP signaling may have their therapeutic effect.

Two other neuronal messenger molecules that have been suggested to have an important role in migraine pathophysiology are pituitary adenylate cyclase-activating polypeptide (PACAP) and glutamate. Recent studies suggest that PACAP may have similar actions as CGRP and has been suggested to be involved in migraine pathogenesis (Kaiser and Russo, 2013; Tuka et al., 2013; Edvinsson, 2014). Also glutamate is implicated

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**Fig. 2** – Immunohistochemistry of CGRP, CLR and RAMP1 in rhesus monkey trigeminal ganglion. (A) CGRP is widely expressed in the neurons (arrows). Thin fibers expressing CGRP is found (arrow heads). (B) CLR and RAMP1 immunoreactivity in neurons (arrows) and satellite glial cells (arrowheads). Negative cells for CLR or RAMP1 are pointed out with small arrows. DAPI, staining nuclei, is used (blue). (C) Double immunolabelling of CGRP and CLR or RAMP1. CGRP is mostly expressed in neurons (small arrows) that lack expression of CLR or RAMP1 (large arrows). Co-localization is rarely found, and then in the smaller neurons (arrow heads). Auto-fluorescence from lipofuscin is detected in some neurons (asterisk). DAPI, staining nuclei, is used in the merged pictures (blue).
In migraine pathophysiology involving trigeminovascular activation, central sensitization and cortical spreading depression. Therefore, the use of glutamate receptor antagonists has been suggested for migraine treatment (Andreou and Goadsby, 2009; Marin and Goadsby, 2010). In relation to the trigeminal system both PACAP and glutamate are found in neurons in the trigeminal ganglion and in the trigemino-cervical complex (Kai-Kai and Howe, 1991; Tajti et al., 1999; Uddman et al., 2002). However, their relation to the CGRP system (mainly the CGRP receptor) in the trigeminal ganglion has not been fully evaluated.

In order to better understand if the trigeminal ganglion may be one possible site of action for drugs such as the CGRP receptor antagonists, we have studied CGRP receptor binding sites with a CGRP receptor antagonist (MK-3207) in rhesus monkey. To confirm expression and cellular distribution of CGRP and its receptor components in rhesus monkey trigeminal ganglion immunofluorescence was used. In addition, the distribution of PACAP and glutamate were analyzed for possible co-expression with CGRP and the CGRP receptor in the rhesus monkey and rat trigeminal ganglion. Additionally, we used Evans blue in rats to

![Fig. 3 – Double staining of CLR and RAMP1 in rhesus monkey trigeminal ganglion (A) Large arrows indicate the co-expression of CLR and RAMP1 in the neurons and arrowheads indicate co-expression in the satellite glial cells. Neurons only expressing RAMP1 are found (small arrows). (B) Vessels of different sizes within the trigeminal ganglion express CLR and RAMP1 in the smooth muscle cell layer (arrows). Auto-fluorescence is detected in lamina elastic interna (arrowheads). DAPI, staining nuclei, is used in the merged pictures (blue).]
study the relation of trigeminal ganglion to the blood brain-barrier (BBB).

2. Results

2.1. Binding of \(^{3}H\)MK-3207 in the rhesus monkey trigeminal ganglion

MK-3207 was labeled with tritium to high specific activity, and used for in vitro autoradiographic studies for binding site localization in rhesus monkey trigeminal ganglia slices. High binding densities of \(^{3}H\)MK-3207 were observed in the trigeminal ganglion of rhesus monkey (Fig. 1). High binding was found in the central parts of the trigeminal ganglion where the ganglion is located and in the trigeminal root, verified by Htx-eosin staining on the same slide (Fig. 1). Areas with low or no binding were also found. Minimal non-displaceable binding was seen in the presence of 1 mM unlabeled MK-3207 on an adjacent slice. The exact orientation of the ganglia was missing and regional variations (V1–V3) in the binding sites could not be determined. Also, the exact cellular binding (in neurons or glial cells) could not be clearly delineated by autoradiography due to low resolution of the technique and therefore the immunofluorescence method was used to demonstrate expression and cellular localization.

2.2. Expression of CGRP, CLR and RAMP1 in rhesus monkey trigeminal ganglion

To correlate binding and the presence of CGRP and its receptor components in rhesus monkey trigeminal ganglion, immunofluorescence was used. Immunoreactivity for CGRP, CLR and RAMP1 were observed in the trigeminal neurons (Fig. 2). The CGRP immunoreactivity was mainly found in the neurons and in thin fibers but not in glial cells (Fig. 2A). The receptor components, CLR and RAMP1, were expressed in neurons and in the satellite glial cells, which surround the neurons (Fig. 2B). Double-staining of CGRP with CLR or RAMP1 showed that the neuropeptide rarely co-expresses with the receptor components (Fig. 2C). The detailed distribution and cellular quantification have been previously described for rat and human trigeminal ganglion (Eftekhari et al., 2010). Double-staining of CLR and RAMP1 revealed co-expression between the receptor components in some of the neurons and in the satellite glial cells (Fig. 3A), supporting expression of a functional CGRP receptor. In addition, RAMP1 and CLR were co-localized in the walls of the vessels within the trigeminal ganglion, suggestive of vascular receptor sites. This was apparent in both large and small vessels in the trigeminal ganglion of rhesus monkey (Fig. 3B).

2.3. Distribution of PACAP, glutamate and their relation to the CGRP system in rhesus monkey and rat trigeminal ganglion

Due to the difficulty in accessing rhesus tissues, rat trigeminal ganglia were used as a surrogate to confirm the primate results. All the double-stainings were confirmed and similar staining patterns were found in primate and rat trigeminal ganglion. Double-staining of PACAP and CGRP revealed co-expression in the trigeminal neurons of rhesus and rat (Fig. 4); however, not all PACAP positive neurons expressed CGRP (Fig. 4). Cell counting in rat trigeminal ganglia showed that immunoreactivity for CGRP was found in 49% of the positive neurons (positive cells/total of number of cells; 1184/2478), PACAP in 29% (713/2478) and co-expression of CGRP and PACAP was found in 23% of the cells (581/2478). Double immunolabeling of PACAP and the CGRP receptor components showed that PACAP did not overall co-localize with RAMP1 or CLR either in rhesus monkey or rat trigeminal ganglion (Fig. 5). Very few neurons showed co-localization of PACAP and RAMP1 (Fig. 5).

There was generally no co-localization between glutamate and CGRP in the trigeminal neurons (Fig. 6) as confirmed in rat trigeminal ganglion (Fig. 6). Double-staining of glutamate and RAMP1 or CLR showed co-localization in the trigeminal neurons of rhesus and rat (Figs. 7 and 9). Neurons expressing only the CGRP receptor components or only glutamate were also found (Figs. 7A

Fig. 4 – Expression of PACAP and CGRP in rhesus monkey and rat trigeminal ganglion Co-expression of PACAP and CGRP in the trigeminal neurons (large arrows). Not all PACAP positive neurons express CGRP (small arrows). Thin CGRP positive fibers or neurons lacking PACAP expression are found (asterisks). Arrowheads point at neurons negative for both markers.
Expression of RAMP1 in satellite glial cells surrounding a glutamate positive neuron was also observed (Fig. 7B).

2.4. Evans blue in rats

In order to evaluate if the trigeminal ganglion is located within or outside the BBB, experiments with Evans blue were performed on rats. Evans blue has high affinity for serum albumin and forms a large complex when given systemically. This complex cannot cross the barrier. Virtually all Evans blue is bound to albumin, and consequently the neural tissue typically remains unstained. Evans blue becomes fluorescent when linked to proteins and can be examined using the fluorescence microscope. The brains from the rats injected with Evans blue displayed as expected no dye uptake (Fig. 9A). However, dye uptake was observed in the pituitary gland (Fig. 9A). Sections from cortex and cerebellum showed no fluorescent signal (Fig. 9B), confirming that Evans blue uptake was not present.
in these parts of the brain. Sections of pituitary gland and choroid plexus, regions that are without BBB, displayed high red fluorescence signal (Fig. 9C). These results confirm that the experiments with Evans blue were adequately performed. Dissected trigeminal ganglia showed uptake of Evans blue (Fig. 9A). Fluorescence signal was observed in trigeminal ganglion sections, where high signal was found in the ganglion where there were numerous cell bodies (Fig. 10). The nerve bundles displayed no or low signals, while some signal was observed around the nerve (Fig. 10A). Evans blue fluorescence appeared proximal to the neurons and/or around the neurons (Fig. 10B). Blood vessels of various diameters within the ganglion, often observed in close proximity to the neurons, displayed red fluorescence signal throughout the vessel wall (Fig. 10C). Sections from trigeminal ganglia without Evans blue injection showed no fluorescent signal (Fig. 10D).

Fig. 6 – CGRP and glutamate double staining in rhesus monkey and rat trigeminal ganglion Most CGRP positive neurons (red, large arrows) do not co-express glutamate. Few CGRP positive neurons co-express glutamate (small arrows). Glutamate (green) is found in separate neurons than those positive for CGRP (arrowheads). Asterisks point at thin CGRP positive fibers lacking glutamate expression. DAPI, staining nuclei, is used in the merged pictures (blue).

3. Discussion

This is the first study to examine CGRP binding sites, expression of CGRP and its receptor in rhesus monkey trigeminal ganglion. In addition, we related the findings to expression of PACAP and glutamate in rhesus monkey trigeminal ganglion and confirmed the staining patterns in rat trigeminal ganglion. We found high binding densities of [3H]MK-3207, a selective CGRP receptor antagonist, and confirmed the presence of CGRP receptor components by immunofluorescence in the rhesus monkey trigeminal ganglion. These results support the presence of functional CGRP receptors in the ganglion that can putatively be occupied by CGRP receptor antagonists. The clinically effective CGRP receptor antagonist, telcagepant, has recently been shown to have low central receptor occupancy (Hostetler et al., 2013). Therefore, we further evaluated if the trigeminal ganglion is protected by the BBB using in vivo administration of Evans blue in rats. These experiments revealed that the trigeminal ganglion is located outside the BBB and can therefore be reached by systemic CGRP receptor antagonists.

The autoradiographic binding experiments revealed high binding densities of the CGRP receptor antagonist MK-3207 in the trigeminal ganglion of rhesus monkey, suggesting the presence of functional CGRP receptors in this region. The pharmacological characterization of this antagonist has been previously described (Bell et al., 2010; Salvatore et al., 2010). Although MK-3207 is selective for the CGRP receptor versus the related adrenomedullin, calcitonin and amylin 3 (AMY3) receptor, it is less selective versus the amylin 1 (AMY1) receptor. In these studies the concentration of [3H]MK-3207 (0.045 nM) was
carefully selected to be approximately 2-fold greater than its affinity value on the CGRP receptor ($K_i=0.024$ nM) to limit the potential for binding on the AMY$_1$ receptor where MK-3207 has a $K_i=0.75$ nM (Salvatore et al., 2010). Although it cannot be completely ruled out that some of the signal in rhesus trigeminal ganglion is a result of AMY$_1$ receptor binding, it is unlikely to be a major contributor to the binding signal.

To further confirm the presence of CGRP and its receptor, immunofluorescence experiments were performed in rhesus monkey trigeminal ganglion. CGRP immunoreactivity was expressed in the neurons of the trigeminal ganglion and in thin fibers distributed among the neurons. This is in agreement with previous studies on rat and human trigeminal ganglion (Tsai et al., 1988; Nozaki et al., 1990; Alvarez et al., 1991; Eftekhari et al., 2010) and very thin unmyelinated nerve fibers (Liu-Chen et al., 1986; Eftekhari et al., 2013c). CLR and RAMP1 were co-expressed mainly in the larger neurons and in satellite glial cells, supporting the existence of functional CGRP receptors in the trigeminal ganglion. The present work is in agreement with studies on human and rat trigeminal ganglion (Lennerz et al., 2008; Eftekhari et al., 2010). Co-expression of CLR and RAMP1 was also found in blood vessels walls of the trigeminal ganglion, opening up the possibility for effects on the microvasculature within the trigeminal ganglion. Since CGRP is a strong vasodilator of cranial vessels (Edvinsson et al., 2010), it is likely that CGRP can act as a local vasodilator on these vessels.

The trigeminal ganglion cells consist of pseudounipolar neurons and two types of glial cells. As observed in human and rat trigeminal ganglion (Lennerz et al., 2008; Li et al.,
there was co-expression of the receptor components in the rhesus satellite glial cells. This cell type has been suggested to have an important role in inflammation and pain (Li et al., 2008; Raddant and Russo, 2011). In primary cultures of rat trigeminal ganglia CGRP treatment increased glial cell iNOS expression and release of NO, which was inhibited by a CGRP receptor antagonist (Li et al., 2008), suggesting that CGRP can activate satellite glial cells. In addition, CGRP may trigger additional inflammatory gene expression changes in glia in vitro (Vause and Durham, 2010). It has been demonstrated that the anti-migraine drug sumatriptan, a 5-HT1B/1D receptor agonist, may inhibit the evoked CGRP release from naïve trigeminal neuron cultures, as well as from cultured trigeminal ganglia (de Corato et al., 2011). The present study suggests that CGRP receptor antagonists may act in the trigeminal ganglion on the satellite glial cells and secondarily influence the neuronal responses. Further functional studies are needed to address this hypothesis.

Several other signaling molecules have been found in the trigeminal ganglion, these include substance P, neurokinin A, dynorphine, nitric oxide and PACAP (Tajti et al., 1999; Hou et al., 2001; Dieterle et al., 2011). Yet clearly there may be many other contributing neuropeptides that could act in concert with CGRP. One interesting candidate is PACAP, which shares some of the actions as CGRP (Kaiser and Russo, 2013). Human studies indicate that PACAP is a potent headache trigger and altered plasma PACAP levels have been shown in migraineurs (Schytz et al., 2009; Tuka et al., 2013). PACAP belongs to the vasoactive intestinal polypeptide (VIP) secretin superfamily (Vaudry et al., 2009) and has been identified in human and rodent sensory and parasympathetic ganglia, as well as in second-order neurons of the TNC (Tajti et al., 2001; Uddman et al., 2002). Three PACAP receptors have been identified, termed PAC1, VPAC1 and VPAC2 (Vaudry et al., 2009). Like CGRP, PACAP dilates cranial blood vessels in humans in association with headache (Amin et al., 2012) and it has some shared actions of CGRP that may contribute to migraine pathophysiology. We observed co-expression of PACAP and CGRP in the trigeminal neurons of rhesus monkey and rat, which is in support of earlier studies showing co-expression of CGRP and PACAP in the dorsal horn of the spinal cord and in the trigeminal ganglion (Moller et al., 1993). In the rat trigeminal ganglion, we found CGRP expression in about 50% of the cells and PACAP in around 20% of the neurons. Co-expression of PACAP and CGRP was found in around 20% of the immunoreactive neurons. Recently we demonstrated nerve fibers in dura mater co-expressing PACAP and CGRP (Eftekhari et al., 2013c). Altogether, this suggests that PACAP may have 2 sites of origin, a minor population co-expressed with sensory CGRP and a larger parasympathetic distribution (Edvinsson et al., 2001).
Our results demonstrate that around 20% of the PACAP neurons co-express with sensory CGRP in the trigeminal ganglion.

The CGRP receptor components are rarely co-expressed with CGRP, suggesting a low presence of putative auto-receptors within the ganglion (Eftekhari et al., 2010). In the present study we did not find co-expression of the CGRP receptor components and PACAP in the trigeminal neurons. Therefore it is unlikely that CGRP receptor antagonists would affect interganglionic PACAP levels. However this needs further evaluation.

Another important neurotransmitter that may contribute to migraine pathogenesis is glutamate. Findings from both animal and human studies suggest a link between glutamate and migraine (Vikelis and Mitsikostas, 2007). Potassium-stimulated cultured trigeminal neurons release both glutamate and CGRP (Xiao et al., 2008). Elevated glutamine levels have been observed in the cerebrospinal fluid of migraine patients (Rothrock et al., 1995). Therefore, it has been suggested that pharmacological management of glutamate receptors may provide further insight into potential migraine treatment (Vikelis and Mitsikostas, 2007; Sprenger and Goadsby, 2009; Marin and Goadsby, 2010). We investigated the localization of glutamate in the rhesus and rat trigeminal ganglion with a focus on its relation to CGRP and the CGRP receptor. Our results demonstrate that glutamate and CGRP are mostly expressed in separate neurons. This suggests that trigeminal neurons that express CGRP do not contain glutamate as a co-transmitter. In support of this observation, most CGRP containing axons in autonomic ganglia and neurons in dorsal root ganglia lack expression of the glutamate transporters VGluT1 and VGluT2, suggesting that most peptide-containing sensory neurons do not use glutamate as transmitter (Morris et al., 2005).

Instead, some RAMP1 and/or CLR positive neurons were shown for the first time to co-express glutamate. Neurons expressing CGRP receptor components only in their satellite glial cells and glutamate in their cell body were also observed. This opens up the possibility of interaction between the glutamate system and CGRP receptors. It remains to be evaluated if CGRP receptor antagonists may have an effect on glutamate release.

The CGRP receptor antagonists represent a potential new group of anti-migraine drugs that may offer a non-vasoconstrictive approach in the treatment of migraine (Chan et al., 2010). Several CGRP receptor antagonists (olecegaptant, telcagepant, MK-3207, BI 44370TA and BMS927711) have displayed efficacy in the treatment of migraine (Durham and Vause, 2010; Negro et al., 2012; Dolgin, 2013). The CGRP receptor antagonists have potentially opened up a new option in migraine treatment; however, there remains active discussion as to where these molecules act to reduce migraine pain. It has been shown that the currently used anti-migraine drugs, the triptans, cannot penetrate the BBB under normal circumstances and a central effect in the trigeminal nucleus can
Fig. 10 – Fluorescence micrograph from trigeminal ganglion following injection of Evans blue (A) Demonstration of a fluorescence microscopic figure showing the trigeminal ganglion with red fluorescent signal from the dye Evans blue, indicating the lack of the blood brain barrier. High signal is detected within the ganglion where the cells are located while no or low signal is detected within the nerve. Arrows demonstrate a higher magnification of the selected areas, from left to right: nerve, ganglion and vessel. (B) The Evans blue signal is detected around the neurons. (C) Vessels within ganglion (arrows), close to the neurons, also shows signal for Evans blue uptake. DAPI, staining nuclei, is used in the merged pictures (blue). (D) Trigeminal ganglion tissue without Evans blue showing no fluorescence signal.
only be seen after BBB disruption (Kaube et al., 1993). Some experimental data have suggested that there might be a minor breakdown or leakage of the BBB during migraine with activation of matrix metalloproteinases (MMPs) following repeated cortical spreading depression (Gursoy-Ozdemir et al., 2004). If the BBB is compromised it would enable the passage of drugs which typically do not enter the CNS. However, these data are indirect and there is no clear proof of breakdown or leakage of the BBB in patients during migraine attacks (Edvinsson and Tfelt-Hansen, 2008).

Clinical studies have shown that CGRP receptor antagonists need to achieve high plasma concentrations in relationship to their intrinsic potency in order to elicit therapeutic effects (Ho et al., 2008a). However, the first PET study in healthy individuals and migraine patients demonstrated that the CGRP receptor antagonist telcagepant achieved low CGRP receptor occupancy (10%) in the CNS at the lowest clinically efficacious dose, whereas a supra-therapeutic dose of telcagepant (1120 mg) resulted in only moderate receptor occupancy in healthy volunteers. These data suggest that CGRP receptor antagonists do not have to act centrally for clinical efficacy (Vermeersch et al., 2012; Hostetler et al., 2013).

If the current CGRP receptor antagonists cannot pass the BBB (or only to a low degree), it is important to determine which of the sites that can be reached by CGRP receptor antagonists contribute to the clinical efficacy. In the periphery the CGRP receptor is found in the smooth muscle cell layer of cerebral arteries, meningeal vessels, nerve fibers and rodent mast cells of the dura mater (Eftekhari et al., 2013c). Peripherally the CGRP receptor antagonists may act on cranial vessels to inhibit CGRP-induced vasodilatation and on dural mast cells to block degranulation and the subsequent release of inflammatory mediators. However, the role of cerebrovascular dilatation is unclear. Recently, it was shown that acute migraine pain was not accompanied by extracranial arterial dilatation and only by minor intracranial dilatation during acute migraine attacks (Amin et al., 2013). In the same study, it was demonstrated that effective treatment with sumatriptan caused no intracranial (cerebral artery) vasconstriction. It is unclear if mast cell degranulation contributes to neurogenic inflammation in humans, since it has been shown that CGRP receptors are not present in human mast cells (Eftekhari et al., 2013c). This agrees with the early finding that CGRP failed to induce release of histamine from human dura mater mast cells (Otsosson and Edvinsson, 1997).

In the present study we demonstrated that the trigeminal ganglion is located outside the BBB and thus accessible to systemic drugs, which to our knowledge has not previously been clearly determined. There is only limited previous work addressing this issue. It has been suggested that capillaries in sensory and sympathetic ganglia are fully permeable to macromolecules (Kiertman, 1996). A study from 1973 showed Evans blue uptake in the trigeminal nerve of rabbits and mice (Arvidsson et al., 1973). In another study, injection of Fluoro-Gold into the tongue showed dense accumulation of the dye in cells of the trigeminal ganglion (Merchenthaler, 1991) and injection of horseradish peroxidase (HRP) showed uptake of HRP in the trigeminal ganglion (Ten Tusscher et al., 1989).

In the present study injection of the dye Evans blue into the circulation of rats showed uptake of the dye in areas known to be outside the BBB such as pituitary gland and choroid plexus, whereas the brain showed no dye uptake. Dye uptake was clearly found in the trigeminal ganglion and the fluorescence signal was found mostly around the cells and vessels in proximity to the cells. Within the trigeminal nerve there was low to no signal observed.

This suggests that the trigeminal ganglion is not protected by the BBB and can be reached by CGRP receptor antagonists regardless of the molecules ability to cross the BBB. The trigeminal ganglion may also be a site of action for the recently developed monoclonal anti-CGRP and anti-CGRP receptor antibodies, which in all likelihood are too large to pass the BBB (Dolgin, 2013). Support for this has been obtained in functional tests in rodents with a CGRP antibody (Edvinsson et al., 2007). CGRP receptor antagonists, anti-CGRP and anti-CGRP receptor antibodies are currently under development (Bigal et al., 2013), and if these modalities are not able to penetrate the BBB, the trigeminal ganglion may be one site of action.

Since the trigeminal ganglion is connected to the periphery and also projects centrally, CGRP signaling may be blocked to these parts by blunting the activation/signaling in the trigeminal ganglion. However, it cannot be ruled out that additional efficacy may be achieved with better access to the CNS since there is wide spread expression of CGRP and its receptor throughout the CNS and the putative role of brain areas such as the brainstem and hypothalamus in the pathophysiology of migraine. Lipophilic triptans with ability to pass the BBB did not show improved efficacy (Pascual and Munoz, 2005). Recently, a series of CNS-penetrant CGRP receptor antagonists have been characterized (Joshi et al., 2014). It will be of great interest to assess if CNS-penetrant CGRP receptor antagonists demonstrate better clinical efficacy. Interestingly, we have shown binding of a CGRP receptor antagonist via in vitro autoradiography in the pineal gland and area postrema of the rhesus monkey (Eftekhari et al., 2013c). These CNS areas are not protected by the BBB suggesting that these areas can also be reached by drugs such as CGRP receptor antagonists.

4. Conclusion

The present study demonstrates for the first time in primate binding sites of a CGRP receptor antagonist and expression of CGRP and its receptor within the rhesus trigeminal ganglion. The results suggest and are in support of the presence of functional CGRP receptors in this area. The receptor components were co-expressed in neurons and satellite glial cells. The study also shows co-localization between PACAP and CGRP, while glutamate co-localizes with the CGRP receptor components in the trigeminal ganglion. The study also reveals that trigeminal ganglion is located outside the BBB and therefore CGRP receptor antagonists do not need to be CNS-penetrant to block receptors in the trigeminal ganglion.

5. Experimental procedure

5.1. Rhesus monkey tissue samples

Rhesus trigeminal ganglion (Macaca mulatta, n=3, age 13–15 years old, females) was harvested in accordance with a Merck
Table 1 – Secondary antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Conjugate and host</th>
<th>Against</th>
<th>Dilution</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>DyLight 549 (goat)</td>
<td>Anti-rabbit</td>
<td>1:200</td>
<td>Jackson ImmunoResearch, West Grove, PA</td>
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<tr>
<td>DyLight 549 (donkey)</td>
<td>Anti-goat</td>
<td>1:200</td>
<td>Jackson ImmunoResearch, West Grove, PA</td>
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<td>DyLight 488 (donkey)</td>
<td>Anti-sheep</td>
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<td>Jackson ImmunoResearch, West Grove, PA</td>
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<tr>
<td>DyLight 488 (donkey)</td>
<td>Anti-goat</td>
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<td>Jackson ImmunoResearch, West Grove, PA</td>
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<tr>
<td>DyLight 488 (donkey)</td>
<td>Anti-rabbit</td>
<td>1:200</td>
<td>Jackson ImmunoResearch, West Grove, PA</td>
</tr>
<tr>
<td>Alexa 594 (goat)</td>
<td>Anti-rabbit</td>
<td>1:400</td>
<td>Invitrogen, La Jolla, CA</td>
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<tr>
<td>Alexa 594 (donkey)</td>
<td>Anti-rabbit</td>
<td>1:400</td>
<td>Jackson ImmunoResearch, West Grove, PA</td>
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<td>Alexa 488 (goat)</td>
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<td>1:200</td>
<td>Cayman Chemical, Ann Arbor, MI</td>
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<td>Anti-rabbit</td>
<td>1:100</td>
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<tr>
<td>Texas-Red (rabbit)</td>
<td>Anti-sheep</td>
<td>1:200</td>
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</table>

Research Laboratories Institutional Animal Care and Use Committee approved protocol. Tissues to be used for autoradiography studies were quickly removed and frozen on dry ice. The samples were cryosectioned at 20 μm (cryostat model CM3050: Leica Microsystems, Inc., Deerfield, IL) and collected on cold Superfrost™ Plus slides, and stored at −80 °C. An additional rhesus monkey (9 years old, female) trigeminal ganglion was harvested for immunohistochemistry. The samples were immersed overnight in 4% paraformaldehyde (PFA) and in 0.1 mol/l phosphate buffer, pH 7.2. After fixation, specimens were paraffin embedded. The sections were cut to the laboratory in Sweden for immunohistochemistry experiments. The rhesus samples were sectioned (5 μm) and stored at room temperature. CITES, import permits, for the Swedish part was approved and given the permit number Dnr34-10088/10 nr 51016-10. CITES permit for export from USA was approved and given the permit number 10US11621A/9. All animal experiments carried out in accordance with EC Directive 86/609/EEC for animal experiments.

5.2. Rat tissue samples

Trigeminal ganglia were removed from 10 male Sprague-Dawley rats weighing 300–350 g (Scanbur, Stockholm, Sweden). The experiments were approved by the University Animal Ethics Committee (M 126-12), Lund University, Sweden. The ganglia were placed in 4% PFA and fixed for 2–3 h. After fixation the ganglia were rinsed in rising concentrations of sucrose in Sörensen’s phosphate buffer, embedded in gelatin medium (30% egg albumin, 3% gelatin in distilled water) and cryosectioned at 12 μm. The sections were stored at −20 °C until use.

5.3. Autoradiography studies

Autoradiography studies were conducted as previously described (Salvatore et al., 2010). Slides were preincubated for 15 min in binding buffer (0.9% NaCl, 50 mM Tris–HCl, pH 7.5, 2 mM KCl, 1 mM MgCl₂ and 1 mM CaCl₂) followed by 90 min incubation with [³H]MK-3207 (0.045 nM; specific activity 76 Ci/nmol) at room temperature (two slides from each animal). Non-displaceable binding was defined by blocking with 1 μM unlabeled MK-3207 using adjacent slides (two slides from each animal). Slides were washed 3 × 1 min in ice-cold buffer (0.9% NaCl and 50 mM Tris–HCl, pH 7.5) followed by rinse in ice-cold water. The slides were air-dried and exposed to phosphorimaging plates (TR2025; Fuji-film Medical Systems USA, Inc., Stamford, CT) for 3 weeks and scanned with a BAS 5000 scanner (Fuji, Tokyo, Japan). Image analysis was conducted with MCID software (MCIC, Linton, Cambridge, UK). After the autoradiography studies, the slides were stained with hematoxylin–eosin following a standard protocol (Htx 3 min, water rinse, eosin 1 min) for orientation and examination of the tissue condition.

5.4. Immunofluorescence studies

Immunofluorescence staining was performed to demonstrate the localization of CGRP, CLR, RAMP1, PACAP and glutamate in the rhesus monkey trigeminal ganglia. Paraffin sections were deparaffinized in xylene followed by serials of alcohol. To unmask the antibody epitopes, heat-induced epitope retrieval was performed, where the sections were heated in a microwave for 10 min in citric acid solution pH 6.0. Rat trigeminal ganglia were used for comparison and confirmation of the staining patterns. The sections were then washed for 10 min in PBS pH 7.2 containing 0.25% Triton X-100 (PBST). The sections were blocked for 1 h in blocking solution of PBS and 5% normal donkey or goat serum (depending on species origin of the secondary antibody). After blocking, the sections were incubated overnight at 4 °C for single or double immunolabelling with primary antibodies against CGRP (mouse, ab81887, Abcam, UK), CLR (rabbit, 3152, sheep, 132, Merck & Co.), RAMP1 (goat, 844, Merck & Co.), PACAP-38 (rabbit, T-4473, Bachem, USA) and glutamate (rabbit, AB5018, Millipore, USA). For all double immunostainings, the antibodies were applied separately and not mixed together. The primary antibodies were diluted in PBST containing 1% BSA and 3% normal serum. After incubation with primary antibodies, sections were equilibrated to room temperature, rinsed in PBST for 3 × 15 min and exposed to secondary antibodies diluted in PBST and 1% BSA for 1 h at room temperature. For detailed description of the secondary antibodies, see Table 1. The sections were subsequently washed with PBST for 3 × 15 min and mounted with an anti-fading mounting medium containing DAPI (Vectorshield, Vector Laboratories). The development and specificity of CLR and RAMP1 antibodies have been demonstrated in our previous study, where the specificity of the antibodies was confirmed in HEK293 cells stably expressing the human CGRP receptor and was confirmed.
by Western blotting (Eftekhari et al., 2010). In our recent study, the antibodies were used on rhesus monkey tissue (Eftekhari et al., 2013b). All stainings were repeated to confirm the results and all slides were analyzed. Omission of the primary antibody served as negative controls for all antibodies.

5.5. Cell-counting

Cell counting was performed to quantify the expression of CGRP, PACAP and their co-expression in rat trigeminal ganglion (n=5). Three slides (with a minimum of five sections between each were used for measurements to avoid double-counting of cells) from each animal were stained with the respective antibody. Four to six images (depending on the size of the ganglia) were taken at magnification 10× in each section to cover the entire trigeminal ganglion. The NIS-elements BR image analysis program (Nikon) was used to calculate the number of positive cells. Only cells with visible nuclei were counted. CGRP, PACAP positive and double-stained cells were counted in each image. The total number of cells positive for CGRP, PACAP, double-stained and total counted cells was calculated for each animal.

5.6. Evans blue experiment

Sprague-Dawley rats weighing 300–350 g (n=4) were used for the Evans blue experiments. Rat experiments were approved by the Animal Ethics Committee of Copenhagen University, Denmark and carried and the experiments conformed to the “European Convention for the protection of vertebrate animals used for experimental and other scientific purpose (council of Europe No 123, Strasbourg 1985). The animals were deeply anaesthetized subcutaneously (dose of 2.5 ml/kg) with a mixture of hypnorm–midazolam (1:1:2) in sterile water (containing 0.079 mg/ml fentanyl, 2.50 mg/ml fluanisone and 1.25 mg/ml midazolam). 2% Evans blue in saline (Sigma-Aldrich, MO, USA) was injected via the tail vein (4 ml/kg).

After 1 h, the animals were perfused transcardially with a prewash of phosphate-buffered saline (PBS, pH 7.2) for 2 min followed by 4% paraformaldehyde (PFA) in PBS for 5 min. The brains and trigeminal ganglia were dissected and the specimens were further fixed in PFA for 2–4 h followed by rinses in rising concentrations of sucrose in Sörensen’s phosphate buffer. The specimens were embedded in a gelatin medium (30% egg albumin, 3% gelatin in distilled water) and cryosectioned (12 μm). The sections were stored at −20 °C until use.

5.7. Microscopic analysis

Immunostained sections were examined and images were obtained using a light- and epifluorescence microscope (Nikon 80i, Tokyo, Japan) coupled to a Nikon DS-2MV camera. Adobe Photoshop CS3 (v.8.0, Adobe Systems, Mountain View, CA) was used to visualise co-labeling by superimposing the digital images and processed for brightness and contrast. Evans blue sections were examined by a filter with settings for excitation 510–550 nm and emission at 590 nm.

Conflict of interest statement

Sajede Eftekhari conducted research at Merck Research Laboratories as a visiting scholar. Christopher Salvatore, Tsing-bau Chen, and Zhizhen Zeng are employees of Merck Sharp & Dohme Corp, and potentially own stock and/or hold stock options in the Company.

Authors’ contributions

SE performed/participated in all the experiments, study design, analyzed all data and drafted the manuscript. CAS participated in designing the study, analyzing the data and helped to draft the manuscript. SJ performed the Evans blue experiments. T-BC performed the autoradiography experiments. ZZ participated in designing the study and analyzing the autoradiography data. LE participated in designing the study and helped to draft the manuscript. All authors have read and approved of the final version of the manuscript.

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References


