Distribution of CGRP and CGRP receptor in the trigeminovascular system and CNS

Sajedeh Eftekhar

DOCTORAL DISSERTATION
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Abstract
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In the trigeminal ganglion, CGRP receptor was localized to neurons and satellite glial cells. CGRP was expressed in small/medium-sized neurons, which lacked CGRP receptor. This suggests that if CGRP is released within the ganglion, then intraganglionic CGRP may act on satellite glial cells and on large sized neurons. It was revealed that the trigeminal ganglion is not protected by the blood-brain barrier (BBB), suggesting that CGRP receptor antagonists may act here.

In the periphery, expression of CLR and RAMP1 was found in the cranial, meningeal and cerebral arteries, and nerve fibers and rodent mast cells within the dura mater. Indeed we have uncovered that sensory nerves exist in two separate fiber populations and this has not been demonstrated before with such clarity.

In the brainstem we identified certain regions expressing CGRP and its receptor. Brainstem regions, outside BBB, showed CGRP receptor binding, suggesting that CGRP receptor antagonists may act there independently from their ability to pass BBB. Rich expression of CGRP and CGRP receptor was detected in the cerebellum, pointing toward a functional role of CGRP in cerebellum.

This research reveals the expression of CGRP receptor both at peripheral and central sites, implicating that many locations may be involved in migraine pathophysiology.

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To my family
With them the Seed of Wisdom did I sow,
And with my own hand labour’d it to grow:
And this was all the Harvest that I reap’d—
I came like Water, and like Wind I go.

Rubaiyat of Omar Khayyam, Persian Poet.
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About the author

I, Sajedeh Eftekhari, was born 1984 in Tehran, Iran. In 1990, my family and I moved to Sweden and I grew up in Lomma, a small town nearby Lund. In 2006, I obtained my Bachelor’s degree in Biomedical Science followed by a Master’s degree in Biomedicine in 2007, at Lund University. Both of my theses were accomplished at the Department of Ophthalmology, Lund University. Subsequent to my studies, I worked at the Blood Center, Department of Transplantation, at Lund University Hospital for one year.

In 2009, I began my PhD studies at the Division of Experimental Vascular Research, Lund University, Sweden, under the supervision of Professor Lars Edvinsson and co-supervision of Professor Karin Warfvinge. During this time I had the privilege to collaborate with Merck & Co. in the United States, where I also did an internship in the Pain and Migraine Department under the supervision of Christopher Salvatore.

Previous research has revealed the importance of the neuropeptide calcitonin gene-related peptide (CGRP) in the pathophysiology of migraine, and more recently CGRP receptor antagonists have demonstrated clinical efficacy. The present research was undertaken to investigate the distribution of CGRP and its receptor within the trigemiovascuclar system and parts of the central nervous system. We believe that this research is important to increase the understanding of CGRP signaling and to explore potential sites of action of CGRP receptor antagonists. During the course of the work presented in this thesis, I have identified the distribution of CGRP and its receptor both at peripheral and central sites, implicating that many locations may be involved in migraine pathophysiology. These regions may serve as sites of action for drug treatments inhibiting CGRP signaling.

I hope you enjoy reading this thesis.

Sajedeh Eftekhari
Original Articles

This doctoral thesis is based on the following articles:


# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CLR</td>
<td>Calcitonin receptor-like receptor</td>
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<td>CSD</td>
<td>Cortical spreading depression</td>
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<td>FHM</td>
<td>Familial hemiplegic migraine</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<tr>
<td>Htx-Eosin</td>
<td>Hematoxylin-Eosin</td>
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<td>LC</td>
<td>Locus coeruleus</td>
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<td>MBP</td>
<td>Myelin basic protein</td>
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<td>NKA</td>
<td>Neurokinin A</td>
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<td>NF</td>
<td>Nerve filament</td>
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<td>nNOS</td>
<td>Neuronal oxide synthase</td>
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<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase activating peptide</td>
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<tr>
<td>PAG</td>
<td>Periaqueductal gray</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered-saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered-saline (PBS) containing 0.25% Triton X-100</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>RAMP1</td>
<td>Receptor activity-modifying protein 1</td>
</tr>
<tr>
<td>RCP</td>
<td>Receptor component protein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>STN</td>
<td>Spinal trigeminal nucleus</td>
</tr>
<tr>
<td>TNC</td>
<td>Trigeminal nucleus caudalis</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
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Abstract

Calcitonin gene-related peptide (CGRP) has a key role in the pathophysiology of migraine and is associated with activation of the trigeminovascular system. Recently, CGRP receptor antagonists have been developed with clinical efficacy. The present thesis aimed therefore to investigate the distribution of CGRP and the CGRP receptor in the trigeminovascular system and in parts of the CNS.

In the trigeminal ganglion, CGRP receptor was localized to neurons and satellite glial cells. CGRP was expressed in small/medium-sized neurons, which lacked CGRP receptor. This suggests that if CGRP is released within the ganglion, then intraganglionic CGRP may act on satellite glial cells and on large sized neurons. It was revealed that the trigeminal ganglion is not protected by the blood-brain barrier (BBB), suggesting that CGRP receptor antagonists may act here.

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In the brainstem we identified certain regions expressing CGRP and its receptor. Brainstem regions, outside BBB, showed CGRP receptor binding, suggesting that CGRP receptor antagonists may act there independently from their ability to pass BBB. Rich expression of CGRP and CGRP receptor was detected in the cerebellum, pointing toward a functional role of CGRP in cerebellum.

This research reveals the expression of CGRP receptor both at peripheral and central sites, implicating that many locations may be involved in migraine pathophysiology.
What is migraine – patients perspective

Patients, members in the Swedish Migraine union, were asked what migraine is for them and how it affects their life. I am grateful to all of you that answered and touched by your descriptions. Below are some of the descriptions.

“A migraine attack feels like all of your senses kick back against your head with tremendous force. Living with migraine is extremely frustrating. It forces me down when all I want to do is to jump, run, play, and work”

“Migraine, to me, is the inability to live a full life. It is to fret having children, planning your life, studies, work, social life, family trips, and travels. Ultimately migraine is something that steals half (at least) of your life, a living hell during the attacks, forcing you to function even though you don’t.”

“The description of migraine for those who suffer from it often: anxiety, anxiety and fear to suffer from an attack that can’t be stopped with medicine… Torment, torment because I have to take migraine tablets all too often! Regards from a hopeless case!”

“Every day I walk with a 5-15kg weight on my head which has to be balanced. The weight drains my energy and if I worry too much the fatigue takes over.”

“Migraine; you just wish you could detach your head and set it down next to you for a while to rest…”

“Migraine, to me, is to be hit by a train over the head. I feel morbidly agonized when I am suffering from migraine and don’t want to live anymore. I find myself in a vacuum and lose track of time and space. I lay in bed, vomiting into a bucket. I don’t know if it’s day
or night and I can’t get up to take my medicine. When I hear the outside world and my children playing I feel miserable, it feels as if life goes on but I am excluded from it.”

From a 12 year old girl: “I can feel it behind my eye, beaming inside. It comes from the temple and pulsates. As soon as you move to hastily it feels like your entire head is pounding for a second. That’s why you can’t move your head. You can’t plan anything because it always comes suddenly. If you have something planned it always has to change. You might fall behind in school as you have to go home and can’t do your homework. Everything becomes so stressful. “

“To have migraine means that you lay in bed while your child graduates from high school or maybe you have to excuse yourself from the midsummer lunch with the family. You are susceptible to sounds, nauseous, and have a thumping headache which allows you do nothing but to close your eyes and lay in the darkness… waiting for it all to end.”

“Migraine to me affects my entire life. Beside the pain, dizziness makes me unable to do anything. You can’t function without a balance… just lay there, still, asking loved ones for help to crawl to the bathroom and to be fed. Worrying when the next fierce attack will strike makes it impossible for me to be by myself. Life has become anxiety.”

“What is real and what is a dream? One eye doesn’t want what the other wants. The tablet starts to work, the pain rescinds. Reality slowly comes back. Your throat is sore from vomiting. You stand up on shaky legs.”
**Svensk sammanfattning**

Migrän är en mycket vanlig sjukdom som drabbar omkring 1 miljon svenskar. Vart fjärde hushåll har minst en individ med migrän, och vart femte kvinna i fértil ålder har migrän. Sjukdomen medför ett stort funktionshinder och en stor ekonomisk bönda för både individen och samhället. Världshälsoorganisationen (WHO) rankade migränssjukdomen på 12:e plats bland kvinnor och på 19:e plats för både könen när det gäller olika sjukdomars grad av funktionshinder.


Under de senaste åren har forskningen kring migrän gjort stora framsteg, dock har sjukdomen visat sig vara mer komplicerad än man anat och orsaken bakom migrän inte helt klarlagt. Studier har visat att både smärtserver till hjärnan och känslerverver runt kraniella blodkärl (hjärnans blodkärl) är kopplade till migränattackerna. En signalsubstans (molekyl som förmedlar en nervsignal) som har väckt stort intresse är CGRP (calcitonin gene-related peptid). Blodprover från migränpatienter har visat att det sker en kraftig ökning av CGRP-frisättning under en migränattack. Flertal studier har visat att det råder ett samband mellan CGRP och migrän.

Upptäckterna kring CGRP har idag lett fram till utveckling av en ny typ av migränmedicin med klinisk effekt vid akut migränbehandling, s.k. CGRP-receptor antagonister. Dessa preparat blockerar CGRP-receptorn, och kan därmed blockera smärtsignalerna till hjärnan. I vår forskning fokuserar vi på att lokalisera CGRP och dess receptorer i kraniella cirkulationen och i centrala nervsystemet. CGRP receptorn består av två komponenter; CLR (calcitonin receptor-like receptor) och RAMP1 (receptor activity-modifying protein 1), som båda kopplar sig intracellulärt till RCP (receptor component protein).
Vårt arbete avser att utreda viktiga frågor som kvarstår; dels ge en förklaring till patofysiologin (läran om sjukdomsmekanismer) vid migrän och dels förklara hur och var CGRP receptor antagonister har sin effekt. Områden vi har undersökt är i det s.k. trigeminovaskulära systemet som är det sensoriska nervsystem vilket innerverar kraniala kärl och dura mater (härda hjärnhinnan) med cellkroppar placerade i trigeminus-gangliet. Vid aktivering av detta system, frisläpps CGRP. Vi har oftast använt biopsier från human vävnad, vilket gör att vår forskning kommer ett steg närmare klinisk verksamhet. För att lokalisera uttrycket av CGRP och receptorkomponenterna har vi använt oss av vävnadssnitt där specifika markörer (antikroppar) mot CGRP och dess receptor tillsätts. Markörerna är bundna till fluorescerande sekundära markörer, vilket gör att dessa kan upptäckas/kartläggas i ett speciellt mikroskop.

Vi har dels visat att 50% av cellerna i trigeminus-gangliet innehåller CGRP och nästan 40% dess receptor. Vi fann att små/mindre nervceller uttrycker CGRP medan de större nervcellerna uttrycker receptorn. Vi fann även att receptor-komponenterna uttrycks i s.k. satellit-gliacell Detta kan innebära om/när CGRP frisläpps i detta ganglion, så kan det ha en effekt på de större cellerna samt satellit-gliaceller. CGRP receptor antagonister kan även verka på dessa celltyper. Vi har även visat bindning av en CGRP receptor antagonist på rhesus apa trigeminus ganglion, vilket stöder antagandet att det uttrycks funktionella CGRP-receptor. Vi visade även att trigeminus-gangliet saknar blod-hjärnbarriär, vilket gör att mediciner som CGRP receptor antagonister kan verka i detta ganglion oavsett medicinens förmåga att passera denna barriär.

Nervfibrerna från trigeminus-gangliet med CGRP som signalmolekyl löper till hjärnans kärl och dura mater. Här fann vi att CGRP-receptorn uttrycks i blodkärlens vägg, nervfibrer och i s.k. mastceller i dura mater. Förekomsten av CGRP receptorer i dessa kärl, fibrer och celler tyder på att CGRP receptor antagonister kan ha effekt där. Centralt löper trigeminusnerven till hjärnstammen, där vi har visat förekomsten av CGRP och dess receptor i nervfibrerna. Vi har genom detaljerade analyser visat att CGRP uttrycks i de tunna C-fibrerna medan receptorn uttrycks i myeliniserade A-fibrer. Vi har även kartlagt bindning av en CGRP receptor antagonist, uttryck av mRNA och CLR- och RAMP1- protein i hjärnstammen i rhesus apa. Det intressanta var att några av dessa områden är områden som saknar blod-hjärnbarriär. Detta kan innebära att CGRP receptor antagonister kan verka där oavsett om de kan passera blod-hjärnbarriären.

I central nervsystemet har vi även påvisat förekomsten av CGRP och CGRP-receptar i lillhjärnan (cerebellum). Aktivering av cerebellum hos patienter under migränattack har visats men aldrig förklarats. Våra fynd tyder på att CGRP kan ha en funktionell roll i cerebellum som kan ha betydelse för migränsjukdomen, vilket kan förklara symptom som yrsel samt balansvårigheter.
Sammanfattningsvis visar denna avhandling en detaljerad distribution av CGRP och dess receptor i migrän-relaterade områden, vilket har ökat vår förståelse för CGRP signalering i det trigeminovaskulära systemet samt centrala nervsystemet. Våra resultat pekar på att ett flertal områden där läkemedel som CGRP receptor antagonister kan verka och ha sin effekt. För närvarande har CGRP receptor antagonister visat god effekt i kliniska studier. Vi hoppas att dessa läkemedel ska inom en kort framtid finnas tillgängliga för migränpatienter.
Vad är migrän - patienters synvinkel

Patienter, medlemmar i Svenska Migränförbundet blev tillfrågade vad migrän är för dem och hur det påverkar deras liv. Jag är oerhört tacksam till er alla som svarade och är rörd över era beskrivningar. Nedan följer några av deras beskrivningar:

"Ett migränanfall känns som om alla ens sinnes sparkar bakåt och träffar en med en stor kraft i huvudet. Att leva med migrän är oerhört frustrerande. Den tvingar mig att ligga lågt när jag egentligen vill hoppa, springa, leka och jobba."

"Migrän för mig är att inte kunna leva ett fullgott liv, inte våga eller orka skaffa barn, inte kunna planera sitt liv, studier, arbete, socialt liv, familjeliv och resor. Slutligen är migrän något som stjäl halva (minst) ens liv och ett helvete medan det pågår att tvinga sig att fungera fast att man inte gör det."

"Beskrivning av migrän för den som har det ofta ofta. Ångest, ångest och skräck för att få ett riktigt anfall som inte går att häva med medicin… Ångest, ångest därför att jag måste ta migräntabletter alldeles för ofta! Hälsningar från ett hopplöst fall!"

"Varje dag går jag med en tyngd 5-15kg extra på huvudet som dessutom helst ska balanseras rätt. Tyngden dränerar mig på energi och om man känner efter för mycket så tar tröttheten överhand."

"Migrän; man vill bara skruva av huvudet och ställa det bredvid sig ett tag och vila..."

"Migrän för mig är som att bli överkörd av ett tåg på huvudet. Jag får dödsängest när jag har migrän och vill inte leva längre. Jag befinner mig i ett vakuum och tappar..."
uppfattningen om tid och rum. Jag ligger i sängen och kräks i en hink, jag vet inte om det är dag eller natt och jag orkar inte gå upp för att ta medicin. När jag hör livet pågå utanför och mina barn leker känner jag mig sorgsen, det känns som livet fortsätter men att jag inte längre kan vara delaktig."


"Att ha migrän innebär att du kan ligga till sängs när ditt barn tar studenten eller kanske lämnar du midsommarlunchen med släkten. Du tål inga ljud, mår illa och har en bultande huvudvärk som gör att du inte kan göra annat än blunda och ligga stilla i mörkret…i väntan på att det går över".

"Migrän för mig påverkar hela min livssituation förutom värken tillkommer en yrsel, som gör mig oförmögen till det mest. Man kan inte fungera utan balans…bara att ligga stilla, be anhöriga on hjälp att försöka vingla till en toalett och få i sig mat. Oron, när kommer nästa svåra anfall som gör att jag inte klarar mig själv. Livet har blivit oro.""

Background

Migraine

Migraine is a painful, incapacitating disease affecting more than 10% of the general population. It is a neurovascular disorder characterized by attacks of severe pulsating headache with autonomic and neurological symptoms. The typical migraine headache is unilateral and pulsating, lasting 4-72 hours with symptoms including nausea, vomiting, scintillations, numbness, fatigue, mood changes, neck stiffness, photophobia and phonophobia. The disease is very complex with a wide spectrum of symptoms that can occur before, during, or after the pain. Migraine can be divided into migraine with aura and migraine without aura. About 20% of the patients experience an aura before the migraine attack, which often manifests as the perception of a strange light or an unpleasant smell. The socio-economic costs are extensive. The total cost of headache disorders in Europe was estimated to €43.5 (in billion €PPP 2010) (Gustavsson et al., 2011).

Scientific work in the last decade has unraveled much of the pathophysiology in migraine but the exact primary cause of migraine is still unknown. Earlier it was thought that migraine was initiated in the cranial blood vessels, but neuroimaging and genetic studies have shown that migraine is primarily a neurological disorder. Currently, migraine is considered to be a neurovascular disorder, which originates in the brain, involving genetics, activation of the trigeminovascular system, changes in function of thalamus and/or dysfunction in the brainstem (Borsook et al., 2006, Goadsby, 2012, Charles, 2013a). The attack is often preceded by prodromal symptoms, which suggest the CNS as a starting point (Figure 1). Cortical spreading depression (CSD) which consists of a spreading wave of depolarization associated with a reduction of cortical activity has also been related to migraine (Noseda and Burstein, 2013). Recently it was demonstrated that patients with premonitory symptoms displayed activation of parts of hypothalamus, cerebellum, certain brainstem regions and various cortical areas during the premonitory phase of migraine, supporting that the brain is involved before the headache starts (F.H. Maniyar et al., 2013).
The symptoms may occur one or two days prior to a migraine attack (premonitory), during the aura or headache phase, or after the migraine attack with symptoms that can last for hours to couple of days (postdrome).

The triptans (serotonin 5-HT\textsubscript{1B/1D} receptor agonists) currently represents the antimigraine therapy of choice. Some patients respond poorly or are unresponsive to these drugs. The triptans are strong vasoconstrictors of coronary and cranial vessels, and can be associated with side-effects such as dizziness, paraesthesia, throat tightness and chest pain. Because of the vasoconstrictor effects, the triptans are contraindicated in patients with cardiovascular disease and uncontrolled hypertension (MaassenVanDenBrink et al., 1998). Therefore, there remains an unmet medical need for improved treatments of migraine.

There has been intense research has to identify signal molecules associated with the activation of trigeminal system and thus far the only neuronal messenger so far reliably identified in migraine attacks is the neuropeptide calcitonin gene-related peptide (CGRP) (Goadsby et al., 1988, Durham, 2006).

The role of CGRP in migraine

CGRP is a 37 amino acid neuropeptide and a potent vasodilatory neuropeptide which also has a role in transmission of nociception information (Edvinsson, 1985, Edvinsson et al., 1987, Poyner, 1992, Gulbenkian et al., 2001, Goadsby, 2007). There are two forms of this peptide, αCGRP, which is predominantly expressed in the nervous system and βCGRP, which is primarily expressed in the enteric sensory
system. Early autoradiographic studies have shown CGRP binding sites in rat cerebellum, hippocampus, amygdala, cortex, brain stem and spinal cord (Inagaki et al., 1986, Sexton et al., 1988). In the CNS, CGRP is expressed in several regions such as the striatum, amygdale, hypothalamus, colliculi, brainstem, cerebellum and the trigeminal complex (Skofitsch and Jacobowitz, 1985, Hokfelt et al., 1992, Eftekhar and Edvinsson, 2010).

The potential role of CGRP in migraine pathophysiology was suggested over 20 years ago (Goadsby et al., 1988, Edvinsson and Goadsby, 1990) and several studies have since then revealed the correlation between migraine pain and cranial release of CGRP (Edvinsson and Uddman, 2005). Experimental and clinical studies have shown that there is an increased level of trigeminal system released CGRP in serum, cerebrospinal fluid, and saliva during migraine attacks (Goadsby et al., 1990, Goadsby and Edvinsson, 1993, Bellamy et al., 2006, Cernuda-Morollon et al., 2013). In addition, infusion of CGRP can trigger 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 C1-2 level of the spinal cord, to transmit pain signals to thalamus and higher cortical pain regions (Goadsby, 2007). Several areas within the trigeminovascular system and CNS have been suggested to be involved in nociception and migraine pathophysiology. The anatomy of some these areas are described in this thesis.

The trigeminovascular system

The trigeminovascular system is part of the system that is responsible for regulation of the cranial vasculature and is a key element in the transmission of pain (Figure 2). This system consists of the trigeminal ganglion, containing neurons, that peripherally innervate the intracranial vasculature, dura mater and centrally projects to spinal trigeminal nucleus (STN) in the brainstem and in related extensions down to the C1-2 level of the spinal cord (Liu et al., 2009, Goadsby, 2012). Neural activity in the trigeminal nociceptive system in migraine patients has been demonstrated using imaging techniques (Borsook et al., 2006).
Figure 2. The trigeminal system.
This system consists of CGRP containing neurons in the trigeminal ganglion (TG) which innervate the cerebral blood vessels and other cranial structures such as the dura mater. In the periphery the release of CGRP results in vasodilation via action on the smooth muscle cell layer. Neurons of the TG project centrally to the brainstem where CGRP functions in the transmission of nociceptive information and subsequently from the brainstem to higher cortical regions.

Trigeminal ganglion

The trigeminal ganglion is of particular interest given the role of the trigeminalvascular system in migraine (Goadsby et al., 1988, May and Goadsby, 1999, Limmroth et al., 2001). The trigeminal ganglion consists of bipolar neurons of different cell sizes and two types of glial cells, satellite glial cells and Schwann cells. The satellite glial cells surround neuronal cell bodies (Figure 3.) and Schwann cells associate with nerve fibers and are responsible for myelin production.
Figure 3. Satellite glial cells in human trigeminal ganglion.
The majority of the cells in the trigeminal ganglion are glial cells, where the satellite glial cells surround the neurons (arrow and insert). Neurons of the human tissue may contain lipofuscin (asterisk).

The trigeminal neurons co-express CGRP with substance P and 5-HT$_{1B/D}$ receptors (Hou et al., 2001). Upon activation of trigeminal ganglion, CGRP and substance P has been shown to be released in both humans or cats (Goadsby et al., 1988). However, the expression of substance P is less marked (18%) than CGRP in the trigeminal ganglion where 40% of the neurons express CGRP (Tajti et al., 1999). Additionally, substance P is not released during migraine attacks (Goadsby and Edvinsson, 1993, Williamson et al., 1997). Other neurotransmitters such as glutamate, neurokinin A (NKA), somatostatin, vasoactive intestinal peptide (VIP) and galanin are expressed in the neurons within trigeminal ganglion (Lazarov, 2002, Edvinsson and Uddman, 2005). Trigeminal fibers innervate cerebral blood vessels and other cranial structures such as large cranial vessels and dura mater.

Cranial vasculature

The CNS is devoid of sensory pain receptors and it is the intracranial blood vessels in the dura mater, including the middle meningeal artery and the large arteries of the circle of Willis that are supplied with sensory nerves (Liu et al., 2004, 2008). The intracranial blood vessels are innervated with nerves that derive from cell bodies in ganglia belonging to the sympathetic, parasympathetic and sensory nervous system. The intracranial sensory fibers originate in the trigeminal ganglion and contain CGRP, substance P and NKA (Edvinsson and Uddman, 2005).

CGRP is one of the most potent vasodilators and almost all vasculatures are innervated by CGRP-containing nerve fibers (Edvinsson et al., 1987). Release of CGRP is thought to act on the vascular smooth muscle cell layer inducing
vasodilation. The vasodilation is independent of endothelium in human cerebral and meningeal blood vessels (Jansen-Olesen et al., 1996, Edvinsson et al., 1998). Immunoreactivity for the CGRP receptor components has been demonstrated in the vascular smooth muscle cell layer of cerebral and meningeal arteries (Oliver et al., 2002).

**Dura mater**

The dura mater is a pain sensitive structure, which stores a wide range of signal molecules. Several of these signal molecules have been suggested to play a role in migraine (Edvinsson and Uddman, 1981). Early studies demonstrated that the dura mater contains nerve fibers expressing substance P and CGRP (sensory system), whereas neuropeptide Y (NPY) fibers are sympathetic. Those positive for VIP and pituitary adenylate cyclase activating peptide (PACAP) are of parasympathetic origin. In addition to blood vessels and nerve fibers, the dura mater contains numerous mast cells (Orr and Pace, 1984). However, few occur in the brain or in the cerebral blood vessels (Edvinsson et al., 1977). The mast cells contain, and can release, numerous vasodilatory, proinflammatory and neurosensitizing molecules (Galli, 1993). Moreover, they are described in close association with CGRP containing fibers and in the proximity of the blood vessels.

The dural mast cells are proposed to be involved in migraine pathophysiology (Rozniecki et al., 1999, Levy et al., 2007). Functional studies have shown that increasing concentrations of CGRP can cause histamine release from the rat dura mater via degranulation of mast cells (Ottosson and Edvinsson, 1997, Schwenger et al., 2007). The sensory nerve fibers in dura mater project to the STN and reciprocal regions at C1-C3 level (Edvinsson, 2011). Interestingly, mast cell degranulation evoked activation of the brainstem as demonstrated by c-fos immunoreactivity (Levy et al., 2007).

**Spinal trigeminal nucleus**

It is thought that CGRP acts post-junctionally on second-order neurons to transmit pain signals centrally via the brainstem and midbrain to the thalamus and higher cortical regions. Processes of the neurons in the trigeminal ganglion synapse in the STN, an area in the brainstem (Liu et al., 2009). The STN is divided into three subnuclei (oral, the interpolar and the caudal part) that in turn may be subdivided into different areas/laminae. The caudal part (Sp5C) is associated with thermal and noxious stimuli (Rusu, 2004).

It has been hypothesized that brainstem stimulation can cause activation of the trigeminovascular system, resulting in CGRP-dependent vasodilation (Just et al.,
Early immunohistochemical studies have revealed the presence of strongly positive CGRP fibers in STN and spinal cord in different species (Gibson et al., 1984, Unger and Lange, 1991, Smith et al., 2002, Uddman et al., 2002). In human STN, a greater proportion of fibers express CGRP compared to substance P or 5-HT₁D receptor expression (Smith et al., 2002). Some of the 5-HT₁D positive fibers co-express CGRP or substance P, suggesting that 5-HT₁D receptor can regulate the release of these neuropeptides (Smith et al., 2002).

The central nervous system

Brainstem and spinal cord

It is supposed that migraine is associated with disturbances in brain and brainstem function (Hargreaves and Shepheard, 1999, Goadsby et al., 2009, Charles, 2012, 2013a). Clinical studies with imaging techniques, such as positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), have demonstrated that certain brainstem areas are activated during migraine attacks (Weiller et al., 1995, Diener, 1997, Bahra et al., 2001, Stankewitz et al., 2011). These areas include the midbrain, pons, substantia nigra, red nucleus, the periaqueductal gray (PAG), nucleus raphe magnus (NRM) and the locus coeruleus (LC) (Lakhan et al., 2013). Investigation of the premonitory phase of migraine revealed that right ventral midbrain, right PAG and right dorsal pons are activated (F.H. Maniyar et al., 2013). However it is not known what causes or drives the activation of the brainstem.

Often patients with migraine experience neck pain, suggesting that the spinal cord and its spinal nerves, the cervical nerves, may have a role in migraine. The trigeminal ganglion and some of the intracranial vessels project to the lamina III/IV of the spinal cord, shown by tracing studies (Edvinsson, 2011). Supporting these findings, stimulation of the first cervical nerve C₁ evoked periorbital (eye pain) and frontal pain in migraine patients, suggesting that the C₁ nerve may serve as an important therapeutic target for headache disorders (Johnston et al., 2013). It remains to be evaluated conclusively how the spinal cord and its nerves are involved in the migraine pain.

The cerebellum

The cerebellum is crucial in modulating many cortical motor and sensory inputs. The cerebellum exerts an inhibitory control in the cerebral cortex, and, thus may play an important role in the filtering of sensory inputs (Strata et al., 2009). A reduction in
cerebellar inhibition in migraineurs with aura has been demonstrated supporting these findings (Brighina et al., 2009).

Interestingly, activation of cerebellar regions has been demonstrated by PET on patients during migraine attacks (Weiller et al., 1995, Bahra et al., 2001). The cerebellum is of interest in migraine research since upcoming studies suggest a role of cerebellum in the migraine pathology (reviewed by Vincent and Hadjikhani, 2007). Vincent and Hadjikhani describe how spreading depression, cerebellar dysfunction and familial hemiplegic migraine (FHM) are connected to cerebellum and migraine (Vincent and Hadjikhani, 2007).

Spreading depression has been related to migraine. This has been recorded in various tissues including the cerebellum (Vincent and Hadjikhani, 2007). FHM is a rare autosomal dominant type of migraine. FHM1 is one subtype that is related to mutations of the CACNA-1A gene, coding for a subunit of the high voltage-gated P/Q calcium channels (Pietrobon, 2007). This channel plays a pivotal role in neurotransmitter release and the influence of neuronal excitability. P/Q calcium channels are expressed throughout the CNS, particularly in the Purkinje cells of cerebellum (Vincent and Hadjikhani, 2007).

CGRP receptor antagonists

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, Ho et al., 2008b). The CGRP receptor antagonists are a new class of antimigraine drug, which act by blocking the action of CGRP on the CGRP-receptor complex (Figure 4). The receptor for CGRP has been identified as a G-protein-coupled receptor of the family B-subtype (Hay et al., 2008). The functional receptor consists of a complex of a seven-transmembrane spanning protein, calcitonin receptor-like receptor (CLR), a single transmembrane-spanning protein designated receptor activity-modifying protein 1 (RAMP1) (McLatchie et al., 1998). RAMP1 is involved in receptor trafficking and is required for CGRP binding to the receptor complex, whereas the interaction of CLR with other RAMP proteins, RAMP2 or RAMP3, forms adrenomedullin receptors (McLatchie et al., 1998, Foord and Marshall, 1999, Zhang et al., 2007). RAMPs can also interact with the calcitonin receptor (CTR) to form amylin receptors. An intracellular protein, receptor component protein (RCP) has been suggested to be a part of the CGRP receptor complex (Evans et al., 2000) as the depletion of RCP from cells inhibits CLR signaling. However, knockdown of RCP did not affect ligand binding to the CGRP receptor, thus RCP seems to primarily be involved in the signal transduction via CLR (Walker et al., 2010).
Olcegepant (BIBN4096BS) was the first developed CGRP receptor antagonist that demonstrated clinical efficacy in the acute treatment of migraine (Doods et al., 2007, Recober and Russo, 2007). Unfortunately due to its low oral bioavailability olcegepant required intravenous administration, which is not practical for the acute treatment of migraine, and therefore the development of this compound was terminated. Telcagepant (MK-0974) was then the first orally active CGRP-receptor antagonist that was effective in the acute treatment of migraine (Ho et al., 2008a, Ho et al., 2008b, Edvinsson, 2009). In clinical trials for the acute treatment of migraine, telcagepant showed clinical efficacy similar to the triptans, but with a better tolerability (Ho et al., 2010). An important differentiator from the triptans was that telcagepant did not constrict human isolated cranial (Edvinsson et al., 2010) or coronary blood vessels (Chan et al., 2010). Telcagepant completed multiple phase III clinical trials, but due to elevations in liver transaminase levels the development of this compound was discontinued.
Other CGRP receptor antagonists that have demonstrated anti-migraine efficacy in clinical studies are MK-3207, BI 44370 TA and BMS927711. At present, antibodies against the CGRP receptor or CGRP itself have been developed and are currently being tested in clinical studies (Dolgin, 2013). The antibody approach has the potential benefit of more specifically blocking CGRP signaling. Additionally, antibodies typically have a much longer half-life in comparison to small molecules which make them ideally suited for chronic or preventative treatments. Compared to the small-molecules CGRP receptor antagonists, it is less likely that the antibodies can cross the blood-brain barrier (BBB) and therefore will not antagonize central CGRP receptors. The inability to cross the BBB could be a pro or a con for this approach as it is currently unknown if central CGRP receptor blockade may provide additional anti-migraine efficacy. A potential safety concern with the antibody approach is the impact of chronically blocking the receptor or systemically depleting CGRP, since the peptide is expressed in many areas outside the CNS and has many functions throughout the body (Toda et al., 2008, Cottrell et al., 2012).

To date no small-molecule or antibody blocking the action of CGRP have reached the market. However, the concept of blocking CGRP signaling for migraine treatment seems to be very promising. The CGRP receptor antagonists have indeed opened a possible new option in migraine treatment (Edvinsson, 2008). Consequently, many scientific questions have arisen, which need to be addressed. It is of great importance to clarify where the CGRP receptor is expressed and on which possible sites drugs blocking CGRP signaling may have their therapeutic effect. Therefore, the overall aim of the present thesis was to study the distribution of CGRP and its receptor within migraine-related areas.
Aims

The general aim of this thesis was to localize the expression of CGRP and its receptor within the trigeminovascular system and parts of the CNS to widen the knowledge about CGRP signaling and potential sites of action for CGRP receptor antagonists. The study-specific aims were:

1. To study the distribution of CGRP and its receptor components, CLR and RAMP1, within the human trigeminal ganglion and estimate to what extent they are expressed.

2. To characterize the effect of telcagepant and the localization of CLR and RAMP1 in human cranial arteries to investigate the possible therapeutic effect mechanism of action.

3. To investigate in detail the expression of CGRP and its receptor in whole-mount rat dura mater, human dural vessels, and their relationship to mast cells, myelin, substance P, neuronal nitric oxide synthase (nNOS), PACAP and VIP.

4. To identify the expression of CGRP and its receptor in regions within the brainstem that process nociceptive information from the trigeminovascular system, such as the STN and the spinal cord, in both human and rat.

5. To map CGRP receptor mRNA, protein expression and CGRP receptor binding throughout the brainstem of rhesus monkey.

6. To localize the expression CGRP, CLR and RAMP1 in the rat cerebellum and the cellular localization.

7. To investigate mRNA expression, protein expression of CLR and RAMP1 and the binding sites of a CGRP receptor antagonist in the primate cerebellum.

8. To assess CGRP receptor expression and receptor binding sites in rhesus trigeminal ganglion and determine if the trigeminal ganglion is protected by the BBB.
Methods

Ethics

All procedures and animal treatments were in accordance with the guidelines of the Ethics Committee of Lund University or the Animal Ethics Committee of Copenhagen University, Denmark (Paper VIII). Animal studies were approved by Lund County Administrative Court under the auspices of the Swedish Department of Agriculture. Human studies were approved by the Regional Ethical Review Board in Lund, Sweden and/or by the local Hungarian Ethics Committee. The human studies conform to the principles outlined in the Declaration of Helsinki. The rhesus monkey tissues were harvested in accordance with Merck Research Laboratories Institutional Animal Care and Use Committee approved protocol. Import of the rhesus monkey tissues were approved by the Swedish Department of Agriculture and given CITES import permits. CITES permit for export from USA was approved by Fish and Wildlife service, division of management authority.

Immunohistochemistry

Sample preparation

An essential part for all histological techniques is the preservation of cells and tissue in a reproducible and life-like manner. Therefore, the tissues must quickly be immersed in a fixative to prevent the breakdown of cellular proteins and tissue architecture. The most common fixative is formaldehyde (PFA), where fixation time, concentration, pH and temperature strongly affect immunostaining (Naish et al., 1989). In paper I, II and IV-VII, immersion fixation with 4% PFA was used. Fixation time was dependent on the tissue size. In paper III and VIII, transcardial perfusion was performed to remove all blood components from the entire animal. The tissues used in all papers, except paper V and VII, were cryoprotected and embedded in a gelatin medium. Tissues in paper V and VII-VIII were paraffin-embedded. In these papers, a standard protocol (xylene, decreasing concentrations of alcohol, and distilled water) was used to remove the paraffin from the sections prior to immunostaining to enable
the antibodies to have access to the target antigens. Often paraffin-embedded sections require treatment to unmask the antibody epitopes. Heat-induced antigen-retrieval with citric acid pH 6.0 was used to unmask epitopes (Paper V and VII).

Staining methods

Hematoxylin-eosin staining was used to study the morphology of the tissues in all papers. This allowed the quality of the fixation and the tissue morphology to be evaluated.

Immunohistochemistry is a method that makes it possible to visualize the distribution and the localization of specific cellular components within the cells and tissues. The method is based on targeting antigens (proteins) in a tissue section with specific antibodies tagged with a visible label. Visualizing the antibody reaction can be performed in a number of ways. In the most commonly used procedures the antibody is conjugated to an enzyme that can catalyze a color reaction or the antibody is tagged with a fluorophore such as fluorescein (FITC) or rhodamine. There are also different detection methods, such as the direct method or the indirect method. The direct method is a one-step method where a labeled primary antibody is directly applied to the target antigen. The indirect method involves a two-step procedure, where an unlabeled primary antibody is applied to the target antigen. Thereafter, a labeled secondary antibody is applied directed against the primary antibody (Naish et al., 1989). In the papers included in this thesis, the indirect method was used with secondary fluorescent dye conjugated antibodies.

Undesired reactions may occur if the secondary antibody cross-reacts with sites on nonspecific proteins that are similar to the binding sites of the target antigen, causing background staining. This can, however, be reduced by applying a buffer containing serum that blocks the reactive sites. Therefore, a blocking step was performed before applying the primary antibody. Blocking buffers included normal serum and bovine serum albumin (BSA). More details regarding the immunofluorescence method are described in the papers.

Antibody characterization and controls

Characterization of the antibodies and the use of controls are necessary for the validation of the immunofluorescence staining results. Without validation the results would be of little value. Omission of the primary antibody served as negative controls. The antibodies against CLR and RAMP1 were developed and evaluated by Merck & Co. USA. The specificity of the antibodies raised against CLR and RAMP1 were evaluated by Western blotting and immunostaining in cell lines with HEK293 cells.
expressing the human CGRP receptor (performed by the Merck co-authors in Paper I).

Further, preabsorption of the primary antibodies (CLR and RAMP1) with the peptides used for immunization (blocking peptides) was used. This indicates that these antibodies are specific for the epitope they are raised against. For CGRP antibody, controls were conducted by preabsorption with the peptide itself, resulting in abolished immunoreactivity.

**Primate tissue**

In the present work, mainly primate tissue has been used. The human material was either obtained as post-mortem (Paper I, IV, VII) or tissue segments were collected from patients undergoing neurosurgical procedures (Paper II-III). Tissues from rhesus monkeys were used in Paper V, VII and VIII. Research on primate tissue is very valuable and is empirically more closely related to the human pathophysiology. However, the histological analysis of primate material is more challenging than that of rodents, in which the tissue collection and processing can be more standardized. Concerning human post-mortem tissue, the investigators have less control over collection and storage. The time between death and tissue fixation may profoundly influence the quality of the tissue. Often longer fixation time is required for large specimens, which can affect the staining properties of the antibodies. In the present papers efforts were made to processes the tissues in a way to achieve good morphology and to be usable for the different experiments.

Working with primate tissue also requires additional controls and analysis to be assured that the given staining is specific. Therefore, rat tissue from corresponding areas was used to compare with the findings on primate materials. Also, after sacrifice rat tissues were kept at 4°C for 24h before dissection to mimic the autopsy situation in humans. In this way it was possible to compare the results and to be more certain that the staining patterns in the human tissues were not due to biological changes during the tissue collection (Paper I and IV).

In addition, when it was possible, several different primary antibodies against the same antigen were evaluated to make sure that similar staining patterns were achieved. Secondary antibodies can also display more unspecific binding in primate tissue. Therefore several secondary antibodies in each study were evaluated. Lipofuscin is autofluorescent and often found in primate tissue, which should be taken into consideration during the analysis.
**Microscopic analysis**

Sections were examined and images were obtained using an epifluorescence microscope (Nikon 80i, Tokyo, Japan) coupled to a Nikon DS-2MV camera. Confocal imaging was performed with Nikon confocal microscope (EZ-cl, Germany). Image analyses were conducted using NIS basic research software (Nikon, Japan). Adobe Photoshop CS3 (v.8.0, Adobe Systems, Mountain View, CA) was used to visualize co-labeling by superimposing the digital images and processed for brightness and contrast.

**In vitro pharmacology**

The functional responses of the effects to αCGRP and the antagonistic effects of telcagepant in human cerebral and meningeal arteries were studied by myograph experiments (Paper II). This technique is based on mounting cylindrical arterial segments in a wire myograph to study contractile responses (Mulvany and Halpern, 1977). The lumens of segments are threaded on two parallel wires, one being connected to a force transducer attached to a digital converter-unit. The other wire is connected to an adjustable micrometer screw setting the distance between the wire and hence to the vascular tone. To study the vascular relaxations the arteries are pre-contracted with a vasoconstrictor prior to cumulative application of αCGRP. Isometric responses to chemical antagonist/agonists, pharmaceutical drugs, etc. can then be investigated. The details for these experiments are described in Paper II.

**In situ** hybridization

This is a method of localizing and detecting specific mRNA sequences in preserved tissue sections by hybridizing the complementary strand of a nucleotide probe to the sequence of interest. This involves a hybridization reaction between a labeled nucleotide probe and complimentary target sequences, which makes it possible to visualize gene sequences in preserved tissue and allows anatomically identification. A number of different types of probes are used for **in situ** hybridization, including cDNA, cRNA and synthetic oligonucleotide probes (Jin and Lloyd, 1997).

In the present work the aim was to detect CLR and RAMP1 mRNA. CLR and RAMP1 cRNA probes were made using cDNA sequences as templates. The cDNA inserts were subcloned into a transcription vector, pBluescript SK+, as 5’EcoRI-3’XbaI fragments. The CLR probe corresponded to bases 1018-1453 of human CLR
and was designed to equally distribute the human and rhesus non-conserved bps. The RAMP1 probe (447 bp) corresponded to the full length rhesus RAMP1 coding sequence. Probes were linearized and labeled for detection. Sense probes were used as controls. In brief, the tissue sections were processed, including pretreatment steps before the hybridization to increase to efficacy and reduce nonspecific background. The hybridizations were performed overnight followed by post-hybridization washes and exposed to films/emulsion. More details regarding the in situ hybridization method is described in paper V and VII.

**Autoradiography**

To further investigate the distribution of CLR and RAMP1 as they correspond to a functional CGRP receptor, in vitro autoradiography was used (Paper V, VII-VIII). With this method radiolabeled ligands, [125I]CGRP or [3H]MK-3207 (a CGRP receptor antagonist), were used to determine the tissue distributions of functional CGRP receptors. The synthesis of [3H]MK-3207 was performed by Merck Sharp and Dohme Research laboratories, USA. Tissue sections were incubated with the radiolabeled ligand, followed by cold washes and exposed to imaging plates for three weeks. Plates were scanned with a BAS 5000 scanner (Fuji, Tokyo, Japan). As control, 1µM self-block with unlabeled ligand was used.

**Evans blue**

Evans blue is often used as a marker to study permeability of the BBB. It is a dye that can be administrated as intravital dye and it binds to serum albumin, which cannot cross the BBB. Virtually all Evans Blue is bound to albumin so typically the neural tissue remains unstained. When Evans blue is linked to proteins it becomes fluorescent and sections can be analyzed with a fluorescence microscope. This method was used in paper VIII to determine if the trigeminal ganglion is protected by the BBB. Two percent Evans blue dye was injected into the circulation of rats, after 1h the rats were perfused transcardially.
Results and comments

The trigeminal ganglion (Paper I and VIII):

The trigeminal ganglion is in the center of interest for studying the expression of CGRP and its receptor components due to the activation of the trigeminovascular system during migraine attacks. Initial studies performed on the rat trigeminal system showed immunoreactivity of CGRP (Uddman et al., 1985, Tajti et al., 1999) and its receptor components in trigeminal ganglion cells (Lennerz et al., 2008). However, the localization of CGRP signaling mechanisms in the human trigeminal ganglion remained unclear. Therefore paper I was designed to evaluate the localization of CGRP and its receptor components CLR and RAMP1, and their association in the human trigeminal ganglion using immunohistochemistry and compared with that of rat.

We observed immunoreactivity for CGRP, CLR and RAMP1, in the human trigeminal ganglion (Figure 5): 49% of the neurons expressed CGRP, 37% CLR and 36% RAMP1. The CGRP immunoreactivity was found in small to medium sized neurons while CLR and RAMP1 were seen to co-localize in large sized neurons. Co-localization of CGRP and the receptor components was rarely found, suggesting low presence of putative auto-receptors in the trigeminal ganglion, which is in agreement with a study performed on rat trigeminal ganglion (Lennerz et al., 2008).

The CGRP receptor was localized to both neurons and satellite glial cells. Expression of CLR or RAMP1 in satellite glial cells has been demonstrated before in rat (Lennerz et al., 2008, Li et al., 2008), and we were able to confirm their presence in human satellite glial cells (Figure 6). In primary cultures of rat trigeminal ganglia, it has been shown that CGRP treatment increased glial iNOS expression and release of NO, which was inhibited by the use of the antagonist CGRP(8-37) (Li et al., 2008), suggesting that CGRP can activate satellite glial cells. It has also been suggested that the communication between neuron-glia in the trigeminal ganglion may occur via gap junctions (Thalakoti et al., 2007). Moreover, it has been shown that, in vitro, CGRP may trigger inflammatory gene expression in rat glial cells (Vause and Durham, 2010). Satellite glial cells also express different kinds of ion channels and the glutamate-aspartate transporter (GLAST), a glutamate recycling component (Ohara et al., 2009). This type of glial cells is also believed to play an important role in many
pain conditions such as chronic pain after nerve injury or inflammation (Watkins and Maier, 2002). Following nerve injury, it has been demonstrated that GFAP expression is increased in the satellite glial cell, suggesting activation of these cells following injury (Ohara et al., 2009). Altogether, these results suggest that the satellite glial cells do not only function as neuronal support, but may also be implicated in pain. Our results indicate that satellite glial cells may be involved in the CGRP signaling within the trigeminal ganglion and be a target for CGRP receptor antagonists which may inhibit the release of local inflammatory molecules from these cells.

Figure 5. Distribution of CGRP, CLR and RAMP1 in the trigeminal ganglion.
Expression of CGRP and its receptor components, CLR and RAMP1, in human and rat trigeminal ganglion (arrows). CGRP is mostly expressed in small to medium sized cells and in thin fibers (asterisk).

Figure 6. Expression of CLR and RAMP1 in satellite glial cells in human trigeminal ganglion.
The satellite glial cells surrounding the neurons (shown with the nuclear staining, DAPI, in blue) express CLR and RAMP1 in their cytoplasm (arrows).

Fibers of different sizes were positive for CGRP and the receptor components. The receptor components were expressed in thicker fibers and co-localized with NF 160/200 marker, suggesting that the CGRP receptor is expressed in A-fibers. CGRP was expressed in thinner fibers and did not co-localize with a marker for smaller neurofilaments, suggesting that CGRP is expressed in C-fibers. One might speculate that these fibers projects further to the brainstem. In nerve fibers, CLR and RAMP1 were expressed in the neuronal processes but not in the surrounding Schwann cells, both in rat and human trigeminal ganglion. In primary culture of trigeminal ganglion, RAMP1 expression was found in satellite glial cells but not the Schwann
cells (Li et al., 2008). These findings are in contrast to the study by Lennerz and coworkers, in which expression of CLR and RAMP1 was demonstrated in the Schwann cells (Lennerz et al., 2008). The dissimilarity may be due to the use of different primary antibodies or other methodological differences and therefore further efforts are needed to evaluate the role of glial cells in CGRP signaling.

In summary, results from paper I suggests that if CGRP is released within the ganglion, then intraganglionic CGRP may act on satellite glial cells and on large sized neurons (Figure 7). This is possible as we have identified CGRP positive fibers in the trigeminal ganglion in relation to neurons and satellite glial cells. Upon stimulation of the fibers and neurons expressing CGRP, the peptide may be released and cause activation of other neurons and satellite glial cells, expressing the CGRP receptor. It could be hypothesized that CGRP receptor antagonists may act on its receptor expressed on larger sized neurons and satellite glial cells, to inhibit the CGRP signaling within the trigeminal ganglion.

Figure 7. CGRP signaling in the trigeminal ganglion.
A summary of the findings in the trigeminal ganglion; CGRP is expressed in small to medium sized neurons (red) which upon activation may be released to act on larger sized neurons and satellite glial cells expressing the CGRP receptor (green).
To further investigate the localization of CGRP and its receptor in the trigeminal ganglion, we aimed to study CGRP receptor binding sites by autoradiography with the use of a radiolabeled CGRP receptor antagonist, [3H]MK-3207, and expression of CGRP and its receptor in rhesus monkey trigeminal ganglion (Paper VIII). In addition, Evans blue experiments were performed in rodents to evaluate if the trigeminal ganglion is protected by the BBB.

This is the first study to examine CGRP binding sites, expression of CGRP and its receptor in rhesus monkey trigeminal ganglion (Paper VIII). In rhesus monkey, high binding densities of [3H]MK-3207 were observed in the trigeminal ganglion, the highest binding was mainly found in the ganglion where the neuronal somata are located (Figure 8). Immunofluorescence revealed expression of CGRP, CLR and RAMP1 in the trigeminal cells. CGRP was expressed in small/medium sized cells whereas the receptor components were mainly co-expressed in larger cells and satellite glial cells (Figure 9). Thus, this correlated well with Paper I on rat and human trigeminal ganglion with no differences in the expression pattern of CGRP and its receptor components. In addition, RAMP1 and CLR were found in the smooth muscle cells of the vessels within the trigeminal ganglion.

Figure 8. Binding densities of a CGRP receptor antagonist in rhesus monkey trigeminal ganglion.
High binding densities of the CGRP receptor antagonist, MK-3207, is displayed within the ganglion where the neurons are located (total binding, red signal). Htx-Eosin staining of the same slice. As control, self-block on the adjacent slide shows no binding (non-displaceable binding).
CLR and RAMP1 are co-expressed in the larger sized neurons (upper panel, arrows) and satellite glial cells (arrow heads). Their co-expression is also found in trigeminal vascular wall (lower panel, arrows).

In early literature, Evans blue uptake in the trigeminal nerve of rabbits and mice has been demonstrated (Arvidsson et al., 1973). In our study, Evans blue dye uptake was clearly found in the rodent trigeminal ganglion. High signal was observed in the ganglion, close to the cell bodies and in the walls of blood vessels within the ganglion, and found close the neurons (Figure 10). Within the nerve bundles, we saw no or low uptake of Evans blue, while some signal was observed around/on the edges of the nerve part. This may be explained by the study on the trigeminal nerve, where Evans blue was found in the epineurium part of the nerve (connective tissue in the outermost layer surrounding a peripheral nerve), while the endoneurium part (the innermost layer of connective tissue in a peripheral nerve) did not display any dye uptake (Arvidsson et al., 1973).
Figure 10. Evans blue in trigeminal ganglion.
A) Evans blue experiments in rodents, displaying no uptake of the dye in the brain while the pituitary gland that is lacking BBB protection is blue from the dye uptake. The trigeminal ganglion becomes blue indicating uptake of Evans blue. B) fluorescence microscopic images showing the trigeminal ganglion of rhesus monkey with red fluorescent signal from the 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 part. C) vessels within ganglion close to the neurons also show signal of Evans blue (arrows). DAPI, staining nuclei, is used in the merged pictures (blue).

These results suggest and support the presence of functional CGRP receptors in this area. Importantly, the experiments with Evans blue in rodents revealed that trigeminal ganglion is located outside BBB and can therefore be reached by the CGRP receptor antagonists to block receptors in the trigeminal ganglion, even if they lack the potential for CNS-penetration (Figure 11).
Since its discovery 3 decades ago it has been shown for almost all parts of the circulation that CGRP is one of the most potent vasodilators on the arterial side (Brain et al., 1985, Poyner, 1992). The cranial vasculatures, both intracranial and extracranial, are innervated by CGRP-containing nerve fibers that originate in the trigeminal ganglion (Edvinsson et al., 1987). Early detailed studies showed that CGRP relaxed arteries via activation of adenyl cyclase and in a non-endothelial dependent manner (Edvinsson et al., 1985). With Boehringer Ingelheim, a series of CGRP specific blockers were studied. Of these olcegepant was found to have antimigraine efficacy (Olesen et al., 2004), but was unsuited for further clinical development. Telcagepant was the first orally available CGRP receptor antagonist to demonstrate clinical efficacy (Ho et al., 2008a, Ho et al., 2008b). However, the site of action of the blockers has remained enigmatic and therefore paper II was set out to compare the functional responses to αCGRP and the antagonistic effects of
telcagepant in human cerebral and meningeal arteries. Furthermore, we examined the cellular localization of CLR and RAMP1 with immunofluorescence in these arteries.

We found that telcagepant had no direct vasoconstrictor or vasodilatation effect per se on human cerebral and meningeal arteries. Telcagepant was able to block, in a competitive manner the vasodilator effect of αCGRP in these intracranial arteries. Co-expression of CLR and RAMP1 was observed in the smooth muscle cell layer, suggesting that CGRP acts via the receptors located on smooth muscle cells (Figure 12).

In a study by Chan and coworkers, it was demonstrated that there is absence of vasoconstriction with telcagepant in human coronary arteries, suggesting a potential cardiovascular safety advantage of the CGRP receptor antagonists in contrast to triptans that produce contraction of coronary arteries (Chan et al., 2010).

The dura mater is considered to play a central role in acute migraine attack pain. Furthermore, neurogenic inflammation is thought to be involved in migraine pathophysiology (Levy, 2009). The hypothesis is that activation and sensitization of primary afferent nociceptive neurons that innervate the cranial meninges and related blood vessels is an early step in promoting pain (Levy, 2009). It is thought that mast cells and inflammatory cells have a key role in this neuronal response. Upon activation they release mediators contributing to local inflammation and migraine genesis (Levy, 2009). Mast cells of the dura mater are of special interest since they are in close proximity to meningeal vessels and pain fibers. It has also been demonstrated that CSD is linked to activation of mast cells and macrophages, resulting in release of inflammatory mediators (Levy, 2012, Karatas et al., 2013). A recent study with in vitro autoradiography, showed binding of a CGRP PET tracer ([³H]MK-4232) in the

![Figure 12. Effect of telcagepant on human cerebral arteries and localization of the CGRP receptor.](image)
meninges of human and rhesus monkey brain (Hostetler et al., 2013). However, the resolution was not sufficient to identify exact localization of the tracer distribution.

We conducted a detailed study of expression of CGRP and its receptor component in the dura mater (Paper III). We evaluated the expression of CGRP, CLR and RAMP1 relative to each other and other neuronal messengers. Moreover, we examined whether dural mast cells express the CGRP receptor in rat and human.

Paper III showed that CGRP is expressed in thin and un-myelinated fibers, suggesting C-fibers (Figure 13). On the contrary, CLR and RAMP1 were expressed in thicker and myelinated fibers, suggesting A-fibers. Lennerz and coworkers found CLR and RAMP1 in dura mater fibers consistent with the shape of Schwann cells (Lennerz et al., 2008). We studied this in detail with confocal analysis with use of a myelin marker (MBP). We found that CLR and RAMP1 were expressed within the axon and not in the surrounding MBP positive sheath (Figure 14). These results are in agreement with the findings in trigeminal ganglion (Paper I), where distinct CGRP and CLR/RAMP1 positive fibers project from the trigeminal ganglion to the dura mater (Figure 15).

Figure 13. CGRP and MBP (myelin) staining in the dura mater.
The thin CGRP fibers (red) are distinct from the myelinated fibers (green) of the dura mater.

Figure 14. CLR and RAMP1 expression within myelinated fibers of the dura mater.
The receptor components, CLR and RAMP1, (red arrows) are expressed within myelin ensheathed axons (green arrows) and not within myelin itself.
Figure 15. CGRP and its receptor are expressed in separate fibers in the dura mater. Confocal analysis of double-staining with CLR or RAMP1 and CGRP. CLR is expressed in cells, within the vessel wall and thicker fibers (green, arrows). CGRP expression (red, arrows) is instead found in thin fibers running across vessels or close to CLR positive cells and fibers. High magnifications of RAMP1 (green, arrows) and CGRP (red, arrows) show positive fibers, that could run close to each other but not expressed in the same fiber.

Additionally, CLR and RAMP1 expression was found in the smooth muscle cell layer of dural vessels and cells within the dura mater. It was confirmed that the receptor components were expressed in mast cells, suggesting expression of CGRP receptors in these cells (Figure 16). However, expression of both CLR and RAMP1 was not found in mast cells of human dura mater. In support of this, it has been shown that CGRP did not induce the release of histamine from human dural mast cells following administration of excess CGRP in contrast to rat dural mast cells (Ottosson and Edvinsson, 1997). Therefore, it is not likely that CGRP acts on human mast cells to cause mast cell degranulation and neurogenic inflammation triggered by CGRP.
Further research is necessary to evaluate if and how neurogenic inflammation is involved in migraine pathology and if this can be confirmed in primates.

![Image of CLR, MAST CELLS, and MERGED views]

**Figure 16. Expression the CGRP receptor components in rat dural mast cells.**
In rat dural mast cells CLR is co-expressed with mast cell tryptase. Similar findings are found between RAMP1 and the mast cell tryptase marker, suggesting expression of CGRP receptor in mast cells of rat dura mater.

Early studies have demonstrated that the parasympathetic innervation of the dura mater of the dura mater were less frequent compared to cholinergic fibers (Edvinsson and Uddman, 1981). Further, it was shown that there is a lower concentration of CGRP and substance P immunoreactivity in human middle meningeal arteries as compared to human cerebral arteries (Jansen et al., 1992). Therefore, we aimed to compare the different innervation patterns of the dura and cerebral vasculatures. Paper III demonstrated that the expression of CGRP seems to be more prominent than that of substance P, nNOS, VIP and PACAP in the rat dura mater. The innervation of nNOS, VIP and PACAP was richer in the cerebral vessels compared to that observed in the dura mater. This might suggest that CGRP have a more prominent role in the dura mater to influence on the vasomotor effects of dural vessels, compared the other messengers.

In summary this study suggest that activation of C-fibers may locally cause release of CGRP, which in turn could act on A-fibers, vascular smooth muscle cells and rodent mast cells which express the CGRP receptor in the dura mater. These sites represent potential pathophysiological targets of novel anti-migraine agents such as the CGRP receptor antagonists.
The STN, brainstem and spinal cord (Paper IV and V)

The trigeminal ganglion, storing CGRP and its receptor components, project further centrally to different regions in the brainstem and to the spinal cord, revealed by tracing studies (Edvinsson, 2011). This constitutes an essential part of the pain pathways activated in migraine attacks. Therefore, it is of importance to identify the regions within the brainstem that processes nociceptive information involving the trigeminovascular system, such as the STN and C1-level of the spinal cord (Paper IV).

In paper IV, we studied the expression of CGRP, CLR and RAMP1 in human and rat STN and the spinal cord at C1-level with immunohistochemistry. In STN, CGRP immunoreactive fibers were found in a network around fiber bundles in the superficial laminae, where CLR and RAMP1 positive fibers were instead found in the spinal trigeminal tract region. Co-expression of CLR and RAMP1 was found in these fibers, suggesting expression of functional CGRP receptor in this area (Figure 17). CGRP fibers did not co-localize with the MBP marker, suggesting that the CGRP fibers belong to un-myelinated fibers. These results are in agreement with paper I and paper III, suggesting that CGRP is expressed in C-fibers and its receptor in myelinated A-fibers.

![Figure 17. Co-expression the CGRP receptor components in STN.](image)

CLR and RAMP1 are co-expressed in the fibers of rat and human STN in the brainstem.
Interestingly, we found no co-expression of CGRP fibers and its receptor, using confocal microscopy allowing a 3-dimension view of the staining. Co-expression of CGRP and its receptor was neither found in human nor in rat STN (Figure 18). In a previous study on rat STN, CGRP was found in glomeruli-like structures that partly co-localized with the CLR (71%) or RAMP1 (37%) in the superficial laminae (Lennerz et al., 2008). The reason for this discrepancy could reside in that different antibodies were used, or other methodological differences.

![Figure 18. Expression of CGRP and its receptor in the STN.](image)

In rat or human STN, no co-expression is found between CGRP (green) and its receptor (red).

At human and rat C1-level of the spinal cord, CLR and RAMP1 expression was found within laminae I and II. CGRP expression was found in the same laminae as the receptor components, however, the CGRP positive fibers were distinct from CLR/RAMP1 positive fibers. This is in agreement with a previous study on rat spinal cord, where CLR was not co-localized with CGRP or substance P containing fibers (Cottrell et al., 2005).

The findings from paper IV suggest that CGRP released from C-fibers in the brainstem may act postjunctionally to modulate the activity in fibers that contain the CGRP receptor in these regions. Tentatively, the two types of fibers may modulate the activity within STN and/or other brainstem levels or CGRP release in the STN may act retrograde on neurons originating from the trigeminal ganglion.

Neuronal activation in the brainstem areas, hypothalamus and the thalamus has been demonstrated in experimental animal studies during trigeminovascular nociception (Akerman et al., 2011). Also, PET studies of patients suffering from spontaneous migraine attacks showed activation in the medulla, midbrain and hypothalamus (Weiller et al., 1995, Afridi et al., 2005, Denuelle et al., 2007). These studies indicate that several brainstem areas may be involved in migraine pathophysiology with involvement of pathways to hypothalamus and thalamus, which are important areas for pain processing. Therefore, we designed a study to map expression of CLR and...
RAMP1 throughout the whole brainstem (Paper V). The mRNA expression (by in situ autoradiography), protein expression (by immunofluorescence) and receptor binding sites (by autoradiography with [³H]MK-3207) were applied to evaluate their detailed expression throughout the rhesus monkey brainstem.

Several brainstem areas expressing CLR and RAMP1 were detected. In general, RAMP1 mRNA seemed to be more prominently expressed compared to CLR and also detected in additional areas within the brainstem. However, immunohistochemistry revealed CLR/RAMP1 co-expression. This was in accordance with the binding sites of [³H]MK-3207. In several areas involving sensory centers, cranial motor nuclei and pathways to the thalamus, expression of the receptor components with binding of the CGRP receptor antagonist were found. Expression and binding densities were detected in the medial mammillary nucleus, which is involved in signaling from the hippocampus and amygdale via the mamillo-thalamic tract to the thalamus. Fibers expressing CGRP have been demonstrated in the medial mammillary nucleus (Covenas et al., 2012). Our results may suggest expression of functional CGRP receptor within this area. The hypothalamus is connected to the pituitary gland via a small tube termed the infundibular stem, where expression of the receptor components and receptor binding were found (Figure 19). The role of CGRP in this area needs to be further evaluated.

A very interesting area that was found to express CLR and RAMP1 with high binding densities of [³H]MK-3207 was the melatonin-producing pineal gland (Figure 19). Melatonin has been suggested to be involved in the migraine pathophysiology documented by reduced levels of melatonin in migraine patients (Peres, 2005). The pineal gland is a region outside the BBB. Our results, with the expression of the CGRP receptor, suggest that CGRP receptor antagonists may act in this region independently of their ability to cross the BBB. Another area outside the BBB that expressed CLR and RAMP1 was area postrema. We found binding of [³H]MK-3207 in this area, suggesting expression of functional CGRP receptor. Area postrema is involved in nausea and vomiting, symptoms that many migraine patients suffer from. One might argue that the CGRP receptor antagonists act in the area postrema and thereby have an impact on these symptoms since it has been shown that CGRP receptor antagonist aborted nausea and vomiting.
Figure 19. Bindings densities of MK-3207, mRNA and protein expression of CLR/RAMP1 in rhesus monkey.

A) Example of a brainstem level of rhesus monkey displaying binding densities of \[^3H\]MK-3207 in the pineal gland, medial mammillary nucleus, infundibular stem, 3rd ventricle, dorsal raphe nucleus, medial lemniscus, PAG and trochlear nucleus. B) RAMP1 mRNA expression in the pineal gland, PAG and dorsal raphe nucleus. C) Immunofluorescence staining of CLR and RAMP1, revealing their co-expression in the pineal gland and D) in PAG.

The PAG is considered to be a key structure in general pain processing, including that of migraine (Knight and Goadsby, 2001). We found mRNA and protein expression of CLR and RAMP1 in this area. High binding of the CGRP receptor antagonist was also found (see figure 19). Several cranial motor nuclei showed expression of the receptor components and binding densities of \[^3H\]MK-3207, such as the trochlear
nucleus, hypoglossal nucleus and dorsal motor nucleus of vagus, centrolateral part. Other areas that revealed expression of the receptor components and [3H]MK-3207 binding were; dorsal raphe nucleus, medial lemniscus, LC, pontine raphe nucleus, pontine nuclei, inferior olive, gracile nucleus, STN and the spinal cord. Thus, the widespread distribution of CGRP receptor in the brain and brainstem suggest an important role of the CGRP mechanism within these regions. The function of CGRP in these areas and the effect of halting the CGRP signaling in these areas remains to be evaluated.

The findings in the present study suggest expression of functional CGRP receptor in many brainstem regions involved in pain processing. Drugs blocking CGRP signaling may act at these central sites in migraine. Interestingly, some of these areas are not protected by the BBB, suggesting that CGRP receptor antagonists may not need to be CNS-penetrant to functionally antagonize receptors in these brain regions.

The cerebellum (Paper VI and VII)

Activation of cerebellar regions during migraine attacks has been demonstrated, but no explanation for these activations has emerged (May and Goadsby, 1999, Weiller, et al., 1995). The cerebellum has been suggested to have a role in migraine pathology (reviewed by Vincent and Hadjikhani 2007). Symptoms such as vertigo and balance changes may occur in migraine patients. These symptoms suggest that migraine affects cerebellar function. CGRP expression in cerebellar Purkinje cells and its elaborated dendrite tree has previously been demonstrated by immunohistochemistry (Kawai et al., 1985). Furthermore, CGRP distribution in different developmental stages in rats has been investigated (Morara et al., 1989, Morara et al., 2000, Morara et al., 2001). It has been shown that CGRP is transiently expressed in cerebellar climbing fibers (Gregg et al., 1999, Morara et al., 2001), while its receptor is suggested to be expressed in cerebellar glial cells (Morara et al., 1998, Morara et al., 2008). However, these studies based on immunohistochemistry and ligand binding sites were performed before the CGRP receptor was fully characterized. Therefore we delineated the expression pattern of CGRP and the CGRP receptor in rodent and primate cerebellum (Papers VI and VII), with the use of specific antibodies against CLR and RAMP1.

Our study on rodent cerebellum revealed that CGRP immunoreactivity is only observed in the cerebellar Purkinje cell bodies as intracellular granular staining (Paper VI). In addition, CLR and RAMP1 were detected on the surface of the Purkinje cell bodies and in their processes (Figure 20). No co-localization between the peptide and its receptor components was found (CGRP was expressed intracellularly while the receptor is expressed on the surface), although they were expressed in the same cell
This finding suggests that CGRP release may then act in an autocrine manner on its receptor expressed on the Purkinje cell surface. It may also suggest that CGRP could act as a paracrine factor to stimulate a nearby neuron expressing its receptor.

The receptor components were co-expressed in the cells, which suggest the presence of functional CGRP receptors within the cerebellum. The glial cells of the cerebellum were also studied in detail with stacking images using confocal microscopy (a 3-D study), revealing that receptor components were not expressed in the glial cells (Figure 21).

**Figure 20. Localization of CGRP and its receptor in rat cerebellum.**

Upper panel demonstrates expression of CGRP and RAMP1 in the Purkinje cells. CGRP is disclosed as intracellular, granular staining, while RAMP1 is expressed on the Purkinje cell surface and processes (arrows). Lower panel demonstrated double-staining of RAMP1 (red) and CGRP (green) CGRP is expressed in the cytoplasm of Purkinje cell bodies as opposite to the RAMP1 staining, which is expressed on the surface of the cell bodies and in the processes. ml – molecular layer, Pc – Purkinje cells.
To further expand these findings to the primate, we performed a study on human and rhesus monkey cerebellum (Paper VII). In this paper we studied the binding of a radiolabeled CGRP receptor antagonist ([³H]MK-3207) and mRNA expression of CLR and RAMP1 in rhesus monkey cerebellum. Binding densities of [¹²⁵I]CGRP was studied in human cerebellum. In addition, protein expression of CGRP and its receptor components were studied. Expression of procalcitonin, the precursor of calcitonin, was also investigated.

In the molecular layer of rhesus cerebellum, high [³H]MK-3207 binding densities were observed while the granular cell layer showed no radiotracer binding (Figure 22). It was not possible to accurately determine if the radiotracer binds to the Purkinje cell layer due to the resolution limit of the autoradiographic method. In human cerebellum, [¹²⁵I]CGRP binding was observed in the molecular layer, in addition to the Purkinje cell layer. These results support the possible expression of functional CGRP receptor in the cerebellum, where CGRP receptor antagonists may act. The in situ hybridization results showed that expression of CLR and RAMP1 mRNA was localized to the Purkinje cell layer. High-resolution localization of the receptor components displayed their mRNA expression mostly located around the Purkinje cells, where some Purkinje cells displayed mRNA expression within the cell body (Figure 23). The low mRNA detection within the soma of the Purkinje cells may be due to artifacts and/or low mRNA levels at the time of the experiment. These findings support the presence of functional CGRP receptor within the primate cerebellum.
Figure 22. Binding of CGRP receptor antagonist in rhesus monkey cerebellum.
High binding density of $[^3H]$MK-3207 in the molecular layer of the cerebellar cortex with minimal-to-no binding in the granular cell layer and white matter (total binding). Htx-Eosin staining of the same slice. Self-block on the adjacent slide with no binding (non-displaceable binding).

Figure 23. mRNA expression of CLR and RAMP1 in rhesus monkey cerebellum.
The mRNAs are mostly expressed around the Purkinje cells, however, some of these cells display mRNA expression within the cell body (arrows).

Immunohistochemistry revealed the more detailed cellular localization of CGRP and its receptor components in primate cerebellum. CGRP expression was found in the cytoplasm of Purkinje cells and in cells in the molecular layer, and was co-expressed with procalcitonin (Figure 24). $\alpha$CGRP is encoded by the CALCA gene, which yields both $\alpha$CGRP and the hormone calcitonin (CT) as alternative splice products (Rosenfeld et al., 1984). Procalcitonin is the precursor of calcitonin. Immunohistochemical and in situ RNA hybridization analyses have shown that CGRP transcripts are selectively expressed in a wide variety of neurons, while calcitonin is expressed predominantly in nonneuronal structures (Crenshaw et al., 1987). Interestingly, in a model of calcitonin/CGRP fusion gene in transgenic mice, calcitonin RNA was primarily found in non-neuronal structures, however, RNA of
both CGRP and calcitonin was observed in the Purkinje layer of the cerebellum (Crenshaw et al., 1987). In our study, we demonstrated the co-expression of CGRP and procalcitonin in cerebellar neurons, suggesting that CGRP may be produced in these cells within the primate cerebellum.

CLR and RAMP1 were co-expressed in the Purkinje cells and in some cells in the molecular layer, supporting the binding and in situ hybridization findings (Figure 25). No obvious difference in expression pattern of CGRP and its receptor was observed between rhesus and human cerebellum. When studying the expression of CGRP together with its receptor, we found some co-localization in the Purkinje cells and cells in the molecular layer. However, not all CLR or RAMP1 positive cells displayed CGRP expression. The findings suggest that CGRP might act on the same cells where it is expressed or cells only expressing its receptor. Compared to our study in rat cerebellum, the CGRP receptor was found on the cell surface of rat Purkinje cells, while it was expressed intracellularly in primate Purkinje cells. This could be due to differences in the quality of tissue or that the receptor expression is so high that the immunoreactivity appears as intracellular. The possibility of CGRP, acting in both autocrine and paracrine manner, was only observed in cerebellum. In the other areas we studied, co-expression of CGRP and its receptor was rarely found, suggesting low presence of putative autoreceptors. The exact signaling mechanism for CGRP in the cerebellum needs to be further evaluated.

Figure 24. Co-expression of CGRP and procalcitonin.
CGRP and procalcitonin immunoreactivity and their co-expression in cerebellar Purkinje cells (arrows) and cells in the molecular layer (arrowheads) in rhesus monkey.
CLR and RAMP1 are co-expressed intracellular in Purkinje cells and cells in the molecular layer (arrows) in rhesus monkey and human cerebellum.

We were able to show that the cells in molecular layer expressing CGRP or CGRP receptor, displayed GAD67 immunoreactivity on their cell surface. GAD67 is an enzyme that converts glutamate into gamma-aminobutyric acid (GABA), which the interneurons of cerebellum use as neurotransmitter. Functional experiments are required to evaluate if and how there may be a connection between the CGRP and GABA system in the cerebellum.

Recent advances in the biology of the cerebellum indicate that there may be a role in nociception and migraine. Our studies demonstrate expression and binding sites of CGRP and CGRP receptor components in rodent and for the first time in primate cerebellum, which suggest a functional role of CGRP in cerebellum. In support of our results, a new PET study demonstrated high concentration of the CGRP receptor antagonist PET tracer, [11C]MK-4232, in rhesus monkey cerebellum (Hostetler et al., 2013). Additional research is needed to reveal the roles of cerebellum and CGRP in response to nociceptive stimuli. This would enrich our understanding for cerebellum as a pain center and as target for new drug therapies.
Major conclusions

Summarizing the results presented in this thesis, following conclusions are made:

**Paper I:** Expression of CGRP, CLR and RAMP1 in the human trigeminal ganglion. CGRP is mainly expressed in smaller neurons lacking expression of the receptor. CLR and RAMP1 are co-expressed in the larger neurons and satellite glial cells. CGRP is expressed in thin fibers, while the receptor is expressed in thicker fibers.

**Paper II:** Telcagepant is able to block CGRP-induced vasodilation in human cerebral and meningeal arteries. The CGRP receptor is expressed in the smooth muscle layer of these vessels.

**Paper III:** CGRP is expressed in C-fibers and may act on A-fibers, rodent mast cells and vascular smooth muscle cells which express the CGRP receptor in the dura mater. These sites represent potential targets of novel anti-migraine agents such as CGRP receptor antagonists.

**Paper IV:** Within rat and human STN and C1-level of the spinal cord, CGRP and the receptor components are expressed in distinct fibers and areas, suggesting that CGRP may act post-synaptic.

**Paper V:** Expression of CLR and RAMP1 is widely distributed in rhesus monkey brainstem, where CGRP receptor antagonist binding densities are found. Some of these areas are not protected by BBB, suggesting that drugs such as CGRP receptor antagonists may act there independent from their ability to pass BBB.

**Paper VI:** CGRP is expressed within the Purkinje cells, while its receptor components are expressed on the Purkinje cell surface and their processes. The rich expression of CGRP and CGRP receptor elements in the rat cerebellum points towards a functional role of CGRP in cerebellar Purkinje cells.

**Paper VII:** Furthermore, mRNA and protein expression of CGRP and its receptor are found in primate cerebellum. High binding densities of a CGRP receptor antagonist are found, supporting expression of functional CGRP receptor in the cerebellum.

**Paper VIII:** High binding densities of a CGRP receptor antagonist are found in the rhesus monkey trigeminal ganglion. The trigeminal ganglion is not protected by the BBB, suggesting that CGRP receptor antagonists do not need to be CNS-penetrant to block receptors in the trigeminal ganglion. The trigeminal ganglion may be a key site of action for agents inhibiting CGRP signaling.
This thesis demonstrates the localization of CGRP and its receptor components within the trigeminovascular system and parts of the CNS. Paper I-VIII covers their distribution from the periphery with cranial vessels and dura mater, continuing into the trigeminal ganglion and further to the CNS with brainstem and cerebellum, suggesting CGRP signaling within and between these regions. We believe that a better understanding of CGRP signaling will enrich the understanding of migraine pathophysiology and drug therapies such as CGRP receptor antagonists. In the future we need to investigate the functional role of CGRP and the effect of inhibiting CGRP signaling within the areas.

Earlier studies have demonstrated expression of CGRP very well (Skofitsch and Jacobowitz, 1985, Inagaki et al., 1986, Unger and Lange, 1991), however CGRP receptor expression has not been fully studied as many of the studies were performed before the CGRP receptor was fully characterized. Subsequently, it has been shown that the CGRP receptor consists of three different components, CLR, RAMP1 and RCP (Hay et al., 2008). We had the privilege to work, for the first time, with newly produced specific antibodies against rat and human CLR and RAMP1. Therefore, we have been able to study expressions of the CGRP receptor in detail and in areas that have not been scrutinized before. By having the advantage to use primate tissues, our research may more easily be translated to the clinical setting.

In all regions we studied, co-expression of CLR and RAMP1 was found, suggesting presence of a functional CGRP receptor. However, proper functional methods are required to evaluate if all functional aspects act in synergy to produce activation. In many of the areas, cells only expressing RAMP1 were found, and RAMP1 mRNA expression seemed to be higher (this needs to be confirmed with RT-PCR). This may be due to several factors: 1) differences in the quality of the antibodies/probes recognizing the epitopes/sequences; 2) higher proportion of RAMP1 compared to CLR; or 3) presence of other RAMP1 containing receptors such as the amylin receptors. However, it has been shown that the cerebellum lacks amylin bindings sites (Sexton et al., 1994, van Rossum et al., 1994) and amylin binding in monkey brain has very little overlap with the pattern of CGRP binding (Christopoulos et al., 1995).

By autoradiography we studied the binding densities with a CGRP receptor antagonist, MK-3207 in the trigeminal ganglion, cerebellum and brainstem. MK-
3207 is considered to be a selective CGRP receptor antagonist with high potency for human and rhesus monkey CGRP receptors (Salvatore et al., 2010). The binding studies using this CGRP receptor antagonist were in good agreement with the immunofluorescence and mRNA findings, supporting the expression of a functional CGRP receptor in these areas.

The role of CGRP in migraine pathophysiology was suggested over 20 years ago (Edvinsson and Goadsby, 1990), and the CGRP receptor has long been regarded as an important target for the development of anti-migraine therapies (Ho et al., 2010). CGRP has also been implicated in other forms of pain, where reducing its action could be useful. It has been suggested that CGRP receptor antagonists may be used as treatment for other diseases involving the trigeminal nerve such as temporomandibular disorders, trigeminal neuralgia and dental pain (Awawdeh et al., 2002, Ambalavanar and Dessem, 2009). CGRP has been suggested to act as a growth or survival factor for a number of tumors (Hay et al., 2011). In a mouse model of cancer pain, it was shown that this was related to elevated CGRP content, which could be blocked with a CGRP receptor antagonist (Wacnik et al., 2005). It remains to be evaluated if the use of CGRP receptor antagonists may be beneficial in other diseases and headache disorders other than migraine.

The CGRP receptor antagonists represent a potential new type of anti-migraine drug that might offer a new non-vasoconstrictive approach in the acute treatment of migraine (Chan et al., 2010). It has been debated for some time if the anti-migraine action of CGRP receptor antagonists is mediated via central or peripheral mechanisms, or both. Our studies reveal the expression of CGRP receptor both at peripheral and central sites, implicating that many locations may be involved in migraine pathophysiology. However, the question is do all these sites need to be blocked by the CGRP receptor antagonists to elicit their anti-migraine efficacy?

Telcagepant is a P-gp substrate (P-glycoprotein, a transporter protein, present in the endothelium of capillaries in the brain), which thereby reduces its brain penetration (Ho et al., 2010). On the other hand, 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). Therefore, it has been suggested that the CGRP receptor antagonists need to penetrate the BBB to achieve acute anti-migraine efficacy (Edvinsson, 2008, Edvinsson and Tfelt-Hansen, 2008). Recent PET studies have revealed that telcagepant has low receptor occupancy in the brain (Vermeersch et al., 2012, Hostetler et al., 2013). In healthy subjects and in migraine patients, it was shown that the CGRP receptor antagonist telcagepant achieved low CGRP receptor occupancy (10%) at the lowest clinically efficacious dose (140mg), whereas a supratherapeutic dose of telcagepant (1120 mg) resulted in only moderate receptor occupancy in healthy volunteers. Generally, antagonists require moderate to high receptor
occupancy to produce a pharmacodynamic effect resulting in efficacy. Therefore low occupancy achieved by an efficacious dose of telcagepant indicates that central occupant of CGRP receptor is not required to for efficacy in migraineurs (Hostetler et al., 2013).

These data suggest that CGRP receptor antagonists do not have to act centrally for clinical efficacy (Vermeersch et al., 2012, Hostetler et al., 2013). However, it cannot be ruled out that additional efficacy may be achieved with better access to the CNS. It would be of great interest to see if a CGRP receptor antagonist which is able to more efficiently pass the BBB would show better clinically efficacy, or if higher CGRP receptor occupancy at well-tolerated doses in migraine patients would result in better/faster migraine relief.

If the current CGRP receptor antagonists cannot pass the BBB (or only to a low degree), it is important to clarify which sites can be reached and may therefore contribute to the clinical efficacy. We have shown that in the periphery the CGRP receptor is expressed in the smooth muscle cell layer of cerebral arteries, middle meningeal vessels, nerve fibers and mast cells of the dura mater. CGRP receptor antagonists may thereby act on cerebral and dural vessels to inhibit CGRP induced vasodilatation and on dural mast cell degranulation. However, we also found that the human mast cells lack expression of both receptor components, suggesting that these cells may lack expression of functional CGRP receptor in human. This is supported by the early finding that CGRP failed to induce release of histamine from dura mater mast cells in human following administration of excess CGRP (Ottosson and Edvinsson, 1997). The role of neurogenic inflammation in primate and the effect of CGRP receptor antagonists on nerve fibers and vessels in the dura mater need to be clarified.

Further in the periphery, a CGRP receptor antagonist could act on vessels of the dura mater such as middle meningeal artery, and on extracranial vessels such as the superficial temporal and extracranial internal carotid, to inhibit CGRP vasodilation. However, the role of extracranial vascular dilatation in migraine is unclear. Recently it was shown that acute migraine pain was not accompanied by extracranial arterial dilatation and by only some intracranial dilatation during attack (Amin et al., 2013). In the same study it was demonstrated that effective treatment with sumatriptan caused no intracranial (cerebral artery) vasoconstriction. Therefore it can be assumed that vasodilation does not have a primary role in spontaneous migraine headache, and any vascular changes (dilation/constriction) seen with migraine is a consequence rather than a cause of headache (Charles, 2013b).

In paper VIII, we demonstrated for the first time in recent literature, that the trigeminal ganglion is devoid of the BBB. Therefore, CGRP receptor antagonists may act in the trigeminal ganglion to block the CGRP receptor regardless of the molecules ability to cross BBB. The recently developed monoclonal anti-CGRP and anti-CGRP
receptor antibodies will not likely cross the BBB due to their large size (Dolgin, 2013). The trigeminal ganglion may also be site of action for these antibodies. If these agents only can act in the periphery, we believe that the trigeminal ganglion is a possible key site of action for drugs inhibiting CGRP signaling due to its importance in migraine pathophysiology and its location. Since the trigeminal ganglion has both peripheral and central connections, it may be that the CGRP signaling to these parts is blunted by blocking the action/signaling in the trigeminal ganglion. However, it is not known if better clinical efficacy can be achieved if central sites are occupied. Interestingly, we found mRNA and protein expression of CLR and RAMP1 with binding of a CGRP receptor antagonist in the pineal gland and area postrema. These brainstem areas are also not protected by BBB and we suggest that these CNS areas can in addition be sites of action for CGRP receptor antagonists.

Even if CGRP receptor antagonists do not need to act within the CNS to demonstrate anti-migraine efficacy, the view that migraine pathophysiology involves many central parts will remain unchanged. The wide distribution of CGRP and its receptor within the CNS, the broad range of symptoms that occur before/during a migraine attack and activation of brain regions during migraine attacks, all together support that migraine is primarily a brain disorder. By focusing research on understanding the function of CGRP signaling and the mechanisms behind the involvement of the brain in migraine pathophysiology, novel therapies for migraine treatment may be developed.
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“As you fill with wisdom, and your heart with love, there’s no more thirst”.

From the The wild rose of praise by Sanai, Persian poet.

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References


DIFFERENTIAL DISTRIBUTION OF CALCITONIN GENE-RELATED PEPTIDE AND ITS RECEPTOR COMPONENTS IN THE HUMAN TRIGEMINAL GANGLION

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Abstract—Calcitonin gene related peptide (CGRP) has a key role in migraine and recently CGRP receptor antagonists have demonstrated clinical efficacy in the treatment of migraine. However, it remains unclear where the CGRP receptors are located within the CGRP signaling pathway in the human trigeminal system and hence the potential antagonist sites of action remain unknown. Therefore we designed a study to evaluate the localization of CGRP and its receptor components calcitonin receptor-like receptor (CLR) and receptor activity modifying protein (RAMP) 1 in the human trigeminal ganglion using immunohistochemistry and compare with that of rat. Antibodies against purified CLR and RAMP1 proteins were produced and characterized for this study. Trigeminal ganglia were obtained at autopsy from adult subjects and sections from rat trigeminal ganglia were used to compare the immunostaining pattern. The number of cells expressing CGRP, CLR and RAMP1, respectively, were counted. In addition, the glial cells of trigeminal ganglion, particularly the satellite glial cell, were studied to understand a possible relation. We observed immunoreactivity for CGRP, CLR and RAMP1, in the human trigeminal ganglion: 49% of the neurons expressed CGRP, 37% CLR and 36% RAMP1. Co-localization of CGRP and the receptor components was rarely found. There were no CGRP immunoreactions in the glial cells; however some of the glial cells displayed CLR and RAMP1 immunoreactivity. Similar results were observed in rat trigeminal ganglia. We report that human and rat trigeminal neurons store CGRP, CLR and RAMP1; however, CGRP and CLR/RAMP1 do not co-localize regularly but are found in separate neurons. Glial cells also contain the CGRP receptor components but not CGRP. Our results indicate, for the first time, the possibility of CGRP signaling in the human trigeminal ganglion involving both neurons and satellite glial cells. This suggests a possible site of action for the novel CGRP receptor antagonists in migraine therapy, © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: trigeminocephalic complex, migraine, immunohistochemistry, calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1).

Migraine is a painful, incapacitating disease affecting more than 10% of the general population. The disorder is being recognized as a fundamental neurological problem, although the primary cause of migraine attacks is still unknown (Goadsby, 2005). There has been intense research to identify signal molecules in nociceptive fibers and modulator systems associated with the trigeminal system (Edvinsson and Uddman, 2005). Signal molecules such as substance P (SP), neuropeptide Y, vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase activating peptide (PACAP) have been studied but the only neuronal messenger so far reliably demonstrated in acute migraine attacks is the neuropeptide calcitonin gene-related peptide (CGRP) (Goadsby et al., 1988; Durham, 2006).

CGRP is a sensory neuropeptide consisting of 37 amino acids, stored in cranial trigeminal C-fibers and Aδ-fibers, and is a potent vasodilatory neuropeptide of intracranial blood vessels (Edvinsson et al., 1987; Gulbenkian et al., 2001). Several studies have revealed a correlation between migraine pain and cranial release of CGRP; it has been reported that CGRP levels in serum, cerebrospinal fluid, and saliva are elevated during migraine attacks (Goadsby et al., 1990; Goadsby and Edvinsson, 1993; Bellamy et al., 2006). A role for CGRP in migraine pathophysiology is supported by clinical evidence including: (1) CGRP infusion caused migraine in a small group of susceptible individuals (Lassen et al., 2002); and (2) CGRP receptor antagonists were shown to be effective in the acute treatment of migraine (Olesen et al., 2004; Ho et al., 2008a,b). CGRP seems therefore to play an important role in the underlying pathology of migraine pain due to its ability to mediate vasodilatation in cerebral and meningeal vessels and to transmit nociceptive information to second order neurons within the CNS (Pietrobon, 2005).

The receptor for CGRP has been identified as a G-protein-coupled receptor of the B-subtype (Hay et al., 2008). The functional receptor consists of a complex of a seven-transmembrane spanning protein, calcitonin receptor-like receptor (CLR), a single transmembrane-spanning protein, and CLR/RAMP1 receptor activity modifying protein 1 (RAMP1).
protein designated receptor activity modifying protein (RAMP1) (McLatchie et al., 1998) and an intracellular protein, receptor component protein (RCP) (Evans et al., 2000). RAMP1 is involved in receptor trafficking and is required for CGRP binding to CLR, whereas the interaction of CLR with other RAMP proteins, RAMP2 or RAMP3, forms adrenomedullin receptors (McLatchie et al., 1998; Foord and Marshall, 1999; Zhang et al., 2007).

CGRP receptor antagonists, unlike the 5HT_{1B/D} agonists (triptans), do not constrict coronary blood vessels (Chan et al., unpublished observation; Lynch et al., 2010) and suggest the potential for CGRP receptor antagonists to exert migraine efficacy in the absence of adverse effects on coronary vessel tone. In this context it is of considerable importance to identify the possible sites of antagonist interaction within the cranial pain systems (Edvinsson, 2008).

The trigeminal ganglion is in the center of interest for studying the expression of CGRP and its receptor components due to the activation of the trigeminovascular system during migraine attacks. The trigeminovascular system transmits signals, via second order neurons in the brain stem, to different parts of the CNS pain regions (Liu et al., 2008). Conversely, it has been hypothesized that brain-stem stimulation can cause activation of the trigeminovascular system, also resulting in CGRP-dependent vasodilatation (Just et al., 2005).

The trigeminal ganglion is comprised of bipolar neurons and two types of glial cells; satellite glial cells and Schwann cells. Earlier it was believed that the main function of glial cells was that of neuronal support; however during the last decade it has been revealed that the satellite glial cells may modulate neuronal function and activity, and could therefore be a factor in migraine pathophysiology (Hanani, 2005; Dublin and Hanani, 2007; Thalakoti et al., 2007; Li et al., 2008).

Immunohistochemical studies performed on the trigeminal system have shown CGRP immunoreactivity in about 50% of the neurons of human and rat trigeminal ganglion (Tahti et al., 1999; Lennerz et al., 2008). The elegant work of Lennerz and co-workers also demonstrated CLR and RAMP1 within the rat trigeminal ganglion. However, the localization of CGRP signaling mechanisms in the human trigeminal ganglion still remains unclear.

The goals of the present study were to (1) generate and comprehensively characterize novel antibodies to human CLR and RAMP1; (2) characterize the distribution of CGRP and its receptor components, CLR and RAMP1, in the human trigeminal ganglion; (3) determine whether CGRP, CLR and RAMP1 are co-localized in the same structure; and (4) generate antibodies to rat CLR and RAMP1 to serve as a point of reference for the human mapping studies.

**EXPERIMENTAL PROCEDURES**

**Generation of antibodies**

Antibodies were generated by Cambridge Research Biochemicals (Billingham, Cleveland, UK). Antigenic sequences were selected for human and rat CLR and RAMP1, purified, and coupled to keyhole limpet haemocyanin (KLH). Human CLR (acetyl-GYSH-DGPSEHLNQK) was coupled to KLH using gluteraldehyde. Rat CLR (C-SIQDIENVALKPEKMDLVI), human RAMP1 (C-Ahx-QSKRTGEV) and rat RAMP1 (C-Ahx-RSKRTGEIV) were coupled using m-Maleimidobenzonic acid N-hydroxysuccinimide ester (MBS) to KLH via an N-terminal cysteine. RAMP1 peptides contained an aminohexanoic acid (Ahx) spacer to space the short peptide away from the KLH carrier. Antibodies (human CLR, rat CLR and rat RAMP1), goat (human RAMP1), and sheep (rat CLR) were immunized with the appropriate peptide (two animals per peptide). Harvest bleeds were purified by affinity chromatography. Antisera were named 3152 (rabbit anti-human CLR), 844 (goat anti-human RAMP1), 3155 (rabbit anti-rat CLR) 132 (sheep anti-rat CLR), and 3158 (rabbit anti-rat RAMP1). In blocking experiments antibodies to human and rat CLR and RAMP1 were preabsorbed by incubation with peptides used for immunization (1:100, 1 h, 4 °C).

**Cell culture and generation of recombinant cell lines**

HEK293 cells were cultured in Dulbecco’s modified eagle medium (DMEM) with 4.5 g/L glucose, 1 mM sodium pyruvate and 2 mM glutamine supplemented with 10% fetal bovine serum (FBS), 100 μM penicillin and 100 μg/ml streptomycin, and maintained at 37 °C, 5% CO₂, and 95% humidity. Cells were subcultured by treatment with 0.25% trypsin with 0.1% ethylenediaminetetraacetic acid (EDTA) in Hank’s balanced salt solution (HBSS).

Human embryonic kidney cells (HEK293 cells) stably expressing the human CGRP (CLR/RAMP1) receptor was previously described in Salvatore et al. (2008). For transient transfections, 24 h prior to transfection HEK293 cells were seeded in 500 cm² dishes. Transfections were performed by combining 60 μg dish Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Rat CLR and RAMP1 cDNAs in the mammalian expression construct pCDNA3.1, described earlier (Mallee et al., 2002), were co-transfected in equal amounts. Transfection cocktail was added directly to the medium and the cells were harvested for membranes 48 h post-transfection.

**Membrane preparation and western blotting**

Transiently and stably transfected cells were washed with phosphate-buffered saline (PBS) and harvested in ice-cold harvest buffer containing 50 mM HEPES, 1 mM EDTA, and Complete protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). The cell suspension was disrupted with a laboratory homogenizer and centrifuged at 48,000 g to isolate membranes. Membranes from untransfected HEK293 cells were prepared similarly and served as a negative control.

Membranes were diluted in gel loading buffer, heated at +70 °C for 10 min, and fractionated on 10% nitrocellulose membranes using the iBlot transfer system according to the manufacturer’s suggested protocol (Invitrogen). Blots were incubated in PBS, 1% bovine serum albumin (BSA), 0.1% nonfat milk powder, 0.1% Tween for 1 h at room temperature. Blots were then incubated overnight at +4 °C with antibodies to CLR (1:100) or RAMP1 (1:100). After overnight incubation membranes were washed and incubated with the appropriate secondary antibody coupled to horseradish peroxidase (HRP) for 30 min at room temperature. Secondary antibodies were as follows: 1:40,000 goat anti-rabbit IgG HRP (Santa Cruz Biotechnol- ogy, Inc., Santa Cruz, CA, USA); 1:50,000 rabbit anti-goat IgG HRP (Millipore, Billerica, MA, USA); and 1:100,000 rabbit anti- sheep IgG HRP (Invitrogen). Blots were then washed and immuno-reactive proteins detected by chemiluminescence (Amersham ECL Plus Kit, GE Healthcare, Piscataway, NJ, USA).
CO2 incubator. The following day the cells were washed with PBS.

Cells were washed and incubated with Alexa Fluor® 488 goat anti-rabbit CLR (1:100) diluted in blocking buffer for 2 h at room temperature. (1:500), the goat antibody to hRAMP1 (1:100) or the sheep antibody were incubated with the rabbit antibody to hCLR, rCLR, and rRAMP1 (hRAMP1, 844; rCLR, 132) for 30 min at room temperature. Cells

Texas-Red (donkey) Anti-rabbit 1:200 Jackson ImmunoResearch, West Grove, PA, USA

CGRP, ab81887b Mouse 1:100 Rat CGRP Europroxima; Arnhem, The Netherlands

Receptor activity modifying protein (RAMP1) 844 Goat 1:100 C-terminal of human RAMP1 Merck & Co., Inc

The samples were immersed overnight in fixative consisting of 2% paraformaldehyde (PFA) and 0.2% picric acid in 0.1 mol/l phosphate buffer, pH 7.2. After fixation, the specimens were rinsed in sucrose-enriched (10%) Tyrode solution overnight, frozen and stored at −80°C. The ganglia were embedded in TissueTek (Sakura Finetek, Europe), sagitally cryosectioned (12 μm) and the sections stored at −20°C until use.

Rat tissue samples

Trigeminal ganglia were removed from 10 male Sprague–Dawley rats weighing 300–350 g (Scanbur, Stockholm, Sweden). The ganglia were placed in 4% PFA and fixed for 2–4 h. After fixation the ganglia were rinsed in raising concentrations of sucrose in Sörensen’s phosphate buffer, embedded in a gelatin medium (30% egg albumin, 3% gelatin in distilled water) and sagitally cryosectioned (12 μm). The sections were stored at −20°C until use. After sacrifice, five animals were kept at +4°C for 24 h before dissection of the ganglia (to mimic the autopsy situation in man). Thereafter they were treated as above.

Hematoxylin–Eosin staining

Human and rat sections were stained with Hematoxylin–Eosin (Htx–Eosin) using a standard protocol (Htx 3 min, water rinse, Eosin 1 min) for orientation and for examination of the tissue condition.

Immunohistochemical studies

Indirect immunofluorescence staining was performed to demonstrate the localization of CGRP, CLR and RAMP1 in human and rat trigeminal ganglia. The sources, characteristics and dilutions of the primary and secondary antibodies used are listed in Tables 1 and 2. In brief, the sections were thawed rapidly at room temperature and 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

| Name and product code | Host | Dilution | Detects | Supplier
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<tr>
<td>Calcitonin receptor-like receptor (CLR) 3152</td>
<td>Rabbit</td>
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<td>CLR 132</td>
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<td>1:100</td>
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<td>RAMP1 3158</td>
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<td>Europroxima; Arnhem, The Netherlands</td>
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<td>Rat α-CGRP</td>
<td>Abcam; UK</td>
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<tr>
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<td>Neurofilament of molecular weight 68 kDa</td>
<td>Sigma; St Louis, MO, USA</td>
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<tr>
<td>NF 160/200</td>
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<td>1:800</td>
<td>Neurofilament of molecular weight 160 and 200 kDa</td>
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<td>Glial fibrillary acidic protein (GFAP)</td>
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<td>Cytoskeleton in glial cells</td>
<td>Dako; Copenhagen, Denmark</td>
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<td>1:200</td>
<td>Myelin, Schwann cells</td>
<td>Dako; Copenhagen, Denmark</td>
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a Cross-reacts with rat receptor at 1:100 dilution.

b Used for double-staining.

Immunostaining of cell lines

HEK293 cells stably expressing the human CGRP (CLR/RAMP1) receptor was described in previous work (Salvatore et al., 2008). Parental HEK293 and cells stably expressing the human CGRP receptor were plated on Poly-D-Lysine 8-well CultureSlides (BD Biosciences, San Jose, CA, USA) and cultured overnight at +37°C in a CO2 incubator. The following day the cells were washed with PBS and then fixed at −20°C for 10 min in methanol followed by 1 min in acetone. Cells were washed a final time in PBS to remove fixative.

Cells were blocked in PBS pH 7.4 containing 2.5% BSA and 5% goat (hCLR, 3152; rCLR, 3155; rRAMP1, 3158) or 5% donkey serum (hRAMP1, 844; rCLR, 132) for 30 min at room temperature. Cells were incubated with the rabbit antibody to hCLR, rCLR, and rRAMP1 (1:500), the goat antibody to hRAMP1 (1:100) or the sheep antibody to rCLR (1:100) diluted in blocking buffer and incubated with the cells for 30 min at room temperature. Cells were washed and incubated with Alexa Fluor® 488 goat anti-rabbit IgG (Invitrogen) for hCLR (3152), rCLR (3155) and rRAMP1 (3158); Alexa Fluor® 488 donkey anti-goat IgG (Invitrogen) for hRAMP1 (844); and Alexa Fluor® 488 donkey anti-sheep IgG (Invitrogen) for rCLR (132). Secondary antibodies were diluted 1:400 in blocking buffer and incubated with the cells for 30 min at room temperature in the dark, washed in PBS, and mounted with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame CA, USA).

Human tissue samples

Trigeminal ganglia were obtained at autopsy from adult subjects in accordance with the Szeged University Medical School guidelines for ethics in human tissue experiments and were approved by the local Hungarian Ethics Committee. Trigeminal ganglia were bila-terally removed from five subjects (four female; one male) with an average age of 75 years (65–86 years). None of the subjects suffered from any central nervous system disease and the cause of death was related to heart failure, septicemia or cancer. The tissue was collected within 24 to 36 h after death.

<table>
<thead>
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<th>Conjugate and host</th>
<th>Against</th>
<th>Dilution</th>
<th>Supplier</th>
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<tr>
<td>FITC (goat)</td>
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<td>Texas-Red (donkey)</td>
<td>Anti-rabbit</td>
<td>1:200</td>
<td>Jackson ImmunoResearch, West Grove, PA, USA</td>
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normal serum, with or without primary antibodies, at
then incubated overnight in PBST containing 1% BSA and 3%
were 100:1. The blocking peptides were resuspended in PBS and
were the same as described in Table 1, peptide concentrations
obtained using a light- and epifluorescence microscope (Nikon 80i,
Thornwood, NY, USA). Sections were examined and images were
were compared. Similar controls were conducted for the CGRP
antibody; CGRP antibody was pretreated with human CGRP
immunostaining protocol was the same as described above. Sec-
tions incubated with antibodies alone versus blocked antibodies
were measured to avoid double-counting of cells) from each subject
controls and blocking peptides
Rat trigeminal ganglia were used for comparison of the staining
pattern for CGRP and its receptor components. The newly pro-
duced primary antibodies against human CLR and RAMP1 were
also tested on rat trigeminal ganglion to see if a similar staining
pattern was produced.
Sections of human and rat trigeminal ganglia, including rats
mimicking the human postmortem situation, were treated in the
same way, except for the dilutions of the primary antibodies (see
Table 1). For all antibodies, omission of the primary antibody served as negative controls.
Preabsorption controls were performed with all of the CLR
and RAMP1 primary antibodies. The newly produced primary antibodies against human CLR and RAMP1 were also tested on rat trigeminal ganglion to see if a similar staining pattern was produced.

Controls and blocking peptides

RESULTS

Specificity of CLR and RAMP1 antibodies

The specificity of the CLR and RAMP1 antibodies were evaluated by Western blotting and immunofluorescence using stably and transiently transfected cells. Antibody 3152 (human CLR) recognized a single protein of approximately 54 kDa in HEK293 cell membranes stably expressing the human CGRP receptor consistent with the predicted mass of the protein (Fig. 1A). Antibodies 3155 and 132 (rat CLR) recognized two proteins with molecular masses of approximately 49 and 60 kDa in HEK293 cells transiently expressing the rat CGRP receptor (Fig. 1C, D). Human (844) and rat RAMP1 (3158) antibodies both recognized a single protein with an approximate molecular mass of 13 kDa in human (Fig. 1B) and rat (Fig. 1E) CGRP receptor expressing cells, respectively. There were no significant signals in parental HEK293.

Fig. 1. Western blot analysis of human and rat CGRP receptors expressed in transfected cells. Detection of human CLR (A), human RAMP1 (B), rat CLR (C, D), and rat RAMP1 (E) in cells stably or transiently expressing the human and rat CGRP receptor, respectively. Lysates of HEK293 CLR/RAMP1 expressing cells (Lane 1) or parental HEK293 cells (Lane 2) were separated by SDS-PAGE and analyzed via Western blotting.

Nikon 80i (Tokyo, Japan) coupled to a Nikon DS-2 MV camera, Adobe Photoshop CS3 (v.8.0, Adobe Systems, Mountain View, CA, USA) was used to visualize co-labelling by superimposing the digital images.

Cell-counting

Cell counting was performed to quantify the expression of CGRP, CLR and RAMP1 in human trigeminal ganglion. Three slides (with a minimum of three sections between each were used for measurements to avoid double-counting of cells) from each subject were stained with the respective antibody. Images were taken at a magnification at 10×.

In each image, a rectangle (26.95 μm^2) was placed on a homogenous stained part of the cytoplasm of immuno-positive cells. A similar procedure was undertaken in immuno-negative cells. The NIS-elements BR image analysis program (Nikon) was used to calculate the number of cells and to measure the fluorescence intensity in each area. Cells were counted and measured in two to three areas (depending on the size of the ganglia). Each area contained 70–100 cells and at least 30 of these cells had visible nuclei, which were the cells that were counted. The average cell count from three sections for each antibody was used for the statistical analysis. The intensity measurements were used to verify that immuno-positive cells were correctly distinguished from immuno-negative cells.

Microscopic analysis

HEK293 cells stable expressing the human CGRP receptor were examined and images obtained using a Zeiss Axiosplan 2 fluorescence microscope with an Axiocam camera (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). Sections were examined and images were obtained using a light- and epifluorescence microscope (Nikon 80i, 10×, Vector Laboratories). Vectashield medium containing DAPI staining nuclei was used on some sections.

Fig. 1A–E. Western blot analysis of human and rat CGRP receptors expressed in transfected cells. Detection of human CLR (A), human RAMP1 (B), rat CLR (C, D), and rat RAMP1 (E) in cells stably or transiently expressing the human and rat CGRP receptor, respectively. Lysates of HEK293 CLR/RAMP1 expressing cells (Lane 1) or parental HEK293 cells (Lane 2) were separated by SDS-PAGE and analyzed via Western blotting.
Immunoreactive human and rat CLR and RAMP1 were detected primarily at the plasma membrane of HEK293 cells stably expressing the human CGRP receptor (Fig. 2A, D, G, J, M). Immunoreactivity was abolished by pre-absorption of antibodies with peptides used for immunization (Fig. 2B, E, H, K, N). No staining was observed in untransfected HEK293 cells (Fig. 2C, F, I, L, O).

The histology of trigeminal ganglion

Htx–Eosin stained section from human (a) and rat (b) trigeminal ganglia are depicted in Fig. 3. In the human and rat trigeminal ganglia, the neurons differed in size and had lighter/darker cytoplasm. The results show that most of the cells in the trigeminal ganglion are glial cells, where the satellite cells are wrapped around the neurons. Due to age alterations, many neurons in the human material contained lipofuscin. We also observed some cell shrinkage. Aside from these changes, the human samples were considered qualitatively adequate for the immunofluorescence technique and the cell counting. Rat ganglia that were dissected 24 h after sacrifice showed some cell shrinkage and splits in the tissue (Fig. 3C).

Immunostaining with CLR and RAMP1 antibodies

A detailed description of all the antibodies used in this study, including cell type or structure recognized, species

Fig. 2. Detection of CLR and RAMP1 in HEK293 cells stably transfected with the human CGRP receptor. Panels on the top row are transfected cells without preabsorption, center rows are corresponding preabsorption controls, and bottom row panels are untransfected control cells. All antibodies detect CLR (A, 3152; G, 3155; J, 132) and RAMP1 (D, 844; M, 3158) predominantly at the plasma membrane. Preabsorption controls and control cells for each antibody are in the center (B, 3152; E, 844; H, 3155; K, 132; N, 3158) and bottom panels (C, 3152; F, 844; I, 3155; L, 132; O, 3158), respectively. There was no detectable staining in the preabsorption controls or the untransfected cells, consistent with a specific signal for all antibodies.

Fig. 3. Hematoxylin -Eosin staining of human (A) and rat (B, C) trigeminal ganglia. The neurons differ in size and dye-uptake in the cytoplasm in both species. The majority of the cells in trigeminal ganglion are glial cells, where the satellite glial cells surround the neurons (arrows and insert). Many neurons (asterisk) in the human material contain lipofuscin (autofluorescent). To mimic the human postmortem situation, rat ganglia were removed 24 h after sacrifice (C), showing cell shrinkage and splits in the tissue.
in which the respective antibody was produced, dilution and source, is found in Table 1. All antibodies were applied in at least three independent staining sessions in order to validate the reproducibility of the staining results. Antibodies were generated against human and rat CLR (human 3152, rat 132 and 3155) and RAMP1 (human 844, rat 3158). For human trigeminal ganglion, the anti-human antibodies were used; in addition they were tested on trigeminal ganglion of rat to compare the staining pattern. The anti-human antibodies also worked well on rat trigeminal ganglion and displayed a similar staining pattern as seen in the human tissues (Fig. 4A). The immunoreactivity of the human anti-CLR antibody was somewhat weaker compared to that of the RAMP1 antibody, in both human and rat sections. The immunoreactivity was homogenously distributed in the cytoplasm, for both anti-human (Fig. 4A) and anti-rat CLR and RAMP1 (Fig. 4B) antibodies, and not in the nucleus. In addition, no notable variability was observed for the localization and distribution of the respective antibody staining pattern (more described in Distribution of CGRP, CLR and RAMP1).

Controls and blocking peptides
The antibodies raised against rat CLR and rat RAMP1 utilized the antigenic sequences identified previously (Cottrell et al., 2005), and allowed comparison between rat and human localization experiments. In the study presented here we compared the findings on the human material with trigeminal ganglia of rat; they were by and large very

![Fig. 4.](image)

(A) Distribution of CGRP, CLR and RAMP1

(B) Anti-rat antibodies

(C) Blocking peptides and negative controls
similar. The primary antibodies for CGRP and the receptor components worked well in both human and rat trigeminal ganglion (Fig. 4A, B). As an additional control for the human material, rat ganglia were removed 24 h after sacrifice. The CGRP antibody and the newly produced receptor component antibodies were tested on these postmortem controls and showed the same immunoreactivity as visualized in the human and in the “normal” rat material (data shown in supplementary file). The morphology of the cells was somewhat poorer, as seen in the Htx–Eosin staining. The negative controls (omission of primary antibody) showed no reactivity, except for the lipofuscin autofluorescence in the neurons, and in connective tissue of human trigeminal ganglion. There was no staining with preabsorbed CLR or RAMP1 antibodies, using their respective blocking peptides (Fig. 4C). The CGRP immunoreactivity was abolished if pretreated with an excess of unlabeled CGRP (data not shown).

Quantitative analysis

We counted cells that stained positive for CGRP and the receptor components in five human trigeminal ganglia (Table 3). Immunoreactivity for CGRP was found in 49% of the neurons (positive cells/total number of cells; 459/946), CLR in 37% (193/521) and RAMP1 in 36% (151/422) of the neurons.

We also measured the fluorescence intensity to assess the probability of selecting cells as positive or negative, respectively. The cells selected as positive or negative varied within its group approximately by 20 on the gray scale (range 0–256). The mean difference between positive and negative cells varied with the primary antibody used. The CLR and RAMP1 antibodies showed the least difference (approx 20) whereas the CGRP antibody displayed the most (approx 60), indicating that our method for cell-counting was reliable. Cells that were judged as outliers were removed from the analysis.

Distribution of CGRP, CLR and RAMP1

We observed immunoreactivity for CGRP, CLR and RAMP1, respectively, in the trigeminal neurons (Fig. 4A). Expression of CGRP was predominantly localized to smaller neurons, whereas CLR and RAMP1 immunoreactivity was expressed primarily in larger neurons. The CGRP immunoreactivity was detected in the cytoplasm in a granular-like staining pattern (Fig. 5A). In addition, we observed CGRP immunoreactivity in fibers proximal to the cell bodies (Fig. 5A). We also observed fibers positive for CLR and RAMP1, although the CGRP positive fibers seemed thinner than those positive for CLR and RAMP1. CGRP did not co-localize with NF 160/200 or NF 68 (Fig. 5B), while the receptor components were expressed in thicker fibers and co-localized with NF 160/200 (Fig. 5C).

Double immunolabeling was performed to investigate co-localization of CLR and RAMP1 in the human samples (Fig. 6A). It was shown that in most cases co-localization occurred between the receptor components, suggesting the presence of the CGRP receptor. However, there were exceptions; Fig. 6A shows a neuron expressing both RAMP1 and CLR while another neuron only expressed RAMP1.

We also evaluated if CGRP and its receptor components were expressed in the same neuron. As shown above we found co-localization of CLR and RAMP1 in most instances. The combination of CGRP with CLR (Fig. 6B) or RAMP1 (Fig. 6C) was uncommon. Occasional neurons (very few) showed co-localization and then only in the small to medium sized cells (Fig. 6C).

Besides neurons within the human trigeminal ganglion, we observed that some satellite glial cells were positive for CLR and RAMP1 (Fig. 7A) but not for CGRP. The neurons are usually surrounded by a string of satellite glial cells; neurons negative or positive for the receptor components sometimes immunoreactive for CLR and RAMP1 in the adjacent satellite glial cells. In rat trigeminal ganglion, similar results were observed (Fig. 7A). Also, the glial cell marker GFAP co-localized with RAMP1 (Fig. 7B). Double immunostaining could only be performed with the GFAP antibody in combination with the RAMP1 antibody due to the host species of the antibodies. Therefore, the combination of CLR and GFAP was studied with the sheep anti-rat CLR antibody in rat trigeminal ganglion (Fig. 7C).

Schwann cells surrounding nerve fibers were studied with the MBP marker in human cross-sectional nerve (Fig. 8A). In rat trigeminal ganglion, both cross-sectioned and length sectioned nerve fibers could be studied (Fig. 8B). Immunoreactivity was never observed for CGRP or the receptor components CLR or RAMP1 in the Schwann cells. In cross-sectioned nerves, the receptor components were found to co-localize with the marker for NF 160/200 (Fig. 8C) but not with MBP.

DISCUSSION

The aim of the present study was to identify CGRP and its receptor components within the human trigeminal ganglion compared with the rat and to evaluate morphologically their relationship using immunohistochemistry. For this purpose, we developed new antibodies against human and rat CLR and RAMP1 and characterized these in detail. The study has, for the first time, demonstrated the detailed distribution and quantification of CGRP receptor components in the human trigeminal ganglion. The CGRP receptor is localized to both neurons (mainly large) and satellite glial cells (surrounding neurons), while CGRP is only ex-

Table 3. Cell-counting in human trigeminal ganglia

<table>
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<tr>
<th>No</th>
<th>CGRP</th>
<th>CLR</th>
<th>RAMP1</th>
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<tbody>
<tr>
<td>1.</td>
<td>49%</td>
<td>37%</td>
<td>29%</td>
</tr>
<tr>
<td>2.</td>
<td>48%</td>
<td>30%</td>
<td>47%</td>
</tr>
<tr>
<td>3.</td>
<td>47%</td>
<td>64%</td>
<td>30%</td>
</tr>
<tr>
<td>4.</td>
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<td>23%</td>
<td>35%</td>
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<tr>
<td>5.</td>
<td>47%</td>
<td>39%</td>
<td>41%</td>
</tr>
<tr>
<td>Mean</td>
<td>49%</td>
<td>37%</td>
<td>36%</td>
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Table showing cell-counting in human trigeminal ganglia. CGRP, CLR and RAMP1 positive cells were counted in five human subjects.
pressed in the neurons of small to medium size and these lack CGRP receptor components. The CGRP immunoreactivity was found in about 50% of the neurons of a small to medium size while the receptor component proteins, CLR and RAMP1, were seen to co-localize in large sized neurons (which were CGRP negative). This suggests that if CGRP is released within the ganglion, then intraganglionic CGRP may act on satellite glial cells and on large sized neurons. We also observed differences in thickness of the fibers positive for CGRP and the receptor components. In nerve fibers within the trigeminal ganglion, the receptor components were expressed in the neuronal processes but not in the surrounding Schwann cells.

**Methodology and technical considerations**

The use of human material is valuable since results from such studies may contribute to our knowledge about the physiology and pathology in human disorders involving the trigeminal system. However, there are some difficulties and limitations when working with human tissue. Human trigeminal ganglia were obtained from adult subjects and the tissue samples were collected within 24–36 h of autopsy. The time lapse between death and postmortem examination allows a series of reactions and autolysis processes to initiate/take place in the tissue. This could cause problems in the immunohistochemical method, such as changes in epitopes, leakage of macrophages and leukocytes from the vessels, unspecific and/or high background staining in the tissue. Due to the relatively high age of the subjects, lipofuscin is accumulated in the neurons, causing auto-fluorescence. Also, the exact orientation of the ganglia is missing and therefore regional variations in CGRP and receptor distribution cannot be determined. The Htx-eosin stained material revealed well-preserved
human ganglia that were adequate for the immunofluorescence technique and cell counting. We performed cell-counting on human trigeminal ganglia for CGRP and the receptor components. Due to the auto-fluorescence in the neurons we decided to perform this task manually, where we counted and measured intensity for positive and negative cells. This was conducted in order to verify that immune-positive cells were correctly distinguished from immuno-negative cells. Use of confocal microscope would probably improve our work; however, this was not within the scope of the present study.

Characterization of CLR and RAMP1 antibodies

In general it is difficult to find antibodies that work specifically in human material, therefore antibodies against human CLR and RAMP1 were produced. Additionally, antibodies against rat CLR and RAMP1 were also generated. The specificity of the antibodies raised against human and rat CLR and RAMP1 was confirmed in HEK293 cells stably expressing the human CGRP receptor. Immunoreactive CLR and RAMP1 were detected at the plasma membrane in stably transfected cells, but no immunostaining was detected in untransfected HEK293 cells. Additionally, preabsorption of the primary antibodies with the peptides used for immunization abolished the staining of cell lines. The specificity of CLR and RAMP1 antibodies was also confirmed by Western blotting. Human and rat CLR antibodies recognized proteins consistent with the molecular weight of CLR. The human CLR antibody recognized a single protein whereas the rat CLR antibody recognized two proteins, most likely representing different glycosylated states of the protein. Human and rat RAMP1 antibodies recognized a single protein of ~13 kDa, which is slightly lower than the predicted mass of 17 kDa. Although slightly lower in mass than predicted, both the human and rat RAMP1 antibodies recognize the same molecular weight protein in stably (human) and transiently expressing (rat) cell lines, therefore this does not appear to be an artifact. Additionally, there were no detectable
The newly produced primary antibodies against CGRP receptor components worked well for immunohistochemistry on the human and rat trigeminal ganglia. The anti-human CLR antibody showed a somewhat weaker staining in both human and rat sections compared to the RAMP1 antibody. Overall the primary antibodies displayed similar localization and distribution of the staining pattern in human and rat trigeminal ganglia. Preabsorption with the blocking peptides resulted in no immunoreactivity for the antibodies against CLR and RAMP1 in the human or rat

Fig. 7. Demonstration of CLR and RAMP1 expression in satellite glial cells of human trigeminal ganglia (anti-human antibodies) and rat ganglia, including nuclear DAPI staining (A). The satellite glial cells surrounding the neurons (arrows) appear to express CLR and RAMP1. The DAPI staining shows that the receptor components are expressed in the cytoplasm of the satellite glial cells. Note that the surrounded neurons can be negative or positive for the receptor components. Similar observations are found in rat (lower panel). Expression of RAMP1 in glial cells is also confirmed with the marker for GFAP (B), double-staining shows clear co-localization (arrows). Co-localization of CLR and GFAP was performed in rat (C), which also shows co-localization (arrows). DAPI is used in the merged picture to clarify the orientation of satellite glial cells.
trigeminal ganglia, which shows that these antibodies are specific for the epitope they are raised against. Similar controls were conducted for the CGRP antibody; exogenous CGRP blocked the immunoreactions as could be expected and verified before (Tajti et al., 2001; Uddman et al., 2002).

**The distribution of CGRP and the receptor components**

CGRP has been shown to be expressed in trigeminal nerve endings, which densely innervate the cerebral blood vessels (Uddman et al., 1985; Edvinsson et al., 1987).
Several studies have shown that the trigeminovascular system has an important role in migraine pathophysiology. Stimulation of the trigeminal ganglion both in humans and in animals resulted in release of CGRP and elevation of CGRP in the external jugular vein followed by vasodilatation (Goadsby et al., 1988; Limroth et al., 2001). Taken together, the release of CGRP from trigeminal neurons seems to play an important role in migraine pathophysiology, in both initiating and sustaining migraine attacks. Previous studies have shown both protein and mRNA levels of CGRP within human and rat trigeminal ganglia (Edvinsson et al., 1998; Tajti et al., 1999; Hou et al., 2001; Li et al., 2008).

The localization of the CGRP receptor elements in the trigeminovascular system has not been fully clarified to date (Lennerz et al., 2008). We have previously reported the expression of CLR and RAMP1 on smooth muscle cells in human cerebral and meningeal arteries (Olive et al., 2002; Edvinsson et al., in press) and of CGRP receptor mRNA in human cerebral vessels and trigeminal ganglion (Edvinsson et al., 1997).

In the present study the distribution of CGRP, CLR and RAMP1 within the human and rat trigeminal ganglia was demonstrated. We observed that neurons positive for CGRP were mostly negative for the receptor components CLR and RAMP1. Almost 50% of human trigeminal neurons expressed CGRP, which is in agreement with previous studies in man (Tajti et al., 1999) and rat (Ma et al., 2001; Lennerz et al., 2008). Expression of CLR and RAMP1 was seen in approximately 40% of the neurons. In a study on rat trigeminal ganglion, it was reported that CLR was more frequently expressed than RAMP1 in the neurons (Lennerz et al., 2008). The dissimilarity between these results and ours could be due to several factors such as the use of different primary antibodies, cell-counting method or differences between human and rat trigeminal ganglia. However, our in parallel study of the rat trigeminal ganglion revealed similar results as we report for the human trigeminal ganglion.

We observed that the CGRP-positive cells were mostly small- to medium-sized while the receptor components were expressed in the larger trigeminal neurons. We decided not to measure the cell diameters due to cell shrinkage in the human material. Different size of the neurons was also observed with Htx–eosin staining, where the dye-uptake in the cytoplasm also differed. The histology of neurons in the human trigeminal ganglion has been described before (Kralev et al., 2007) where they classified human trigeminal neurons into different groups according to their shape, size and light or dark cytoplasm. Here we present a variation in size between neurons expressing CGRP and those that store the receptor components, the function of this differential distribution remains to be clarified. The CGRP immunoreactivity displayed a granular-like staining pattern in the neuronal cell bodies, which could be due to the localization of CGRP in the endoplasmatic reticulum. In addition, thin “pearl-like” staining of the fibers was observed. This type of granular staining pattern of CGRP in human trigeminal neurons has been described previously (Quartu et al., 1992). In addition to the difference in cell-size of neurons positive for CGRP, CLR and RAMP1, we found a difference in thickness of the fibers positive for CGRP and the receptor components; this was confirmed using nerve filament antibodies for different molecular weights. It was clearly shown that CGRP was expressed in thinner fibers, which did not co-localize with the marker for smaller diameter neurofilaments. It is tempting to speculate that the thin CGRP positive fibers belong to the C-fibers and project to the lamina I/II in the trigeminal complex of the brainstem while the CGRP negative fibers are Aδ-fibers that contain CLR/RAMP1 project to lamina III/IV (Liu et al., 2004, 2008). This hypothesis may unravel with studies of the trigeminal complex using the present novel antibodies.

In order to verify neurons expressing CGRP and the receptor components, double-immunolabelling was performed. The results revealed that co-localization between the neuropeptide and its receptor component occurs rarely, suggesting low presence of putative auto-receptors (CGRP receptors on cells storing CGRP) within the human and rat trigeminal ganglion. The combination of CLR and RAMP1 was considerably more frequent on CGRP-negative neurons; whereas neurons expressing only a single receptor component were rarely observed. This might indicate that there exist situations when the CGRP receptor is not expressed as a functional receptor, however, this needs to be further evaluated. Our results are in agreement with those of Lennerz and colleagues (2008), who showed that a low percent of the trigeminal neurons express CGRP and RAMP1 together. They found that the combination of CGRP and CLR was more frequent; however a functional CGRP receptor requires RAMP1 expression. As described earlier, CLR can interact with other RAMP proteins, RAMP2 or RAMP3 to form adrenomedullin receptors (McLachie et al., 1998; Forooz and Marshall, 1999; Zhang et al., 2007). This could explain the finding of cells being immuno-positive for CLR but not for RAMP1. However, the functional role of RAMP1 only positive neurons remains unclear.

The CGRP receptor has long been proposed as target for antimigraine drugs (Edvinsson, 2004; Doods et al., 2007; Durham, 2008). Today the CGRP receptor antagonist telcagepant is being developed (Paone et al., 2007; Edvinsson and Linde, 2010). Clinical studies conducted to date have found telcagepant to be superior to placebo in reducing the pain and symptoms associated with acute migraine (Ho et al., 2008a,b). Results from our study indicate that the human trigeminal ganglion could be one possible target for CGRP receptor antagonists.

The glial cells

The physiological function of the satellite glial cells within the trigeminal ganglion is not yet fully understood. However, recent studies show that neuron-glial signaling may occur via gap junctions, and the release of CGRP within the ganglion could further stimulate the release of nitric oxide and pro-inflammatory cytokines from the satellite glial cells (Thalakoti et al., 2007; Li et al., 2008; Capuano et
al., 2009; Vause and Durham, 2009). We observed that the CGRP receptor components could be located on satellite glial cells within their cytoplasm. This observation was confirmed with the co-localization of a glial cell marker and the distinct organizational pattern around the neurons. Studies performed on rat trigeminal ganglion also demonstrated the expression of the CGRP receptor components on satellite glial cells (Lennerz et al., 2008; Li et al., 2008).

The function of this localization needs to be evaluated, however expression of receptors in satellite glial cells suggest that they can receive signals from CGRP positive neurons and potentially respond to activate local inflammatory signals. Also, it could be hypothesized that CGRP receptor antagonists could have an effect on the satellite glial cells and thereby preventing the release of inflammatory cytokines and nitric oxide.

We found no immunoreactivity for CGRP or the receptor components in Schwann cells. In cross-sectioned nerves, it was clearly shown that CLR and RAMP1 were expressed in neuronal processes, rather than Schwann cells. This suggests that these cells originate from the large neurons and belong to the Aδ-fibers.

CONCLUSION

The neuropeptide CGRP is implicated in the underlying pathology of migraine and the CGRP receptor has long been regarded as a useful target for the development of novel antimigraine therapies. Using immunohistochemistry and new antibodies specifically recognizing CLR and RAMP1, we studied in detail CGRP and its receptor components in the human trigeminal ganglion and compared with that of rat. Our study contributes to a better understanding of CGRP signaling in the trigeminal ganglion. In summary, we found that small to medium sized neurons express CGRP and not CLR and RAMP1, while larger neurons contain only CLR and RAMP1, suggesting that the trigeminal ganglion could be a possible site of action for CGRP receptor antagonists. Interestingly, the receptor components were also located on satellite glial cells and neuronal fibers. Further efforts are essential to evaluate the role of trigeminal ganglion and satellite glial cells in the pathology of migraine.

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REFERENCES


APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroscience.2010.05.016.
Effect of the calcitonin gene-related peptide (CGRP) receptor antagonist telcagepant in human cranial arteries

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Abstract
Introduction: Calcitonin gene-related peptide (CGRP) is a neuronal messenger in intracranial sensory nerves and is considered to play a significant role in migraine pathophysiology.

Materials and methods: We investigated the effect of the CGRP receptor antagonist, telcagepant, on CGRP-induced cranial vasodilatation in human isolated cerebral and middle meningeal arteries. We also studied the expression of the CGRP receptor components in cranial arteries with immunocytochemistry. Concentration response curves to CGRP were performed in human isolated cerebral and middle meningeal arteries in the absence or presence of telcagepant. Arterial slices were stained for RAMP1, CLR and actin in a double immunofluorescence staining.

Results: In both arteries, we found that: (i) telcagepant was devoid of any contractile or relaxant effects per se; (ii) pretreatment with telcagepant antagonised the CGRP-induced relaxation in a competitive manner; and (iii) immunohistochemistry revealed expression and co-localisation of CLR and RAMP1 in the smooth muscle cells in the media layer of both arteries.

Conclusions: Our findings provide morphological and functional data on the presence of CGRP receptors in cerebral and meningeal arteries, which illustrates a possible site of action of telcagepant in the treatment of migraine.

Keywords
CGRP receptors, cerebral arteries, meningeal arteries, telcagepant, MK-0974

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Introduction
The intracranial circulation is supplied by calcitonin gene-related peptide (CGRP)-containing nerve fibres that originate in the trigeminal ganglion (1,2). Activation of the intracranial trigeminovascular system results in release of CGRP (3); this occurs in primary headaches and following subarachnoid hemorrhage (4–7). CGRP is a potent vasodilator in several species (8–10), increases heart rate (11) and has positive inotropic effects on isolated human trabeculae (12). CGRP initiates vascular responses through interaction with G-protein coupled receptors of the B-type that are primarily coupled to the activation of adenyl cyclase and is independent of endothelium in human cerebral, meningeal (1,13,14) and coronary (10) vessels. CGRP receptor characterisations have relied on the use of the antagonist CGRP8–37; more recently, however, several small dipeptide CGRP receptor antagonists have been reported such as olcegepant (BIBN4096BS) (15).
Although this dipeptide derivative is effective in migraine (16), due to its molecular structure it has until now only been parenterally. Recently, an orally bio available CGRP receptor antagonist, telcagepant (MK-0974), was described (17) and shown to be effective as an antimigraine drug (18,19).

The aims of the present study were to: (i) compare the functional responses to αCGRP and the antagonistic effects of telcagepant in human cerebral and meningeal arteries; and (ii) examine the expression of the receptor elements calcitonin-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1) with immunohistochemistry in human cerebral and meningeal arteries.

Materials and methods

Human isolated arteries

Human cerebral (cortex) arteries (5 male, 4 female; age 45–76 years; internal diameter 300–500μm) were removed at neurosurgical tumour operations in Lund, Sweden. Human meningeal arteries (2 male, 2 female; aged 42–62 years; internal diameter 500–750μm) were obtained peri-operatively from patients undergoing neurosurgical procedures at Erasmus MC, Rotterdam, The Netherlands. All vessels were placed in buffer solution, for cerebral arteries, composition in mM: NaCl, 119; KCl, 4.7; CaCl2, 1.5; MgSO4, 1.17; NaHCO3, 25; KH2PO4, 1.18; EDTA, 0.027; glucose, 5.5, pH 7.4; for meningeal arteries, composition in mM: NaCl, 119; KCl, 4.7; CaCl2, 1.25; MgSO4, 1.2; KH2PO4 1.2; NaHCO3 25 and glucose 11.1; pH 7.4 aerated with 5% CO2 in O2 (carbogen) and transported to the laboratory for investigation. The Swedish part of the study was approved by Lund University Ethics Committee (LU99) and had the individual patients’ approval, while the ethics committee dealt with human experimentations at Erasmus Medical Centre, Rotterdam, approved the Dutch part of the study.

Functional experiments

The arteries were cut into cylindrical segments of 1 or 2mm in length for in vitro pharmacological experiments. Each segment was mounted on two metal prongs, one of which was connected to a force displacement transducer and attached to a computer, and the other to a displacement device. The position of the holder could be changed by means of a movable unit allowing fine adjustments of vascular tension by varying the distance between the metal prongs (20,21).

The mounted specimens were immersed in temperature-controlled tissue baths (37°C) containing the buffer solution continuously gassed with carbogen, and the artery segments were allowed to equilibrate for approximately 30 min. The vessel tension was continuously recorded and the distance between the pins or wires was adjusted to maintain a resting tone of 4 mN for cerebral arteries or stretched to a tension normalised to 90% of 100 (the diameter when transmural pressure equals 100 mmHg (21)) for the meningeal segments.

Following the 30-min equilibration period, the contractile capacity of each vessel segment was examined by exposure to a potassium-rich (60 mM) buffer solution which had the same composition as the standard solution except that the NaCl was exchanged for an equimolar concentration of KCl for the experiments performed in the cerebral artery. In the experiments performed in the meningeal artery, vessel segments were exposed to 30 mM KCl once. Subsequently, the tissue was exposed to 100 mM KCl to determine the maximum contractile response to KCl.

The relaxant effect of human αCGRP was examined by cumulative application of increasing concentrations of the peptide in the absence or presence of various concentrations of the antagonist telcagepant. Segments were precontracted with 1 μM U46619 (cerebral arteries) or 30 mM KCl (meningeal arteries) before αCGRP was added. In the meningeal artery, each segment was exposed to a single cumulative concentration-effect curve and a matched pair’s protocol was used where one segment acted as control (no antagonist present) while in another segment from the same artery, the agonist response was assessed following equilibration (20–30 min) with 1 μM of the antagonist. After wash out, the functional integrity of the endothelium was verified by observing relaxation to substance P (1–10 nM) after precontraction with the thromboxane A2 analogue U46619 (10 nM). In the cerebral artery, due to the scarcity of the tissue, cumulative concentration-effect curves in the absence or presence of the antagonist were performed in the same segments. The first curve acted as control (no antagonist present). After washout, the next curve was then performed in the presence of the antagonist (10 nM, 100 nM or 1 μM).

Compounds

The following materials were used in the in vitro experiments: human αCGRP (NeoMPS S.A., Strasbourg, France, and Sigma, St Louis, MO, USA, for the Dutch and Swedish experiments, respectively) and U46619 (Sigma). Telcagepant (MK-0974) was synthesized by the Medicinal Chemistry Department, Merck Sharp and Dohme Research Laboratories, USA. The αCGRP and U46619 were dissolved in water and stored in aliquots at −20°C. Telcagepant was dissolved in dimethylsulphoxide (DMSO) and stored...
in aliquots at −20°C. When the compounds were to be used, they were diluted in saline.

**Analysis of data**

The vasodilator response to CGRP was expressed relative to the contraction evoked by U46619 or KCl, respectively (=100%). For each segment, the maximum vasodilator effect (E\text{max}) was calculated. The concentration-response curves for all agonists were analysed using non-linear regression analysis and the potency of agonists was expressed as pEC\text{50} (i.e. negative logarithm of the molar concentration of agonist inducing half maximum response) using Graph Pad Prism v.4.0 (Graph Pad Software Inc., San Diego, CA, USA). The blocking potency of the antagonists was estimated by calculating EC\text{50} ratios and plotting a Schild-plot (22) using linear regression to get the slope value. The pA\text{2} represents the negative logarithm of the concentration of antagonist that induces a 2-fold shift of the concentration response curve to the right. This parameter can be calculated in the case of competitive antagonism, i.e. when the slope of the Schild-plot is equal to unity. In meningeal arteries, only one concentration of telcagepant was studied; in these cases, ‘apparent pK\text{B}’ (a parameter similar to the pA\text{2}, which is used in cases where antagonism has not been demonstrated to be competitive in nature) values were calculated, constraining the Schild slope to unity. Since it was not feasible to use agonist concentrations higher than 3 \( \mu \)M, concentration response curves in the presence of higher concentrations of antagonist did not always reach a plateau. In these cases, the concentration response curves were extrapolated, considering the maximal response in the absence of antagonist as E\text{max}.

Data are expressed as mean values ± SEM and ‘n’ refers to the number of patients from whom the vessels were collected. Statistically significant differences in pEC\text{50} values were examined by Mann–Whitney U-test.

**Immunohistochemistry**

For immunofluorescence, the cerebral and meningeal artery segments obtained peri-operatively from patients were directly snap-frozen immediately after arrival in the laboratories and were then embedded in Tissue TEK (Gibco, Invitrogen A/S, Taastrup, Denmark), frozen at −80°C and subsequently sectioned into 10-µm thick slices. Cryostat sections were fixed for 10 min in ice-cold acetone (−20°C) and thereafter rehydrated in phosphate-buffered saline (PBS, pH 7.2) containing 0.25% Triton X-100 (PBST), for 3 × 5 min. The sections were then permeabilised with PBST and blocked for 1 h in blocking solution containing PBS and 5% normal donkey serum and then incubated overnight at 4°C with either of the following primary antibodies: rabbit anti RAMP1 (Santa Cruz Biotechnology, CA, USA; sc-11379) diluted at 1:50, or rabbit anti CLR (Alpha Diagnostic International, SA, USA; CRLR-11A) diluted at 1:100. The dilutions of the primary antibodies were done in PBST, 1% bovine serum albumin (BSA) and 3% normal donkey serum. On the second day, sections were washed at room temperature and rinsed in PBST (3 × 15 min). Sections were subsequently incubated with the secondary antibody (1 h, room-temperature). The secondary antibody used was Cy\text{TM2} conjugated donkey anti rabbit (Jackson ImmunoResearch, West Grove, PA, USA; 711-165-152) diluted 1:200 in PBST and 1% BSA. The sections were washed subsequently with PBST and mounted with Crystal mounting medium (Sigma). To determine the cellular localisation of RAMP1 and CLR, double immunofluorescence was performed by addition of a mouse anti-smooth muscle actin antibody (Santa Cruz; sc-53015) diluted 1:200 in PBST, 1% BSA and 3% normal donkey serum. As secondary antibody, Texas Red-conjugated donkey anti-mouse was used (Jackson ImmunoResearch; 715-076-150), diluted 1:200 in PBST and 1% BSA. In order to co-localise RAMP1 and CLR, a new CLR antibody was used (anti-goat, 1:50; Santa Cruz; sc-18007). Vectashield medium containing 4',6-diamidino-2-phenylindole (DAPI) staining nucleuses was used on some sections (Vectashield, Vector Laboratories Inc., Burlingame, CA, USA).

Immunoreactivity was visualised and photographed with an Olympus microscope (BX 60, Japan) at the appropriate wavelength (FITC Filterblock N B-2EC, EX465–495, EM515–555; Filterblock N G-2EC, EX540/25, EM605/55; DAPI Filterblock N UV-2EC, EX540–495, EM605/55; Filterblock N G-2EC, EX465–495, EM515–555; Filterblock N B-2EC, EX465–495, EM515–555). Adobe Photoshop CS3 was used to visualise co-labelling by superimposing the digital images. Negative controls for all antibodies were made by omitting primary antibodies. In all cases, no specific staining was found; only autofluorescence in lamina elastica interna was seen (not shown). To evaluate the autofluorescence in lamina elastica interna, controls were made with only primary antibodies.

**Results**

**Functional studies to αCGRP in human isolated arteries**

The cumulative administration of αCGRP caused a concentration-dependent relaxation of cerebral arteries precontracted with U46619, yielding a pEC\text{50} value of 9.0±0.2 and an E\text{max} value of 56±6% (Figure 1). Also, in the meningeal artery, αCGRP induced a concentration-dependent relaxation, the E\text{max} amounting to 50±11%
of precontraction with 30 mM KCl and pEC$_{50}$ value of 8.7 ± 0.1 (Figure 2).

**Effects of telcagepant in human isolated arteries**

The CGRP receptor antagonist telcagepant, tested in concentrations up to 10 µM, did not show any marked vasomotor responses in any of the isolated vessel segments at basal tone (contraction response with E$_{max}$ of 2.0 ± 2.9%; $n = 9$). Pretreatment with telcagepant at increasing concentrations (10 nM to 1 µM) shifted the concentration response curves to aCGRP to the right without changing the maximal effect (Figure 1). In the meningeal arteries, CGRP induced an E$_{max}$ of 55 ± 7% in the presence of 1 µM telcagepant (Figure 2). The pA$_2$ value amounted to 9.37 ± 0.12 in cerebral artery; the slope of the Schild plot did not differ from unity (0.97 ± 0.17). In the meningeal arteries, the apparent pK$_B$ value was 8.03 ± 0.16 (pA$_2$ could not be calculated since only one concentration of telcagepant was studied, the slope of the plot was constrained to unity to calculate the apparent pK$_B$).

**Immunohistochemistry of human arteries**

The distributions of RAMP1 and CLR in human cerebral (Figure 3A) and meningeal (Figure 3B) arteries were studied by immunohistochemistry. We observed positive immunoreactions for RAMP1 and CLR in the smooth muscle cell layer (media layer) of cerebral and meningeal artery segments. Localisation of the CGRP receptor components in the smooth muscle layer was confirmed by double staining with an antibody specific for actin. In separate experiments using another CLR antibody, we verified that the two receptor components RAMP1 and CLR co-localised in the smooth muscle cells (Figure 4). There were no obvious positive immunoreactions in the endothelium or in the

![Figure 1](image1.png)

**Figure 1.** Relaxant effect of aCGRP on human cerebral arteries that were precontracted with U46619. Concentration response curves to aCGRP in the absence or presence of increasing concentrations of telcagepant (left). There was a clear shift to the right in the concentration effect curve. The average Schild plot of the concentration response curves in the cerebral arteries (right). Values given represent mean ± SEM; number of subjects, $n = 9$.

![Figure 2](image2.png)

**Figure 2.** Relaxant effect of aCGRP on human meningeal arteries that were precontracted with 30 mM KCl. Concentration response curves to aCGRP in the absence or presence of 1 µM telcagepant (left). There was a clear shift to the right in the concentration effect curve. The apparent pK$_B$ value was calculated with a slope (dotted line) constrained to the unity (right). Values given represent mean ± SEM; number of subjects, $n = 4$. 
Figure 3. Immunohistochemistry of human cerebral (A) and meningeal (B) artery segments. Antibodies specific for RAMP1 and CLR showed positive staining in the walls of the artery segments (insert, a higher magnification). Co-staining with actin-specific antibody revealed the localisation of the immunoreactions in the smooth muscle cells. We observed no staining in endothelium or in the adventitial layers. Marker, 100 μm.

Figure 4. Immunohistochemistry of human cerebral (A) and meningeal (B) artery segments. Antibodies specific for RAMP1 and CLR showed positive staining in the cytoplasm of the smooth muscle cells in walls of the artery. The receptor components co-localised (merged, arrows). DAPI (blue), staining of the nuclei. Marker, 100 μm.
Discussion

CGRP receptors have long been regarded as a useful target for the development of novel antimigraine therapies (23). In this study, we have shown that CGRP induced vasodilatation and that telcagepant had no direct vasoconstrictor or vasodilator effect per se on isolated human cerebral and meningeal arteries. However, telcagepant was able to block, in a competitive manner, the vasodilator effect of αCGRP on these blood vessels. We also showed that the CGRP receptor complex, consisting of CLR and RAMP1, is co-expressed in the smooth muscle cells of human cerebral and meningeal arteries and not present in the endothelium or in the adventitia, which is in agreement with a previous study of human intracranial arteries (24).

As stated above, telcagepant did not show any evidence of direct vasoconstrictor or vasodilator effect when given alone. If the same will apply to observations made in vivo, these findings suggest that CGRP receptors normally do not have a tonic role (25). This profile of telcagepant agrees well with previous studies of CGRP receptor antagonists such as CGRP\textsubscript{8–37}, Compound 1 (26), and olecegant (10,13,27).

The relaxant responses to αCGRP were not different in cerebral and meningeal arteries and were in accordance with previous studies (13,26,28). Telcagepant antagonised relaxations induced by αCGRP with a potency that seemed somewhat lower in the middle meningeal than in the cerebral artery. Because of the small methodological differences between the protocols used for the both tissues, no statistical comparison was made. However, since the potency of telcagepant was determined using the control curves in each respectively tissue, the results obtained in the two laboratories should be comparable. Further, in pilot experiments we demonstrated that the different precontractions used in the two laboratories do not affect the response to CGRP (data not shown). When comparing the effects of telcagepant with those of the CGRP receptor antagonist olecegant, telcagepant shows activity in higher concentrations (about one to two log units) (13,26), thus being less potent than olecegant which agrees with the \( K_i \) of respective compounds (telcagepant, 0.77 nM; olecegant, 0.014 nM) (29,30).

However, telcagepant has the advantage that it can be given as an oral medication (19), whereas olecegant is not orally available (16).

In comparing the clinical effects, it is interesting that both compounds required substantially higher plasma concentrations relative to their \( \text{in vitro} \) \( pA_2 \) to achieve clinical efficacy for the acute treatment of migraine (16,19). For example, plasma concentrations of telcagepant associated with clinical efficacy are in the micromolar range, which is substantially higher than the \( pA_2 \) that we have seen for the cranial vascular effect (in the nanomolar range). Several factors may be involved in explaining this discrepancy, namely:

1. The difference may be due, in part, to the high protein binding of these compounds.
2. A concentration of drug equal to the \( pA_2 \) value may not be sufficient to decrease functional responses since it only shifts the concentration response curves 2-fold to the right – most likely a concentration of at least 10 times \( pA_2 \) would functionally inhibit relaxations to CGRP.
3. As nerve terminals releasing CGRP are located in the adventitia close to the media layer of the blood vessels, the concentration of telcagepant at the receptors may be substantially smaller than that at the lumen of the blood vessel, i.e. the plasma concentration. This phenomenon is unlikely to occur in \textit{vivo}, where the antagonist can reach the CGRP receptors from both the luminal and abluminal sides.
4. The therapeutic effect of CGRP receptor antagonists could obviously also be mediated via other pathways than only inhibition of blood vessel dilation induced by CGRP. Penetration of telcagepant through the blood–brain barrier may be necessary in addition to the peripheral blockade to achieve anti-migraine efficacy (31).

Arguments in favour of a neuronal mechanism are the lack of presynaptic CGRP receptors in the meninges, which suggests that exogenous CGRP is unlikely to modify the innervating sensory nerve fibres directly (32). This finding is also in agreement with \textit{in vivo} data obtained in the rat, suggesting that an action of CGRP on the dura mater cannot account for the activation of peripheral afferents during migraine (33). In this study, the effects of CGRP in the meninges, including meningeal vasodilatation, were not sufficient to activate or sensitize meningeal nociceptors. Clearly, further studies are needed to resolve the therapeutic mechanisms involved of CGRP receptor antagonists.

Conclusions

Telcagepant antagonises relaxations induced by αCGRP with a potency that is consistent between human cerebral and meningeal arteries, without affecting the vascular tone per se. To predict potential vascular side effects, the effects of telcagepant obviously have to be investigated in more arteries, including the

Lamina elastica interna; the latter is strongly autofluorescent, especially in the green filter.
human coronary artery, as well as an in vitro model. Also, it remains to be demonstrated whether inhibition of vasodilatation by CGRP mediates the therapeutic action of telcagepant or that central penetration is also required. Our findings on the vascular properties of telcagepant provide insight into a possible site of action in the treatment of migraine.

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Lars Edvinsson and Kayi Y. Chan contributed equally to this study.

References


Differentiation of Nerve Fibers Storing CGRP and CGRP Receptors in the Peripheral Trigeminovascular System

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Abstract: Primary headaches such as migraine are postulated to involve the activation of sensory trigeminal pain neurons that innervate intracranial blood vessels and the dura mater. It is suggested that local activation of these sensory nerves may involve dural mast cells as one factor in local inflammation, causing sensitization of meningeal nociceptors. Immunofluorescence was used to study the detailed distribution of calcitonin gene–related peptide (CGRP) and its receptor components calcitonin receptor–like receptor (CLR) and receptor activity–modifying protein 1 (RAMP1) in whole-mount rat dura mater and in human dural vessels. The relative distributions of CGRP, CLR, and RAMP1 were evaluated with respect to each other and in relationship to mast cells, myelin, substance P, neuronal nitric oxide synthase, pituitary adenylate cyclase-activating polypeptide, and vasoactive intestinal peptide. CGRP expression was found in thin unmyelinated fibers, whereas CLR and RAMP1 were expressed in thicker myelinated fibers coexpressed with an A-fiber marker. CLR and RAMP1 immunoreactivity colocalized with mast cell tryptase in rodent; however, expression of both receptor components was not observed in human mast cells. Immunoreactive substance P fibers coexpressed CGRP, although neuronal nitric oxide synthase and vasoactive intestinal peptide expression was very limited, and these fibers were distinct from the CGRP-positive fibers. Few pituitary adenylate cyclase-activating polypeptide immunoreactive fibers occurred and some colocalized with CGRP.

Perspective: This study demonstrates the detailed distribution of CGRP and its receptor in the dura mater. These data suggest that CGRP is expressed in C-fibers and may act on A-fibers, rodent mast cells, and vascular smooth muscle cells that express the CGRP receptor. These sites represent potential pathophysiological targets of novel antimigraine agents such as the newly developed CGRP receptor antagonists.

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Key words: Migraine, calcitonin gene–related peptide (CGRP), calcitonin receptor–like receptor (CLR), receptor activity–modifying protein 1 (RAMP1), dura mater, mast cells.

Activation of sensory trigeminal pain neurons is postulated to play a central role in migraine attacks. This activation assembly takes place in deep-brain structures and involves many central neurons. There are 2 opposing views regarding the peripheral nociceptors in the dura mater and in cerebral arteries: 1) early in the migraine attack, these sensory fibers propagate signals from the central nervous system to dural vessels that activate the peripheral nociceptors, which is a referred type of pain, and 2) the headache phase begins with the activation of peripheral nociceptors that then results in activation of central aspects of pain. The latter view defines the rationale for administering systemic vasoactive drugs to induce migraine-like attacks and studying the mechanisms involved. One caveat is that this may only occur in the sensitized brains of migraineurs, who are already susceptible to a migraine attack, and not in nonmigraineurs.

Whatever the cause, the abnormal activation of peripheral nociceptors in the intracranial vasculature and meninges results in headache (peripheral sensitization). Electrical stimulation of dural and cerebral arteries, but...
not the brain parenchyma itself, evoked nausea and referred pain in conscious humans. Interestingly, stimulation of smaller arteries of the brain or in the pia mater distal to the major arteries did not show referred pain response, despite the fact that these vessels also contain sensory fibers and receptors.

The dura mater is a pain-sensitive structure, and early studies suggested that the dura mater contains nerve fibers expressing substance P and calcitonin gene-related peptide (CGRP) belonging to the sensory system. Further, whereas neuropeptide Y fibers are sympathetic, those positive for vasoactive intestinal peptide (VIP) and pituitary-adrenocorticotropin (ACTH) are of parasympathetic origin.

The sensory fibers originate in the trigeminal ganglion, whereas the sympathetic fibers originate in the superior cervical ganglion and the parasympathetic fibers in the sphenopalatine and otic ganglia. Mast cells are especially plentiful in the dura mater, but few occur in the brain or in the cerebral blood vessels. They contain and can release numerous vasodilatory, proinflammatory, and neurosensitizing molecules and are proposed to be involved in migraine pathophysiology.

In addition, quantitative studies have shown a rich supply of mast cells containing histamine in the dura mater. Functional studies have shown that increasing concentrations of CGRP can cause histamine release from the rat dura via degranulation of mast cells.

Because the dura mater is considered to play a central role in acute migraine attack pain, we have conducted a detailed study of expression of CGRP and its receptor components. We evaluated CGRP and the CGRP receptor components relative to each other and other neuronal messengers in dura mater. Additionally, we compared the expression in dura mater to other migraine-relevant structures, which included both cerebral vessels and trigeminal ganglion. Our aim was to examine the type of sensory nerves that express CGRP and its receptor components in the dura mater of rat and human using immunohistochemistry with well-characterized specific antibodies. Finally, because neurogenic inflammation is thought to be involved in migraine pathophysiology, we determined whether or not dural mast cells express the CGRP receptor in rat and humans.

Methods

Rat Tissue Preparation

Eight Sprague Dawley rats (Scanbur, Stockholm, Sweden) weighing 300 to 350 g were used for the study. The experiments were approved by the University Animal Ethics Committee (M8-09), Lund University, Sweden. The animals were anesthetized with an intraperitoneal dose of 2.5 mL/kg with a mixture of Hypnorm-midazolam (1:1:2) in sterile water (containing 0.79 mg/mL fentanyl, 2.50 mg/mL fluanison, and 1.25 mg/mL midazolam). The animals were then perfused transcardially with a prewash of phosphate-buffered saline (PBS, pH 7.2) for 2 minutes followed by 4% paraformaldehyde in PBS for 5 minutes. The dura mater, cerebral vessels, and trigeminal ganglia were directly dissected. The dura mater was cut into smaller pieces (0.5–1 cm) and processed as whole-mount preparations on glass slides. Immediately after perfusion-fixation, the specimens were further fixed in paraformaldehyde for 1 to 3 hours and washed in PBS pH 7.2 containing 0.25% Triton X-100 (PBST) prior to immunohistochemistry.

Human Dura Vessels

Human dura vessels were obtained from 3 patients (ages 23, 76, and 76, sex; 2 men, 1 woman) undergoing neurologic surgery for subarachnoid hemorrhage and epilepsy. The vessels (mainly meningeal artery) were dissected during surgery and immediately immersed in cold, sterile Dulbecco’s modified Eagle’s medium (Gibco; Invitrogen, Carlsbad, CA). Each vessel was cut into 2 or 3 segments and fixed in 4% paraformaldehyde for 1 to 2 hours. After fixation, the vessels were rinsed in rising concentrations of sucrose in Sörensen’s phosphate buffer. The samples were embedded in Tissue-Tek (Sakura Fine-tek, Torrance, CA) and cryosectioned at 10 μm. The sections were stored at −20°C until use. The human study was approved by the Regional Ethical Review Board in Lund, Sweden (LU-818-01).

Immunohistochemistry

Rat dura mater whole mounts, cerebral vessel, trigeminal ganglia, and human dura vessels were washed for 10 minutes in PBST, followed an incubation with a blocking solution of PBS and 5% normal donkey serum for 1 hour. After blocking, the specimens were incubated overnight at 4°C with primary antibodies against CGRP, calcitonin receptor–like receptor (CLR), and receptor activity–modifying protein 1 (RAMP1) (Table 1). The specificity of CLR and RAMP1 has been evaluated in a previous study. Double immunostainings were performed with CGRP, CLR, or RAMP1 in combination with mast cell tryptase, actin, myelin basic protein (MBP), nerve filament high molecular weight 68 or 160/200 (NF68 and NF160/200), synaptosomal-associated protein of 25 kDa (SNAP-25), substance P, neuronal nitric oxide synthase (nNOS), PACAP, or VIP. For double immunolabeling, the antibodies were applied separately and not mixed as a cocktail. The primary antibodies were diluted in PBST containing 1% bovine serum albumin and 3% normal serum. After incubation with primary antibodies, sections were washed with PBST for 3 × 15 minutes and exposed to secondary antibodies in PBST and 1% bovine serum albumin for 1 hour at room temperature (Table 2). The specimens were subsequently washed with PBST for 3 × 15 minutes and mounted with an antifading mounting medium containing nuclei staining DAPI (VECTORSHIELD; Vector Laboratories, Burlingame, CA). All stainings were repeated 3 to 5 times to confirm the results and all slides were analyzed. The trigeminal ganglion experiments were focused on the main body of the trigeminal ganglion.

Microscopic Analysis

Sections were examined and images were obtained using an epifluorescence microscope (Nikon 80i; Nikon, Tokyo, Japan) coupled to a Nikon DS-2 MV camera. The
stainings were also examined in Nikon confocal microscope (EZ-cl, Nikon, Dusseldorf, Germany), using 20x or 60x oil immersion lenses. Z (frame) stacks were acquired using different laser channels 1 by 1 before next z position was acquired. The thickness (depth) of the Z-stack images were 12.60 to 72 μm. Laser channels used were 488 nm excitation (filter 515/30) and 543 nm excitation (filter 605/75).

All double stainings were viewed 3-dimensionally to examine possible colocalizations. Image analyses were conducted using NIS Basic Research software (Nikon, Tokyo, Japan). Adobe Photoshop CS3 (version 8.0; Adobe Systems, Mountain View, CA) was used to visualize colabeling by superimposing the digital images, and images were processed for brightness and contrast.

Results

Rat Dura Mater and Trigeminal Ganglion

CGRP, CLR, and RAMP1

Numerous CGRP immunoreactive fibers were identified in rat dura mater (Fig 1A). These nerve fibers ran parallel to or across the vessels, but not in the vessel walls or in any visible cell somata. The CGRP immunoreactivity resembled pearl-like structures and was found in thin, long, branched nerve fibers (Fig 1A). In addition, we investigated if CGRP immunoreactivity varied between periosteal and meningeal sides of the dura mater. No obvious differences were found in CGRP expression in the various sides of the dura mater.

CGRP receptor components immunoreactivity was detected in nerve fibers, vessels, and cells (Figs 1B and 1C), but not all RAMP1-positive nerve fibers expressed CLR (Fig 1B). CLR and RAMP1 were found in thicker and less-branched nerve fibers, compared to those containing CGRP, and were seen both as single fibers and as fiber bundles (Fig 1B). These fibers were found along the vessels and in the adjacent dural tissue. In addition, CLR and RAMP1 expression was detected within the vessel walls corresponding to the medial smooth muscle cell layer. CLR and RAMP1 immunoreactivity was found in cells throughout the dura mater (Fig 1C). CLR and RAMP1 colocalized in the smooth muscle cell layer of the dura vessels (data not shown).

Colocalization of CGRP and the receptor components, CLR and RAMP1, was assessed using double labeling. CGRP did not colocalize with CLR or RAMP1 (Fig 2; for details see Videos 1 and 2, available at www.jpain.org). Delicate fibers expressing CGRP and thicker fibers expressing CLR or RAMP1 could be found running close together in a parallel manner, but they did not coexpress in the same fiber (Fig 2). Additionally, double staining identified CGRP-positive fibers close to CLR- or RAMP1-positive vessels (Fig 2A). Confocal microscopy confirmed that CGRP and the receptor components were not expressed in the same fibers (Figs 2A and 2D).

Mast Cell Tryptase

Double staining of mast cell tryptase marker and CLR or RAMP1 showed colocalization in the cytoplasm (Fig 3; for details see Video 3, available at www.jpain.org), suggesting that mast cells express functional CGRP receptors.
SNAP-25

Double immunolabeling of CGRP and the synaptic vesicle marker, SNAP-25, revealed their coexpression in thin, long fibers (Figs 4A and 4B). In addition, fibers expressing only SNAP-25 or CGRP were found (Fig 4A). In trigeminal ganglion, SNAP-25 immunoreactivity was observed as vesicular profiles in the neurons (Fig 4C). Double staining of SNAP-25 and CGRP showed coexpression in the vesicular structures in the cytoplasm of the cells (Fig 4C), but not all SNAP-25-positive cells were positive for CGRP.

NF68 and NF160/200

To establish the type of fibers that express CGRP and the CGRP receptor components, double staining was performed with markers for neurofilaments sized 68 kDa (NF68) or 160/200 kDa (NF160/200). Because CGRP was found in thinner fibers, we first examined possible coexpression of CGRP and the NF68 marker. This revealed no colocalization (data not shown). Furthermore, we observed that CLR and RAMP1 were detected in thicker nerve fibers coexpressing with NF160/200 (used as a marker for A-fibers) (Fig 5). This was verified by confocal microscopy (see Video 4, available at www.jpain.org).

MBP

To determine if CGRP and the CGRP receptor components were expressed in myelinated or unmyelinated fibers, double staining was performed with MBP. Double labeling with CGRP and MBP showed no coexpression (Fig 6; also see Video 5, available at www.jpain.org).
Double staining with CLR or RAMP1 and MBP showed presumed colocalization in fibers (Fig 7A). Detailed confocal analysis showed that CLR and RAMP1 were expressed within the axon and not in the surrounding MBP-positive sheath (Fig 7B; also see Video 6, available at www.jpain.org).

Figure 2. CLR/RAMP1 and CGRP double staining in rat dura mater. (A) Confocal analysis of double staining with CLR and CGRP. CLR (green arrows) is expressed in cells, within the vessel wall, and in thicker fibers. CGRP expression (red arrows) is instead found in thin fibers running across vessels or close to CLR-positive cells and fibers. No colocalization is found between CLR and CGRP. (B) Double staining of CGRP (red, thin arrows) and RAMP1 (green, thick arrows), where CGRP and RAMP1 are expressed in separate fibers. (C) High magnifications of RAMP1-positive (green arrows) and CGRP-positive (red arrows) fibers, which could run close to each other but not expressed in the same fiber. (D) Confocal image of CLR-positive (green, thick arrow) and CGRP-positive (red, thin arrow) fibers with no colocalization. For more details, see Videos 1 and 2, available at www.jpain.org.

Substance P

All thin immunoreactive substance P fibers were found to coexpress with CGRP (Fig 8A). However, the CGRP-positive fibers outnumbered the substance P–positive fibers, and consequently many CGRP fibers

Figure 3. Double staining with mast cell tryptase and CLR or RAMP1. (A) Double staining of CLR (green) and mast cell tryptase (red). CLR is found in fibers (thick arrow) and cells, which colocalize with mast cell tryptase (thin arrows). Insert shows their colocalization in the cytoplasm. (B) Coexpression of RAMP1 and mast cell tryptase in cells of dura mater. Nuclei staining (DAPI, blue) is used in the merged pictures. For more details, see Video 3, available at www.jpain.org.
displayed no substance P immunoreactivity. Double staining of substance P and CGRP in the trigeminal ganglion showed colocalization only in the smaller neurons (Fig 8 B). The medium-sized CGRP-positive neurons did not express substance P, although all substance P-positive neurons were CGRP positive. CGRP-positive neurons outnumbered the substance P immunoreactive cells.

**nNOS**

Immunostaining with nNOS revealed a very limited number of fibers displaying immunoreactivity. These fibers were thin and were very few in number. The nNOS immunoreactive fibers did not coexpress CGRP, although the nNOS- and CGRP-positive fibers could be found running close to each other (Fig 9A).

**VIP**

Thin and sparse VIP-positive fibers were expressed in the dura mater. These did not colocalize with CGRP (Fig 9B).

**PACAP**

There were very few fibers expressing PACAP in the dura mater. When detected, only a single PACAP-expressing fiber was found. Double staining of PACAP and CGRP showed coexpression in fibers of dura mater (Fig 9C).

**Rat Cerebral Vessels**

The expression of CGRP, nNOS, PACAP, and VIP in dura mater was compared to that observed in cerebral vessels. We found no obvious difference in the density of the network of nerve fibers expressing CGRP; however, the
innervation of nNOS, VIP, and PACAP was richer in the cerebral vessels compared to that observed in the dura mater (Fig 10).

**Human Dura Vessels**

**CGRP, CLR, and RAMP1**

Pearl-like, immunoreactive CGRP fibers were found in the adventitia of human dural vessels (mainly middle meningeal artery) (Fig 11A). Expression of the receptor components was observed in the smooth muscle longitudinal and cross-sectioned cell layers (Fig 11B). CLR and RAMP1 were coexpressed in the smooth muscle cell layer (Fig 11C). In addition, occasional fibers in the adventitia displayed immunoreactivity for the receptor components (Fig 11D).

**Mast Cell Tryptase**

To determine if the human material contained mast cells, staining was performed with mast cell tryptase. We observed mast cell tryptase–positive cells close to the adventitia in some of the human dural vessels (Fig 12A). Double staining with mast cell tryptase showed colocalization with CLR (Fig 12B) but not with RAMP1 (Fig 12C).

**Negative Controls**

In the negative controls, where the primary antibodies were omitted, no fluorescence was observed in the rat material. In human dura vessels, fluorescence observed in the endothelium was a result of nonspecific binding of the secondary antibodies and auto-fluorescent lamina elastic interna. The generation and specificity of the antibodies against CLR and RAMP1 have been described in our previous study, in which their specificity was confirmed in HEK293 cells stably expressing the human CGRP receptor. The specificity was also confirmed by Western blotting.

**Discussion**

CGRP and its receptor are widely expressed in the trigeminovascular system and play an important role in migraine pathophysiology. Recently, CGRP receptor antagonists have been developed with clinical efficacy. Intense activation of the sensory system in rodents may result in neurogenic inflammation along with extravasation of plasma proteins and sensitization of nociceptors, all considered to be an important part of the migraine attack. This has not been verified in humans. It is suggested that the neuropeptides located in the dura mater can be released from sensory nerve endings, causing vasodilation, plasma protein exudation, and degranulation of mast cells. Over the last decades, our scientific understanding of the pathophysiology of migraine has advanced. This understanding coupled with the advent of novel methods and specific antibodies provides us a more intricate description of the anatomic relations in dura mater.

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**Figure 5.** Double staining of CLR or RAMP1 with NF160/200. Confocal images showing coexpression of CLR or RAMP1 with NF160/200 (used as a marker for A-fibers) in the thicker nerve fibers of dura mater (arrows). For more details, see Video 4, available at www.jpain.org.
In order to provide more insight into the innervation of the dura mater, we examined in detail the association between CGRP and its receptor, other signal molecules, mast cells, and both myelinated and unmyelinated fibers in the peripheral trigeminovascular system.

**Figure 6.** Double staining of CGRP and MBP (myelin). (A) CGRP (red) and MBP (myelin, green) double staining in rat dura mater. CGRP (thick arrows) are distinct from MBP-positive fibers (thin arrows). Arrowheads point at CGRP-positive fibers/areas where no MBP immunoreactivity is found. (B) Detailed confocal analysis of CGRP (red arrows) and myelin expression (green arrows), showing no co-expression. The image in the middle is also shown in Video 5, available at www.jpain.org.

**Figure 7.** RAMP1/CLR and MBP (myelin) double staining. (A) Double staining of RAMP1 with MBP in nerve fibers of rat dura mater. (B) The receptor components (red arrows) are expressed within myelin (green arrows) ensheathed axons, and not within myelin itself. Also see Video 6, available at www.jpain.org.

**CGRP, CLR, and RAMP1 in Rat Dura Mater**

The dura mater contains 2 separate layers, an outer periosteal layer (attached to the inner face of the skull) and an inner meningeal layer. In the present study, we found CGRP expression in thin, branched nerve
fibers, with no obvious difference between periosteal and meningeal sides of the dura mater.

The receptor components, CLR and RAMP1, were coexpressed in thicker fibers that were separate from the CGRP-positive fibers. Moreover, CLR and RAMP1 were found in vessel walls and cells. This suggests that CGRP acts postjunctionally in the dura mater on CGRP receptors in the vascular smooth muscle, on mast cells, and more speculatively to modulate signaling in the associated A-fibers. This is in agreement with our previous studies on trigeminal ganglion and the brainstem in which CGRP and the receptor components were found in different neurons and separate fibers.16,17 These results provide anatomic evidence for expression of CGRP receptors in these elements of rat dura mater.

To evaluate the CGRP expression in more detail, it was examined with SNAP-25, which is a membrane-bound presynaptic nerve terminal protein that has a role in synaptic vesicle fusion and exocytosis.23,52 Double staining of SNAP-25 and CGRP showed coexpression in fibers of the dura mater and in vesicular structures in neurons of the trigeminal ganglion. Therefore, CGRP-containing vesicles are present and can be released upon activation of the C-fibers. These results are in agreement with a previous study showing that SNAP-25 was localized in the majority of trigeminal neurons in all regions of the trigeminal ganglion, suggesting the capability of neuronally mediated paracrine signaling within the ganglia.11

Additionally, we examined the type of fibers that express CGRP and the receptor components using NF markers of different sizes. Three differently sized neurofilaments (light, NF-L, 61 KDa; medium, NF-M, 90 kDa; and heavy, NF-H, 115 kDa) are the most abundant structural components of large myelinated axons.19,28 We found that CGRP did not colocalize with the light NF68 marker, which more convincingly points toward CGRP expression in C-fibers. Our results are in agreement with earlier studies demonstrating CGRP and substance P in very thin nerve fibers32 and in small ganglion cells.39,55 The NF160/200, used as a marker for A-fibers,44 colocatalized with the receptor components. These results are in accordance with a previous study on trigeminal ganglion in which thin CGRP-positive fibers did not colocalize with NF68, and the thicker fibers positive for CGRP receptor components colocatalized with NF160/200.17 Taken together, these results suggest that the CGRP receptor is expressed in A-fibers.

Rat dura mater is richly supplied by myelinated A-fibers and unmyelinated C-fibers and contains approximately 3 times more unmyelinated fibers than myelinated.1 In the present study, we examined if
CGRP and its receptor are expressed in myelinated or unmyelinated fibers. CGRP did not colocalize with the myelin-specific marker MBP. This is in agreement with detailed structure analysis of CGRP-positive nerve fibers in cat dura mater, in which it was demonstrated that small-diameter unmyelinated fibers expressed CGRP.35

CLR and RAMP1 were expressed in fibers that were also positive for MBP. Detailed confocal microscopy analysis showed that the receptor components were localized within the axon and surrounded by a myelin-positive sheath (see details in Fig 7). Lennerz and coworkers29 previously described CLR expression in Schwann cells, a result that we could not confirm.

In conclusion, our results demonstrate that CGRP is expressed in unmyelinated fibers and the receptor components in myelinated, which is in agreement with our previous study on the spinal trigeminal nucleus.16

**Mast Cells**

It has been suggested that CGRP receptors are expressed on cells of the dura mater, putatively mast cells.29 In the present study, we identified expression of both receptor components in rat dura mater cells. Double staining with mast cell tryptase confirmed that these cells were mast cells. The results further validate the presence of functional CGRP receptors in rat dural mast cells as CLR and RAMP1 are coexpressed.

Mast cells were also found in some of the human vessels, close to or outside the adventitia. CLR and mast cell tryptase coexpression was observed, but not mast cell tryptase and RAMP1. The expression of only CLR in human mast cells could reflect the presence of other receptors that contain CLR, such as the adrenomedullin receptors, which are formed when CLR is coexpressed with RAMP2 or RAMP3.34

These results suggest that CGRP receptors may not be present in human mast cells and agree with the early finding that CGRP failed to induce release of histamine from dural mast cells in humans following administration of excess CGRP.43 To our knowledge, there is no previous report of the expression of CGRP receptors in human dural mast cells. The limitation with this study is that we only could study human peri-arterial mast cells rather than whole dura mater. CLR/RAMP1 expression in mast cells in whole-mount primate dura mater remains to be studied.

![Figure 9](image-url)

**Figure 9.** Double staining of nNOS, VIP, or PACAP with CGRP in dura mater. (A) Few nNOS-positive fibers (thick arrows) were found, and these fibers were separate from CGRP-positive fibers (thin arrows). (B) VIP-positive fibers (thick arrow) are thin and sparse in dura mater and do not colocalize with CGRP (thin arrow). (C) Single PACAP-positive fiber that is coexpressed with CGRP (thicker arrows). Not all CGRP-positive fibers expressed PACAP (thin arrows).
Nerve Fibers Containing Substance P, nNOS, VIP, and PACAP in Dura Mater and Cerebral Vessels

In the present study, we studied the nerve fiber distribution in the dura mater with that of the cerebral circulation. As can be seen in Fig 10, the supply of CGRP fibers in brain vessels correlated well with the density of supply in dura mater. The parasympathetic nerve markers VIP, nNOS, and PACAP were markedly weak (few immunopositive fibers) in the dura mater, whereas a considerably richer supply is seen in cerebral vessels. This is consistent with the innervation of the tributing artery, the middle meningeal artery, and the dura mater of guinea pigs, which also has very little parasympathetic nerve supply.  

The present study demonstrates that the expression of CGRP seems to be more prominent than that of
substance P, nNOS, VIP, and PACAP in both dura mater and trigeminal ganglion. More CGRP-positive fibers and neurons were observed in comparison to the other neuropeptides. This is in agreement with a study on cat dura mater, in which CGRP nerve fibers were more abundant than substance P and less VIP innervation has been demonstrated in rat dura mater. In agreement, it has been shown that there is a lower concentration of CGRP and substance P immunoreactivity in human middle meningeal arteries as compared to human cerebral arteries. Recently, measurements of mRNA and protein showed very low nNOS expression in dura mater and trigeminal ganglion as compared to pial arteries and trigeminal nucleus caudalis. RTX-induced neuropathy caused depletion of CGRP and substance P, and this was associated with thermal hypoalgesia. Intradermal injection of capsaicin produced morphologic changes in cutaneous nerve fibers and rapid degeneration of epidermal nerve fibers with a loss of immunoreactivity for CGRP and a decrease of substance P-positive fibers. 

The study suggests that depletion of C-fibers of neuropeptides, such as CGRP and substance P, results in desensitization of nociceptors. RTX-induced neuropathy permanently depleted substance P neurons and cutaneous terminals, whereas CGRP neurons and their cutaneous terminals were reversibly affected by RTX. These results suggest that CGRP is more resistant to RTX treatment than substance P and to the possibility of reinnervation of CGRP after RTX-induced neuropathy. During the pain phase of migraine, there is an elevation of CGRP but not of substance P. This lends scientific support to the concepts currently being tested in the clinical setting, such as reducing the circulating concentration of CGRP (currently with an antibody) or by blocking CGRP receptor function with receptor antagonists or with antibodies.

Occasional PACAP-positive fibers were found in the dura mater, and these colocalized with CGRP. PACAP has been found to be coexpressed with a subpopulation of CGRP-positive fibers in the dorsal horn of the spinal cord and neurons in the trigeminal ganglion. This suggests that PACAP has 2 sites of origin; 1 minor population is coexpressed with sensory CGRP and has a...
larger parasympathetic distribution.9 Taken together, the results suggest that the parasympathetic innervation of the dura is less prominent and may therefore play a minor role in migraine pathophysiology. However, clinical studies have suggested a link between the sensory trigeminal system and the parasympathetic ganglia, mainly containing VIP, PACAP, and NOS. Recently, CGRP-positive fibers were demonstrated in the human sphenopalatine ganglion along with the CGRP receptor components.3 These results suggest a possible sensory influence in the parasympathetic cranial ganglia.3

CGRP, CLR, and RAMP1 in Human Dura Vessels

Almost all vasculatures are innervated by CGRP-containing nerve fibers.5 We found pearl-like CGRP-positive fibers in the adventitia of human dura mater vessels. The receptor components, CLR and RAMP1, were expressed in the smooth muscle cell layer, which is in agreement with previous studies of meningeal, cerebral, pial, and superficial temporal arteries.5,8,41 Some fibers in the adventitia expressed the receptor components. No expression was found in the endothelial cells, supporting the knowledge that these cells have a minor role in CGRP signaling. Functional studies have demonstrated that CGRP-induced relaxation is independent of the endothelium.10,11 These findings support that released CGRP may act postjunctionally on its receptor expressed in vascular smooth muscle and on adventitial A-fibers.

Conclusions

We have described in detail the distribution of CGRP and its receptor components in rat dura mater and human dural vessels using immunohistochemistry. Here we show that CGRP is expressed in thin, unmyelinated fibers, suggesting C-fibers. The receptor components, CLR

Figure 12. CLR/RAMP1 and mast cell tryptase double staining in human dura vessels. (A) Cells positive for mast cell tryptase are found close to the adventitia of human dura vessel. (B) CLR and mast cell tryptase are coexpressed in human mast cells (arrows). (C) RAMP1 and mast cell tryptase are not colocalized in the cells. Arrows point at cells positive for mast cell tryptase lacking RAMP1 expression. Nuclei staining (DAPI, blue) is used in the merged pictures.
and RAMP1, were instead expressed in thicker, myelinated fibers, suggesting A-fibers. This supports the view that activation of C-fibers may locally cause release of CGRP, which in turn could act on A-fibers and rodent mast cells in the dura mater. Interestingly, CLR and RAMP1 expression was found in rat dural mast cells, suggesting expression of CGRP receptors in these cells. However, expression of both receptor components was not found in mast cells of human dura mater vessels. The role of CGRP and its receptor in the dura mater needs to be further evaluated.

References


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Thanks are due to Christopher A. Salvatore, at Merck West Point USA, for providing us with the antibodies against CLR and RAMP1 and for his valuable comments and reviewing the manuscript.

Supplementary Data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpain.2013.03.010.
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Paper IV
Calcitonin gene-related peptide (CGRP) and its receptor components in human and rat spinal trigeminal nucleus and spinal cord at C1-level

Sajedeh Eftekhari and Lars Edvinsson

Abstract

Background: Calcitonin gene-related peptide (CGRP) has a key role in migraine pathophysiology and is associated with activation of the trigeminovascular system. The trigeminal ganglion, storing CGRP and its receptor components, projects peripheral to the intracranial vasculature and central to regions in the brainstem with Aδ- and C-fibers; this constitutes an essential part of the pain pathways activated in migraine attacks. Therefore it is of importance to identify the regions within the brainstem that processes nociceptive information from the trigeminovascular system, such as the spinal trigeminal nucleus (STN) and the C1-level of the spinal cord. Immunohistochemistry was used to study the distribution and relation between CGRP and its receptor components - calcitonin receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1) - in human and rat STN and at the C1-level, using a set of newly well characterized antibodies. In addition, double-stainings with CGRP and myelin basic protein (MBP, myelin), synaptophysin (synaptic vesicles) or IB4 (C-fibers in general) were performed.

Results: In the STN, the highest density of CGRP immunoreactive fibers were found in a network around fiber bundles in the superficial laminae. CLR and RAMP1 expression were predominately found in fibers in the spinal trigeminal tract region, with some fibers spanning into the superficial laminae. Co-localization between CGRP and its receptor components was not noted. In C1, CGRP was expressed in fibers of laminae I and II. The CGRP staining was similar in rat, except for CGRP positive neurons that were found close to the central canal. In C1, the receptor components were detected in laminae I and II, however these fibers were distinct from fibers expressing CGRP as verified by confocal microscopy.

Conclusions: This study demonstrates the detailed expression of CGRP and its receptor components within STN in the brainstem and in the spinal cord at C1-level, and shows the possibility of CGRP acting postjunctionally in these areas putatively involved in primary headaches.

Background

Migraine is considered as a neurovascular disorder affecting more than 10% of the general population. Calcitonin gene-related peptide (CGRP) has a key role in migraine, where levels of CGRP are increased during acute migraine attacks [1]. CGRP is expressed throughout the central and peripheral nervous systems, consistent with control of vasodilatation and transmission of nociceptive information. In migraine, CGRP is released from the trigeminal vascular system. At peripheral synapses, CGRP results in vasodilatation via receptors on the smooth muscle cells. At central synapses, CGRP has been suggested to act postjunctionally on second-order neurons to transmit pain centrally via the brainstem and midbrain to higher cortical pain regions [2].

There are two forms of this peptide; (i) αCGRP, which is predominantly expressed in the nervous system, and (ii) βCGRP, which is primarily expressed in the enteric sensory system. In the central nervous system (CNS), CGRP is expressed in several regions such as the striatum, amygdalae, hypothalamus, colliculi, brainstem, cerebellum and the trigeminal complex [3-7]. Moreover, CGRP is found in primary spinal afferent C- and Aδ-
fibers, which project to the brainstem. However, CGRP and its receptor components have not fully been studied in man due to the fact that the receptor components only fairly recently were characterized.

The receptor for CGRP consists of a complex of a seven transmembrane-spanning protein, calcitonin-receptor-like receptor (CLR), a single transmembrane-spanning protein designated receptor activity modifying protein 1 (RAMP1) [8] and an intracellular protein, receptor component protein (RCP) [9]. Recently, CGRP antagonists have been developed with clinical efficacy for the treatment of acute migraine attacks [10–12]. Consequently, it is of considerable importance to clarify where the CGRP receptor is expressed which would indicate possible sites for the therapeutic effect of these antagonists. Hence, studies have focused on mapping CGRP and its receptor components in the trigeminal vascular system and in the brainstem as recently reviewed [13].

A migraine active region has been demonstrated in the brainstem with positron emission tomography (PET) [14–16]. It has been hypothesized that brainstem stimulation can cause activation of the trigeminal vascular system, resulting in CGRP-dependent vasodilatation [17].

We have investigated in detail the distribution and relationship of CGRP and its receptor components within human and rat spinal trigeminal nucleus (STN) in the brainstem and in the spinal cord at C1-level, using a set of newly characterized antibodies for immunohistochemistry [7]. Our main findings in the present work were that CGRP and the receptor components appear in different structures/regions of STN, and in lamina I and II at C1-level, but do not co-localize. This suggests that C-fiber released CGRP acts post-functionally on fibers expressing CLR/RAMP1 in these regions.

Methods
Postmortem human tissue samples
Samples of STN and C1 were obtained at autopsy from adult subjects in accordance with the Faculty of Medicine University of Szeged guidelines for ethics in human tissue experiments and were approved by the local Hungarian Ethics Committee. The tissue was bilaterally removed from 6 subjects (3 female; 3 male) with an age span of 65 to 86 years. None of the subjects suffered from any central nervous system disease and the cause of death was related to heart failure, septicemia or cancer. The tissues were collected within 24 to 36 hrs after death.

The samples were immersed overnight in fixative consisting of 4% paraformaldehyde (PFA) and in 0.1 mol/l phosphate buffer, pH 7.2. After fixation, the specimens were rinsed in sucrose-enriched (10%) Tyrode solution overnight, frozen and stored at -80°C. The samples were embedded in a gelatin medium (30% egg albumin and 3% gelatin in distilled water) and cryosectioned at 12 μm. The sections were stored at -20°C until use.

Rat tissue samples
Brainstems were quickly removed from 5 male Sprague-Dawley rats weighing 300–350 g (Scanbur, Stockholm, Sweden). The STN samples were dissected at bregma -14.08, corresponding the caudal part of STN, Sp5C (using brain atlas Paxinos and Watson, second edition, 1986) and C1 were dissected at C1 vertebra. The tissues were immediately placed in 4% PFA and fixed for 2–4 hrs. After fixation the tissues were rinsed in raising concentrations of sucrose in Sörensen’s phosphate buffer, embedded, sectioned and stored as the human samples.

Brainstems from 2 additional rats were after removal kept in the refrigerator at +4°C for 24 hrs before they were treated as above (to mimic the autopsy situation in man).

Also, comparison was made to tissue obtained from 2 additional rats that were perfusion fixed with 4% PFA (data not shown). We found no difference in the immunostaining patterns between the two procedures. The experiments were approved by the University Animal Ethics Committee (M8-09), Lund University, Sweden.

Hematoxylin-Eosin staining
Human and rat sections were stained with Hematoxylin-Eosin (Htx-Eosin) using a standard protocol (Htx 3 min, water rinse, Eosin 1 min) for orientation and examination of the tissue condition. The areas within the rat and human brainstem were identified by the use of a brain atlas (Paxinos and Watson, second edition, 1986, and Koutrackov et al. chapter 10 in The Human Nervous System). Sections with STN (the caudal subdivision) or C1, were used and adjacent sections were employed for immunohistochemistry.

Immunohistochemistry
Sections were thawed and washed for 10 min in PBS pH 7.2 containing 0.25% Triton X-100 (PBST). The sections were blocked for 1 hr 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, CLR and RAMP1. Anti-human CLR (3152) and RAMP1 (844) were used for the human material, anti-rat CLR (3155, 132) and RAMP1 (3155) were used for the rat material. For detailed description of the primary antibodies, see Table 1.

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 (for details, see Table 2) in PBST and 1% BSA for 1 hr at room temperature. The sections were subsequently washed with PBST for 3 × 15 min. Vectashield, an anti-fading medium, containing DAPI (Vectashield, Vector Laboratories, Burlingame CA) or glycerol in PBS were used as mounting media.

In addition, double immunostainings for CGRP together with either myelin basic protein (MBP), synaptic vesicle protein (synaptophysin) or Isolectin IB4 (IB4) were performed (Table 1). For all double immunostainings, the antibodies were applied separately and not mixed as a cocktail.

Controls and DAB staining
Omission of the primary antibody served as negative controls for all antibodies. To evaluate secondary antibody staining, three different secondary antibodies (Table 2) were tested together with CLR or RAMP1, respectively.

Preabsorption controls with blocking peptides (details on these have been described before in [7]) were performed with all CLR and RAMP1 primary antibodies. Concentrations of the antibodies were the same as described in Table 1, peptide concentrations were 100:1. The blocking peptides were resuspended in PBS and then incubated at +4°C overnight in PBST containing 1% BSA and 3% normal serum, with or without primary antibodies. The immunostaining protocol was the same as described above. Sections incubated with antibodies alone versus blocked antibodies were compared.

In order to evaluate the fluorescence technique staining, 3,3’-diaminobenzidine (DAB) substrate together with Vectastain ABC kit standard PK-6100 (Vector Laboratories) was performed. In brief, sections were rinsed in PBST followed by incubation with methanolic hydrogen peroxidase (3% H2O2 and 10% MetOH in PBS) to remove endogenous activity. After incubation for 1 h with blocking solution of PBS and 5% normal swine or rabbit serum, the sections were incubated with primary antibodies against CGRP, CLR and RAMP1, at +4°C overnight. At the second day, sections were incubated with biotin-conjugated secondary antibodies, anti-rabbit or anti-goat for 1,5 h (1:400, Dako, Glostrup, Denmark). Visualization was achieved through the ABC kit using DAB/H2O2. Omission of primary antibody served as negative controls.

Microscopic analysis
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-labelling by superimposing the digital images and to adjust brightness and contrast. In addition, confocal microscopy was

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**Table 1 Details on primary antibodies used for immunohistochemistry**

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**Table 2 Secondary antibodies used for immunohistochemistry**

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performed using Nikon confocal microscope (EZ-cl, Germany), where detailed localization and/or co-localization of immunoreactivity were recorded using the confocal microscope. All pertinent questions were addressed with examination with confocal microscope analysis (see figures). The confocal microscopy was carried out using 20× or 60× oil immersion lenses. Z (frame) stacks were acquired using different laser channels one by one before next z position was acquired. Laser channels used were 488 nm excitation (filter 515/30) and 543 nm excitation (filter 605/75). Image analyses were conducted using NIS basic research software (Nikon, Japan). Briefly, z stacks were 3-dimensionally examined for detailed localization and distribution of immunoreactivity, and for possible co-localization of the antibodies used. The opportunity to move around 3-dimensionally within the 12 μm thick section allows for scrutinizing the detailed localization of the immunoreactivity, but also for thorough evaluation of the immunoreactive structures.

**Results**

**Histology**

Htx-Eosin stained sections from human STN (A), C1 (B) and rat STN (C) and C1 (D) are depicted in Figure 1. The human samples were considered qualitatively adequate for the immunofluorescence technique by and large comparable in structures to that found in rat.

**STN CGRP immunoreactivity**

In human STN, the highest density of CGRP immunoreactive fibers was found in a network around fiber bundles in the superficial laminae (Figure 2A). In this area, few fibers extended into the spinal trigeminal tract region and into the mid part of the brainstem (Figure 2A). The CGRP immunoreactive fibers were thin and displayed “pearl-like” structures (Figure 2A). No CGRP positive cells were detected in the STN or in other regions within the brainstem at the examined level. Double-staining did not reveal any co-localization of CGRP and MBP (myelin marker) (Figure 2B,C).

In rat STN, similar staining pattern of CGRP immunoreactivity was observed in the superficial laminae as in man, with some fibers extending into the spinal trigeminal tract (Figure 3A). The expression of CGRP was limited to fibers. However, in the region of the inferior olive and the hypoglossal nucleus, CGRP positive neurons were found. Here, CGRP immunoreactivity was revealed as intracellular, granular staining in the cytoplasm, visualized with the two different CGRP antibodies (Figure 3B). Double-staining did not reveal any co-
localization of CGRP and MBP (Figure 4A,B). CGRP and synaptic vesicle protein (synaptophysin) double-staining showed co-expression of these two markers (Figure 4C). CGRP and synaptophysin revealed same results in human STN (data not shown).

**CLR and RAMP1 immunoreactivity**

In human STN, the staining of the receptor components was not as distinct as the CGRP staining. Nevertheless, CLR and RAMP1 positive fibers were found in the spinal trigeminal tract region (Figure 5A). Double-
staining of CLR and RAMP1 showed co-localization in the spinal trigeminal tract region (Figure 5B). No CLR or RAMP1 positive neurons were found. Double-staining did not reveal any co-localization of CGRP and RAMP1 (Figure 5C) or CGRP and CLR (data not shown).

Similar fiber staining pattern was seen in rat STN, however, the staining was more intense. In contrast to that of the human STN, positive CLR and RAMP1 staining was observed around fiber bundles within the spinal trigeminal tract (Figure 6A). Double-staining of CLR and RAMP1 showed co-localization in the spinal trigeminal tract region, in both fiber bundles (Figure 6B) and fibers spanning from the spinal trigeminal tract (Figure 6C). Double-staining of CGRP and the receptor components showed that CGRP is not expressed in the same fibers as CLR (Figure 6D) or RAMP1 (data not shown). No CLR or RAMP1 positive neurons were found. Interestingly, the receptor components were detected in the walls of capillaries within the rat brainstem (Figure 6).

C1

CGRP immunoreactivity
At the C1-level of the human spinal cord, CGRP positive fibers were detected within the posterior horn of the gray matter, notably in laminae I and II (Figure 7A). In addition, few fibers reaching the deeper laminae were detected. No CGRP immunoreactive neurons were found in the human C1.

Double-staining revealed no co-localization of CGRP and MBP, as the expression of the two markers was observed in different structures at the C1-level (Figure 7B,C). CGRP was detected in areas that were absent from MBP expression. Furthermore, CGRP and synaptophysin were expressed in the same laminae and structures (Figure 8A), which was confirmed by confocal microscopy (Figure 8B). Not all synaptophysin positive fibers expressed CGRP. Expression of synaptophysin was detected in all laminae within C1-level (Figure 8C).

The CGRP staining was similar in rat C1 compared to man (Figure 9A). In rat, transverse positive CGRP fibers were detected close to the central canal (Figure 9B).
addition, CGRP positive neurons were found close to the central canal (Figure 9B). Double-staining with CGRP and MBP (Figure 9C,D), CGRP and synaptophysin (Figure 10A) revealed similar results as those seen in the human samples. Synaptophysin expression was detected in all laminae, including circular immunoreactive fiber formations around neurons (Figure 10B). Double-staining with CGRP and the IB4 marker revealed CGRP and IB4 immunoreactivity in laminae I and II (Figure 10C). IB4 staining was brighter in laminae II/III, compared to laminae I and II. Confocal microscopy disclosed that CGRP and IB4 were most often not expressed in the same fibers. However, occasionally the slender CGRP positive fibers co-localized with the more stubby IB4 immunoreactive fibers (Figure 10D, additional file 1, movie).

CLR and RAMP1 immunoreactivity
In human C1, the receptor components were detected in laminae I and II, although the staining was weaker compared to the CGRP staining (Figure 11A). No
positive cells for CLR or RAMP1 were found. Double-staining of CGRP and RAMP1 showed no co-localization (Figure 11B). Same results were observed with CGRP and CLR (data not shown).

In rat C1, the staining for CLR and RAMP1 was more prominent, although, the CLR staining was weaker compared to that of RAMP1. The receptor components were detected in fibers within laminae I and II (Figure 12A,B). Double-staining of CLR and RAMP1 showed co-localization (Figure 12C). These fibers were different from fibers containing CGRP, revealed by double-staining of CGRP and the receptor components. Confocal
microscopy disclosed that CGRP and the receptor components were not expressed in the same fibers (Figure 12D, additional file 2, movie). The receptor components, especially RAMP1, were also detected close to the central canal (laminae X) (Figure 12E). As for the STN of rat, the receptor components were found in capillary walls within the spinal cord at C1-level (Figure 12).

Controls and DAB staining
The negative controls (omission of primary antibody) showed no immunoreactivity, except for the lipofuscin autofluorescence in human samples (Figure 5, 7).

There was no immunoreactivity observed with preabsorbed CLR or RAMP1 antibodies, using their respective blocking peptides (additional file 3).
DAB stained sections showed similar staining pattern for CGRP, CLR and RAMP1 as seen with the immunofluorescence technique. DAB staining revealed CLR and RAMP1 expression in the walls of capillaries in rat (Figure 12E) and in laminae X, close to the central canal. Furthermore, three different secondary antibodies were tested to ensure that the capillary staining was not due to unspecific binding of the secondary antibody.

Two additional rat brainstems were kept at +4°C for 24 hrs before dissection, fixation and immunostaining to mimic the autopsy situation in man. As in our previous paper on the trigeminal ganglion [7]; this procedure did
not differ in immunoreactivity for CGRP, CLR and RAMP1 as compared to fixation directly of fresh brain-stems (data not shown).

Discussion
We have previously reported in detail the distribution of CGRP and its receptor components in the human trigeminal ganglion [7]. Small to medium sized neurons of the trigeminal ganglion store CGRP and have further connections with STN in the brainstem, and in related extensions down to the C1-2 level via un-myelinated C-fibers [18]. CLR and RAMP1 are expressed in large neurons and thick fibers, which are co-expressed with a marker for Aδ-fibers. These fibers from the trigeminal ganglion extend further to the brainstem. Several studies point to the involvement of the brainstem in migraine pathophysiology [1,19] and a region within the brainstem has been demonstrated to be active during attacks in migraine patients [14,20].

The present study has revealed the detailed distribution and relations between CGRP and its receptor components in human and rat projection regions from the trigeminal ganglion. The study demonstrates for the first time the expression of CLR and RAMP1 within human STN in the brainstem and in the spinal cord at C1-level, using a series of novel well characterized antibodies. Thus, this indicates CGRP signaling in areas of the brainstem/spinal cord putatively involved in the migraine pathophysiology. The reason for choosing these regions in the present study is based on a series of detailed neuroanatomical mappings in rat showing pain pathways that may be involved in primary headaches [21].

Distribution of CGRP, CLR and RAMP1 in STN
We observed CGRP expression around nerve fiber bundles in the superficial laminae of human and rat STN. We found no CGRP expressing neurons in the STN
neither in man nor in rat. However, in rat we observed a small group of CGRP positive neurons located close to the central canal, in the inferior olive and in the hypoglossal nucleus of the brainstem. Notably these neurons were absent in man. The results were confirmed using 2 different antibodies against CGRP. The functional role of CGRP in these regions remains to be disclosed. The presence of CGRP in the brainstem is supported by
Figure 10 Double-staining of CGRP and synaptophysin or IB4. (A) Double-staining of CGRP (red) and synaptophysin (green) in rat C1. (B) Synaptophysin immunoreactivity is found all laminae, with fibers extending into the white matter. Circular immunoreactive fiber formations are seen around neurons (arrows). (C) Double-staining of CGRP (green, thin arrows) and IB4 (red, thick arrow). IB4 is mostly expressed in laminae II/III. IB4 is also expressed in laminae I/VI which could be seen as co-localization with CGRP. However, confocal microscopy (D) revealed that CGRP (green, thin arrow) and IB4 (red, thick arrow) are most often not expressed in the same fibers. Occasionally the two markers co-localize in fibers (arrow head).
early autoradiography studies [22,23]. In addition, CGRP expression in STN and spinal cord of different species has been studied [6,24-26].

The expression of CGRP in the brainstem differs between species [6]. It has been shown that the distribution of CGRP fibers is similar in rat and alpaca brainstem. However, CGRP containing neurons are more widespread in rat than in alpaca. In addition, the localization of CGRP positive neurons in the cat and alpaca brainstem differs [27]. Hence, the distribution pattern observed between rat and man in our study is likely due to species differences.

CGRP and MBP double-staining showed no co-localization, indicating that CGRP is as expected expressed in un-myelinated fibers in STN and this is in agreement with a previous study on the trigeminal ganglion [7]. Synaptophysin, used as a marker for synaptic vesicles, and CGRP were expressed in the same structures within the STN. This supports that CGRP is stored and released from nerve terminals as supported by a study on rat dorsal horn [28] and acts post-synaptic at CLR/RAMP1 expressing fibers.

In human STN, we found CLR and RAMP1 positive fibers mostly in the spinal trigeminal tract, spanning towards the superficial laminae. In rat STN, expression of CLR and RAMP1 were also found mostly in the spinal trigeminal tract, around fiber bundles and fibers in the spinal trigeminal tract spanning towards the superficial laminae. These results are in contrast to an earlier study by Lennerz et al., 2008, where CLR and RAMP1 expression was detected in the superficial laminae, partially co-localizing with CGRP. The reason for this difference could reside in that different antibodies were used.

There were no neuronal cell bodies immunopositive for CLR and RAMP1 in the human or rat STN. CLR and RAMP1 were observed to be co-expressed in the fibers, suggesting the presence of functional CGRP receptor in both types of species. Interestingly, we found that the receptor components were co-expressed on the walls of capillaries in rat STN.

In rat STN, it was suggested that CGRP and its receptor components are localized in terminals from primary afferents [29]. In contrast, CLR did not co-localize with neuropeptides of primary spinal afferents in the dorsal horn of rat [30]. In the present work, we observed no co-localization between the receptor components and CGRP positive fibers, neither in man nor in rat. These results suggest that CGRP and the receptor components appear in nerve terminals, where C-fiber released CGRP may act post-synaptic at CGRP receptors on second-order neurons or modify the responses of trigeminal Aδ-fibers.

**Figure 11** CLR/RAMP1 and CGRP double-staining in human C1
(A) Expression of RAMP1 and CLR in human C1. The receptor components are found within laminae I and II. (B) Double-staining of CGRP (red) and RAMP1 (green) shows that CGRP positive fibers (arrows) within laminae I and II do not co-localize with RAMP1. Asterisks point at autofluorescent lipofuscin.

**Distribution of CGRP, CLR and RAMP1 in C1**

We examined the localization and expression of CGRP and its receptor components in the spinal cord at the C1-level, since the main part of the trigeminovascular projection occurs at this level [21]. CGRP positive fibers were found in laminae I and II. Similar staining pattern has been demonstrated previously in the chick, quail dorsal horn of the spinal cord [31] and in the cat [32]. In addition, we observed some CGRP positive neurons and fibers close to the central canal (laminae X). This finding is in agreement with a previous study in rat spinal cord with neurons being positive for CGRP [6].

To determine which fibers express CGRP, co-localization experiments were performed with CGRP and IB4. IB4 has previously been used as a marker for C-fibers [30]. In the present study we found that IB4 and CGRP could be expressed in the same laminae, but most often in different types of fibers. This observation was confirmed by confocal microscopy. The IB4 staining was more prominent in the deeper laminae of the spinal cord, which is in agreement with a study of rat spinal cord [30]. It has been shown that the degree of co-expression of CGRP and IB4 in neurons vary in the rat. More neurons expressing both markers are found in dorsal root ganglia compared to the trigeminal ganglion of rat [33]. Electron microscopy showed that IB4 and CGRP expressing axons were distinct, but both could be present in the same bundle of un-myelinated fibers [34].

The MBP marker showed that in some areas within laminae I and II myelinated fibers are absent. Co-staining experiments with CGRP showed that CGRP is
Figure 12 CLR/RAMP1 and CGRP double-staining in rat C1. (A) RAMP1 and (B) CLR expression in rat C1. The receptor components are found from Lissauer's tract and within laminae I and II (arrows). Higher magnification discloses expression in the walls of the capillaries. (C) Co-expression of CLR (red) and RAMP1 (green) in the laminae and the capillaries. (D) Double-staining of CGRP (red, thick arrows) and RAMP1 (green, thin arrows) using confocal microscopy. CGRP and RAMP1 are not co-expressed. (E) Fluorescence and DAB staining of the receptor components. Positive fibers are displayed close to the central canal (arrows), where also positive capillaries are found (arrow heads).
expressed in areas that are absent of MBP, suggesting that CGRP is indeed expressed in un-myelinated fibers. To further scrutinize the CGRP positive fibers, double-staining of CGRP and synaptophysin was performed. In laminae I and II, where CGRP staining was found, co-expression of CGRP and synaptophysin was observed. With confocal microscopy, we obtained a detailed three-dimensional view of the staining pattern. This clearly showed that CGRP and synaptophysin were detected in the same fibers.

Reportedly, CLR and RAMP1 are expressed in fibers within laminae I and II in the dorsal horn of rat spinal cord [30]. Similar observation but in different tissue was seen in our study; CLR and RAMP1 expressions were found within laminae I and II of human and rat C1. No CLR or RAMP1 positive neuronal cell bodies were observed at the C1-level. In rat, CGRP was expressed in the same laminae, but it did not co-localize with CLR or RAMP1. CLR and RAMP1 were co-expressed, suggesting expression of functional CGRP receptor in fibers within laminae I and II. The presence of receptor components in the spinal cord is supported by the mRNA expression of RAMP1 and RCP, detected with specific oligonucleotides for in situ hybridization [35].

CLR and RAMP1 were in addition detected in fibers close to the central canal. As described above, CGRP positive fibers were also detected in this area. Interestingly, tracing experiments in cat have revealed projections from the periaqueductal gray (PAG) region to the spinal cord. Horseradish peroxidase (HRP) injections into the PAG region resulted in labeled fibers close to the central canal, terminating in laminae X of C1 and C2-levels [36]. The same authors found labeled fibers in segments C4 to T8 adjoining the ependyma layer of the central canal and next to the basal membrane of the nearby capillaries. Histochemical studies in different species have revealed neurons, axons and terminals within laminae X containing neuropeptides such as substance P [37]. Thus, our results demonstrate fibers containing CGRP and its receptor components close to the central canal. The function of this is not known; one may speculate that these fibers can release CGRP directly into the cerebrospinal fluid or stimulate the ependymal cells of the central canal.

Methodology and technical considerations

The hematoxylin-eosin stained material revealed well-preserved human tissue adequate for immunofluorescence technique, even though the tissue samples were collected 24 to 36 hrs after death. Due to the relatively high age of the subjects, lipofuscin is accumulated in the tissue, causing auto-fluorescence. We have earlier examined, in rat trigeminal ganglion, if storage of the animals for 24 hrs at +4°C prior fixation would affect the immunohistochemistry; which was not the case [7]. Similar results were obtained in the present study on the rat brainstem (data not shown). In rat we also performed a direct comparison of perfusion-fixed and immersion-fixed brainstems, and found no observable difference in antibody expression pattern.

The C1-level compared well in the Paxinos atlas for rat and man. The STN is a structure that is distributed for a considerable length in the brainstem and could therefore not be examined for its entire distribution. It is a limitation of the present study that we only studied a portion of the STN; the exact part is given in the method part.

In our previous study, antibodies against human and rat CLR and RAMP1 were generated, and the specificity of the antibodies was confirmed in HEK293 cells stably expressing the human CGRP receptor. The specificity of the raised antibodies was also confirmed by Western blotting [7]. The same antibodies were used in this study.

The staining of the receptor components was weaker and a bit more diffuse in the human tissue compared to rat. This could be due of several factors: differences in antibodies recognizing the epitopes, tissue condition, or low level of CLR and RAMP1 in human tissue. In rat STN and C1, the RAMP1 antibody displayed a stronger staining pattern compared to the CLR antibody. If this was due to differences in antibody recognizing the epitopes or more expression of RAMP1 in these areas could not be verified.

Within the rat brainstem, we found expression of RAMP1 and CLR in the capillary walls. This was blocked with the specific blocking peptides (epitopes used in the production of the respective antibodies). The staining of the capillaries was similar in the endogenously activity blocked DAB-stained sections.

Conclusions

The neuropeptide CGRP is implicated in the pathophysiology of migraine and the CGRP receptor has long been regarded as a useful target for the development of novel antimigraine therapies. We have described in detail CGRP and its receptor components in the STN and C1 of man and rat using immunohistochemistry. Fibers expressing CGRP and its receptor components occur in STN and C1, however they were not co-expressed in the different areas and laminae. This suggests that CGRP released from C-fibers in the brainstem may act postjunctionally to modulate the activity in fibers that store the CGRP receptor in these regions. Differences in the CGRP expression between the species were observed in other parts of the brainstem. We have also demonstrated fibers and neurons expressing CGRP close to the central canal which suggests that CGRP
may have a function within this area. Further efforts are essential to understand CGRP signaling and its function within the brainstem.

Additional material

Additional file 1: Movie: 3-dimensionally view of staining with CGRP (green) and IB4 (red) in rat C1. The two markers are most often not expressed in the same fibers. Occasionally the two markers co-localize in fibers (yellow).

Additional file 2: Movie: 3-dimensionally view of staining with CGRP (red) and RAMP1 (green) in rat C1. The neuropeptide CGRP and the receptor component RAMP1 are found within the same laminae, but they are not co-expressed in the same structures.

Additional file 3: Blocking peptide experiments. CLR and RAMP1 antibodies were pre-absorbed with their respective blocking peptides. No positive immunoreactivity is found when the blocking peptides are used Asterisks point at autofluorescent lipofuscin in the human samples. Human STN (A) preabsorption for CLR, (B) preabsorption for RAMP1. Rat STN (C) preabsorption for CLR, (D) preabsorption for RAMP1. C1 rat C1 (E) preabsorption for CLR, (F) preabsorption for RAMP1. Rat C1 (G) preabsorption for CLR, (H) preabsorption for RAMP1.

Abbreviations

BSA: Bovine serum albumin; CLR: Calcitonin receptor-like receptor; CGRP: Calcitonin gene-related peptide; DAB: 3,3'-diaminobenzidine; HtX-EoXin: Hematoxylin-Eosin; MBP: Myelin basic protein; NF: Nerve filament; PAG: Periaqueductal gray; PBS: Phosphate buffered-saline; PBST: Phosphate buffered-saline (PBS) containing 0.25% Triton X-100; PFA: Paraformaldehyde; RAMP1: Receptor activity-modifying protein 1; RCP: Receptor component protein; STN: Spinal trigeminal nucleus

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Authors' contributions

SE participated in the design of study, performed the experiments, analysed the data, prepared the figures and wrote the manuscript. LE conceived the study, guided the experimental procedures, and participated in writing the manuscript. Both authors read and approved the final manuscript.

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Paper V
Localization of CGRP receptor components and receptor binding sites in rhesus monkey brainstem: a detailed study using *in situ* hybridization, immunofluorescence and autoradiography

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Abstract

**Background and aims**

Functional imaging studies have revealed that certain brainstem areas are activated during a migraine attack. Calcitonin gene-related peptide (CGRP) has a key role in migraine pathophysiology and is associated with activation of the trigeminovascular system. The trigeminal ganglion, storing CGRP and its receptor, project peripherally to the intracranial vasculature and centrally to different regions in the brainstem; these routes are essential parts of the pain pathways activated in migraine attacks. Therefore, it is important to identify the regions within the brainstem that processes nociceptive information. A mapping study was performed to identify CGRP receptor mRNA, protein expression and CGRP receptor binding throughout the brainstem of rhesus monkey.
Methods

In situ hybridization was performed to detect mRNA expression of calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1). Immunofluorescence was used to define cellular localization of CLR and RAMP1. To define CGRP receptor binding sites, in vitro autoradiography were performed with [³H]MK-3207 (a CGRP receptor antagonist).

Results

CLR and RAMP1 mRNA and protein expression were detected in the pineal gland, medial mammillary nucleus, infundibular stem, periaqueductal gray, area postrema, potine raphe nucleus, gracile nucleus and spinal trigeminal nucleus. In addition, CLR and RAMP1 expression were detected in the spinal cord. RAMP1 mRNA expression was also detected in the trochlear nucleus, dorsal raphe nucleus, medial lemniscus, potine reticular nucleus, vagus nerve (10N, centrointermediate), inferior olive, abducens nucleus, motor trigeminal nucleus, and potine raphe nucleus, where co-expression of CLR and RAMP1 was observed with immunofluorescence. [³H]MK-3207 showed high binding densities concordant with mRNA and protein expression.

Conclusions

This is the first mapping study demonstrating CLR and RAMP1 mRNA and protein localization in combination with CGRP receptor binding throughout the brainstem of rhesus monkey. The present study suggests that several regions in the brainstem may be involved in CGRP signaling and migraine pathophysiology. Interestingly, we found expression and antagonist binding in areas that are not protected by the blood brain-barrier, suggesting that CGRP receptor antagonists may not need to be CNS-penetrant to functionally antagonize receptors in these brain regions.

Keywords

CGRP, CLR, RAMP1, CGRP receptor antagonists, brainstem, primate.
Introduction

Migraine is a complex neurovascular disorder affecting more than 10% of the global population with a prevalence of 15% to 18% in females and 6% to 9% in males (Stovner et al., 2007). The disorder is characterized by attacks of severe pulsating headache associated with nausea and vomiting and, in some cases, preceded by aura (migraine with aura). Neurologic symptoms including photophobia, phonophobia, tingling, numbness and weakness are common and occur before, during, or after headache (Society, 2004).

The neuropeptide calcitonin gene-related peptide (CGRP) is a potent vasodilator neuropeptide which also has a role in transmission of nociceptive information (Edvinsson, 1987, Poyner, 1992, Gulbenkian et al., 2001, Goadsby, 2007). CGRP has been implicated in the pathogenesis of migraine, where experimental and clinical studies have shown that there is an increased level of trigeminal-system-released CGRP during migraine attacks (Goadsby et al., 1990, Goadsby and Edvinsson, 1993, Bellamy et al., 2006). The most important evidence for the role of CGRP in migraine pain came from the development of CGRP receptor antagonists (Olesen et al., 2004, Edvinsson, 2008, Ho et al., 2008, Ho et al., 2010, Luo et al., 2012). The CGRP receptor antagonists act by blocking the action of CGRP at the CGRP-receptor complex, which is composed of the calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1) (McLatchie et al., 1998) and an intracellular protein receptor component protein (RCP) (Evans et al., 2000). There are three different RAMP proteins: RAMP1, RAMP2 and RAMP3. Interaction of CLR with RAMP2 or RAMP3 forms adrenomedullin receptors (McLatchie et al., 1998, Zhang et al., 2007). RAMPs can also interact with the calcitonin (CT) receptor to form amylin receptors.

It is generally believed that migraine is associated with disturbances in brain function, including the brainstem (Hargreaves and Shepheard, 1999, Goadsby et al., 2009, Charles, 2012, 2013). In patients, migraine-active regions in the brainstem have been demonstrated with imaging techniques such as positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) (Weiller et al., 1995, Diener, 1997, Stankewitz et al., 2011). Also, the brainstem is an important site for sensory signals from the periphery, including the trigeminal ganglion. It has been demonstrated that brainstem stimulation can cause activation of the trigeminovascular system, resulting in CGRP-dependent vasodilatation (Just et al., 2005).

Several brainstem areas have been shown to be activated during a migraine attack, including the midbrain, pons, substantia nigra, red nucleus and the periaqueductal gray
Therefore it is of importance to identify the regions within the brainstem that processes nociceptive information. The distribution of CGRP and its receptor has only been elucidated in a few regions of the brainstem, mainly the spinal trigeminal nucleus (STN) (Lennerz et al., 2008, Eftekhari and Edvinsson, 2011). In the present study, we aimed to localize mRNA and protein expression of the CGRP receptor components by in situ hybridization and immunofluorescence, and identify binding sites of a CGRP receptor antagonist using in vitro autoradiography in rhesus monkey brainstem. The combination of all three methods provides accurate validation of functional CGRP receptor expression in the primate brainstem.

Material and Methods

Rhesus monkey tissue samples
Rhesus monkey brainstem (Macaca mulatta, n=3, age; 13-15 years old, 2 females, 1 male) was harvested and divided into pieces (midbrain, pons and medulla) in accordance with a Merck Research Laboratories Institutional Animal Care and Use Committee approved protocol. The tissue were frozen and stored at -80°C. For each tissue and animal, sets with adjacent sections were prepared for in situ hybridization and autoradiography. The tissue was placed in the cryostat to equilibrate to -20°C for 15-20 min. Cerebellum from the same animals served as controls for both methods. The samples were cryosectioned at 20 μm (cryostat model CM3050: Leica Microsystems, Inc., Deerfield, IL). Sections were collected on cold Superfrost® Plus slides, thawed and then placed on a slide warmer (37°C) to dry for 5-10 min. Slides were placed in closed desiccated boxes (arranged with dricap dehydrators) and stored at -80°C until use. One rhesus monkey brainstem (Macaca mulatta, age 10 years old, female) was divided into pieces and immersed overnight in fixative consisting of 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer, pH 7.2 and paraffin embedded. The paraffin blocks were sent to the laboratory in Sweden for immunohistochemistry experiments. Paraffin sections were cut at 5 μm and incubated at +60°C for 1h. 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.

In situ hybridization
Probes were designed to be specific for CLR and RAMP1. The CLR probe corresponds to bases 1018-1453 of human CLR (accession number L7630) and was designed to equally distribute the human and rhesus non-conserved bps. The RAMP1 probe (447 bp) corresponds to the full length rhesus RAMP1 coding sequence. Both probes were synthesized by
GENEWIZ (South Plainfield, NJ) and subcloned into pBluescript SK+ vectors as 5'EcoRI-3'XbaI fragments. The rhesus RAMP1 and CLR sequences have been disclosed previously and deposited in GenBank with accession numbers KC855758 and KC855759, respectively (Eftekhari et al., 2013).

The CLR and RAMP1 vector constructs were used to generate sense and antisense 35S-UTP labeled cRNA probes for the in situ hybridization. For a complete description of the in situ hybridization method, see Eftekhari et al., 2013. Briefly, tissue sections processed for in situ hybridization were slowly warmed to room temperature, fixed in ice-cold 4% PFA, followed by washes in 1X PBS, 100mM triethanolamine (TEA), 0.25% acetic anhydride in 100mM TEA and 2X saline-sodium citrate (SSC). The sections were delipidated and dehydrated with chloroform and ethanol and allowed to air-dry. Sections were hybridized overnight at 55°C with a hybridization solution containing the 35S-labeled cRNA probe at a concentration of 6x10^6 counts/slide and hybridization buffer.

After hybridization, sections were washed in 2X SSC containing DTT, treated with RNase A (5ml/L) at 37°C, washed in 1X SSC, then finally incubated at 72°C in 0.1X SSC. Slides were equilibrated to room temperature, dehydrated with increasing concentrations of ethanol and air-dried. Sections were exposed to Biomax film (Kodak # IB 8715187) for 7 days. The films were developed using a Kodak Min-R mammography processor and digitized using a Vidar film scanner. The tissue sections were then dipped in NTB-2 liquid emulsion (Kodak # IB1654433) and stored at 7 weeks at 4°C in light-tight desiccated slide boxes. After 7 weeks, these sections were processed for silver grain deposition with developer and fixer (Kodak #1464593 and 1971746), stained with hematoxylin (Htx) and coverslipped. Scanned images of the tissue sections were taken using an Aperio brightfield slide scanner.

Fluorescent in situ hybridization

Fluorescent in situ hybridization was performed on selected slides displaying intense in situ hybridization signal. By using this technique, it was possible to determine if the mRNA expression of the receptor components was localized in the same neurons. The CLR probe was labeled with DIG-UTP and the RAMP1 probe was labeled with FITC-UTP (Roche Applied Science). Briefly, 1μg of the linearized plasmids were mixed with labeling mix containing transcription buffer, RNA polymerase, RNase inhibitor and RNAase/DNAase free water, incubated for 2 hr at 37°C. Subsequently, DNAse I was added, incubated for 15min at 37°C, followed by addition of EDTA. Purification was done using NucAway Spin columns (Invitrogen) and concentration of the probe was estimated using a Nanodrop. The hybridization and washing steps of the
sections were performed in the same way as the in situ hybridization described above. After the last wash, the sections were equilibrated to room temperature in Tris-NaCl-Tween buffer (TNT; 0.1M Tric-HCl, pH 7.5, 0.15M NaCl, 0.05% Tween 20), followed by a 15 min wash in TNT containing 3% H$_2$O$_2$ and 2x5 min wash in TNT. Slides were incubated for 30 min at room temperature with blocking solution containing TNB (0.1M Tric-HCl, 0.15M NaCl) and 1% PerkinElmer blocking reagent. After the blocking step, sections were incubated at 4°C overnight with the primary antibody anti-FITC (Roche Applied Science), diluted 1:1000 in TNB containing 0.1% blocking reagent. Sections were washed 3x5 min in TNT, incubated with FITC 1:50 diluted in 1X plus tyramide reagent for 30 min at room temperature, followed by 3x5 min wash in TNT. The same steps were repeated (except the blocking step) to apply a new antibody, anti-DIG 1:500 (Roche Applied Science) and fluorophore Cy$^3$ diluted 1:50 in tyramide reagent. Slides were cover slipped with Prolong Gold containing DAPI. The sections were examined with confocal microscope and scanned with a fluorescent Aperio slide scanner.

Hematoxylin-Eosin staining
One set of the adjacent slides for each method were stained with Htx using a standard protocol. This was performed for the purposes of orientation and for examining the condition of the tissue. Paraffin sections were deparaffinized in xylene followed by serials of alcohol and stained with Htx-Eosin (Htx 3 min, water rinse, Eosin 1 min). For orientation, The Rhesus Monkey Brain in Stereotaxic Coordinates (Paxinos G, 2000) was used.

Immunofluorescence
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 a citric acid solution, pH 6.0. Sections were washed for 10 min in PBS pH 7.2 containing 0.25% Triton X-100 (PBST), followed by incubation in blocking solution for 1 hr (5% normal donkey or goat serum in PBS). The sections were thereafter incubated overnight at 4°C for single or double immunolabelling with primary antibodies against CLR (3152) and RAMP1 (844), diluted in PBST containing 1% BSA and 3% normal serum 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 by Western blotting (Eftekhari et al., 2010). On the following day, sections were equilibrated to room temperature, rinsed in PBST for 3x15 min and exposed to secondary antibodies, Alexa 594 (donkey) anti-rabbit 1:400 or Alexa 488 (donkey) anti-goat 1:400, diluted in PBST and
Localization of CGRP receptor components and receptor binding sites in rhesus monkey brainstem: a detailed study using in situ hybridization, immunofluorescence and autoradiography

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Abstract

Background and aims
Functional imaging studies have revealed that certain brainstem areas are activated during a migraine attack. Calcitonin gene-related peptide (CGRP) has a key role in migraine pathophysiology and is associated with activation of the trigeminovascular system. The trigeminal ganglion, storing CGRP and its receptor, project peripherally to the intracranial vasculature and centrally to different regions in the brainstem; these routes are essential parts of the pain pathways activated in migraine attacks. Therefore, it is important to identify the regions within the brainstem that processes nociceptive information. A mapping study was performed to identify CGRP receptor mRNA, protein expression and CGRP receptor binding throughout the brainstem of rhesus monkey.
Methods

*In situ* hybridization was performed to detect mRNA expression of calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1). Immunofluorescence was used to define cellular localization of CLR and RAMP1. To define CGRP receptor binding sites, *in vitro* autoradiography were performed with \(^{3}H\)MK-3207 (a CGRP receptor antagonist).

Results

CLR and RAMP1 mRNA and protein expression were detected in the pineal gland, medial mammillary nucleus, infundibular stem, periaqueductal gray, area postrema, perine raphe nucleus, gracile nucleus and spinal trigeminal nucleus. In addition, CLR and RAMP1 expression were detected in the spinal cord. RAMP1 mRNA expression was also detected in the trochelear nucleus, dorsal raphe nucleus, medial lemniscus, perine reticular nucleus, vagus nerve (10N, centrointermediate), inferior olive, abducens nucleus, motor trigeminal nucleus, and perine raphe nucleus, where co-expression of CLR and RAMP1 was observed with immunofluorescence. \(^{3}H\)MK-3207 showed high binding densities concordant with mRNA and protein expression.

Conclusions

This is the first mapping study demonstrating CLR and RAMP1 mRNA and protein localization in combination with CGRP receptor binding throughout the brainstem of rhesus monkey. The present study suggests that several regions in the brainstem may be involved in CGRP signaling and migraine pathophysiology. Interestingly, we found expression and antagonist binding in areas that are not protected by the blood brain-barrier, suggesting that CGRP receptor antagonists may not need to be CNS-penetrant to functionally antagonize receptors in these brain regions.

Keywords

CGRP, CLR, RAMP1, CGRP receptor antagonists, brainstem, primate.
Introduction

Migraine is a complex neurovascular disorder affecting more than 10% of the global population with a prevalence of 15% to 18% in females and 6% to 9% in males (Stovner et al., 2007). The disorder is characterized by attacks of severe pulsating headache associated with nausea and vomiting and, in some cases, preceded by aura (migraine with aura). Neurologic symptoms including photophobia, phonophobia, tingling, numbness and weakness are common and occur before, during, or after headache (Society, 2004).

The neuropeptide calcitonin gene-related peptide (CGRP) is a potent vasodilator neuropeptide which also has a role in transmission of nociceptive information (Edvinsson, 1987, Poyner, 1992, Gulbenkian et al., 2001, Goadsby, 2007). CGRP has been implicated in the pathogenesis of migraine, where experimental and clinical studies have shown that there is an increased level of trigeminal-system-released CGRP during migraine attacks (Goadsby et al., 1990, Goadsby and Edvinsson, 1993, Bellamy et al., 2006). The most important evidence for the role of CGRP in migraine pain came from the development of CGRP receptor antagonists (Olesen et al., 2004, Edvinsson, 2008, Ho et al., 2008, Ho et al., 2010, Luo et al., 2012). The CGRP receptor antagonists act by blocking the action of CGRP at the CGRP-receptor complex, which is composed of the calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1) (McLatchie et al., 1998) and an intracellular protein receptor component protein (RCP) (Evans et al., 2000). There are three different RAMP proteins: RAMP1, RAMP2 and RAMP3. Interaction of CLR with RAMP2 or RAMP3 forms adrenomedullin receptors (McLatchie et al., 1998, Zhang et al., 2007). RAMPs can also interact with the calcitonin (CT) receptor to form amylin receptors.

It is generally believed that migraine is associated with disturbances in brain function, including the brainstem (Hargreaves and Shepheard, 1999, Goadsby et al., 2009, Charles, 2012, 2013). In patients, migraine-active regions in the brainstem have been demonstrated with imaging techniques such as positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) (Weiller et al., 1995, Diener, 1997, Stankewitz et al., 2011). Also, the brainstem is an important site for sensory signals from the periphery, including the trigeminal ganglion. It has been demonstrated that brainstem stimulation can cause activation of the trigeminovascular system, resulting in CGRP-dependent vasodilatation (Just et al., 2005).

Several brainstem areas have been shown to be activated during a migraine attack, including the midbrain, pons, substantia nigra, red nucleus and the periaqueductal gray
Therefore it is of importance to identify the regions within the brainstem that processes nociceptive information. The distribution of CGRP and its receptor has only been elucidated in a few regions of the brainstem, mainly the spinal trigeminal nucleus (STN) (Lennerz et al., 2008, Eftekhari and Edvinsson, 2011). In the present study, we aimed to localize mRNA and protein expression of the CGRP receptor components by in situ hybridization and immunofluorescence, and identify binding sites of a CGRP receptor antagonist using in vitro autoradiography in rhesus monkey brainstem. The combination of all three methods provides accurate validation of functional CGRP receptor expression in the primate brainstem.

**Material and Methods**

**Rhesus monkey tissue samples**
Rhesus monkey brainstem (Macaca mulatta, n=3, age; 13-15 years old, 2 females, 1 male) was harvested and divided into pieces (midbrain, pons and medulla) in accordance with a Merck Research Laboratories Institutional Animal Care and Use Committee approved protocol. The tissue were frozen and stored at -80°C. For each tissue and animal, sets with adjacent sections were prepared for in situ hybridization and autoradiography. The tissue was placed in the cryostat to equilibrate to -20°C for 15-20 min. Cerebellum from the same animals served as controls for both methods. The samples were cryosectioned at 20 μm (cryostat model CM3050: Leica Microsystems, Inc., Deerfield, IL). Sections were collected on cold Superfrost® Plus slides, thawed and then placed on a slide warmer (37°C) to dry for 5-10 min. Slides were placed in closed desiccated boxes (arranged with dricap dehydrators) and stored at -80°C until use. One rhesus monkey brainstem (Macaca mulatta, age 10 years old, female) was divided into pieces and immersed overnight in fixative consisting of 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer, pH 7.2 and paraffin embedded. The paraffin blocks were sent to the laboratory in Sweden for immunochemistry experiments. Paraffin sections were cut at 5 μm and incubated at +60°C for 1h. 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.

**In situ hybridization**
Probes were designed to be specific for CLR and RAMP1. The CLR probe corresponds to bases 1018-1453 of human CLR (accession number L7630) and was designed to equally distribute the human and rhesus non-conserved bps. The RAMP1 probe (447 bp) corresponds to the full length rhesus RAMP1 coding sequence. Both probes were synthesized by
and subcloned into pBluescript SK+ vectors as 5’EcoRI-3’XbaI fragments. The rhesus RAMP1 and CLR sequences have been disclosed previously and deposited in GenBank with accession numbers KC855758 and KC855759, respectively (Eftekhari et al., 2013).

The CLR and RAMP1 vector constructs were used to generate sense and antisense 35S-UTP labeled cRNA probes for the in situ hybridization. For a complete description of the in situ hybridization method, see Eftekhari et al., 2013. Briefly, tissue sections processed for in situ hybridization were slowly warmed to room temperature, fixed in ice-cold 4% PFA, followed by washes in 1X PBS, 100mM triethanolamine (TEA), 0.25% acetic anhydride in 100mM TEA and 2X saline-sodium citrate (SSC). The sections were delipidated and dehydrated with chloroform and ethanol and allowed to air-dry. Sections were hybridized overnight at 55°C with a hybridization solution containing the 35S-labeled cRNA probe at a concentration of 6x10^6 counts/slide and hybridization buffer.

After hybridization, sections were washed in 2X SSC containing DTT, treated with RNase A (5ml/L) at 37°C, washed in 1X SSC, then finally incubated at 72°C in 0.1X SSC. Slides were equilibrated to room temperature, dehydrated with increasing concentrations of ethanol and air-dried. Sections were exposed to Biomax film (Kodak # IB 8715187) for 7 days. The films were developed using a Kodak Min-R mammography processor and digitized using a Vidar film scanner. The tissue sections were then dipped in NTB-2 liquid emulsion (Kodak # IB1654433) and stored at 7 weeks at 4°C in light-tight desiccated slide boxes. After 7 weeks, these sections were processed for silver grain deposition with developer and fixer (Kodak #1464593 and 1971746), stained with hematoxylin (Htx) and coverslipped. Scanned images of the tissue sections were taken using an Aperio brightfield slide scanner.

**Fluorescent in situ hybridization**

Fluorescent in situ hybridization was performed on selected slides displaying intense in situ hybridization signal. By using this technique, it was possible to determine if the mRNA expression of the receptor components was localized in the same neurons. The CLR probe was labeled with DIG-UTP and the RAMP1 probe was labeled with FITC-UTP (Roche Applied Science). Briefly, 1μg of the linearized plasmids were mixed with labeling mix containing transcription buffer, RNA polymerase, RNase inhibitor and RNAase/DNAase free water, incubated for 2 hr at 37°C. Subsequently, DNase I was added, incubated for 15min at 37°C, followed by addition of EDTA. Purification was done using NucAway Spin columns (Invitrogen) and concentration of the probe was estimated using a Nanodrop. The hybridization and washing steps of the
sections were performed in the same way as the in situ hybridization described above. After the last wash, the sections were equilibrated to room temperature in Tris-NaCl-Tween buffer (TNT; 0.1M Tris-HCl, pH 7.5, 0.15M NaCl, 0.05% Tween 20), followed by a 15 min wash in TNT containing 3% H$_2$O$_2$ and 2x5 min wash in TNT. Slides were incubated for 30 min at room temperature with blocking solution containing TNB (0.1M Tric-HCl, 0.15M NaCl) and 1% PerkinElmer blocking reagent. After the blocking step, sections were incubated at 4°C overnight with the primary antibody anti-FITC (Roche Applied Science), diluted 1:1000 in TNB containing 0.1% blocking reagent. Sections were washed 3x5 min in TNT, incubated with FITC 1:50 diluted in 1X plus tyramide reagent for 30 min at room temperature, followed by 3x5 min wash in TNT. The same steps were repeated (except the blocking step) to apply a new antibody, anti-DIG 1:500 (Roche Applied Science) and fluorophore Cy3 diluted 1:50 in tyramide reagent. Slides were cover slipped with Prolong Gold containing DAPI. The sections were examined with confocal microscope and scanned with a fluorescent Aperio slide scanner.

**Hematoxylin-Eosin staining**

One set of the adjacent slides for each method were stained with Htx using a standard protocol. This was performed for the purposes of orientation and for examining the condition of the tissue. Paraffin sections were deparaffinized in xylene followed by serials of alcohol and stained with Htx-Eosin (Htx 3 min, water rinse, Eosin 1 min). For orientation, The Rhesus Monkey Brain in Stereotaxic Coordinates (Paxinos G, 2000) was used.

**Immunofluorescence**

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 a citric acid solution, pH 6.0. Sections were washed for 10 min in PBS pH 7.2 containing 0.25% Triton X-100 (PBST), followed by incubation in blocking solution for 1 hr (5% normal donkey or goat serum in PBS). The sections were thereafter incubated overnight at 4°C for single or double immunolabelling with primary antibodies against CLR (3152) and RAMP1 (844), diluted in in PBST containing 1% BSA and 3% normal serum. 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 by Western blotting (Eftekhari et al., 2010). On the following day, sections were equilibrated to room temperature, rinsed in PBST for 3x15 min and exposed to secondary antibodies, Alexa 594 (donkey) anti-rabbit 1:400 or Alexa 488 (donkey) anti-goat 1:400, diluted in PBST and
1 % BSA for 1 hr at room temperature. The sections were subsequently washed with PBST for 3x15 min and cover slipped with Vectashield, an anti-fading medium, containing DAPI (Vectashield, Vector Laboratories., Burlingame, CA). For all double immunolabelling, the antibodies were applied separately and not mixed as a cocktail. Several secondary antibodies were tested and evaluated to confirm that same staining pattern was generated. Omission of the primary antibody served as a negative control for all antibodies.

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-labelling by superimposing the digital images and processed for brightness and contrast.

Autoradiography studies

Autoradiography studies were conducted as previously described (Salvatore et al., 2010, Effekhari et al., 2013). Slices (20 μm) were prepared using a cryostat (model CM3050; Leica Microsystems, Inc., Deerfield, IL). Slices 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/mmol) at room temperature. Nondisplaceable binding was defined by blocking with 1 μM unlabeled MK-3207 on cerebellum slides (Salvatore et al., 2010). Slides were washed 3-times in ice-cold buffer (0.9% NaCl and 50 mM Tris-HCl, pH 7.5) followed by an ice-cold rinse. Rhesus brainstem slices were air-dried and exposed to phosphorimaging plates (TR2025; Fujifilm 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).

Results

Histology and controls

The brainstem tissues prepared for the different techniques were not always cut at the same angle as the sections illustrated in the primate atlas used for orientation, thus the depth in some parts of the sections did not correspond to the atlas. Htx-Eosin stained sections from rhesus monkey brainstem prepared for immunofluorescence were considered qualitatively adequate for the immunofluorescence technique. The sections used for immunofluorescence spanned more regions in the brainstem, since the paraffin sections were cut thinner than the ones used for the other techniques. Additionally, the nature of the immunofluorescent technique provided more clearly defined boundaries of the brainstem
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regions than the other techniques. Overall, however, many comparable structures could be delineated throughout the different methods.

Slides containing cerebellar sections served as controls for the *in situ* hybridization and autoradiography studies, since in a previous study we found high binding of [3H]MK-3207 and expression of CLR and RAMP1 in rat and primate cerebellum (Edvinsson et al., 2011, Eftekhari et al., 2013). The sense probes displayed no binding (Fig. 1A). [3H]MK-3207 displayed minimal nondisplaceable binding defined by 1 μM self-block (Fig. 1B). The *in situ* hybridization experiments showed in general lower CLR mRNA expression as compared to that of RAMP1 mRNA, except from few areas were CLR expression seemed to be higher. However, this would need to be confirmed with RT-PCR. One can also speculate that since other receptors rely on RAMP1 in their formation, RAMP1 may be functionally rate limiting and an excess might be necessary for high density CGRP binding. This may also be related to how well the probes recognize the sequences, or it may be that CLR mRNA levels were low at the time of the experiment. Further efforts are needed to clarify this issue. The negative controls used for the immunofluorescence displayed no reactivity, although lipofuscin autofluorescence was seen in some neurons (Fig. 1C).
Figure 1. Controls used for the different methods.
A) Sense probes served as controls for the in situ hybridization and displayed no binding. B) Control for autoradiography with non-labeled compound (cold compound) on the adjacent slide with no binding. C) Negative controls used for immunofluorescence, where primary antibodies were omitted. Use of only the secondary antibodies, Alexa 594 for CLR and Alexa 488 for RAMP1, no immunoreactivity is found, except for lipofuscin auto-fluorescence.

mRNA and protein expression of CLR and RAMP1

mRNA and protein expression were detected within these brainstem regions, starting from the midbrain down to the medulla and the spinal cord:

CLR and RAMP1 mRNA expression were detected in the pineal gland (Pi), medial mammillary nucleus (MM), infundibular stem (InfS), and around the PAG area (Fig. 2 and 3). CLR mRNA expression seemed to be higher than RAMP1 in the MM. In addition, RAMP1 mRNA expression was detected in the 3rd ventricle, dorsal raphe nucleus (DR), medial lemniscus (ml), and trochlear nucleus (4N) (Fig. 3). Fluorescent in situ hybridization revealed expression of
CLR and RAMP1 in the Pi and the MM (Fig 4A). Immunofluorescence data showed co-expression of CLR and RAMP1 in the Pi, MM, 3rd ventricle and infS (Fig. 5). Further co-expression of CLR and RAMP1 was found in PAG, DR, ml, 4N and potine nuclei (Pn) (Fig. 6 and 7). Immunoreactivity of CLR and RAMP1 was found in neurons located in all areas of the PAG (Fig. 6).

Figure 2. *In situ* hybridization displaying CLR mRNA expression. White boxes in the lower resolution images represent the areas shown in high resolution images. Expression CLR mRNA is found in the MM, infS, Pi and around PAG. Example of an area with no mRNA expression is also shown. *Medial mammillary nucleus (MM), Infundibular stem (InfS), Pineal gland (Pi), Periaqueductal gray (PAG).*
Figure 3. *In situ* hybridization displaying RAMP1 mRNA expression. White boxes in the lower resolution images represent the areas shown in high resolution images. RAMP1 mRNA expression is found in the MM, around the 3rd ventricle, infS, Pi, around PAG, DR, ml and 4N. Example of an area with no mRNA expression is also shown. Medial mammillary nucleus (MM), Infundibular stem (InfS), Pineal gland (Pi), Periaqueductal gray (PAG) dorsal raphe nucleus (DR), medial lemniscus (ml), trochlear nucleus (4N).
Figure 4. Localization of CLR and RAMP1 mRNA with in situ fluorescent.
A) 1. Expression of CLR and RAMP1 mRNA in the pineal gland. 2. CLR and RAMP1 mRNA expression in the medial mammillary nucleus, where co-expression of their mRNA is found in the cells (arrows). B) Box number 1 presents the area postrema, where co-expression of CLR and RAMP1 is found. Box 2 displays co-expression of CLR and RAMP1 mRNA in the inferior olive. C) In motor trigeminal nucleus only RAMP1 mRNA is found.
Figure 5. Double-staining of CLR and RAMP1 in rhesus monkey brainstem.
Htx-Eosin staining showing the areas expressing CLR and RAMP1. Co-expression of the receptor components is found in neurons of Pi, MM, 3V and infS (arrows). Neurons only expressing RAMP1 are also found (arrow heads). For nuclei staining DAPI (blue) is used. Pineal gland (Pi), Medial mammillary nucleus (MM), third ventricle (3V), Infundibular stem (InfS).
Figure 6. Distribution of CLR and RAMP1 in rhesus monkey brainstem.
Immunofluorescence results showing co-expression of CLR and RAMP1 in PAG, DR and ml. Expression is found in the neurons (arrows). Insert is showing their co-expression in higher magnification. Neurons expressing CLR and RAMP1 are found in all the areas of PAG. Neurons only expressing RAMP1 are found in DR (arrow heads). For nuclei staining DAPI (blue) is used. Periaqueductal gray (PAG), dorsal raphe nucleus (DR), medial lemniscus (ml).
Moreover, mRNA expression of both receptor components was detected in potine raphe nucleus (PnR), area postrema (AP) and dorsal motor nucleus of vagus, centrointermediate part (10NCeI) (Fig. 8 and 9). In addition, RAMP1 mRNA expression was found in motor trigeminal nucleus (Mo5), abducens nucleus (6N), inferior olive (IO) and hypoglossal nucleus (12N) (Fig. 9 and 4C). By fluorescent in situ hybridization, expression was found in AP and IO, where the receptor components were expressed in the same cells (Fig. 4B). Protein expression of CLR and RAMP1 was found in neurons of the locus coeruleus (LC), PnR and Mo5 (Fig. 10 A). Immunoreactivity of the receptor components was also found in 6N (Fig. 10 B), 10NCeI, 12N and IO (Fig. 11). Cells in the AP area co-expressed CLR and RAMP1 (Fig. 12).
Figure 8. mRNA expression of CLR.
Expression of CLR mRNA is demonstrated by in situ hybridization in PnR, AP and 10NCEI, centrointermediate (arrows). Potine raphe nucleus (PnR), area postrema (AP), dorsal motor nucleus of vagus, the centrointermediate part (10NCEI).
Figure 9. *In situ* hybridization, mRNA expression of RAMP1.

RAMP1 mRNA is found in Mo5, PnR, AP, IO, 12N and 10NCEI (arrows). White boxes in the lower resolution images represent the areas shown in high resolution images. Motor trigeminal nucleus (Mo5), potine raphe nucleus (PnR), area postrema (AP), inferior olive (IO), hypoglossal nucleus (12N) dorsal motor nucleus of vagus, the centrointermediate part (10NCEI).
Figure 10. Protein expression of CLR and RAMP1 in rhesus monkey brainstem.
A) CLR and RAMP1 are expressed in the neurons of LC, PnR and Mo5 (arrows). Smaller neurons co-expressing the receptor components in PnR are found. B) Larger neurons in 6N co-express CLR and RAMP1 (arrows). For nuclei staining DAPI (blue) is used. Locus coeruleus (LC), pontine raphe nucleus (PnR), motor trigeminal nucleus (Mo5), abducens nucleus (6N).
Figure 11. Double-staining of CLR and RAMP1.
Htx-Eosin staining showing the areas with CLR and RAMP1 expression. Co-expression of the receptor components are found in neurons of 10N, 12N and IO (arrows). DAPI (blue), nuclei staining is used in the merged pictures. dorsal motor nucleus of vagus, the centrointermediate part (10NCeI), hypoglossal nucleus (12N), inferior olive (IO).
CLR and RAMP1 mRNA were expressed in the gracile nucleus (Gr) and spinal trigeminal nucleus (Sp5C) (Fig. 13 and 14). In the spinal cord, the receptor components were detected in ventral and dorsal horns, as well around the central canal (Fig. 13 and 14). Interestingly, it seemed that CLR mRNA expression was higher in the dorsal horn while RAMP1 expression was higher in the ventral horn. Immunoreactivity of the receptor components was located in neurons around the Gr and central canal (Fig. 15). In the Sp5C, expression of CLR and RAMP1 was detected in neurons. RAMP1 expression was also found around fiber bundles, but this was not seen for CLR. Double-staining of CLR and RAMP1 revealed co-expression in the neurons of the dorsal and ventral horns (Fig. 16). RAMP1 immunoreactive fibers were found in laminae I and II; however, this was not distinct with the CLR staining.

Figure 12. Double-staining of CLR and RAMP1 in area postrema.
CLR and RAMP1 are co-expressed in cells in the area postrema (arrows). Neurons only expressing CLR are found in this area (arrow heads). DAPI (blue), nuclei staining is used in the merged pictures.
Figure 13. CLR mRNA expression in rhesus monkey.
mRNA expression of CLR is detected by *in situ* hybridization in Gr, STN, around the central gray and in the ventral and dorsal horns of the spinal cord (arrows). *Gracile nucleus (Gr), spinal trigeminal nucleus (STN).*
Figure 14. Expression of RAMP1 mRNA in rhesus monkey.
mRNA expression of RAMP1 is detected by *in situ* hybridization in Gr, STN, around the central gray and in the ventral and dorsal horns of the spinal cord (arrows). *Gracile nucleus* (Gr), *spinal trigeminal nucleus* (STN).
Figure 15. CLR and RAMP1 double-staining.
CLR and RAMP1 are co-expressed in cells of Gr and within Sp5C (arrows). RAMP1 positive fibers are also found in Sp5C (arrow heads). The receptor components are expressed in cells around the central canal of rhesus monkey. For nuclei staining DAPI (blue) is used.
Figure 16. Double-staining of CLR and RAMP1 at the spinal cord level of rhesus monkey. Small neuron expressing CLR and RAMP1 are found within laminae I/II of the dorsal horn (arrows). Co-expression of the receptor components are also found in larger neurons of the ventral horn (arrows).

Autoradiographic distribution of $[^{3}H]$MK-3207 binding sites
$[^{3}H]$MK-3207 binding densities were observed in the Pi, MM, 3rd ventricle and infS (Fig. 17A). Further, binding densities were detected in the DR, ml, around the PAG area and in the 4N (Fig. 17A). $[^{3}H]$MK-3207 binding was also found in PnR, Pn, IO, AP, 10NCeI and 12N (Fig. 17B). In some areas, such as 6N and Mo5, antagonist binding could not be detected due to tissue damage. High $[^{3}H]$MK-3207 binding densities were observed in the Gr and Sp5C (Fig. 17C). At the spinal cord level, binding was detected in the ventral and dorsal horns, and around the central canal (Fig. 17C).
Figure 17. Distribution of \[^{3}H\]MK-3207 binding to rhesus monkey brainstem.
A) \[^{3}H\]MK-3207 displayed high binding density in Pi, mm, infS, 3rd ventricle, ml, DR, PAG and 4N.
B) Binding densities in PnR, Pn, AP, IO, 10N and 12N. C) Areas that also display high binding densities of \[^{3}H\]MK-3207 are; Gr, STN, around the central gray and in dorsal and ventral horn of the spinal cord.

Discussion
Brainstem activation has been demonstrated in many pain conditions, including migraine (May, 2013). Several studies have demonstrated the role of the brainstem in acute migraine and potentially in chronic migraine as well (Bahra et al., 2001, Afridi et al., 2005, Stankewitz et al., 2011). These observations consistently showed an increase of regional cerebral blood flow in the rostral brainstem that persisted even after sumatriptan had induced relief from headache, nausea, phonophobia and photophobia (Weiller et al., 1995). The role of the brainstem in migraine pathophysiology is supported by studies that showed non-headache patients developed migraine-like attacks after stereotactic placement of electrodes in the PAG (Raskin et al., 1987, Veloso et al., 1998). Interestingly, activity of
the spinal trigeminal nuclei and increased activation of the rostral pons was observed in response to nociceptive information, with a cycling behavior over the migraine interval. The activity increased toward the next migraine attack, which may reflect the susceptibility of the brain to generate the next attack, as these areas increase their activity long before the headache starts (Stankewitz et al., 2010, May, 2013).

Together, these studies support the hypothesis that the brainstem has a pivotal role in migraine pathophysiology. Given the role of the brainstem and the clinical efficacy of CGRP receptor antagonists in migraine treatment, knowledge about the distribution of CLR and RAMP1 in the brainstem will provide a better understanding of CGRP signaling and the potential sites of action of treatments. Therefore, we designed the present study to map the expression pattern of the CGRP receptor components and in vitro binding of a CGRP receptor antagonist throughout the entire brainstem of rhesus monkey. To our knowledge, this study is the first to demonstrate CLR and RAMP1 expression in several of these brainstem regions many which correspond with receptor binding.

Several areas within the brainstem displayed mRNA and protein expression of CLR and RAMP1. [³H]MK-3207 showed binding densities concordant with expression of the receptor components. The widespread distribution of the CGRP receptor in the brainstem suggests that CGRP receptor antagonists may need to act on central sites. The first PET studies in both healthy and migraine patients suggested that inhibition of central CGRP receptors is not essential for the relief of migraine pain by telcagepant (Vermeersch S, 2012, Hostetler et al., 2013). Additional studies with more brain penetrant CGRP receptor antagonists will be required to see if additional anti-migraine efficacy can be gained by accessing central receptors. The present study also identified CLR and RAMP1 expression in brainstem areas outside the BBB, where CGRP receptor antagonists do not need to be CNS-penetrant to block receptors in these areas. The areas displaying mRNA, protein expression and receptor binding are discussed below.

The medial mammillary nucleus (MM)
The MM is one part of the mammillary body, considered to be part of the limbic system. It is linked to the hypothalamus via a nerve path called the fornix. The mammillary bodies act as a relay for signal coming from the hippocampi and amygdale via the mamillo-thalamic tract to the thalamus. These structures are important for memory processing (Aggleton et al., 2011). CGRP immunoreactive fibers have been demonstrated in the MM (Covenas et al., 2012) and in the present study we found mRNA expression of CLR and RAMP1. Immunofluorescence de-
monstrated co-expression of CLR and RAMP1 in the neurons of the MM. The fibers may release CGRP to act on neurons expressing its receptor in the MM. Binding of the CGRP receptor antagonist was clearly observed in this area, supporting the expression of functional CGRP receptors. The results are in agreement with a study showing binding of \(^{[125]}\text{I-Tyr}\) hCGRP\(_{8-37}\) in the MM of rodents (Van Rossum et al., 1994).

3rd ventricle
The third ventricle is situated between the two halves of the diencephalon. In this area, we found RAMP1 mRNA expression, while CLR mRNA could not be detected. Using immune-fluorescence it was observed that both CLR and RAMP1 proteins are expressed in neurons around the third ventricle. Similarly, \(^{[3}\text{H}]\text{MK-3207}\) binding was detected around the area of the third ventricle.

Infundibular stem (InfS)
The pituitary gland is functionally connected to hypothalamus via a small tube called the InfS. It carries axons from cells of the hypothalamus down to the posterior pituitary gland where they release oxytocin and vasopressin into the blood. CGRP positive nerve fibers have been demonstrated in the pituitary gland of rat and human (Skofitsch and Jacobowitz, 1985, Liu, 2004). Interestingly, we found mRNA expression and protein expression of the CGRP receptor components in the InfS. CLR and RAMP1 were co-expressed in neurons, and binding of the CGRP receptor antagonist was detected in this area. It remains to be evaluated if, and in which cell type, the CGRP receptor is expressed within the hypothalamus and the pituitary gland and what role CGRP may have in this system.

The pineal gland (Pi)
The Pi lies in the center of the brain, behind the third ventricle. The gland consists of two types of cells, pinealocytes and neuroglial cells. Pi produces melatonin and serotonin (Reiter, 1991). Melatonin has been linked to migraine and cluster headache in several ways (Toglia, 2001, Peres, 2005). Some patients reported their headaches to occur predominantly or specifically at a certain period of the day, either waking up in the morning with headache or being woken up at night by the headache (Reiter, 1991). The first study showing lower plasma levels of melatonin in migraine patients was in 1989 (Claustrat et al., 1989) and thereafter supported by several studies (Peres, 2005). Melatonin has been suggested to be involved in headache pathophysiology by several mechanisms such as anti-inflammatory effects, reducing up-regulation of proinflammatory cytokines and inhibition of the activity of nitric oxide synthase (Peres, 2005). In addition, CGRP positive fibers have been detected in the Pi (Nowicki et al., 2007) and it has been demonstrated that the trigeminal ganglion innervates Pi (Reuss, 1999).
Interestingly, we found mRNA expression of the CGRP receptor components in the rhesus monkey Pi. CLR and RAMP1 mRNA and protein were expressed in the Pi, as demonstrated with FISH and immunofluorescence. High binding densities were displayed with [³H]MK-3207, supporting the expression of functional CGRP receptor in the Pi. The functions of CGRP and its receptor in the Pi are currently unknown. What is very interesting with this brain area is that the Pi is not protected by the BBB, which makes it a possible site of action for drugs like CGRP receptor antagonists, regardless of their ability to pass the BBB. The effects of blocking CGRP signaling in the Pi is not known, however the relief of certain symptoms with CGRP receptor antagonist treatment may be explained by its action in the Pi.

Dorsal raphe nucleus (DR)
The DR gives rise to an ascending serotonergic projection to the striatum and has extensive projections to most of the cerebral cortex and other forebrain nuclei (Martin, 2003). The DR is known to play an important role in nociceptive processing and in anti-nociception (Wang and Nakai, 1994). During trigeminal nociception, noxious trigeminal stimulation with capsaicin, it was found that the DR is activated, suggesting that the DR has a role in migraine (Ter Horst et al., 2001). High densities of neurons expressing CGRP have been demonstrated in the DR (de Souza et al., 2008). Protein expression of RCP has also been shown in neurons of the DR (Ma et al., 2003). In the current work, we demonstrated expression of the CGRP receptor components, CLR and RAMP1, with [³H]MK-3207 binding in the DR of rhesus monkey. Our results support that the DR may be involved in migraine pathology and that CGRP signaling may have a function in this area.

Medial lemniscus (ml)
The ml is a brainstem tract that contains axons traveling from the dorsal column nuclei to the thalamus, called the dorsal column-medial lemniscal system. This system brings information from sensory receptors in the periphery to the brainstem, and to higher levels, such as the thalamus (Martin, 2003). Axons in the medial lemniscus synapse in the thalamus, from where neurons project to the internal capsule, which then sends axons to synapse on neurons in the primary somatic sensory cortex (Martin, 2003). The receptor components of the CGRP receptor have been revealed in the rat thalamus (Summ et al., 2010), and this works reveals the presence of CLR and RAMP1 in the medial lemniscus. High binding densities of the CGRP receptor antagonist were also found, supporting the expression of functional CGRP receptors. CLR and RAMP1 have also been identified in the dorsal root ganglia, the first neurons transmitting signals to the dorsal column (Cottrell et al., 2005). Altogether, these results demonstrate
the presence of the CGRP receptor within the dorsal column-medial lemniscal system, suggesting that CGRP signaling may occur in this system.

**Periaqueductal gray (PAG)**

This brainstem area is a known modulator of somatic pain transmission. The ventrolateral part of periaqueductal gray has been shown to be activated during migraine (Weiller et al., 1995). This area has been suggested to modulate the trigeminovascular input, where the PAG inhibits afferent trigeminal nociceptive traffic (Knight and Goadsby, 2001). Therefore, it is believed that PAG is a key structure in the pain processing of migraine. CLR and RAMP1 mRNA expression was found in the PAG of rhesus monkey. Their co-existence in neurons was demonstrated in all areas of PAG and the autoradiography results showed binding densities in the PAG. An early study demonstrated the existence of CGRP in the fibers of PAG (Conti and Sternini, 1989). Perhaps CGRP is released from these fibers to act on the neurons expressing its receptor.

**Locus coeruleus (LC)**

The LC sends projections to higher brain structures and to the TNC and spinal cord dorsal horn. It contains noradrenergic neurons and its projections are thought to play an important role in response of the brain to stressful stimuli, particularly fear (Martin, 2003). The LC is suggested to have an important role in nociceptive processing and migraine pathophysiology since it is often activated after painful stimuli (Ter Horst et al., 2001). A high number of LC neurons express CGRP (Tajti et al., 2001). We found co-expression of CLR and RAMP1 in the neurons of LC, suggesting expression of CGRP receptors in this area. Due to technical reasons, we were not able to study mRNA expression or receptor binding in LC. However, our results suggest that CGRP may have a function in LC neurons.

**Pontine raphe nucleus (PnR)**

The PnR is one of the raphe nuclei, which projects to different brainstem nuclei. We found that the PnR small neurons displayed both mRNA and protein expressions of CLR and RAMP1. Binding densities of [³H]MK-3207 were also found in the PnR. The function of CGRP in this area remains to be assessed.

**Pontine nuclei (Pn)**

Pn transmit information from the cerebral cortex to the cerebellum, and participate in skilled movement control. In Pn, protein expression of CLR and RAMP1 was found in the small-sized neurons. Co-expression of the receptor components was seen, suggesting expression of a functional CGRP receptor. This was supported by the autoradiography experiments, showing binding of [³H]MK-3207 in Pn. Our results are further reinforced by a binding study with the CGRP receptor antagonist CGRP₈₋₃₇, where high densities of [¹²⁵I-Tyr] hCGRP₈₋₃₇
were detected in Pn (Van Rossum et al., 1994).

**Cranial nerve motor nuclei**

Several motor nuclei in the brainstem were found to express CLR and RAMP1. The trochlear nerve is a motor nerve that innervates muscles that move the eyes (Martin, 2003). The trochlear motor neurons are found in the 4N, trochlear nucleus. CGRP expressing neurons have been found in the 4N (de Souza et al., 2008) and the existence of CGRP mRNA (Smith et al., 1994). In the present study, we found RAMP1 mRNA expression and co-existence of CLR and RAMP1 protein expression in the 4N. The autoradiography experiment with \[^{3}H\]MK-3207 supported the expression of functional CGRP receptors in this motor nuclei.

In the Mo5 (motor trigeminal nucleus), RAMP1 mRNA expression and neurons co-expressing CLR and RAMP1 were found. The motor neurons in Mo5 innervate the muscles of mastication (masseter, temporalis, and external and internal pterygoid muscles). CGRP positive neurons have been found in the Mo5 (de Souza et al., 2008).

Further, we found mRNA expression of RAMP1 in the 6N (abducens nucleus) and 12N (hypoglossal nucleus), and co-expression of the receptor components in neurons were revealed by immunofluorescence. The 6N contains motor neurons that project to the periphery by the abducens (VI) nerve and innervate the lateral rectus muscle (Martin, 2003). The axons of motor neurons in 12N course through the hypoglossal (XII) nerve and innervate intrinsic tongue muscles (Martin, 2003). Due to artifacts in the tissue, binding of \[^{3}H\]MK-3207 in Mo5 and 6N could not be determined. However, binding densities were detected in 12N, and in support, binding densities of CGRP\(^{-37}\) have been demonstrated in rat Mo5 (Van Rossum et al., 1994). These results suggest a role of CGRP in cranial (Mauskop, 2005) motor nuclei, where CGRP receptor antagonists may modulate their activity.

Expression of CLR and RAMP1 was furthermore found in the dorsal motor nucleus of the vagus, the centrointermediate part (10CeI) that is a part of the parasympathetic nervous system. In this region, binding of the CGRP receptor antagonist was detected, supporting the presence of a functional CGRP receptor. Stimulation of the vagus nerve has been suggested as a treatment for chronic migraine (Mauskop, 2005). In this study, we suggest that CGRP receptor antagonists may act in the centrointermediate part of the vagus nerve and modulate its activity. However functional studies are necessary to investigate this idea.

**Area postrema (AP)**

The AP, a part of caudal medulla, is important in the control of vomiting and nausea. This area belongs to the
circumventricular organs of the brain that do not have a BBB. It is known that about a third of migraine sufferers experience vomiting, suggesting that the AP may be involved in migraine pathophysiology. Activation of the AP has been demonstrated in a trigeminal stimulation-induced rat model, suggesting that the AP may have role in migraine headache (Ter Horst et al., 2001). Interestingly, we found mRNA and protein expression of CLR and RAMP1 in the AP. This is in agreement with earlier observations showing RAMP1 mRNA in the AP (Ueda et al., 2001, Barth et al., 2004). Furthermore, this study demonstrates binding of $[^3H]MK-3207$ in the AP. Our results suggest expression of functional CGRP receptors in these areas, and CGRP receptor antagonists may be able to act on this site independently from their ability to cross BBB. In clinical studies, CGRP receptor antagonists were able to abort symptoms such as nausea (Ho et al., 2008). Perhaps this could be due to the action of CGRP receptor antagonists in the AP.

**Inferior olive (IO)**

Neurons in the IO project to the cerebellum, where they form strong excitatory synapses (Martin, 2003). In our previous study, we demonstrated expression of the CGRP receptor, as well as binding of CGRP and a CGRP receptor antagonist, in the primate cerebellum (Eftekhari et al., 2013). In the present study, we found expression of the CGRP receptor components in the IO with binding densities of $[^3H]MK-3207$. In support of this, binding of sites $[^{125}\text{I}]\text{hCGRP}_{\alpha}$ and $[^{125}\text{I-Tyr}]\text{hCGRP}_{8-37}$ have been shown in the IO and cerebellum of rat (Inagaki et al., 1986, Van Rossum et al., 1994). These results suggest CGRP signaling between the IO in the brainstem to the cerebellum.

**Gracile nucleus (Gr)**

The Gr is involved in sensation of fine touch and transmission of information to the legs and lower trunk. This nucleus contains second-order neurons that receive inputs from dorsal root ganglia and send axons to the thalamus via the ml. As in the ml, we found mRNA and protein expression of CLR and RAMP1 in the Gr. $[^3H]MK-3207$ binding was also demonstrated in Gr. This supports our proposal that the dorsal column-medial lemniscal system may involve CGRP signaling where CGRP receptor antagonists may interact.

**Spinal trigeminal nucleus (STN)**

The STN processes nociceptive information from the trigemino-vascular system and contains CGRP binding sites (Inagaki et al., 1986). Studies have therefore investigated the expression of CGRP and its receptor components in the STN (Smith et al., 2002, Lennerz et al., 2008, Eftekhari and Edvinsson, 2011). We found mRNA expression of CLR and RAMP1 in the STN of rhesus monkey. In contrast to our previous study on rat and human STN, we detected co-expression of the receptor components in cells within STN of
The present study provides accurate validation of functional CGRP receptor expression throughout the brainstem and the spinal cord. Several areas in the brainstem were revealed to express mRNA and protein expression of CLR and RAMP1, which were in accordance with binding of a CGRP receptor antagonist. Many of these areas involve sensory centers, cranial motor nuclei and pathways to the thalamus, suggesting that drugs blocking CGRP signaling may act at these central sites during migraine. Interestingly, some of these areas are not protected by the blood brain-barrier, suggesting that effective...
CGRP receptor antagonists may not need to be CNS-penetrant.

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Ho TW, Ferrari MD, Dodick DW, Galet V, Kost J, Fan X, Leibensperger H,


Paper VI
Cerebellar distribution of calcitonin gene-related peptide (CGRP) and its receptor components calcitonin receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1) in rat

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A B S T R A C T
Clinical and experimental results have revealed a fundamental role of calcitonin gene-related peptide (CGRP) in primary headaches. CGRP is widely expressed in neurons both in the central nervous system (CNS) and in peripheral sensory nerves. In the CNS there is a wide distribution of CGRP-containing neurons with the highest levels seen in striatum, amygdala and cerebellum. Moreover, in acute attacks of migraine there is evidence of cerebellar activation. To understand the role of CGRP, antibodies towards the CGRP receptor components calcitonin receptor-like receptor (CLR) and receptor activity modifying protein type 1 (RAMP1) have been developed. In the present study we therefore examined immunohistochemically the distribution of CGRP and its receptor components in the cerebellum. CGRP immunoreactivity was only found intracellullarly in the cerebellar Purkinje cell bodies, whereas CLR and RAMP1 were detected on the surface of the Purkinje cell bodies and in their processes. The elaborate dendritic tree of Purkinje cell fibers was distinctly visualized with the RAMP1 antibody. In addition, profoundly stained fibers spanning from the molecular layer into the medulla was observed with the RAMP1 antibody. Judged from the high density of immunoreactive cells expressing CGRP, RAMP1 or CLR, and from the double staining of CGRP and RAMP1 it is likely that most, if not all, Purkinje cells express both the peptide and the receptor components. Double staining with RAMP1 and the glial cell markers glial fibrillary acidic protein (GFAP) and S-100 revealed an almost identical staining pattern of the antibodies in the area of the cell body surfaces. However, as judged by confocal microscopy, no double staining was present. Instead, it was discovered that the glial cells tightly surrounded the Purkinje cell bodies which easily could be interpreted as co-localization in the epifluorescence microscope.

Our observations demonstrate that there is a rich expression of CGRP and CGRP receptor elements in the cerebellum which points towards a functional role of CGRP in cerebellar Purkinje cells. Recent advances in the biology of the cerebellum indicate that there may be a role in nociception; hence a target of the recently discovered CGRP receptor antagonists that have demonstrated improvement in migraine pain and associated symptoms could be cerebellar CGRP receptors.

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Introduction

Positron emission tomography (PET) studies of patients during migraine attacks demonstrated activation of cerebellar regions (May and Goadsby, 1999; Weiller et al., 1995; Bahra et al., 2001). However, no explanation for this activation has emerged (Goadsby et al., 2002). Migraine is considered to originate in the central nervous system (CNS), and as a result of an acute attack the trigeminovascular system is activated resulting in both central and peripheral release of calcitonin gene-related peptide (CGRP) (Edvinsson and Uddman, 2005). Increased levels of CGRP occur in the cranial venous outflow following an acute migraine attack (Edvinsson, 2004) and CGRP receptor antagonists have demonstrated improvement in migraine pain and associated symptoms (Ho et al., 2008a,b; Olesen et al., 2004). The site of action of the CGRP receptor antagonists is debated but presently both the peripheral and central ends of the trigeminovascular system are considered likely targets (Edvinsson, 2008a,b).

Early immunohistochemical studies have demonstrated CGRP in the cerebellum (Kawai et al., 1985; Lee et al., 1985). The CGRP immunoreactivity was localized in the Purkinje cell and in its elaborated dendrite.
Morara and collaborators have since then investigated CGRP distribution in different developmental stages in rats (Morara et al., 1989, 2000, 2001, 1998). It has been shown that CGRP is transiently expressed in cerebellar climbing fibers (Gregg et al., 1999) while its receptor is suggested to be expressed in cerebellar glial cells (Morara et al., 1998). Climbing fiber afferents to the cerebellum from the inferior olivary complex have powerful excitatory effects on Purkinje cells. CGRP may suppress both the spontaneous firing rate of olivary neurons and the enhanced activity induced by excitatory amino acids. The receptor for CGRP is composed of a G-protein-coupled receptor known as the calcitonin receptor-like receptor (CLR), a single trans-membrane domain protein called receptor activity-modifying protein 1 (RAMP1) and an intracellular protein, receptor component protein (RCP) (McLatchie et al., 1998). A previous study on RAMP1 revealed localization in the cerebellum (Ueda et al., 2001) while the detailed localization remains unclear.

The detailed examination of CGRP receptor components have been made possible with the recent development of a series of specific antibodies against CLR and RAMP1 (Eftekhari et al., 2010). This is of particular interest in understanding how the recent CGRP receptor antagonists exert their antimigraine effects. In the present study we examined the distribution of CGRP and its receptor components in the cerebellum.

**Results**

The negative controls displayed no immunoreactivity.

CGRP immunoreactivity was found in all of the cerebellar Purkinje cell bodies as intracellular granular staining. CGRP immunoreactivity was sometimes identified in combination with thin, twisted and thread-like immunoreactive formations (Fig. 1A–C), resembling the tree. Morara and collaborators have since then investigated CGRP distribution in different developmental stages in rats (Morara et al., 1989, 2000, 2001, 1998). It has been shown that CGRP is transiently expressed in cerebellar climbing fibers (Gregg et al., 1999) while its receptor is suggested to be expressed in cerebellar glial cells (Morara et al., 1998). Climbing fiber afferents to the cerebellum from the inferior olivary complex have powerful excitatory effects on Purkinje cells. CGRP may suppress both the spontaneous firing rate of olivary neurons and the enhanced activity induced by excitatory amino acids. The receptor for CGRP is composed of a G-protein-coupled receptor known as the calcitonin receptor-like receptor (CLR), a single trans-membrane domain protein called receptor activity-modifying protein 1 (RAMP1) and an intracellular protein, receptor component protein (RCP) (McLatchie et al., 1998). A previous study on RAMP1 revealed localization in the cerebellum (Ueda et al., 2001) while the detailed localization remains unclear.

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**Results**

The negative controls displayed no immunoreactivity.

CGRP immunoreactivity was found in all of the cerebellar Purkinje cell bodies as intracellular granular staining. CGRP immunoreactivity was sometimes identified in combination with thin, twisted and thread-like immunoreactive formations (Fig. 1A–C), resembling the
appearance of endoplasmic reticulum. No immunoreactivity was found in the dendrites or axons, nor in other neurons or glial cells.

CLR immunoreactivity was found on the surface of the Purkinje cell bodies and in their processes (Fig. 1D–F). However, the staining results were somewhat inconsistent compared to the results obtained with the CGRP and RAMP1 antibodies, with weakly stained or even immuno-negative areas.

Similar to the CGRP staining, RAMP1 was found in all of the Purkinje cells. The elaborated tree of Purkinje cell fibers, especially the proximal parts, displayed intense and homogenous RAMP1 immuno-positivity (Fig. 1G–I). In addition, profoundly stained fibers spanned from the molecular layer into the medulla.

Staining with rat CLR and RAMP1 displayed similar results as for human CLR and RAMP1, i.e. immunoreactivity localized to the region of the cytomembrane of the Purkinje cells (Fig. 2).

Double staining with CGRP and RAMP1 revealed no double-labeling of the peptide and its receptor component, but all cells positive for CGRP seemed to be positive for RAMP1 (Fig. 3A–F).

CLR-RAMP1 double staining showed somewhat variable results due to inconsistency with the CLR antibody, as also was the case with

![Images of staining results](image)

**Fig. 3.** RAMP1 and CGRP, and RAMP1 and CLR double staining. CGRP was exclusively expressed in the cytoplasm of Purkinje cell bodies as opposite to the RAMP1 staining (A–F), which was expressed on the surface of the cell bodies and in the processes throughout all the cerebellar layers. (G–I) CLR and RAMP1 were expressed on the surface of the Purkinje cells ml — molecular layer, Pc — Purkinje cells, DAPI — blue.
the CLR single staining. However, Purkinje cells positive for both CLR and RAMP1 were found in some regions with cell surface and fiber staining for both CLR and RAMP1 (Fig. 3G–I).

Double staining with RAMP1 and GFAP disclosed an almost identical staining pattern of the two antibodies in the area of the cell body surfaces, which would suggest RAMP1 in the glial cells.

**Fig. 4.** RAMP1 and GFAP double staining. Images A–C represent low magnifications of the cerebellar layers. The RAMP1 image demonstrates the distribution of immuno-positive fibers spanning through the cerebellar layers. Also the GFAP staining reveals immunopositivity in all the layers. Images D–F show the staining of the granular layer and the white matter. Arrow points at RAMP1 positive fiber and asterisk at a GFAP positive astrocyte. Images G–I show the molecular and Purkinje cell layer. Arrow points at a horizontal RAMP1 positive fiber and asterisk at GFAP positive vertical process. Images J–L demonstrate the Purkinje cell layer. The staining with RAMP1 and GFAP showed similar pattern of cell surface immunoreactivity. However, there was a difference: the GFAP staining distinctly outlined the cell surface whereas the RAMP1 staining seemed more diffuse. Large arrows point at regions where either of the antibodies showed positivity and small arrow at a Bergmann glial cell outlined with RAMP1 immunoreactivity, which could be mistaken for RAMP1 immunoreactivity in Bergmann cells. However, confocal microscopy revealed that no co-localization exists (M), which means that RAMP is exclusively expressed in neurons and GFAP in glial cells. Image 3M is also shown as video data, see supplementary data. ml — molecular layer, Pc — Purkinje cells, gc — granular layer, wm — white matter, DAPI — blue.
In the molecular layer, RAMP1 positive horizontal fibers, judged as fibers of the Purkinje cells, were a common finding whereas GFAP stained vertical fibers typical for glial cell processes. True double staining could not be established with the epifluorescence microscope, however, confocal microscopy revealed that such double staining did not occur, which means that RAMP1 is not expressed in the cerebellar glial cells.

Double staining with RAMP1 and the glial cell marker, S-100, revealed a similar pattern as for RAMP1 and GFAP. In addition, Bergmann glial cell stained positive with the S-100 antibody.

Double staining with RAMP1 and the glial cell marker, S-100, revealed a similar pattern as for RAMP1 and GFAP. In addition, Bergmann glial cell stained positive with the S-100 antibody.

**Fig. 5.** RAMP1 and S-100 double staining. Images A–C demonstrate low magnifications of the molecular, Purkinje cell and granular layers. Thin arrows point at a Purkinje cell seemingly positive for both antibodies and thick arrow at a fiber traversing through the granular layer. Images D–F show the staining of the Purkinje cells: RAMP1 intense, but diffuse and the S-100 outlining the cells. Thin arrows point at a region where the RAMP1 and S-100 staining went parallel, arrowheads at areas with distinct S-100 staining but diffuse RAMP1. Thick arrows point at Bergmann glial cells. Images G–I demonstrate a high magnification of Purkinje cells. Thin arrows point at the cell surface with distinct RAMP1 staining and with missing S-100 staining. Arrowheads point at the outlining of a process, the RAMP1 and S-100 running parallel. (J) Confocal microscopy in combination with NIS Element software revealed no co-localization of RAMP1 and S-100. Arrows point at Bergmann glial cells. ml—molecular layer, Pc—Purkinje cells, gc—granular layer, DAPI—blue. Image 4J is also shown as video data, see supplementary data.
and J), a staining that was not present with the GFAP antibody. Also in this experiment, the glial cell marker seemed to outline the cell surface of the Purkinje cells (Fig. 4G–I), but again confocal microscopy revealed no double staining (Fig. 4J, video data).

In summary, CGRP and its receptor components RAMP1 and CLR could be found in an individual Purkinje cell (Fig. 6). However, no peptide- or receptor immunoreactivity was found in the glial cells.

Discussion

The aim of the present study was to identify CGRP and its receptor components within the rat cerebellum since there are PET data revealing activation of the cerebellum in migraine attacks. For this purpose, we used a set of newly developed and in depth characterized antibodies against CLR and RAMP1 (Eftekhari et al., 2010).

The study demonstrates for the first time the detailed mapping of CGRP together with its receptor components in the rat cerebellum. The study shows that CGRP is exclusively found in the cytoplasm of cerebellar Purkinje cell bodies, which confirms results from an early study of Kawai and co-workers (Kawai et al., 1985). RAMP1 and CLR, which collectively represent the two components of the mature CGRP receptor, are expressed on the surface of the Purkinje cell bodies and in the fibers, spanning through the entire cerebellum. In the CGRP receptor complex the CLR component is a 7 transmembrane component that needs to be associated with RAMP1 in order to form the functional CGRP receptor. Overall there was a larger RAMP1 distribution compared to CLR. There are several possible explanations for this; (i) it might reflect differences in the quality of the antibodies recognizing the epitopes. (ii) The slightly higher proportion of RAMP1 compared to CLR may also reflect the presence of other RAMP1-containing receptors such as the amylin receptors (Oliver et al., 2001). Since there are few amylin binding sites in the cerebellum, another receptor could be involved. (iii) Hypothetically, since other receptors use RAMP1 in their formation, RAMP1 may well be functionally rate limiting in receptor formation and an excess would be necessary for high density CGRP binding. This issue may be clarified in future studies.

Double-staining with CGRP and RAMP1 revealed no co-localization between the peptide and its receptor components, although they were expressed in the same cell suggesting that the neuronal messenger might act on sites where it is produced. RAMP1 and CLR double staining revealed that both are expressed in the same cell which indicates that the functional receptor is present. In addition, double staining with RAMP1 and the glial cell markers did not reveal any co-localization. This was studied in detail using confocal microscopy and the NIS Element software; this allows the full use of confocal microscopy where stacks of 100–200 sections can be viewed and analyzed 3-dimensional. Hence, the adjacent glial cells were observed not to be the target of the Purkinje cell CGRP. Gialal cells were in the rat cerebellum devoid of both CGRP and CGRP receptors.

CGRP has a key role in migraine, where levels of trigeminal system released CGRP is increased during migraine attacks (Ho et al., 2010). Activation of regions in the cerebellum has been demonstrated by PET in patients during acute migraine attacks (May and Goadsby 1999; Weiller et al., 1995; Bahra et al., 2001). The cerebellum is of interest in migraine research as studies suggest a role of cerebellum in symptoms of migraine such as vertigo (reviewed by Vincent and Hadjikhani, 2007). Our observations reveal that there is a rich expression of CGRP and CGRP receptor elements in the cerebellum clearly pointing towards a functional role of CGRP in the cerebellum; this is of course not necessarily related to primary headaches.

Table 1
Details on primary antibodies used for immunohistochemistry.

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<th>Name and product code</th>
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<th>Supplier</th>
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<td>Rat CGRP</td>
<td>Europroxima; Arnhem, The Netherlands.</td>
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<tr>
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<td>C-terminal of human CLR</td>
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</tr>
<tr>
<td>Calcitonin receptor-like receptor (CLR) 3155</td>
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<td>1:500</td>
<td>C-terminal of rat CLR</td>
<td>Merck &amp; Co., Inc., (Eftekhari et al., 2010)</td>
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<tr>
<td>Receptor activity modifying protein (RAMP1) 844</td>
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<td>1:100</td>
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<td>Merck &amp; Co., Inc., (Eftekhari et al., 2010)</td>
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<tr>
<td>Receptor activity modifying protein (RAMP1) 3158</td>
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<td>1:500</td>
<td>C-terminal of rat RAMP1</td>
<td>Merck &amp; Co., Inc., (Eftekhari et al., 2010)</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
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</tr>
<tr>
<td>S-100, ab66041</td>
<td>Rabbit</td>
<td>1:300</td>
<td>Glial cells</td>
<td>Abcam; Cambridge, UK</td>
</tr>
</tbody>
</table>
**Experimental methods**

Adult male Sprague-Dawley rats (weighing 300–400 g; Scanbur, Stockholm, Sweden) were exsanguinated during CO2 sedation, and the cerebellum removed (n = 8). The experiments were approved by the University Animal Ethics Committee (M161-07), Lund University, Sweden.

The tissue pieces were immersed into 4% paraformaldehyde (PFA) for 2–4 h and subsequently rinsed in raising concentrations of sucrose in Störeen’s phosphate buffer, embedded in a gelatin medium (30% egg albumin and 3% gelatin in distilled water) and cryosectioned at 12 μm. The sections were stored at −20 °C until use.

Sections were thawed and rinsed in phosphate buffered saline (PBS, pH 7.2) containing 0.25% Triton X-100 (PBST). The sections were then exposed to primary anti-sera against CGRP, CLR or RAMP1 (for detailed description of antibodies, see Table 1) for 16–18 h in a moist chamber at 4 °C, following rinsing in PBST. In addition, double immunostainings with CGRP and RAMP1, RAMP1 and CLR, RAMP1 and GFAP, RAMP1 and S-100 were performed. Tissue sections were incubated with secondary antibodies (Table 2) for 1 h in the dark at room temperature and thereafter mounted with an antifading mounting medium (Vectashield, Sigma). DAPI (Vectashield medium containing 4,6-diamidino-2-phenylindole, Sigma) was used to stain nuclei in some sections.

Omission of the primary antibody served as negative controls.

In addition, to compare the results achieved with the human RAMP1 and CLR antibodies used, we examined the outcome of rat RAMP1 and CLR immunohistochemistry (for antibody details, see (Eftekhari et al., 2010)).

**Disclosure statement**

All authors participated in design, performing the study, and writing of the manuscript. All authors participated in the writing of the manuscript and approved the final manuscript.

**References**


Localization of CGRP Receptor Components, CGRP, and Receptor Binding Sites in Human and Rhesus Cerebellar Cortex

Sajedeh Eftekhari • Christopher A. Salvatore • Renee C. Gaspar • Rhonda Roberts • Stacey O’Malley • Zhizhen Zeng • Lars Edvinsson

Abstract The cerebellum is classically considered to be mainly involved in motor processing, but studies have suggested several other functions, including pain processing. Calcitonin-gene-related peptide (CGRP) is a neuropeptide involved in migraine pathology, where there is elevated release of CGRP during migraine attacks and CGRP receptor antagonists have antimigraine efficacy. In the present study, we examined CGRP and CGRP receptor binding sites and protein expression in primate cerebellar cortex. Additionally, mRNA expression of the CGRP receptor components, calcitonin receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1), was examined. In addition, expression of procalcitonin was studied. We observed high [3H]MK-3207 (CGRP receptor antagonist) binding densities in the molecular layer of rhesus cerebellar cortex; however, due to the limit of resolution of the autoradiographic image the exact cellular localization could not be determined. Similarly, [125I]CGRP binding was observed in the molecular layer and Purkinje cell layer of human cerebellum. CLR and RAMP1 mRNA was expressed within the Purkinje cell layer and some expression was found in the molecular layer. Immunofluorescence revealed expression of CGRP, CLR, and RAMP1 in the Purkinje cells and in cells in the molecular layer. Procalcitonin was found in the same localization. Recent research in the biology of cerebellum indicates that it may have a role in nociception. For the first time we have identified CGRP and CGRP receptor binding sites together with CGRP receptor expression through protein and mRNA localization in primate cerebellar cortex. These results point toward a functional role of CGRP in cerebellum. Further efforts are needed to evaluate this.

Keywords Cerebellum • Nociception • CGRP • CLR • RAMP1 • Primate

Introduction

The cerebellum is classically considered an area of the brain mainly involved in motor processing, but recent studies have suggested several other functions, including pain processing [1]. The deep cerebellar nuclei receive inhibitory inputs from Purkinje cells in the cerebellar cortex and excitatory inputs from mossy fiber and climbing fiber pathways. The deep cerebellar nuclei project to brainstem nuclei and the thalamus, which relays these projections to different parts of the cerebral cortex [1]. A detailed description of the organization of the connections from the cerebellar nuclei to the brainstem in rat showed that widespread areas in the brainstem receive cerebellar projections [2]. Some of these regions are red nucleus, inferior olive, deep layers of spinal trigeminal complex, periaqueductal grey (PAG), and ventrolateral thalamic nuclei [2]. The pons receives input from cerebral cortical efferents that classically relate to motor function but there are also cortical inputs from association areas involved in cognition, such as the prefrontal cortex [3], and in pain modulation [4],...
which distributes the information via mossy fibers to the cerebellum. The inferior olive sends information via climbing fibers from both cortical and subcortical sources to the cerebellum [5].

The cerebellum receives inputs from cutaneous primary afferent connections as based both on neuroanatomical tracing [6] and electrophysiology [7, 8]. Thus, stimulation of cutaneous A-β and C-fiber nociceptors activates climbing fibers that terminate on Purkinje cells [8]. Early neurophysiology and anatomy literature report that sensory modalities arise in the trigeminal nucleus in mid pons and end in the anterior lobule and the tonsils [9]. It is possible that both crossed and uncrossed fibers arise in the spinal nucleus of the trigeminal and ascend to the same areas of the cerebellum. It has been suggested that C-fiber nociceptors may act through mossy fibers to reach the Purkinje cells, the final decoders of information in the cerebellar cortex [10]. In particular, the cerebellum may engage pain modulation circuitry in the brainstem that involves PAG and the rostral ventromedial medulla [11].

Pain neuroimaging studies often show cerebellar activation [1]. Stimuli such as thermode, electric, laser, and capsaicin injection evokes cerebellar activation. Neuroimaging studies have demonstrated that, during pathological pain conditions such as neuropathy, cluster headache, back pain, and fibromyalgia, activation of the cerebellum is observed [11]. The first PET study of acute migraine attacks revealed activation both of the cerebellum and brainstem area around the PAG [12]; this finding has been substantiated and extended by others [13]. Additionally, this response was unrelated to pain relief by subcutaneous sumatriptan. In human pain imaging studies, activation of the cerebellum is almost always observed [14] suggesting a role in nociception. However, relatively little is known about the details of signaling and distribution of pain-relevant receptors in cerebellum.

Calcitonin gene-related peptide (CGRP) is a neuropeptide consisting of 37 amino acids and is involved in transmitting nociceptive information to second order neurons within CNS [15]. CGRP is widely expressed in the CNS, in regions such as the striatum, amygdale, hypothalamus, brainstem, trigeminal ganglion, and the cerebellum [16, 17]. CGRP is so far the only neuropeptide consistently shown to be involved in migraine pathophysiology, where there is elevated release of CGRP during migraine attacks [18, 19]. The receptor for CGRP is a G-protein-coupled receptor, consisting of a seven transmembrane-spanning protein, calcitonin receptor-like receptor (CLR), a single transmembrane-spanning protein, receptor activity modifying protein 1 (RAMP1), and an intracellular protein, receptor component protein (RCP) [20, 21]. There are three different RAMP proteins: RAMP1, RAMP2, and RAMP3. Interaction of CLR with RAMP2 or RAMP3 forms adrenomedullin receptors [20, 22]. RAMPs can interact with the calcitonin receptor to form amylin receptors. For a functional CGRP receptor, co-expression of CLR and RAMP1 is required. Recently, CGRP receptor antagonists have been developed with clinical efficacy [23].

In a recent study on rodents, we observed that the Purkinje cells store both CGRP and the CGRP receptor components, CLR and RAMP1 [24]. In support of this observation, Salvatore et al., 2010, reported that tritium-labeled MK-3207, a selective CGRP receptor antagonist with clinical antimigraine efficacy [25], displayed high binding densities in the rhesus cerebellum cortex but the cellular distribution remained unclear. The present study was designed to examine in detail cellular localization of CGRP and CGRP receptors. We addressed this issue in monkey and human, using immunohistochemistry, in vitro autoradiography, and in situ hybridization mapping studies to define the cerebellar localization of CGRP and its receptor binding sites in order to unravel their putative role in cerebellum physiology and in migraine.

Materials and Methods

Post-mortem Human Tissue Samples

Samples of cerebellum were obtained at autopsy from adult subjects in accordance with the University of Szeged guidelines for ethics in human tissue experiments and were approved by the Hungarian Ethics Committee. The tissue was bilaterally removed from six subjects (three female; three male) with an age span of 65 to 86 years. None of the subjects suffered from any central nervous system disease and the causes of death were related to heart failure, septicemia, or cancer. The tissues were collected within 24 to 36 h after death. The samples were immersed overnight in fixative consisting of 4 % paraformaldehyde (PFA) and in 0.1 mol/l phosphate buffer, pH 7.2. After fixation, the specimens were rinsed in sucrose-enriched (10 %) Tyrode solution overnight, frozen, and then stored at −80 °C. The samples were embedded in a gelatin medium (30 % egg albumin and 3 % gelatin in distilled water) and cryosectioned at 12 μm. The sections were stored at −20 °C until use. In addition, two paraffin-embedded human cerebella were obtained from the Department of Pathology Lund, Lund University (one male 67, years old; one female, 74 years old, cause of death were due to cardiac reasons). This part was approved by the Regional Ethical Review Board in Lund, Sweden (LU-801-01). The tissue was collected at autopsy, prior to which the bodies were kept at refrigerator temperature (+4 °C). The autopsy procedure lasted approximately 2 h, during which the body temperature approached room temperature. Paraffin sections were cut at 5 μm and incubated at +60 °C for 1 h. Human cerebellum (Analytical Biological Services Inc., Wilmington, DE) utilized for autoradiography studies was collected 7 h postmortem from a brain donor (male, 46 years old) without any known neurological disorders. The procedures for the human
samples were conducted according to the principles outlined in the Declaration of Helsinki.

**Rhesus Monkey Tissue Samples**

Rhesus cerebellum (*Macaca mulatta*, *n*=3, age; 13–15 years old, females) was harvested and divided into pieces in accordance with a Merck Research Laboratories Institutional Animal Care and Use Committee approved protocol. The samples were placed in a fixative solution consisting of 4 % PFA in 0.1 mol/L phosphate buffer, pH 7.2, approximately 20 min after sacrifice and fixed overnight. After fixation, specimens were either rinsed in sucrose-enriched (25 %) Tyrode solution overnight, frozen, and stored at −80 °C or paraffin embedded. The tissues were sent to the laboratory in Sweden for immunohistochemistry experiments. The rhesus samples were then gelatin-embedded, sectioned, and stored as the human samples. 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. Tissues to be used for in situ hybridization were quickly removed and frozen over 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 until hybridization.

**Autoradiography Studies**

Autoradiography studies were conducted as previously described [26]. Briefly, rhesus and human cerebellum slices (20 μm) were prepared using a cryostat (model CM3050; Leica Microsystems, Inc., Deerfield, IL) from a fresh frozen rhesus monkey or human brain. Slices were precultured 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 73.7 Ci/mmol) or [125I]CGRP (80 pM; specific activity 2,200 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) at room temperature for rhesus and human, respectively. Non-displaceable binding was defined by blocking with 1 μM unlabeled MK-3207 [26] or 30 nM MK-2918 [27] using an adjacent slice for rhesus and human, respectively. Slices were washed three times in ice-cold buffer (0.9 % NaCl and 50 mM Tris–HCl, pH 7.5) followed by an ice-cold rinse. Rhesus brain slices were air-dried and exposed to phosphorimaging plates (TR2025; Fujifilm 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). Human brain slices were air-dried, exposed to X-ray film for 2 days, developed, and scanned images analyzed as above.

Rhesus CLR and RAMP1 cDNA Cloning

A partial rhesus RAMP1 cDNA was previously isolated from rhesus forebrain ([28]; accession number AY587017). Rapid amplification of cDNA ends (RACE) experiments were conducted using the GeneRacer Kit (Invitrogen, Carlsbad, CA) according the manufacturer’s recommended protocol. 5’ RACE gene-specific primers (5’-GCAGCCTAGCTTCTC CGCCATGTG and 5’GTGCAGTGGCCAGGCTCCCTGTAG) and for 3’ RACE gene-specific primers (5’-CTGGCGGACTG CACCTGCAATTG and 5’-CTGGACTGCCAGGGGACCT ACTTC) were designed based on the partial RAMP1 sequence.

A partial rhesus CLR cDNA was isolated from rhesus forebrain cDNA using PCR. The PCR primers were based upon human CLR (5’-CCAATGCAAAAGCAAGGGC and 5’-CATCAGTGTGACACTGTC). Amplification reactions consisted of 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 90 s at 68 °C and were carried out according to the manufacturer’s recommended protocol for Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA). Amplification products were subcloned using TOPO TA Cloning Kit (Invitrogen) and multiple clones were sequenced bidirectionally.

**In Situ Hybridization**

Probes were designed to be specific for CLR and RAMP1. The CLR probe corresponds to bases 1018–1453 of human CLR (accession number L7630) and was designed to equally distribute the human and rhesus non-conserved bps. The RAMP1 probe (447 bp) corresponds to the full length rhesus RAMP1 coding sequence. Both probes were synthesized by GENEWIZ (South Plainfield, NJ) and subcloned into pBluescript SK+ vectors as 5’ EcoRI-3’XbaI fragments.

The CLR and RAMP1 vector constructs were linearized with EcoR1 (antisense) and Xba1(sense) and used to generate 35S-UTP-labeled cRNA probes for in situ hybridization. The slide-mounted sections were processed for in situ hybridization. Briefly, section-mounted slides were slowly warmed to room temperature, fixed in ice-cold 4 % PFA, pH 9 for 5 min, followed by washes in: 1× PBS (2 min), 100 mM triethanolamine (TEA, 1 min), 0.25 % acetic anhydride in 100 mM TEA (10 min), and 2× saline-sodium citrate (SSC, for 2 min). The sections were then delipidated and dehydrated with chloroform and ethanol and allowed to air-dry. Sections were hybridized overnight at 55 °C with hybridization solution, 200 μl/slide. The hybridization solution was 1/5 a mix of 1 M dithiothreitol (DTT) in TEA combined with 35S-labeled cRNA probe at a concentration of 6×106 counts/slide and hybridization buffer. The other 4/5 of the hybridization solution was a mix of 50 % formamide, 5 % dextran, 0.3 M
NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 1× Denhardt’s, and 0.2 M DTT, Q.S. with double-distilled water.

After hybridization sections were washed in 2× SSC containing DTT at room temperature, treated with RNase (5 ml/L RNase) for 30 min at 37 °C, and washed in 1× SSC for 15 min at RT, followed by 40 min at 72 °C in 0.1× SSC. Slides were equilibrated to room temperature, dehydrated with increasing concentrations of ethanol, and air-dried. Sections were placed in film cassettes and exposed to Biomax film (Kodak # IB 8715187) for 7 days. The films were developed using a Kodak Min-R mammography processor and digitized using a Vidar film scanner. The tissue sections were then taken using an Aperio brightfield slide scanner.

Hematoxylin–Eosin Staining

Paraffin sections were deparaffinized in xylene followed by serials of alcohol. Human and rhesus monkey sections were stained with hematoxylin–eosin (Htx–eosin) using a standard protocol (Htx 3 min, water rinse, eosin 1 min) for orientation and examination of the tissue condition. Adjacent slides used for in situ hybridization were also stained with Htx.

Immunohistochemistry

Before the immunohistochemistry experiments, paraffin sections were deparaffinized in xylene followed by serials of alcohol. To unmask the antibody epitopes, heat-induced epitope retrieval were performed, where the sections were heated in a microwave for 10 min in citric acid solution pH 6.0.

All sections were 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 immunolabeling with primary antibodies against CGRP, CLR (3152), and RAMP1 (844). 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 [29]. In addition, double immunostaining for CGRP and procalcitonin, GFAP, S-100, GAD67 with CGRP and the receptor components was performed. For detailed description of the primary antibodies, see Table 1. For all double immunostainings, the antibodies were applied separately and not mixed as a cocktail. Omission of the primary antibody served as negative controls for all antibodies. 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 (for details, see Table 2) in PBST and 1 % BSA for 1 h at room temperature. The sections were subsequently washed with PBST for 3×15 min. Vectashield, an anti-fading medium, containing DAPI (Vectashield, Vector Laboratories, Burlingame, CA).

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 visualize co-labeling by superimposing the digital images and processed for brightness and contrast.

Results

Autoradiographic Studies

High [3H]MK-3207 binding densities were observed in the molecular layer of rhesus cerebellum while the granular cell layer

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Table 1 Details on primary antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Name and product code</th>
<th>Host</th>
<th>Dilution</th>
<th>Detects</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcitonin receptor-like receptor (CLR) 3152</td>
<td>Rabbit</td>
<td>1:500</td>
<td>C-terminal of human CLR</td>
<td>Merck &amp; Co., Inc, USA</td>
</tr>
<tr>
<td>Receptor activity modifying protein (RAMP1) 844</td>
<td>Goat</td>
<td>1:100</td>
<td>C-terminal of human RAMP1</td>
<td>Merck &amp; Co., Inc, USA</td>
</tr>
<tr>
<td>Calcitonin gene-related peptide (CGRP, PA1-36017)</td>
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<td>1:500</td>
<td>Human and rat CGRP</td>
<td>Thermo Scientific, IL, USA</td>
</tr>
<tr>
<td>Calbindin (ab11426)</td>
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<td>1:200</td>
<td>Human, mouse, and rat calbindin D28k</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>Procalcitonin (ab53897)</td>
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<td>1:500</td>
<td>Amino acids 69–82 of human procalcitonin</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>GFAP (z0334)</td>
<td>Rabbit</td>
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<td>Glial cells</td>
<td>Dako, Copenhagen, Denmark</td>
</tr>
<tr>
<td>S-100 (ab66041)</td>
<td>Rabbit</td>
<td>1:200</td>
<td>Glial cells</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>GAD67 (MAB5406)</td>
<td>Mouse</td>
<td>1:500</td>
<td>67 kDa isoform of glutamate decarboxylase</td>
<td>Millipore, Billerica, USA</td>
</tr>
</tbody>
</table>
showed no radiotracer binding (Fig. 1). Due to the limit of resolution of the autoradiographic image it was not possible to accurately determine if the radiotracer binds to the Purkinje cell layer. [3H]MK-3207 displayed minimal nondisplaceable binding defined on an adjacent brain slice by 1 μM self-block (Fig. 1). Similarly, high-density [125I]CGRP binding was observed in the molecular layer, in addition to the Purkinje cell layers of human cerebellum (Fig. 2a). Moderate nondisplaceable binding was observed in the granular layer and white matter of the human cerebellum. To identify the granular and molecular layers the slides were counter-stained with Htx-eosin (Figs. 1 and 2b).

Isolation of Rhesus CLR and RAMP1 cDNAs

The full-length rhesus RAMP1 cDNA encodes a protein of 148 amino acids in length, which shares 96.4 % identity with human RAMP1 nucleotide sequence. The partial rhesus CLR cDNA is 1,076 bp in length which shares 98.9 % identity with human CLR on the nucleotide sequence. The rhesus RAMP1 and CLR sequences have been deposited in GenBank with accession numbers KC855758 and KC855759, respectively.

In Situ Hybridization

Expression of CLR and RAMP1 mRNA was localized to the Purkinje cell layer (Fig. 3a, b). It appears that there was less CLR mRNA expression as compared to that of RAMP1 mRNA, but this would need to be confirmed with RT-PCR. CLR and RAMP1 mRNA expression was mostly expressed around the Purkinje cells (Fig. 3a, b). Some Purkinje cells displayed mRNA expression within the cell body. It could not be determined if the expression of CLR and RAMP1 was located on the dendrites/processes of the Purkinje cell and/or other cell types in the Purkinje cell layer. Low mRNA detection within the soma of Purkinje cells could be due to artifactual changes such as cell shrinkage and loss of Purkinje cells in these slides (as visualized by Htx staining). This may also be due to that the mRNA levels in the Purkinje cells were low at the time of the experiment.

Histology

Some cell shrinkage was observed in the rhesus and human materials used for immunohistochemistry (Fig. 4a, b). Areas where Purkinje cells were absent or lost were seen. Paraffin-embedded cerebellum displayed better morphology.
Therefore these samples were primarily used for the immunohistochemistry experiments. To examine the condition of the Purkinje cells, a specific marker for these cells calbindin, was used (Fig. 4c, d). The calbindin marker showed strong immunoreactivity in all Purkinje cells and their dendrite elaborated tree of rhesus and human cerebellum which allowed the integrity of these cells to be verified.

Immunohistochemistry

The negative controls displayed no immunoreactivity. Some of the secondary antibodies revealed unspecific staining in the white matter. Autofluorescence was observed in basket cells, both in rhesus and human cerebellum. For each primary antibody, at least two secondary antibodies were tested to make sure that the same results were obtained. The human material showed higher background staining with all secondary antibodies as compared to the rhesus material.

CGRP immunoreactivity was found in the Purkinje cell bodies (cytoplasm) and in their branches (Purkinje cell dendrites) of human and rhesus cerebellum (Fig. 5). Not all Purkinje cells expressed CGRP. In some Purkinje cells the CGRP staining was especially strong in the cell nuclei. CGRP positive cells were found in the molecular layer.

Procalcitonin expression was found in the Purkinje cell bodies and in cells in the molecular layer (Fig. 5a, b). Like CGRP, not all Purkinje cells expressed procalcitonin. Often, several positive Purkinje cells were found in row in the same folia. The human cerebellum showed similar staining pattern as observed in rhesus monkey (Fig. 5b). Co-expression was found between CGRP and procalcitonin (Fig. 5a, b).

Expression of CLR and RAMP1 was found in the Purkinje cells of rhesus (Fig. 6a) and human (Fig. 6b) cerebellum. The RAMP1 staining was more prominent compared to that of CLR. CLR and RAMP1 positive cells were also observed in the molecular layer (Fig. 6). Expression of the receptor components were also observed in the processes of the Purkinje cells (Figs. 6 and 7). Co-expression of the receptor components was found in the Purkinje cells and cells in the molecular layer.
layer of rhesus (Fig. 7a) and human cerebellum (Fig. 7b). Some Purkinje cells only expressed RAMP1 (Fig. 7a).

Double-staining with CGRP and CLR (Figs. 8a and 9a) or RAMP1 (Figs. 8b and 9b) showed co-localization in some Purkinje cells and cells in the molecular layer of rhesus (Fig. 8) and human cerebellum (Fig. 9). Almost all CGRP positive cells were also positive for RAMP1 or CLR. However, not all CLR or RAMP1 positive cells showed CGRP immunoreactivity (Figs. 8 and 9).

In order to identify the other cells expressing CGRP and its receptor, double-staining with GFAP, S-100, or GAD 67 was performed. Double-staining with GFAP and S-100 with CGRP or the receptor components revealed no co-expression (data not shown). GAD67 expression was found mostly on the surface of Purkinje cells and neurons in the molecular layer. GAD67 immunoreactivity was found on the surface of some of the cells expressing CGRP or its receptor components in the molecular layer (Fig. 10a, b). Purkinje cells expressing CGRP or its receptor in the cytoplasm showed GAD67 expression on the cell surface (Fig. 10). The GAD67 did not work on the human sections.

Discussion

Clinical and experimental studies suggest that the cerebellum is involved in pain conditions, including migraine [1, 30, 31]. CGRP has an important role in migraine pathogenesis, and the recently discovered CGRP receptor antagonists have demonstrated efficacy in the treatment of migraine [23, 32]. Therefore, we have delineated the expression pattern of CGRP and the CGRP receptor in primate cerebellar cortex. Additionally, CGRP receptor binding sites were defined using both a radiolabeled CGRP receptor antagonist and CGRP itself. This is the first report to show in detail the localization of CGRP and the CGRP receptor in primate cerebellar cortex. Additionally, CGRP receptor binding sites were defined using both a radiolabeled CGRP receptor antagonist and CGRP itself. This is the first report to show in detail the localization of CGRP and the CGRP receptor in primate cerebellar cortex. Additionally, CGRP receptor binding sites were defined using both a radiolabeled CGRP receptor antagonist and CGRP itself. This is the first report to show in detail the localization of CGRP and the CGRP receptor in primate cerebellar cortex.
expressed in Purkinje cells and cells in the molecular layer. These results demonstrate the presence of CGRP and its receptor in primate cerebellar neurons, and suggest a role of CGRP in cerebellum, which could be involvement in pain and migraine pathophysiology.

Pain neuroimaging often show activation in the cerebellum, where patterns of cerebellar responses to innocuous and noxious thermal stimuli have been identified [33, 34]. Also, cerebellar activation is observed in patients who have neuropathic pain that affects the maxillary division of the trigeminal nerve (V2) [14]. Activation of cerebellar regions has been demonstrated by PET in patients during migraine attacks [12, 13]. However, there is at present no explanation for this activation. Interestingly, a recent study showed activation of the cerebellum during the premonitory phases of migraine [35]. The cerebellum is of interest in migraine research since studies suggest a role of cerebellum in migraine pathophysiology. Spreading depression, cerebellar dysfunction, and familial hemiplegic migraine have suggested a connection between the cerebellum and migraines [30]. Symptoms such as vertigo and balance changes may occur in migraine patients, and these suggest that migraine affects cerebellar function [36]. CGRP is an important player in migraine pathogenesis and CGRP receptor antagonists have been shown to be effective in the acute treatment of migraine [23, 37, 38]. In this report binding sites of MK-3207 were examined by in vitro autoradiography. MK-3207 is a selective CGRP receptor antagonist, which in vitro is a potent antagonist of the human and rhesus monkey CGRP receptors [26]. We observed high binding density of $[^3H]MK-3207$ to be mainly located in the molecular layer of rhesus cerebellum. No binding was found in the granular layer. However, due the limit of resolution of the autoradiographic image, it could not be excluded that the antagonist binds to the Purkinje cells as well. In human cerebellum, high-density $[^{125}I]CGRP$ binding was also found in the molecular layer and most likely the Purkinje cell layer. By examining the binding sites of both the small molecule antagonist MK-3207 and the endogenous ligand CGRP, the receptor expression

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Fig. 5 CGRP and procalcitonin expression in rhesus and human monkey cerebellum. CGRP and procalcitonin immunoreactivity and their co-expression in cerebellar Purkinje cells (arrows) and cells in the molecular layer in rhesus monkey (a) and human cerebellum (b). The CGRP staining is found in the cytoplasm and the elaborated tree of Purkinje cells. The human material contains lipofuscin, which is autofluorescent (asterisks). For nuclei staining DAPI (blue) is used. Pc: Purkinje cells, ml: molecular layer.
pattern could be confirmed. These results suggest the possibility of a functional role of CGRP in primate cerebellar cortex. Due to the low limit of resolution of the autoradiographic image, the exact cellular localization could not be determined. Immunohistochemistry was used to define the cellular/subcellular localization of CGRP and its receptor components.

Earlier studies have shown CGRP immunoreactivity in the Purkinje cells of rat [39] and recently by our group [24]. CGRP has been shown to be expressed in the cerebellar climbing fibers [40]. In primates, we demonstrate that CGRP is expressed in the cytoplasm of Purkinje cells and their branches. The climbing fibers did not show expression of CGRP. Some cells in the molecular layer displayed CGRP immunoreactivity. It cannot be determined if these minor variations between rat and primate are related to species differences or technical reasons such as material collection.

CGRP is produced by alternative mRNA splicing of the calcitonin gene and belongs to the calcitonin family peptides. Procalcitonin, the precursor of calcitonin was co-expressed with CGRP in the Purkinje cell body. This suggests that CGRP is produced in these cells within the cerebellum. Early studies have revealed expression of the CGRP receptors in several regions of the brain [41] including cerebellar glial cells [42]. However, these studies were based on immunohistochemistry and bindings sites, and were performed before the CGRP receptor was fully characterized. Recently, we found CLR and RAMP1 expression on the surface of rat Purkinje cell bodies and in fibers spanning through the entire cerebellum [24]. Detailed confocal analysis showed no co-expression of the CGRP receptor and glial cell markers in rat cerebellum [24]. In the present study, we observed expression of the receptor components in the cytoplasm of Purkinje cells and in cells within the molecular layer. Compared to our previous study on rat cerebellum, we did not find any fibers expressing CLR or RAMP1 in the primate cerebellum. The expression of the receptors components in primate Purkinje cells appeared to be intracellular rather than only on the surface of Purkinje cells. This might reflect differences in the quality of tissue or that the receptor expression is so high that the immunoreactivity appears as intracellular. No obvious difference in expression pattern of CGRP and its receptor was observed between rhesus and human cerebellum. Similar to the CGRP
staining, the receptor components displayed immunoreactivity in some cells in the molecular layer. We found that some of these cells in the molecular layer expressing CGRP or its receptor expressed GAD67 on their cell surface. GAD67 is one of major isoforms of the enzyme that converts glutamate into GABA, where the interneurons of cerebellum use GABA as neurotransmitter. The interneurons provide feed-forward and lateral inhibition to Purkinje cells, thus controlling their firing rate, the precise timing of action potential firing, and the spread of activity [43]. Functional experiments are needed to evaluate if and how there is a connection between the CGRP and the GABA system in cerebellum. CLR and RAMP1 were co-expressed in the Purkinje cells and cells in the molecular layer, suggesting expression of functional CGRP receptors in

Fig. 7 Double-staining of CLR and RAMP1 in rhesus monkey and human cerebellum. Co-expression of CLR and RAMP1 in rhesus monkey (a) and human cerebellum (b) in the Purkinje cells (large arrows) and cells in the molecular layer (small arrows). Not all RAMP1 positive cells expressed CLR (arrowheads). DAPI (blue), nuclei staining, is used in the merged pictures

Fig. 8 CGRP and CLR/RAMP1 double-staining in rhesus monkey cerebellum. Double-staining of CGRP and CLR (a) or RAMP1 (b) in the Purkinje cells (large arrows) and cells in the molecular layer (small arrows). Not all CLR or RAMP1 positive cells express CGRP (arrowheads). DAPI (blue), nuclei staining, is used in the merged pictures
these cerebellar cells. However, this needs to be evaluated with proper functional methods. RAMP1 positive cells, negative for CLR, were also observed. This could be due differences in the quality of antibodies recognizing the epitopes, slightly higher proportion of RAMP1 compared to CLR or the presence of other RAMP1 containing receptors, such as amylin receptors. The latter suggestion is less likely since it has been shown that the cerebellum is devoid of amylin binding sites [44, 45]. The in situ hybridization results also showed slightly higher RAMP1 mRNA expression. One can also speculate that since other receptors rely on RAMP1 in their formation, RAMP1 may be functionally rate-limiting and an excess might be necessary for high-density CGRP binding. This may also be related to how well the probes

Fig. 9 CGRP and CLR/RAMP1 double-staining in human cerebellum. Double-staining of CGRP and CLR (a) or RAMP1 (b) in the Purkinje cells (large arrows) and cells in the molecular layer (small arrows). Not all CLR or RAMP1 positive cells express CGRP (arrowheads)

Fig. 10 Double-staining of GAD67 with RAMP1 or CGRP in rhesus monkey cerebellum. RAMP1 with GAD67 (a) and CGRP with GAD67 (b). RAMP1 and CGRP is found intracellularly in Purkinje cells and cells in the molecular layer (large arrows) while GAD67 is expressed on the surface of the same cells (small arrows). Inserts show higher magnification
recognize the sequences. Further efforts are needed to clarify this issue.

Double-staining of CGRP and the receptor components showed co-localization in Purkinje cells and in cells in the molecular layer. However, not all CLR or RAMP1 immunoreactive cells displayed CGRP expression. This suggests that the neuronal messenger might act on the same cell or on cells only expressing the receptor.

The functional role of CGRP in the cerebellum is unknown. It has been shown that the expression of CGRP and its receptor undergoes marked variations during development in rat. The number of CGRP receptors in the Purkinje cells/molecular layer is low in early stages and greatly increases with maturation and decreases from high to low levels during the same time in the white matter [46]. The same group demonstrated that CGRP stimulates Purkinje cell dendrite growth in vitro, suggesting that CGRP influences Purkinje cell dendrite growth [47]. Further efforts are needed to evaluate the role of CGRP and its receptor in the cerebellum.

Conclusion

Recent studies clearly indicate that the cerebellum might have a role in noiception and primary headache disorders. The present study demonstrates that there is a rich expression of the neuropeptide CGRP and CGRP receptor components in primate cerebellar cortex, which together suggest a functional role of CGRP in cerebellum. The roles of cerebellum and CGRP in response to nociceptive stimuli warrant additional research which may in turn lead to a better understanding of the cerebellum as a pain control center and as a target for new drug therapies.

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Conflict of Interest

The authors do not have any conflicts of interest related to this work.

References


Trigeminal ganglion- a site of action for CGRP receptor antagonists

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Abstract

Background
Calcitonin gene-related peptide (CGRP) receptor antagonists have demonstrated antimigraine efficacy. The remaining question is do the antagonists act peripherally or centrally? In this study we examined CGRP receptor protein expression and binding sites in rhesus trigeminal ganglion. Additionally, we examined if the trigeminal ganglion is protected by the blood-brain barrier.

Methods
Autoradiography studies were performed with [3H]MK-3207 to define receptor binding sites. Immunofluorescence was used to delineate CGRP and its receptor components, calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein 1 (RAMP1). Experiments with Evans blue were performed on rodents.

Results
High receptor binding densities were found in the trigeminal ganglion. Immunofluorescence revealed expression of CGRP, CLR and RAMP1 in the trigeminal cells. CGRP was expressed in small/medium sized cells whereas the receptor components were mainly co-expressed in larger cells and satellite glial cells.
Evans blue revealed that the trigeminal ganglion is not protected by the blood-brain barrier.

**Conclusion**
The present study demonstrates CGRP receptor binding sites and protein expression of CGRP and its receptor in rhesus trigeminal ganglion. In rodents we demonstrated that the trigeminal ganglion is located outside the blood-brain barrier, suggesting that CGRP receptor antagonists do not need to be CNS-penetrant to block receptors in the trigeminal ganglion.

**Keywords**
Trigeminal ganglion, CGRP receptor antagonists, CLR, RAMP1, CGRP, Evans blue.
Introduction

Migraine is today recognized as a neurovascular disorder (1) which originates in the brain, involving the hypothalamus and thalamus, as well as certain brainstem regions. The attack is often preceded by prodromal symptoms which suggest the central nervous system as a starting point. The pain in the migraine attack has been shown to be associated with the release of the peptide calcitonin gene-related peptide (CGRP) which appears to have a key role in migraine pathophysiology, supported by studies showing that CGRP is released during migraine attacks (2), infusion of CGRP can trigger migraine-like headache in patients (3) and CGRP receptor antagonists can abort the migraine pain (4, 5). The CGRP receptor consists of a G protein-coupled receptor, the calcitonin receptor-like receptor (CLR), which associates with receptor activity-modifying protein (RAMP) 1 to yield a functional receptor for CGRP (6). CGRP and its receptor are widely expressed throughout the trigeminovascular system and central nervous system, consistent with control of vasodilatation and transmission of nociceptive information (7).

Recent findings have identified the blockade of the CGRP receptor as a mechanism to reduce migraine pain (8). Clinical studies using CGRP receptor antagonists have demonstrated clinical efficacy comparable to triptans in the treatment of acute migraine attacks (9). Therefore it is of importance to determine where the CGRP receptor antagonists act: 1) is inhibition of CGRP released peripherally sufficient for the antimigraine action? 2) or is inhibition of CGRP acting centrally in trigeminal pain-relay nuclei of the brainstem the key contributor to clinical effectiveness?

In this study we have focused on the trigeminal ganglion to determine if CGRP receptor antagonists may act there. The trigeminal ganglion is of particular interest given the role of the trigeminovascular system in migraine (2, 10). Trigeminal fibers innervate cerebral blood vessels and contain CGRP and substance P, where release of CGRP causes vasodilatation (1). Further, the trigeminal ganglion, storing CGRP and its receptor components, projects to parts of the central nervous system where the nociceptive information is processed to higher cortical regions.

In order to define if trigeminal ganglion could be a site of action for CGRP receptor antagonists we studied the binding sites of a CGRP receptor antagonist and the cellular distribution of CGRP and its receptor components by immune-fluorescence in rhesus monkey trigeminal ganglion. Additionally, we used Evans blue in rodents to determine if the trigeminal
ganglion is located within or outside the blood brain-barrier (BBB).

Material and methods

Rhesus monkey tissue samples
Rhesus trigeminal ganglion (Macaca mulatta, n=3, age; 13-15 years old, females) was harvested in accordance with a Merck Research Laboratories Institutional Animal Care and Use Committee approved protocol. Tissues to be used for autoradiography studies were quickly removed and frozen over 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 until hybridization. An additional rhesus monkey (9 years old, female) trigeminal ganglion was harvested for immunohistochemistry. The samples were immersed overnight in fixative consisting of 4% PFA and in 0.1 mol/L phosphate buffer, pH 7.2. After fixation, specimens were paraffin embedded. The tissues were sent to the laboratory in Sweden for immunohistochemistry experiments. The rhesus samples were then sectioned (5μm) 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.

Autoradiography studies
Autoradiography studies were conducted as previously described (11). 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/mmol) at room temperature. Non-displaceable binding was defined by blocking with 1 μM unlabeled MK-3207 using adjacent slides. Slides were washed 3x1min 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; Fujifilm 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 Htx-Eosin following a standard protocol (Htx 3 min, water rinse, Eosin 1 min) for orientation and examination of the tissue condition.

Immunofluorescence studies
Immunofluorescence staining was performed to demonstrate the localization of CGRP, CLR and RAMP1 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 were performed,
where the sections were heated in a microwave for 10 min in citric acid solution pH 6.0. 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 (ab81887, Abcam, UK), CLR (3152, Merck & Co.) and RAMP1 (844, Merck & Co.). 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 3x15 min and exposed to secondary antibodies 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 3x15 min, thereafter mounted with an anti-fading mounting medium containing DAPI (Vectashield, 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 (12).

**Rat tissue samples and 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. 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 fluanison 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 1h, the animals were perfused transcardially with a prewash of phosphate-buffered saline (PBS, pH 7.2) for 2 minutes followed by 4% paraformaldehyde (PFA) in PBS for 5 minutes. The brains and trigeminal ganglion were dissected and the specimens were further fixed in PFA for 2-4 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.

**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,
Results

Binding of $[^3H]MK$-3207 in the rhesus monkey trigeminal ganglion
High binding densities of $[^3H]MK$-3207 were observed in the trigeminal ganglion of rhesus monkey (Fig. 1). The highest binding was mainly found in the ganglion where the neurons are located, verified by Htx-Eosin staining on the same slide (Fig. 1). Areas with low or no binding were also found. Minimal nondisplaceable binding was seen in the presence of 1 μM unlabeled MK-3207 on an adjacent slice. Due to low resolution, the cellular binding could not be determined. Also, the exact orientation of the ganglia was missing and regional variations (V1-V3) in the binding sites cannot be determined. In general, all parts of the trigeminal ganglion showed homogenous binding.

Figure 1.
$[^3H]MK$-3207 displayed high binding density in the rhesus monkey trigeminal ganglion (total binding). High binding is found within the ganglion where the neurons are located (red signal). Htx-Eosin staining of the same slice. Self-block on the adjacent slide with no binding (non-displaceable binding)
Distribution of CGRP, CLR and RAMP1 the rhesus monkey trigeminal ganglion

Immunoreactivity for CGRP, CLR and RAMP1 were observed in the trigeminal neurons (Fig. 2). The CGRP immunoreactivity was mainly found in the smaller neurons and displayed granular-like staining in the cytoplasm (Fig. 2A). The receptor components, CLR and RAMP1, were mostly expressed in larger neurons, and in addition were found in the cytoplasm of satellite glial cells (Fig. 2B). Double-staining of CLR and RAMP1 revealed co-expression between the receptor components in the neurons and the cytoplasm of satellite glial cells (Fig. 3A). However, some neurons only displayed RAMP1 immunoreactivity (Fig. 3). In addition, RAMP1 and CLR were found in the walls of the vessels within the trigeminal ganglion, expressed in the smooth muscle cells. This was apparent in both large and small vessels (Fig. 3B). Double-staining of CGRP with the CLR or RAMP1 showed that the neuropeptide is rarely co-expressed with the receptor components (Fig. 4). CLR and RAMP1 were expressed in the larger neurons lacking CGRP expression (Fig. 4A, B). Very few small-sized neurons expressed CGRP and the receptor components (Fig. 4). Thin CGRP positive fibers were observed proximal to the neurons. These thin fibers did not display immunoreactivity for CLR (Fig. 4A) or RAMP1 (Fig. 4B).

Figure 2.
(A) Expression of CGRP in rhesus monkey trigeminal ganglion. CGRP is widely expressed in the neurons, mainly in the small to medium sized neurons (arrows). Thin fibers expressing CGRP is found (arrow head). (B) Expression of CLR and RAMP1 in rhesus monkey trigeminal ganglion. Immunoreactivity is found in the larger neurons (arrows) and satellite glial cells (arrow heads). DAPI, staining nuclei, is used in the merged pictures (blue).
Figure 3.
(A) Double-staining of the receptor components CLR and RAMP1. Large arrows indicate their co-expression in the larger neurons and arrow heads indicate CLR and RAMP1 co-expression in the satellite glial cells. Neurons only expressing RAMP1 are found (small arrows). DAPI, staining nuclei, is used in the merged pictures (blue). (B) Vessels of different sizes within the trigeminal ganglion expressing CLR and RAMP1 in the smooth muscle cell layer (arrows). Auto-fluorescence is detected in lamina elastic interna (arrowheads).
Evans blue in rodents
In order to evaluate if the trigeminal ganglion is located within or outside the BBB, experiments with Evans blue were performed on rodents. Evans blue has high affinity for serum albumin, which cannot cross the barrier. Virtually all Evans Blue is bound to albumin so normally the neural tissue remains unstained. Evans blue becomes fluorescent when linked to proteins. The brains from the Evans blue rats displayed no dye uptake (Fig. 5A), instead dye uptake was observed in the pituitary gland (Fig. 5A). Sections from cortex and cerebellum showed no fluorescence signal (Fig. 5B), confirming that Evans blue was not taken up into these parts of the brain. Sections of pituitary gland and choroid plexus, regions that are

Figure 4.
(A) Double-staining of CGRP with CLR or RAMP1 (B) in rhesus monkey trigeminal ganglion. CGRP is mostly expressed in the smaller neurons (small arrows) while the larger neurons express of CLR or RAMP1 (large arrows). Co-localization is rarely found, and then only in the smaller neurons (arrow heads). DAPI, staining nuclei, is used in the merged pictures (blue).
without BBB, displayed high red fluorescence signal (Fig. 5C). These results confirm that the experiments with Evans blue were properly performed. Dissected trigeminal ganglion showed uptake of Evans blue (Fig. 5A). Fluorescence signal was observed in the trigeminal ganglion sections, where high signal was found in the ganglion where there were numerous cell bodies (Fig. 6). The nerve bundles displayed no or low signals, while some signal was observed around the nerve part (Fig. 6A). Evans blue fluorescence appeared proximal to the neurons and/or around the neurons (Fig. 6B). In addition, the blood vessels within the ganglion, found close the neurons, clearly displayed the red fluorescence signal in their entire walls (Fig. 6C).

Figure 5.
(A) Evans blue experiments in rodents, showing no uptake of the dye in the brain while the pituitary gland that is lacking BBB protection is blue from the dye uptake. The trigeminal ganglion becomes blue indicating uptake of Evans blue. (B) No fluorescent signal from Evan blue is detected in cortex or cerebellum, however red fluorescent signal is detected in tissues located outside BBB such as the pituitary gland and choroid plexus (C).
Figure 6.
(A) Demonstration of a fluorescence microscopic figure showing the trigeminal ganglion of rhesus monkey 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 part. Higher magnification is showing Evans blue detection in a larger vessel close to the ganglion. (B) The red color signals extravasation of the Evans blue in the trigeminal ganglion, where the 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).
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 evaluated if the trigeminal ganglion is protected by the BBB or not by using in vivo administration of Evans blue. We found high binding densities of [3H]MK-3207, a CGRP receptor antagonist, (by in vitro autoradiography) and expression of CGRP and its receptor components (by immunofluorescence) in the rhesus monkey trigeminal ganglion. The experiments with Evans blue in rodents revealed that trigeminal ganglion is located outside BBB and can therefore be reached by the CGRP receptor antagonists that lack the potential for CNS-penetration.

The CGRP receptor antagonist binding sites were mainly located in the ganglion where the cells were located. This supports the presence of functional CGRP receptor within the ganglion that can be blocked by CGRP receptor antagonists. The cellular localization could not be determined due to low resolution and therefore immunofluorescence was used.

CGRP immunoreactivity was mainly found in the small-medium sized cells of the trigeminal ganglion while the receptor components were expressed in the larger neurons. In addition, thin CGRP containing fibers distributed among the neurons were found. CLR and RAMP1 were co-expressed in the larger neurons, suggesting expression of functional CGRP receptors in the trigeminal ganglion. Some cells only expressing RAMP1 were found. The peptide, CGRP, and its receptor components were rarely expressed in the same cells. This suggests low presence of putative auto-receptors within the ganglion, where CGRP is most likely released from one cell to act on another cell. These results are all in accordance with our previous study on human and rat trigeminal ganglion (12). Co-expression of CLR and RAMP1 was also found in the trigeminal vascular wall, opening up the possibility for effects on the microvasculature of the trigeminal ganglion.

The cells of trigeminal ganglion consist of bipolar neurons and two types of glial cells. Interestingly we found co-expression of the receptor components in the satellite glial cells that surround the cell bodies. This cell type has been suggested to have an important role in inflammation and pain (13, 14). Studies have shown that release of CGRP within the ganglion stimulates the release of nitric oxide and pro-inflammatory cytokines from satellite glial cells and CGRP treatment of satellite glia cells increases proinflammatory cytokine release and actions that can sensitize sensory neurons (15-17). In addition, CGRP may trigger additional inflammatory gene expression changes in glia in vitro (15). It has been demonstrated that the anti-migraine drug, sumatriptan,
inhibited the evoked CGRP release from naïve trigeminal neuron cultures, as well as from trigeminal culture (18). In the present study we suggest that CGRP receptor antagonists also may act on the satellite glial cells and thereby this could influence the neuronal responses. Further functional studies are needed to address this hypothesis in detail.

The CGRP receptor antagonists are a new type of anti-migraine drug that might offer a new non-vasoconstrictive approach in the acute treatment of migraine (19). Four CGRP receptor antagonists (olcegepant, telcagepant, MK-3207, BI 44370 TA and BMS927711) have displayed efficacy in the treatment of migraine (20, 21). The CGRP receptor antagonists have opened a possible new option in migraine treatment, and consequently many scientific questions have arisen which need to be addressed. One question that has been discussed is if the anti-migraine action of CGRP receptor antagonists are mediated via central or peripheral mechanisms, or both. 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 (22). Therefore it has been suggested that the CGRP antagonists need to penetrate the blood-brain barrier to achieve acute anti-migraine efficacy (23, 24). Recently, the first PET study in healthy and migraine patients demonstrated that the CGRP receptor antagonist telcagepant achieved low CGRP receptor occupancy (10%) at the lowest clinically efficacious dose, whereas a supratherapeutic 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 (25). However, it cannot be ruled out that additional efficacy may be achieved with better access to the CNS.

If the current CGRP receptor antagonists cannot pass the BBB (or only to a low degree), it is important to clarify which sites can be reached and may therefore 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 mast cells of the dura mater (26). Peripherally the CGRP receptor antagonists may act on cerebral vessels to inhibit CGRP vasodilatation and on dural mast cell degranulation. However the role of cerebrovascular dilatation is unclear. Recently it was shown that acute migraine pain was not accompanied by extracranial arterial dilatation and by only some intracranial dilatation during attack (27). In the same study it was demonstrated that effective treatment with sumatriptan caused no intracranial (cerebral artery) vasoconstriction. It is also unclear if mast cell degranulation contributing to neurogenic inflammation exists in humans, since it has been suggested
that CGRP receptors may not be present in human mast cells (26). This agrees with the early finding that CGRP failed to induce release of histamine from dura mater mast cells in man following administration of excess CGRP (28).

In the present study we demonstrate that the trigeminal ganglion is located outside the BBB, which to our knowledge has not been clearly demonstrated before in recent literature. A study from 1973 showed Evans blue uptake in the trigeminal nerve of rabbits and mice (29). Dye uptake was found in the epineurium part of the nerve while the endoneurium part did not contain Evans blue. In the present study, injection of the dye Evans blue into the circulation of rodents showed up-take of the dye in areas outside the BBB such as pituitary gland and choroid plexus while the brain showed no dye up-take. Dye up-take was clearly found in the trigeminal ganglion and the fluorescent signal was found mostly around the cells and vessels in proximity to the cells. Within the nerve part low/no signal was observed. Some signal was found around the nerve. 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 BBB. The trigeminal ganglion may also be site of action for the recently developed monoclonal anti-CGRP and anti-CGRP receptor antibodies, which will in all likelihood not cross the BBB due to their large size (21). Support for this has been obtained in functional tests in rodents with a CGRP antibody (30).

Since the trigeminal ganglion is connected both to the periphery and also centrally, CGRP signaling may be blocked in these parts by blunting the activation/signaling in trigeminal ganglion. However, it is not known if better clinical efficacy can be achieved if central sites are occupied, since the wide expression of CGRP and its receptor within CNS and the role of brain areas such as the brainstem and hypothalamus in the pathophysiology of migraine. Interestingly, we have shown mRNA expression of CLR and RAMP1 and binding of a CGRP receptor antagonist via \textit{in vitro} autoradiography in the pineal gland and area postrema (31). These brainstem areas are devoid from BBB and we suggest that these areas can also be sites of action for CGRP receptor antagonists.
Conclusions

The present study demonstrates binding of CGRP receptor antagonists and expression of CGRP and its receptor within the rhesus trigeminal ganglion. These results suggest and support the presence of functional CGRP receptors in this area. CGRP expression was found in small-medium sized cells, lacking expression of the receptor. The receptor components were instead co-expressed in larger neurons and satellite glial cells. 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. CGRP receptor antagonists and anti-CGRP and anti-Cgrp receptor antibodies are currently under development, if these are not able to penetrate BBB then the trigeminal ganglion may represent a key site of action.

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