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Primary trigeminal afferents are the main source for stimulus-induced CGRP release into jugular vein blood and CSF

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Abstract

Background: Administration of inflammatory soup (IS) leads to a significant release of calcitonin gene-related peptide (CGRP). Whether IS-induced CGRP release originates in primary or secondary neurons of the trigeminovascular system has not been clarified.

Methods: We determined CGRP release into the external jugular vein and in cerebrospinal fluid (CSF) following intracisternal IS administration using an in vivo rat model. We further performed polymerase chain reaction (PCR) and immunohistochemistry of the trigeminal ganglion and brainstem (trigeminal nucleus caudalis). To further elucidate a primary vs. secondary origin, experiments were repeated after neonatal capsaicin treatment (NCT) as this treatment destroys primary trigeminal afferents.

Results: IS-induced CGRP release into the external jugular vein and CSF were significantly reduced after NCT in both compartments but inhibition was more pronounced in jugular vein blood than in CSF. Baseline CGRP levels were not affected by NCT. PCR results show that following NCT, CGRP mRNA was significantly reduced in the trigeminal ganglion but not in the brainstem. Immunohistochemistry of the TG and brainstem support these results.

Conclusions: We conclude that resting state CGRP levels can be maintained after trigeminal denervation of the meninges. However, for functional purposes primary trigeminal afferents are mandatory as they are the major source for stimulus-induced CGRP release.

Keywords

Calcitonin gene-related peptide, trigeminal activation, inflammatory soup, capsaicin

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Introduction

Calcitonin gene-related peptide (CGRP) is a crucial neurotransmitter in migraine pathophysiology. Data of experimental animal models show that the activation of the trigeminal nerve system leads to a release of CGRP (1) which causes vasodilation and modulates neuronal activity in the trigeminal nucleus caudalis (TNC) (2).

Final proof for a key role of CGRP in the pathophysiology of migraine was obtained from clinical trials in which the CGRP receptor antagonist BIBN4096BS (olcegepant), MK0974 (telcagepant) and BI44370TA were effective in the acute treatment of migraine (3–6).

In addition to trigeminal activation, sensitization of the trigeminal nerve system is important in migraine pathophysiology. Sensitization is considered to be the pathophysiological correlate of cutaneous allodynia (pain resulting from a non-noxious stimulus to normal skin), a phenomenon described by 70% of migraineurs during attacks (7). Experimental and clinical data from an open-label study show a reduced efficacy of triptans once sensitization is established, thereby highlighting the importance of this phenomenon (7–9). These findings are controversial as several

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randomized, double-blind, placebo-controlled trials do not support these findings (10–12). Another clinical study indicates that triptans may also have sensitizing effects, which could be involved in the generation of triptan-induced medication overuse headache (13). Moreover, the findings of Burstein and Jakubowski rely on the assumption that triptans mainly act by blocking the transmission of pain signals from primary nociceptors to secondary neurons, although other mechanisms of action of triptans within the central nervous system (CNS) were also demonstrated (2,14).

Trigeminal sensitization can be induced experimentally in animal models by topical application of inflammatory soup (IS) onto the exposed dura mater (8). Recently, we were able to demonstrate that the intracisternal administration of IS also causes the release of CGRP into the extracerebral circulation (15), suggesting a link between CGRP, a marker of trigeminal activation, and the establishment of trigeminal sensitization. Interestingly, administration of CGRP on the dura mater in experimental rat studies did not cause trigeminal nerve activation or sensitization (16).

Although CGRP receptor antagonists are effective anti-migraine drugs, their exact mode of action is not entirely understood. In addition, the relative contribution of primary and secondary trigeminal neurons to induced CGRP release has not been described in detail.

To address these questions, we studied stimulus-induced CGRP release in jugular vein blood and CSF and also assessed the source of induced CGRP release into both compartments. We used rats which had been treated as neonates with a single subcutaneous capsaicin injection (neonatal capsaicin treatment, NCT). This approach reliably destroys primary afferents (17–19) and allows the effects of primary and secondary trigeminal components to be distinguished.

Methods

All experiments were approved by the Landesamt für Gesundheitsschutz und Tierschutz (G268/05 and G317/09), the body responsible for animal experimentation in Berlin.

Surgical procedures and drug administration

Male Sprague–Dawley rats (240–300 g, Charles River, Sulzfeld, Germany) were anaesthetized with intraperitoneally administered thiopental-sodium (60 mg/kg body weight, Trapanal®, Altana, Wesel, Germany). When necessary, supplemental doses were administered during the experiments. The rats were placed on a heating blanket and the body temperature was maintained at $37 \pm 0.5^{\circ}$C. Temperature monitoring was performed with a rectal probe.

After a midline skin incision from the occipital protuberance to the cervical area the neck muscles were carefully retracted. A soft flexible catheter (Portex polythene tubing 0.28 mm i.d., neoLab GmbH, Heidelberg, Germany) was inserted into the cisterna magna for intracisternal administration of IS and fixed with cyanoacrylate adhesive (Contact VA100, Weicon, Münster, Germany). A second catheter was placed into the left jugular vein for the collection of blood samples and determination of CGRP levels. The methods have been described in detail elsewhere (15). After the surgical procedure the animals were kept anaesthetized and undisturbed for 2 hours to avoid unspecific effects. Afterwards, a blood sample (1.5 ml) was obtained from the jugular vein to determine CGRP baseline levels. After 30 minutes, 70 µl IS (histamine 1 mM, serotonin 1 mM, bradykinin 1 mM and prostaglandin E_2 0.1 mM, pH 5.5; adapted from Burstein et al. (8)) was injected over a 5 minute period via the intracisternal catheter using a 100 µl Hamilton syringe into the cisterna magna. Blood samples (1.5 ml each) were obtained 2 and 15 minutes after the start of the IS infusion. All blood samples were collected without aspiration in pre-chilled Eppendorf tubes containing ethylenediaminetetraacetic acid (EDTA) disodium salt solution 0.5 M (Sigma Aldrich, Munich, Germany) and the protease inhibitor aprotinin (aprotinin from bovine lung, 0.55 trypsin inhibitor units (TIU) per ml blood; Sigma Aldrich, Munich, Germany).

Because the volume of CSF in rats is insufficient for multiple sampling we were limited to collecting one sample per experiment to determine CGRP levels. Samples were collected 15 minutes after IS administration or from rats after instrumentation (for baseline analysis). CSF samples for controls were collected in a similar time period.

Our study protocol required a minimum volume of CSF of 50 µl to reliably determine CGRP levels. CSF was collected via the intracisternal catheter into pre-chilled Eppendorf tubes containing aprotinin (0.55 TIU). Blood samples were immediately centrifuged with 2000 *g* at 3°C for 30 minutes. Plasma and CSF samples were stored at −80°C until further analysis. CGRP concentration in plasma and CSF was determined using a commercially available Rat CGRP Enzyme Immunoassay Kit (SPI-Bio, Montigny le Bretonneux, France).

Neonatal rats (day 2) received one subcutaneous capsaicin injection (Sigma Aldrich, Munich, Germany) at a dose of 50 mg/kg under light isoflurane anaesthesia and isoproterenol nebulization (17–19). Experiments were performed when the animals reached a weight of 240–300 g.

Quantitative real time polymerase chain reaction (RT-PCR)

RT-PCR was used to determine the effect of NCT on CGRP mRNA expression. In some experiments capsaicin treated and naïve rats were perfused transcardially with 350 ml cold saline 0.9% (within 3 minutes after the last blood sampling) and the trigeminal ganglia were removed. Total cellular RNA was extracted using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's manual. RNA preparation and cDNA synthesis were performed as described previously by our laboratory (20). RT-PCR was carried out on a Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany) using the LC-Fast Start DNA Master SYBR Green I Kit as recommended by the manufacturer. The following specific primers (MWG, Ebersberg, Germany) were used: CGRP_forward: 5′-AAG TTC TCC CCT TTC CTG GT-3′, CGRP_reverse: 5′-GGT GGG CAC AAA GTT GTC CT-3′; rod_Actin_forward: 5′-ACC CAC ACT GTG CCC ATC TA-3′ and rod_Actin_reverse: 5′-GCC ACA GGA TTC CAT ACC CA-3′. The reaction mix (20 µl) contains 3 mM $MgCl_2$ dissolved in fast start mix 18 µl (Roche, Basel, Switzerland) plus 2 µl cDNA. For CGRP PCR we used the following protocol: pre-incubation at 95°C (600 seconds); amplification at 95°C for 15 seconds, 66°C (10 seconds), 72°C (15 seconds) and 94°C. Cooling was performed for 30 seconds at 40°C. For actin PCR: pre-incubation at 95°C for 600 seconds, followed by the amplification process at 95°C (15 seconds), 68°C (10 seconds) and 72°C (15 seconds); cooling 40°C for 30 seconds. The primers have been used previously in PCR of rat trigeminal ganglion cell culture tissue and the reaction product has been sequenced. Sequencing analysis (BLAST) revealed a product consistent with the rat CGRP gene.

Histochemistry of trigeminal ganglion

For histological staining animals with or without NCT were perfused transcardially with ice cold saline followed by 300 ml paraformaldehyde 4% and picric acid 0.2%. Extracted trigeminal ganglia were cut in 20 µm sections using a Cryostat at −20°C. Phosphate-buffered saline (PBS, 0.1 M, pH 7.4) was used for antibody dilutions and washes. For CGRP DAB staining, sections were placed in 0.3% hydrogen peroxide (Sigma Aldrich, Munich, Germany) for 30 minutes followed by 2-hour blocking at 4°C with 10% normal goat serum (NGS, Chemicon, Schwalbach, Germany) and 0.3% Triton X-100 (Sigma Aldrich, Munich, Germany). Sections were then incubated with the primary polyclonal rabbit anti-rat CGRP antibody (1:1000; C8198; Sigma Aldrich, Munich, Germany) dissolved in 3% normal goat serum and 0.3% Triton X-100 overnight at 4°C (Sigma Aldrich, Munich, Germany). As a secondary antibody a biotinylated goat anti-rabbit IgG antibody (1:600) was applied at room temperature for 2 hours in 3% NGS + 0.3% Triton X-100 (Vector Labs, Burlingame, CA, USA).

After multiple washes the sections were incubated with a biotin horseradish peroxidase complex (ABC Elite Kit, Vector Labs, Burlingame, CA, USA) for 1 hour at room temperature and finally in a solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma Aldrich, Munich, Germany) for visualization. Sections were then dehydrated with ethanol in ascending concentrations and mounted with rotihistol medium. For Nissl staining cresyl violet was used before dehydration. Sections were covered with Histokitt (Carl Roth, Karlsruhe, Germany).

For immunohistochemistry, CGRP and NF-200 staining of the trigeminal ganglion was performed. To block unspecific binding 10% donkey serum and 0.3% Triton X-100 was used, as well as 3% donkey serum plus 0.3% Triton X-100 when the secondary antibody was applied.

As primary antibodies, a polyclonal rabbit anti-rat CGRP antibody in a concentration of 1:1000 as well as a mouse NF-200 antibody (1:600) were used. As secondary antibody Alexa Fluor 594 donkey anti-rabbit antibody against NF-200 (1:600) was used for 90 minutes at room temperature.

Light microscopy of dura mater and brainstem tissue

For light microscopy of CGRP in whole mount dura mater we also used the polyclonal rabbit anti-rat CGRP antibody (C8198; Sigma Aldrich, Munich, Germany) in a concentration of 1:1000. The incubation times are described above. As a secondary antibody a biotinylated goat anti-rabbit IgG antibody (1:600) was applied at room temperature for 2 hours in 3% NGS + 0.3% Triton X-100. The incubation in hydrogen peroxide (Sigma Aldrich, Munich, Germany) and the staining were performed as described by Reuter et al. (21).

For DAB staining of brainstem sections we used a standard methodology as described by our group previously (22). One difference was that the primary antibody was a polyclonal rabbit anti-rat CGRP antibody in a concentration of 1:800 which was incubated overnight at 4°C and the secondary antibody was a biotinylated goat anti-rabbit IgG antibody (1:600) which was incubated for 2 hours at room temperature.

Statistical analysis

To calculate the normal distribution of values the Shapiro–Wilk test was used. All values were normally distributed. Changes within one treatment group were analysed using an ANOVA with repeated measures. Where the assumption of sphericity was violated we used the Greenhouse–Geisser corrected degrees of freedom. To determine specific changes within one treatment group the paired *t*-test was used. Differences between the different treatment groups were analysed using the unpaired *t*-test. All data were expressed as mean ± SEM. Statistical significance was assumed when $p < 0.05$. Statistical analysis was performed using IBM SPSS 19 software (SPSS Inc., Chicago, IL, USA).

Results

Intracisternal IS administration induces CGRP release into extracerebral circulation and CSF

Intracisternal administration of IS led to a significant increase of plasma CGRP in the jugular vein ($F_{1.2,10.8} = 38.84$; $p < 0.001$). CGRP levels were significantly higher after 2 minutes (35.24 ± 3.44 pg/ml, $n = 10$, $p < 0.001$) and 15 minutes (21.56 ± 1.78 pg/ml, $n = 10$, $p < 0.001$) when compared to baseline levels (13.84 ± 0.80 pg/ml, $n = 10$). The rise of CGRP after IS was also significantly different to controls (vehicle injection); 2 and 15 minutes after vehicle injection the CGRP levels were 16.31 ± 2.17 pg/ml ($n = 5$, $p = 0.003$) and 15.16 ± 1.47 pg/ml ($n = 5$, $p = 0.037$), respectively. Baseline CGRP levels were not different between groups (Figure 1).

Baseline CGRP levels in the CSF (125.45 ± 11.57 pg/ml; $n = 5$) were significantly higher compared to the levels measured in the extracerebral circulation (13.84 ± 0.80 pg/ml, $n = 10$, $p = 0.001$). In parallel to the release of CGRP into the jugular vein intracisternal administration of IS led to a significant increase of CGRP levels in the CSF (222.60 ± 13.34 pg/ml, $n = 5$; $p = 0.001$) after 15 minutes (Figure 2). Due to limited CSF volumes we could perform CGRP measurements only once in each experiment.

Neonatal capsaicin administration significantly reduced IS-induced CGRP release into extracerebral circulation and the CSF

NCT did not affect baseline CGRP levels in jugular vein blood (13.84 ± 0.80 pg/ml, $n = 10$ (control) vs. 15.34 ± 1.04 pg/ml, $n = 12$ (capsaicin group), $p = 0.283$). In contrast, NCT almost completely abolished IS-induced neuropeptide release after 2 minutes (19.08 ± 1.08 pg/ml, $n = 12$) when compared to positive controls (intracisternal IS, no capsaicin pre-treatment:

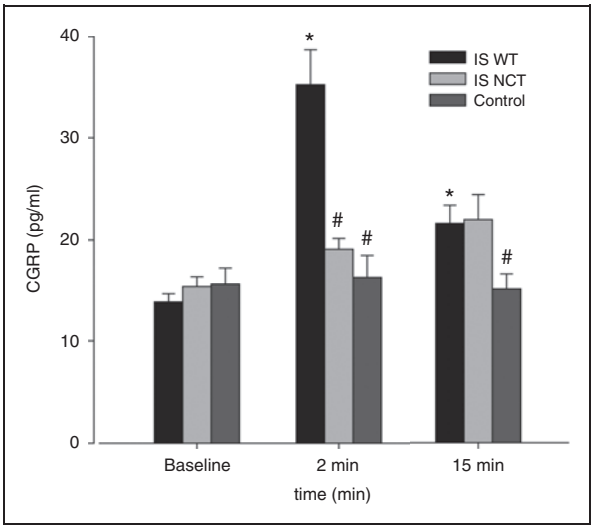

Figure 1. CGRP plasma concentration in jugular vein blood. Intracisternal IS administration induced a significant increase in CGRP concentration in the jugular vein when compared to baseline (*). This increase was immediate (2 minutes) after IS and although already decreasing, still significant after 15 minutes. The comparison between treatment groups at each point of time revealed that subcutaneous NCT almost completely abolished early IS-induced CGRP increase (#).
NCT: neonatal capsaicin treatment; WT: without neonatal capsaicin treatment.

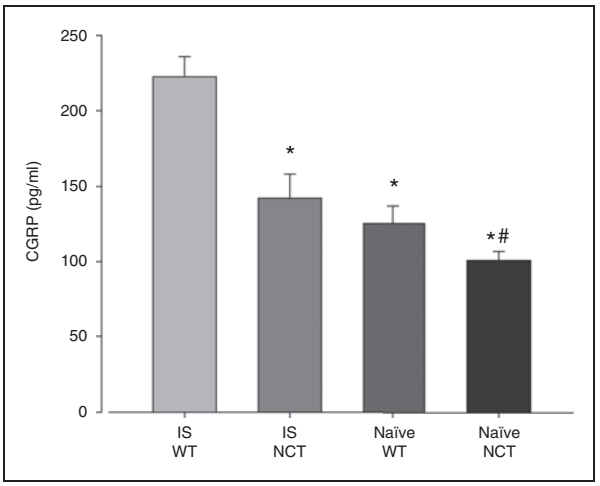

Figure 2. CGRP concentration in the CSF. Intracisternal IS administration caused a significant increase in CGRP concentration in the CSF (measured 15 minutes after IS administration) when compared to all other treatment groups (*). NCT treatment attenuated this increase with CGRP levels now ranging close to the naïve groups. However, CGRP levels before IS were significantly lower in the NCT group when compared to the WT group (#).
NCT: neonatal capsaicin treatment; WT: without neonatal capsaicin treatment.

35.24 ± 3.44 pg/ml, $n = 10$, $p = 0.001$). There was no difference 15 minutes after IS (21.56 ± 1.78 pg/ml, $n = 10$ vs. 21.93 ± 2.48 pg/ml, $n = 12$, $p = 0.907$) (Figure 1).

NCT also did not affect baseline CGRP levels in CSF (control: 125.45 ± 11.57 pg/ml, $n = 6$; NCT group: 100.95 ± 5.89 pg/ml, $n = 6$, $p = 0.09$). However, stimulus-induced CGRP concentrations in the CSF were strikingly reduced in the NCT group; 15 minutes after IS mean CGRP levels were 142.22 ± 15.64 pg/ml ($n = 6$) in the NCT group vs. the neonatal control group (222.60 ± 13.34 pg/ml, $n = 5$, $p = 0.004$). The CGRP concentration in the CSF in NCT animals was still significantly higher after intracisternal IS administration (142.22 ± 15.64 pg/ml, $n = 6$) compared to baseline levels (100.95 ± 5.89 pg/ml, $n = 5$, $p < 0.05$) (Figure 2). In comparison to corresponding baseline CGRP levels, IS led to a CGRP increase of 40.88% in the NCT group vs. 77.44% in the neonatal control group.

NCT reduces CGRP mRNA in the trigeminal ganglion, but not in the TNC

We used PCR and histochemistry for CGRP to determine the efficacy of NCT as this neuropeptide is the main content of trigeminal pain fibres and also functionally important. CGRP mRNA and protein was determined in the tissues of interest (TG and TNC). Analysis was performed in tissues from animals that were used for the functional experiments described above.

In the trigeminal ganglion CGRP mRNA levels were strikingly reduced in the NCT group (0.021 ± 0.008, $n = 5$) when compared to naïve rats (0.078 ± 0.016, $n = 5$, $p = 0.012$). CGRP levels in the TNC were significantly lower than in the TG but most importantly and in contrast to TG not affected by neonatal capsaicin (NCT group: 0.006 ± 0.003, $n = 4$; naïve group: 0.007 ± 0.004, $n = 4$, $p = 0.763$) (Figure 3).

Immunohistochemistry indicates a massive reduction of CGRP-positive neurons in the TG in the NCT group and thereby supports our PCR data. In contrast, neonatal naïve animals show strong staining of CGRP in this tissue (Figure 4A and B). Due to the absence of CGRP-positive neurons in the TG Nissl-stained perikarya are more prominent (Figure 4A).

In order to demonstrate that capsaicin delivery to newborn rats only affects nociceptors we performed double staining with a marker for neurofilament (NF200) and CGRP protein. Double staining of TG slices in Figures 4C and D does not reveal any reduction of NF200 positive fibres, indicating no loss of thickly myelinated, A-beta-fibres in both groups. However, the quantity of CGRP-positive pain fibres and neurons is considerably reduced in the NCT group.

DAB staining of dura mater whole mounts also shows scarce CGRP staining in capsaicin neonates whereas controls show rich CGRP staining around the meningeal blood vessels and throughout the tissue (Figure 4E and F). In line with findings from the TG these experiments show a clear reduction of CGRP protein in trigeminal nociceptors after capsaicin.

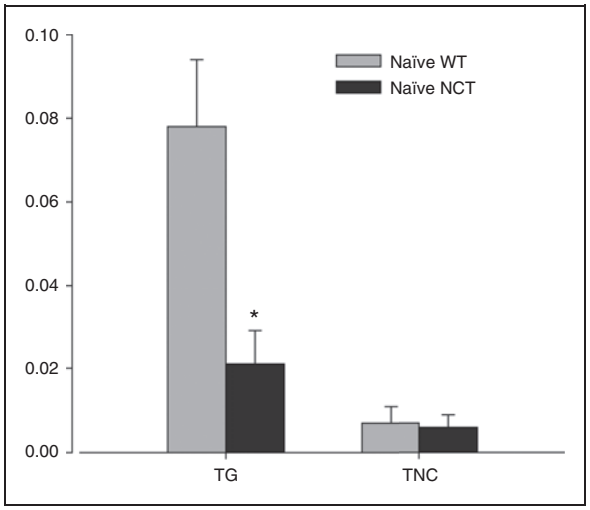

Figure 3. CGRP mRNA levels in the TG and TNC. PCR of the TNC and the TG demonstrated that NCT significantly reduced the amount of CGRP mRNA in the TG (*) but not in the TNC. NCT: neonatal capsaicin treatment; WT: without neonatal capsaicin treatment.

Finally, we also investigated brainstem tissue to dissect out the effects of NCT on secondary trigeminal neurons. Immunohistochemistry for CGRP did not reveal any difference in CGRP content in the TNC throughout all levels and laminae (Figure 4G and H). This finding is in line with the PCR data.

Discussion

The findings of this study confirm our previously published data and expand upon these. Intracisternal administration of IS leads to a significant increase of the CGRP concentration in the jugular vein (15). Now, we show that intracisternal administration of IS also leads to a rise in CGRP levels in the CSF. IS-induced CGRP release in the extracerebral circulation and in the CSF could be attenuated by subcutaneous capsaicin treatment in neonates, thereby indicating that a large portion of stimulus-induced CGRP in both compartments originates from trigeminal afferents. To determine the efficacy of NCT and to dissect out the contribution of primary or secondary trigeminal neurons, RT-PCR and immunohistochemistry were carried out in selected tissues showing that capsaicin reduced the concentration of CGRP mRNA in the trigeminal ganglion but not in the TNC. Immunohistochemistry data are in line. Together our data point to a significant role of primary trigeminal

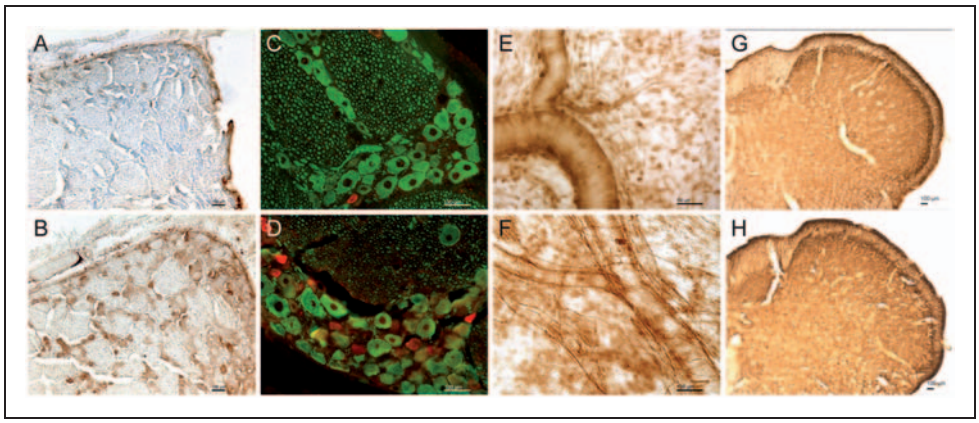

Figure 4. Nissl staining in the TG shows the absence of CGRP-positive neurons in the NCT animals (A) compared to naïve rats (B). In line with this result immunofluorescent double staining for CGRP (red) and NF200 (green) demonstrates the loss of CGRP-positive fibres in the TG (C [NCT] and D). Correspondingly to the results in the TG, DAB staining of dura mater whole mounts shows the loss of the CGRP-positive fibres surrounding the meningeal blood vessels in NCT animals (E [NCT] and F). In contrast, in sections of the TNC no difference could be observed in immunohistochemistry between WT and NCT animals (G and H). WT: without neonatal capsaicin treatment. NCT: neonatal capsaicin treatment; WT: without neonatal capsaicin treatment.

afferents for event-related induced CGRP release. However, a contribution of spinal afferents to CGRP release in this study is possible.

CGRP in migraine

CGRP is a crucial neuropeptide in migraine and in experimental animal models of trigeminally mediated pain. In the past it was shown that electrical stimulation of the trigeminal ganglion as well as the superior sagittal sinus leads to a significant release of CGRP into the circulation (1,23–25). Blockade of CGRP receptors attenuates stimulus-induced vasodilation and inhibits neuronal activity in the TNC (2,26). The neurochemical mediators that lead to the release of CGRP in spontaneous migraine attacks remain largely unknown. Experimental and clinical data addressing possible mediators (e.g. nitric oxide) remains scarce and inconclusive (22,27). Despite all the controversies, CGRP is considered a reliable marker of trigeminal activation.

To our knowledge this is the first study to compare CGRP levels in CSF and peripheral venous blood in relation to activation of the trigeminal system. Baseline data show that CGRP concentrations are significantly higher in CSF as compared to jugular vein blood and this difference remains stable after trigeminal nerve stimulation. If this finding applies to the human, one can understand that studies in migraine subjects can fail to detect CGRP changes in jugular vein blood during attacks. The determination of CGRP in CSF in humans during attacks may lead to direct evidence regarding its role in migraine. However, our work does not point in any way to CGRP as a biomarker of migraine. Instead this approach was chosen to better understand the pathophysiology of CGRP after trigeminal stimulation.

CGRP released from trigeminal nerves probably enhances nociceptive signalling at the level of central terminals rather than peripherally within the meninges. Recent studies indicate that when CGRP is applied directly onto the dura mater it neither induces primary afferent trigeminal nociceptor nor activation or sensitization (16) but when applied centrally by microiontophoresis it can enhance neuronal transmission (2). Previously, we have shown that intracisternal administration of IS leads to a significant release of CGRP into the circulation (15), and the current study demonstrates that stimulus-induced CGRP release from primary afferents can be reliably measured in the CSF. Taken together, these findings suggest an important role for CGRP signalling from the primary afferent terminals in the medullary dorsal horn that may lead to sensitization of second-order neurons.

Origin of CGRP release

Based on the literature one would assume the trigeminal system as the main source of CGRP following intracisternal IS infusion, a novel approach to study CGRP levels in CSF and blood. Here we provide experimental evidence for our hypothesis. We treated newborn rats with subcutaneously administered capsaicin, a method that has repeatedly been shown to destroy most of the pain-transmitting C-fibres (17). Experimental work in mice shows no difference in TNC with respect to CGRP immunoreactivity between capsaicin and vehicle after several weeks (28). This is in line with our CGRP PCR and immunohistochemistry data. IS-induced CGRP release is strikingly attenuated due to neonatal capsaicin, indicating that CGRP is derived from the trigeminal nerve system. The remaining CGRP in CSF and blood could be derived from secondary trigeminal

neurons in the brainstem and the remaining primary trigeminal fibres, as some fibres are left intact. Other structures of the brain may also contribute to CGRP concentrations. However, our data of strikingly reduced stimulus-increased CGRP levels in NCT indicate only a very minor contribution of non-trigeminal structures to stimulus-dependent CGRP levels.

CGRP is not capable of crossing the blood–brain barrier (29) and therefore cannot migrate into CSF from arterial blood. Hence, a non-trigeminal primary source for CGRP increase into CSF is very unlikely. These data are in line with data from our previous work. We demonstrated that pre-treatment with 5-$HT_{1B/D}$ agonists inhibits CGRP release into jugular vein blood, also pointing to the secondary and primary trigeminal system as the source for CGRP in jugular vein blood after IS administration (15).

The contribution of spinal afferents to CGRP release is a possibility we cannot entirely rule out. However, because spinal afferents have only limited – if any – role in migraine headache, this contribution is probably irrelevant. Moreover, it is questionable whether CGRP derived from spinal afferents accumulates in significant concentration in the cisterna magna and jugular vein blood as the neuropeptide quickly degrades after release. In case spinal afferents contribute to neuropeptide release in this model, the results pointing to a significant role of primary afferents would be unchanged.

Capsaicin treatment and CGRP release

CGRP is released into CSF and the circulation from primary afferents of the trigeminal nerve system after intracisternal IS. However, secondary trigeminal neurons also contain CGRP (30–32). Strikingly reduced stimulus-induced CGRP levels in NCT animals support the hypothesis that trigeminal afferents are the main source of CGRP in CSF and blood. Capsaicin does not cross the blood–brain barrier and thereby only destroys primary trigeminal afferents. Our CGRP PCR data in trigeminal ganglia tissues and the TNC confirm this fact, as do the immunohistochemistry data. Both techniques show a strong reduction of CGRP in peripheral tissues but no change in central trigeminal tissues in the brainstem. These observations lead to the conclusion that the majority of stimulus-induced CGRP release is derived from trigeminal afferents. Secondary trigeminal neurons may contribute, but based on our data only to a limited extent.

We have used CGRP PCR as a marker of the effect of NCT. Although very obvious, this approach is new in this regard. CGRP is found in 50% of trigeminal neurons (33), including c- and a-delta fibres. The destruction of c-fibres also seems to finally destroy the cell bodies as demonstrated by CGRP PCR. Our histological and immunofluorescence studies support this observation. We were able to demonstrate that subcutaneous pre-treatment with capsaicin significantly reduces the amount of CGRP-positive fibres in the primary end of the trigeminal nerve system. Capsaicin pre-treatment only affected the IS-induced increase of plasma and CSF-CGRP levels while baseline CGRP concentrations were unaffected. This leads to the conclusion that the remaining primary trigeminal neurons and secondary trigeminal neurons seem to be sufficient to guarantee baseline CGRP levels.

A few studies have shown elevated CGRP levels in jugular vein blood after trigeminal ganglion or sagittal sinus stimulation (1,24). None of the studies has measured CGRP in CSF and blood in the same experimental set-up. In fact, TG stimulation is not a suitable model to determine CGRP in CSF as the blood–brain barrier is destroyed and electrodes are drilled through the brain (25). CGRP levels in the CSF are significantly higher than in jugular vein blood. There may be several reasons for this. First, CGRP is a protein that degrades quickly after release. By the time CGRP is reabsorbed into venous blood it may have degraded to a large extent and therefore impossible to completely detect. Secondly other absorption for CSF such as the arachnoid villi, the transependymal route and the lymph system may also drain some CGRP (34). Together this may result in significantly lower CGRP levels in the peripheral blood. Finally, CGRP derived from other structures than primary trigeminal afferents may also contribute to CGRP levels in the CSF.

The increase in CGRP in jugular vein blood in this study is comparable to the data we have published previously (15). Limmroth et al. also published evidence of an increase in CGRP in the extracranial circulation of 130% following trigeminal ganglion stimulation in the rat (24). Amrutkar et al. recently demonstrated that stimulated CGRP release is significantly higher from isolated TNC than from isolated dura mater, thereby showing a quantitatively different response to a stimulus depending on the tissue (35). Human data also indicate a 100% increase in CGRP during spontaneous migraine attacks (36). Data from in vitro models are different and show a four-fold increase in CGRP after IS (37). The concentration of IS in this study is identical to the IS concentration we used before and, according to published data by Burstein et al., from numerous experimental animal studies (8). We explicitly chose this IS concentration and composition in order to be able to relate effects of intracisternal administration to topical administration. Considering the distribution of IS in the CSF space, the concentration of IS must be at least as strong as if IS was applied topically onto the dura mater or into a skull preparation. Given the fact that the rise in CGRP is comparable to the rise in

human studies and other rat in vivo studies the IS composition and concentration are sufficient to cause effects within a physiological previously demonstrated range.

In summary, our data suggests that the intracisternal administration of IS leads to an activation of the trigeminal nerve system, which in turn leads to an immediate release of CGRP, mainly from the primary end of the trigeminal nerve system into the circulation and the CSF.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

References

1. Zagami AS, Goadsby PJ and Edvinsson L. Stimulation of the superior sagittal sinus in the cat causes release of vasoactive peptides. *Neuropeptides* 1990; 16: 69–75.
2. Storer RJ, Akerman S and Goadsby PJ. Calcitonin gene-related peptide (CGRP) modulates nociceptive trigeminovascular transmission in the cat. *Br J Pharmacol* 2004; 142: 1171–1181.
3. Olesen J, Diener HC, Husstedt IW, et al. Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migraine. *N Engl J Med* 2004; 350: 1104–1110.
4. Ho TW, Ferrari MD, Dodick DW, et al. Efficacy and tolerability of MK-0974 (telcagepant), a new oral antagonist of calcitonin gene-related peptide receptor, compared with zolmitriptan for acute migraine: a randomised, placebo-controlled, parallel-treatment trial. *Lancet* 2008; 372: 2115–2123.
5. Ho TW, Mannix LK, Fan X, et al. Randomized controlled trial of an oral CGRP receptor antagonist, MK-0974, in acute treatment of migraine. *Neurology* 2008; 70: 1304–1312.
6. Diener H-C, Barbanti P, Dahlöf C, et al. BI 44370 TA, an oral CGRP antagonist for the treatment of acute migraine attacks: Results from a phase II study. *Cephalalgia* 2011; 31: 573–584.
7. Burstein R, Collins B and Jakubowski M. Defeating migraine pain with triptans: a race against the development of cutaneous allodynia. *Ann Neurol* 2004; 55: 19–26.
8. Burstein R and Jakubowski M. Analgesic triptan action in an animal model of intracranial pain: a race against the development of central sensitization. *Ann Neurol* 2004; 55: 27–36.
9. Dodick D and Silberstein S. Central sensitization theory of migraine: clinical implications. *Headache* 2006; 46(Suppl 4): S182–S191.
10. Cady R, Martin V, Mauskop A, et al. Symptoms of cutaneous sensitivity pre-treatment and post-treatment: results from the rizatriptan TAME studies. *Cephalalgia* 2007; 27: 1055–1060.
11. Goadsby P, Zanchin G, Geraud G, et al. Early vs. non-early intervention in acute migraine – 'Act when Mild (AwM)'. A double-blind, placebo-controlled trial of almotriptan. *Cephalalgia* 2008; 28: 383–391.
12. Schoenen J, De Klippel N, Giurgea S, et al. Almotriptan and its combination with aceclofenac for migraine attacks: a study of efficacy and the influence of auto-evaluated brush allodynia. *Cephalalgia* 2008; 28: 1095–1105.
13. De Felice M, Ossipov MH, Wang R, et al. Triptan-induced latent sensitization: a possible basis for medication overuse headache. *Ann Neurol* 2010; 67: 325–337.
14. Bartsch T, Knight YE and Goadsby PJ. Activation of 5-HT(1B/1D) receptor in the periaqueductal gray inhibits nociception. *Ann Neurol* 2004; 56: 371–381.
15. Hoffmann J, Neeb L, Israel H, et al. Intracisternal injection of inflammatory soup activates the trigeminal nerve system. *Cephalalgia* 2009; 29: 1212–1217.
16. Levy D, Burstein R and Strassman AM. Calcitonin gene-related peptide does not excite or sensitize meningeal nociceptors: implications for the pathophysiology of migraine. *Ann Neurol* 2005; 58: 698–705.
17. Markowitz S, Saito K and Moskowitz MA. Neurogenically mediated leakage of plasma protein occurs from blood vessels in dura mater but not brain. *J Neurosci* 1987; 7: 4129–4136.
18. Jancso G and Kiraly E. Sensory neurotoxins: chemically induced selective destruction of primary sensory neurons. *Brain Res* 1981; 210: 83–89.
19. Jancso G, Kiraly E and Jancso-Gabor A. Pharmacologically induced selective degeneration of chemosensitive primary sensory neurones. *Nature* 1977; 270: 741–743.
20. Ruscher K, Freyer D, Karsch M, et al. Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model. *J Neurosci* 2002; 22: 10291–10301.
21. Reuter U, Bolay H, Jansen-Olesen I, et al. Delayed inflammation in rat meninges: implications for migraine pathophysiology. *Brain* 2001; 124: 2490–2502.
22. Offenhauser N, Zinck T, Hoffmann J, et al. CGRP release and c-fos expression within trigeminal nucleus caudalis of the rat following glyceryltrinitrate infusion. *Cephalalgia* 2005; 25: 225–236.
23. Goadsby PJ and Edvinsson L. The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. *Ann Neurol* 1993; 33: 48–56.
24. Limmroth V, Katsarava Z, Liedert B, et al. An in vivo rat model to study calcitonin gene related peptide release following activation of the trigeminal vascular system. *Pain* 2001; 92: 101–106.
25. Reuter U, Sanchez del Rio M and Moskowitz MA. Experimental models of migraine. *Funct Neurol* 2000; 15(Suppl 3): 9–18.
26. Dux M, Santha P and Jancso G. Capsaicin-sensitive neurogenic sensory vasodilatation in the dura mater of the rat. *J Physiol* 2003; 552: 859–867.

27. Juhasz G, Zsombok T, Modos EA, et al. NO-induced migraine attack: strong increase in plasma calcitonin gene-related peptide (CGRP) concentration and negative correlation with platelet serotonin release. *Pain* 2003; 106: 461–470.
28. Aita M, Maeda T and Seo K. The effect of neonatal capsaicin treatment on the CGRP-immunoreaction in the trigeminal subnucleus caudalis of mice. *Biomed Res* 2008; 29: 33–42.
29. Villalon CM and Olesen J. The role of CGRP in the pathophysiology of migraine and efficacy of CGRP receptor antagonists as acute antimigraine drugs. *Pharmacol Ther* 2009; 124: 309–323.
30. Eftekhari S and Edvinsson L. Possible sites of action of the new calcitonin gene-related peptide receptor antagonists. *Ther Adv Neurol Disord* 2010; 3: 369–378.
31. Brain SD and Grant AD. Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol Rev* 2004; 84: 903–934.
32. Ho TW, Edvinsson L and Goadsby PJ. CGRP and its receptors provide new insights into migraine pathophysiology. *Nat Rev Neurol* 2010; 6: 573–582.
33. Reuss S, Riemann R and Vollrath L. Substance P- and calcitonin gene-related peptide-like immunoreactive neurons in the rat trigeminal ganglion – with special reference to meningeal and pineal innervation. *Acta Histochem* 1992; 92: 104–109.
34. Kawajiri H, Furuse M, Namba R, et al. Effect of internal jugular vein ligation on resorption of cerebrospinal fluid. *J Maxillofac Surg* 1983; 11: 42–45.
35. Amrutkar DV, Ploug KB, Olesen J, et al. Role for voltage gated calcium channels in calcitonin gene-related peptide release in the rat trigeminovascular system. *Neuroscience* 2011; 172: 510–517.
36. Goadsby PJ, Edvinsson L and Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Ann Neurol* 1990; 28: 183–187.
37. Ebersberger A, Averbeck B, Messlinger K, et al. Release of substance P, calcitonin gene-related peptide and prostaglandin E2 from rat dura mater encephali following electrical and chemical stimulation in vitro. *Neuroscience* 1999; 89: 901–907.