Increase in CGRP- and nNOS-immunoreactive neurons in the rat trigeminal ganglion after infusion of an NO donor

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Cephalalgia

31(1) 31–42

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DOI: 10.1177/0333102410375725

cep.sagepub.com

Abstract

Background: Nitrovasodilators, such as glyceroltrinitrate (GTN), which produce nitric oxide (NO) in the organism, are known to cause delayed headaches in migraineurs, accompanied by increased plasma levels of calcitonin gene-related peptide (CGRP) in the cranial venous outflow. Increases in plasma CGRP and NO metabolites have also been found in spontaneous migraine attacks. In a rat model of meningeal nociception, infusion of NO donors induced activity of neurons in the spinal trigeminal nucleus.

Methods: Isoflurane-anaesthetised rats were intravenously infused with GTN (250 µg/kg) or saline for two hours and fixed by perfusion after a further four hours. Cryosections of dissected trigeminal ganglia were immunostained for detection of CGRP and neuronal NO synthase (nNOS). The ganglion neurons showing immunofluorescence for either of these proteins were counted.

Results: The proportions of CGRP- and nNOS- as well as double-immunopositive neurons were increased after GTN infusion compared to saline treatment in all parts of the trigeminal ganglion (CGRP) or restricted to the ophthalmic region (nNOS). The size of immunopositive neurons was not significantly different compared to controls.

Conclusion: High levels of NO may induce the expression or availability of CGRP and nNOS. Similar changes may be involved in nitrovasodilator-induced and spontaneous headache attacks in migraineurs.

Keywords

Calcitonin gene-related peptide, nitric oxide, immunohistochemistry, headache

Date received: 26 January 2010; revised: 7 April 2010; accepted: 7 April 2010

Introduction

Various clinical studies in humans (1–3) and experimental observations in animals (4–6) have shown that nitric oxide (NO) may play an important role in the pathophysiology of primary headaches. Intravenous application of the NO donor glyceroltrinitrate (GTN, 2.5–20 µg/kg over 20 min) to healthy subjects provoked headaches, which could be prevented by sumatriptan 6 mg given subcutaneously (SC) (7). Comparing healthy subjects with patients suffering from migraine with aura, GTN infusion (0.5 µg/kg over 20 min) caused immediate headaches, which were more severe in headache patients (1). Headaches gradually decreased in the control subjects, while they peaked in headache patients five to eight hours later and fulfilled migraine criteria in half of the patient group.

In a rat model of meningeal nociception, infusion of the NO donors sodium nitroprusside (SNP, 25 µg/kg)

or GTN (250 µg/kg) increased the spontaneous neuronal activity of neurons in the spinal trigeminal nucleus (STN) with meningeal afferent input (4,8). This increase in activity was biphasic in that an immediate weak activation was followed by a second phase of activity slowly developing within one to two hours (4), and it could be reversed by infusion of the CGRP receptor antagonist BIBN4096BS (8). Thus the comparably high dose of NO donor may have converted a normal rat into an animal model reflecting some similarities to migraine. The pathophysiological processes behind

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these findings are not known. However, regarding the delayed onset of migraine-like headaches, NO might be able to trigger migraine attacks by initiating changes in gene expression. Endogenous NO is synthesised by three distinct isoforms of NO synthase (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (9). The nNOS isoenzyme is widely distributed in the mammalian central nervous system and has been detected in the trigeminal ganglion as well (10,11). As the inhibition of endogenous NO synthesis in migraineurs has been reported to attenuate migraine attacks (12) and to decrease the spontaneous spinal trigeminal activity in rats (4), a tonic NO effect on the mediation of migraine attacks has been suggested. This theory was supported by clinical observations demonstrating an increase of NO metabolites such as nitrites and nitrates in internal jugular vein blood of migraineurs during spontaneous migraine attacks (13). Thus, NO may play a role in both initiating and mediating migraine attacks.

Calcitonin gene-related peptide (CGRP) is one of the most common neuropeptides in the central and peripheral nervous systems (14). In migraineurs clinical studies have revealed increased plasma levels of CGRP during both spontaneous attacks and nitrovasodilator-induced headaches (2,13,15). Moreover, recent clinical trials have shown that inhibition of CGRP receptors with the CGRP-receptor antagonists olcegepant (BIBN4096BS) and telcagepant (MK-0974) is effective in the acute treatment of migraine attacks (16,17). In addition, BIBN4096BS reduced NO-induced increases in neuronal activity in the STN in the mentioned rat model of meningeal nociception (8). As both CGRP and NO are involved in spontaneous and induced migraine headaches, there may be a link between NO infusion and expression of CGRP- and NO-producing enzymes. An immunohistological analysis of the trigeminal ganglion demonstrated coexpression of CGRP and NOS in the majority of NOS-immunopositive neurons (10). Moreover, various in vitro experiments have shown that NO stimulation causes CGRP release from the spinal cord, meninges and trigeminal ganglion cells (18–20). Bellamy et al. (21) demonstrated in primary cultures of rat trigeminal neurons that NO donors are even able to increase transcription of the CGRP gene by promoter activation. However, these findings have not yet been confirmed in vivo.

The aim of this study was to investigate whether NO donors increase the expression of CGRP and nNOS in neurons within the rat trigeminal ganglion. Thus, after infusion of GTN, the number of CGRP-, nNOS-immunoreactive and double immunopositive trigeminal neurons was analysed and compared to those of saline-treated animals.

Material and methods

Animals

Twelve adult male inbred Wistar rats (290–310 g) were used for the study. Animals were housed at a 12 hour:12 hour light-dark cycle with ad libitum access to food and water. All procedures were conducted in accordance with principles of Laboratory Animal Care (National Institutes of Health [USA] publication no. 86–23, revised 1985), the European Communities Council Directive and German regulations of animal welfare and treatment. Experimental protocols were approved by the local government.

Treatment, perfusion, fixation, preparation and tissue processing

Rats were initially anaesthetised in a closed box by inhalation of 5% isoflurane (Abbott, Wiesbaden, Germany). During surgery animals breathed oxygen-enriched (30%) and humidified air with 2.5% isoflurane supplied by a loosely fitted mask. A catheter was inserted into the femoral vein for application of substances. Isoflurane was reduced to 1.5% and the animals were placed in a prone position on a feedback-controlled warming plate in order to maintain body temperature. Six animals were slowly intravenously (IV) infused with the NO donor glyceroltrinitrate (GTN, Schwarz Pharma, Monheim, Germany) at a dose of 250 µg/kg over a period of two hours. After testing different infusion rates and doses of GTN in previous experiments, the infusion parameters were adjusted so that no significant lowering of the arterial pressure occurred (8). Six control animals received isotonic saline with an identical infusion rate and duration. All substances were diluted in isotonic saline and infused by a syringe pump (Harvard Apparatus, March-Hugstetten, Germany). After infusion all animals were under anaesthesia for a further four hours. Then the concentration of isoflurane in the inspiration gas was increased to 5%, the animals were quickly thoracotomised and perfused through the left ventricle with isotonic saline at room temperature for about two minutes, followed by a solution of 4% paraformaldehyde in 10 mM phosphate-buffered saline (PBS, pH 7.4) for 20 minutes. After an extended craniectomy and cervical laminectomy, the supratentorial dura mater was dissected from the brain. Cerebrum and brainstem were removed and the trigeminal ganglia excised from the skull base. Trigeminal ganglia were postfixed by immersion in 4% paraformaldehyde for two hours and stored in 10 mM PBS for 24 hours. For cryoprotection the ganglia were incubated in a 30% solution of sucrose in PBS for one day.

After mounting on Tissue-Tek (GSV1, Slee Technik, Mainz, Germany), the ganglia were transferred to a plastic micro test tube, rapidly frozen at -70°C by immersing the tube in methylbutane cooled in liquid nitrogen and stored in the freezer at -20°C . A series of 20- μm thick longitudinal sections were cut from the ganglia with a cryostat (Leica, Bensheim, Germany). Sections were mounted on poly-L-lysine-coated slides and dried for one hour at room temperature prior to staining.

Double-labelling immunohistochemistry

The sources, characteristics and dilutions of the primary and secondary antibodies used in this study are listed in Tables 1 and 2. For immunostaining the mounted sections were rinsed in a solution of PBS containing antimicrobial sodium azide (NaN_3) and preincubated with a solution of 5% normal donkey serum (Dako, Hamburg, Germany) containing 0.5% Triton X-100 and 1% bovine serum albumine (BSA) (both from Sigma-Aldrich, Steinheim, Germany) in PBS/ NaN_3 for one hour at room temperature. After rinsing in PBS/ NaN_3 for five minutes, the sections were incubated with primary antibodies directed against nNOS and CGRP at room temperature overnight. All primary antibodies were diluted in PBS/ NaN_3 , containing 1% BSA and 0.5% Triton X-100. Sections were washed with PBS/ NaN_3 three times for 5 minutes and incubated with donkey anti-rabbit immunoglobulin G (IgG), coupled to Alexa 488 for detection of nNOS, and donkey anti-goat IgG, coupled to Alexa 555 for detection of CGRP, for one hour at room temperature. All secondary antibodies were diluted in PBS with NaN_3 containing 1% BSA and 0.5 % Triton X-100. Sections were rinsed with PBS/ NaN_3 three times, coverslipped in PBS-glycerol (pH 8.6) and

stored in the refrigerator where they were prevented from light.

Specificity of the immunocytochemical reactions was verified by sections incubated in solutions lacking primary antibodies. No specific staining was found with ALEXA Fluor 488 (green) or with ALEXA Fluor 555 (red) but in the green channel all neurons showed a faint background staining, which was used to count the whole cell number. The pattern of CGRP-immunoreactivity (-ir) was identical to previous descriptions (22–24). Enzyme-linked immunosorbent assay (ELISA) was used to evaluate the anti-CGRP primary antibody (manufacturer's technical information). The rabbit anti-nNOS antibody has been used in various rat preparations (25,26) and its specificity was tested by pre-absorption with an excess of the relevant antigen (27).

Azur methylene blue staining

Four overview micrographs representative sections of the trigeminal ganglion were stained with an azur methylene blue solution containing 1 g/l azur II (Merck, Germany; catalogue # 1.09211) and 1 g/l methylene blue (Merck, Germany; # 1283) in distilled water with 1% sodium tetraborate. The slides with the mounted sections were rinsed for 10 minutes in distilled water, then placed on a heating plate at 50°C , stained with drops of the solution for 30 seconds and rinsed again in distilled water, before they were embedded in Kaiser's glycerine and coverslipped.

Light microscopy and cell counting

The quantitative analysis of the sections was performed using a Leica Aristoplan epifluorescence microscope (Leica, Bensheim, Germany) equipped with an HBO 100 W-2 lamp (Osram, Munich, Germany), connected

Table 1. Primary antibodies used for immunohistochemistry

Name	Host	Source	Dilution
Calcitonin gene-related peptide (CGRP)	Goat	Biotrend, Cologne, Germany; BT 17-2090-07	1 : 100
Neuronal nitrogenmonooxyde synthase (nNOS)	Rabbit	Dr. Mayer, University of Graz	1 : 800

Table 2. Secondary antibodies used for detection of primary antibodies

Secondary antibodies	Conjugated to	Source	Dilution
Donkey anti-rabbit	ALEXA Fluor 488	Molecular Probes, Eugene, OR, USA; A21206	1 : 1000
Donkey anti-goat	ALEXA Fluor 555	Molecular Probes, Eugene, OR, USA; A21432	1 : 1000

to a CCT camera (Spot RT, Visitron Systems, Puchheim, Germany). For detecting fluorescence we used filter block N2.1 for red emission (ALEXA Fluor 555) and filter block I3 for green emission (ALEXA Fluor 488).

In each ganglion the 34 central sections of the whole series were selected, from which every second section was analysed in order to avoid double counting; that is, 17 sections per ganglion were analysed. Using a 40x dry objective lens, neurons with clearly visible nuclei were counted. CGRP- and nNOS-immunoreactive (-ir) cells as well as double-stained cells were analysed. As a reference, the total cell count of all neurons visible in the

background of the green channel was used. Neurons located in the medial region (ophthalmic, V1) of the trigeminal ganglion and in the lateral regions (mandibular, V3, and maxillary, V2) were separately counted (see Figure 1C). The investigator counting the neurons (AD) was blinded to the experiments from which the sections were derived.

Confocal microscopy and image processing

In addition to the quantitative light microscopic analysis, images were obtained using a BioRad MRC 1000 confocal laser scanning system (Bio-Rad, Hemel

Figure 1. CGRP and nNOS immunofluorescence in the trigeminal ganglion. (A, B) Light microscope images showing neurons in the maxillary region immunopositive for CGRP (A, red arrows) and nNOS (B, green arrow) as visualised for cell countings. CGRP- and nNOS-ir are co-localised in one neuron. The cell marked by an arrowhead is not counted in this section, because it lacks a visible nucleus. (C) Low-power micrograph of a trigeminal ganglion section stained with azur methylene blue showing nerve fibre plexus (blue) and neuronal cell bodies (purple) in the three trigeminal partitions, V1–V3. Neurons were counted separately in the V1 half of the V1/2 region, as indicated by a broken line. (D–F) Confocal images of the ophthalmic region of a trigeminal ganglion after saline pretreatment. CGRP (D) and nNOS immunofluorescence (E) is co-localised in one cell (arrow), visible in the merged image (F) as yellow granules within the neuron (inset at higher magnification). (G–I) Confocal images of the ophthalmic region of a trigeminal ganglion after GTN pretreatment. One relatively large CGRP- and nNOS-positive neuron is visible in the upper left, one small exclusively nNOS-positive neuron in the middle. In another neuron (arrow), weak CGRP (G) and nNOS immunofluorescence (H) is seen but not strictly co-localised, visible as red granules (CGRP-ir) on a green background (nNOS-ir) within the cell in the merged image (I, inset at higher magnification). Size bars 100 μ m, panel C 500 μ m. CGRP = calcitonin gene-related peptide. nNOS = neuronal nitric oxide synthase. nNOS-ir = nNOS immunoreactivity. GTN = glycerol trinitrate.

Hempstead, UK) equipped with a three line krypton-argon laser (American Laser Technology, Salt Lake City, UT, USA) and attached to a Nikon Diaphot 300 microscope (Nikon, Düsseldorf, Germany). The filter settings of the BioRad confocal scanner for double label were: 488-nm excitation for ALEXA 488 (filter 522 DF32), 568-nm excitation for ALEXA 555 (filter 605 DF322). A 20x dry objective lens (numerical aperture 0.75) was used. Electronic zoom factors varied between 1.0 and 3.6.

Images of 768x512 pixels were obtained, and the channels were merged into a 24-bit red-green-blue (RGB) tif file by using confocal assistant software 4.02. To test for co-localisation, single optical sections at the same focus plane were taken and the two corresponding channels were merged. Only adjustment for contrast, brightness and evenness of illumination was performed. To apply text and scale bars and organise the final layouts, CorelDraw (Corel, Dublin, Ireland) was used.

Statistical analysis

The number of CGRP-, nNOS- as well as co-localised cells was compared between GTN and saline-treated animals. Further, the number of nNOS- and CGRP-positive cells in medial and lateral regions of the trigeminal ganglion were analysed. Statistical analysis was performed with STATISTICA (Tulsa, OK, USA). The number of neurons depended on the plane of sections with the maximum of cells counted in the central slices ($R=0.33$, $p=.001$ in saline-treated animals). Therefore, the number of cells in corresponding slices from animals pretreated with saline and GTN were compared as matched pairs. T-test for dependent samples was used for $n \geq 10$, the Wilcoxon rank test for smaller samples and analysis of variance (ANOVA) for three or more groups. Significance was accepted for $p < .05$. The data are reported as mean $\pm$ standard error of the mean (SEM).

Results

In the selected sections of the trigeminal ganglion, the total number of neurons and the number of neurons showing CGRP-, nNOS-immunoreactivity or co-localisation of both markers were separately quantified using light microscopy (Figures 1A and 1B). In the first countings the number of nNOS-immunoreactive neurons seemed to be especially high in the medial (ophthalmic) region (V1) of the trigeminal ganglion. Therefore, in three animals per group the immunoreactivity was separately counted in the V1 region and in the mandibulo-maxillary division (V2/3, lateral) of the trigeminal ganglion (Figure 1C). Confocal imaging was used to visualise the intracellular distribution of

immunoreactivity and to take representative micrographs (Figure 1D–I).

As a reference, the total cell count was detected by counting all neurons visible in the background of the green channel. A similar number of cells was counted in the groups treated by saline and GTN ($p=0.25$; Figure 2D).

CGRP immunoreactive neurons

CGRP-ir neurons were either completely stained or marked by granular immunofluorescent structures around the nucleus (Figures 1A, 1D and 1G). Cells containing this marker were widely distributed throughout the trigeminal ganglion. The average number of CGRP-positive neurons in the saline-treated animals was 53 ± 5 per slice, in the GTN-treated animals 93 ± 4 ($p=.028$; Figure 2A). The increase in CGRP-immunopositive cells after GTN treatment was found both in V1 and in V2/3 (ANOVA $F_{(1,8)}=6.4$, $p=.035$, HSD post-hoc tests both $p < .001$; Figure 3). The size of CGRP-ir and nNOS-ir neurons was not significantly different between GTN-treated and control animals.

nNOS immunoreactive neurons

Cells immunoreactive for nNOS mostly showed a homogeneous green staining (Figures 1B, 1E and 1H). The average number of nNOS-ir neurons per slice was 3.6 ± 0.8 in the saline-treated animals, and 8.0 ± 0.8 in the GTN-treated group ($p=.028$, Figure 2B). After infusion of GTN an increase in the number of nNOS positive neurons was observed in the V1 division (ANOVA $F_{(1,8)}=7.7$, $p=.024$, HSD (honestly significant difference) post-hoc test $p=.004$), but not in the V2/3 region ($p=.61$, HSD post-hoc test; Figure 3).

Double immunopositive cells

Co-localised neurons were identified by a CGRP-immunopositive staining in the red channel and an nNOS-immunopositive staining in the green channel. In the merged confocal images the double positive neurons appeared homogeneously or partly yellow (Figure 1F, I). They were rarely found throughout the trigeminal ganglion. On average 1.0 ± 0.3 cells per slice in the saline-treated and 2.5 ± 0.5 in GTN-treated animals were detected ($p=.028$, Figure 2C).

Discussion

In this study we found that pretreatment with the NO donor GTN increased the number of trigeminal ganglion neurons immunoreactive to CGRP as well as the number of nNOS-immunopositive and

Figure 2. Number of immunoreactive cells for CGRP (A), nNOS (B) and both (C) in animals infused with either saline or GTN. The left panels show the mean $\pm$ SEM, the right panels are histograms (number of observations) of the difference (Δ , GTN minus saline-treated animals) in cells per slice of corresponding sections. (D) Total cell count (in thousands) from saline- and GTN-treated animals. CGRP = calcitonin gene-related peptide. nNOS = neuronal nitric oxide synthase. SEM = standard error of the mean. GTN = glycerol trinitrate.

Figure 3. Number of CGRP- (A) and nNOS- (B) immunopositive neurons in the medial (V1) and lateral (V2/3) divisions of the trigeminal ganglion. CGRP = calcitonin gene-related peptide. nNOS = neuronal nitric oxide synthase.

double-immunopositive cells. Furthermore, nNOS-positive neurons were particularly increased in the ophthalmic region of the trigeminal ganglion. This may be functionally important, as the ophthalmic division is considered to be the most frequently affected region in migraine, although animal studies suggest that all divisions of the trigeminal ganglion receive afferent projections from the dura (28). Our results suggest that NO produced by GTN is able to induce the expression of both CGRP and nNOS in neurons previously immunonegative or below the immunohistochemical detection level for these proteins. Alternatively, it may be assumed that the immunoreactivity of CGRP and nNOS was increased by changes in intracellular storing or the molecular structure of these proteins such as nitrosylation induced by the GTN infusion (see below).

Structural and functional effects caused by NO donors

In a recent electrophysiological study in the rat, in which we recorded from neurons in the spinal trigeminal nucleus caudalis with afferent input from the cranial dura mater, the same dose of GTN as used in the present study (250 µg/kg IV) as well as the NO donor sodium nitroprusside (SNP, 25 µg/kg IV) increased the ongoing neuronal activity within one hour (8),

confirming the results of an older study with SNP infusion (4). To see if these functional changes are associated with structural changes, we examined histochemical markers for NOS isoforms in the spinal trigeminal nucleus after infusion of SNP (50 µg/kg) and found that the number of NADPH-diaphorase-positive neurons increased continuously from three to eight hours after SNP infusion and the number of nNOS-immunoreactive neurons increased significantly four hours after SNP infusion (29). This was in line with reports about an increase in nNOS levels in the spinal trigeminal nucleus caudalis four hours after subcutaneous injection of 10 mg/kg GTN (6,30). In addition, we recognised an increase in the density of NADPH-diaphorase- and nNOS-positive nerve fibres in the spinal trigeminal tract, assuming that nNOS in primary trigeminal afferents may also be upregulated after NO donor infusion. This initiated the present study to look at the trigeminal ganglion four hours after the SNP infusion, and we determined not only nNOS- but also CGRP-immunoreactive neurons, as it was reported that NO donors can increase the CGRP expression in trigeminal ganglion cell cultures (21).

Regulation of gene expression of CGRP and nNOS

CGRP is expressed by alternative splicing of the primary gene product of CALCA and in addition by CALCB, resulting in two CGRP isoforms, alpha- and beta-CGRP, the first of which is predominant in neurons (31). It has previously been demonstrated that the alpha-CGRP gene is regulated by intracellular signalling cascades, in particular by mitogen-activated protein kinase (MAPK) pathways (32,33). Bellamy et al. (21) have shown in an in vitro model of primary cultures of rat trigeminal neurons that superfusion of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) causes an increase in the release of CGRP concomitant with an activation of the CGRP gene promoter. Using immunocytochemistry they found that this treatment also increased the number of neurons that contain JNK and p38 in their nuclei, suggesting that these two MAPKs are involved in the induction of CGRP expression by NO donors. The present study suggests that similar processes are induced by NO donors in vivo confirming changes in this trigeminal cell culture system (21), which could have been influenced by the culture conditions.

The transcription of nNOS in cultivated cortical neurons has been shown to be dependent on calcium influx and the cAMP response element-binding (CREB) transcription factor (34). The expression of nNOS in human neuroblastoma cells is activated by acetylation of the nuclear factor kappa B (NF-kappaB) (35). Activation of this factor is also involved in nNOS expression preceding the

development of experimental hyperalgesia (36). NO induces calcium influx, possibly by membrane depolarisation or cGMP-dependent calcium channels (37), activates multiple protein kinases in various tissues (38) and together with calcium promotes activation of CREB in neuronally derived cell lines (39). Thus, NO could stimulate the expression of nNOS, and the intracellular machinery for a NO donor to induce nNOS may be present also in trigeminal ganglion neurons.

Alternative regulation of CGRP and nNOS immunoreactivity

Messenger RNA for neuronal and endothelial (but not inducible) NOS has been identified in rat trigeminal ganglia not subjected to culture (40). Thus, CGRP and nNOS production can be expected to be regulated on the transcriptional as well as translational level. However, in a series of recent experiments two or six hours after pretreatment of rats with GTN, we found no evidence for an upregulation of CGRP mRNA in the trigeminal ganglion using real-time polymerase chain reaction (PCR) or of total CGRP protein using an ELISA. The same treatment caused an increase in CGRP release from the isolated ganglion stimulated by inflammatory mediators (41). Thus, the present data could also reflect subcellular changes in the distribution of CGRP, which may modify the access or binding of antibodies. The CGRP immunofluorescence appeared in different forms, either as conspicuous granular structures usually surrounding the nucleus, or as homogeneous staining of the whole cell (see Figures 1A, 1D and 1G). The granular structures are reminiscent of intracellular stores such as endoplasmic reticulum or Golgi apparatus. Neuropeptides such as CGRP are thought to be stored in, transported by and released from large vesicles with or without dense cores (42–45). Thus, an alternative explanation for the increase in numbers of immunoreactive neurons may be an opening of these intracellular stores caused by infusion of the NO donor, so that more neurons gain sufficient immunoreactivity to be detected by immunofluorescence.

Apart from the most common intracellular mechanism of NO via activation of guanylate cyclase and increase in cGMP, other direct reactions of NO have been described, such as nitration and nitrosylation (reviewed in 46). Thus, it could also be speculated that nitrated or nitrosylated CGRP and nNOS are more immunoreactive to the respective antibodies.

Possible role of NO and CGRP in meningeal nociception and headache

Although it is unclear which process underlies the described changes in immunoreactivity of trigeminal

ganglion neurons, they are accompanied by dramatic functional changes of the intracranial trigeminal system. Infusion of NO donors causes increased activity and responsiveness to meningeal stimulation of spinal trigeminal neurons in animals (4,8) and induces delayed migraine-like attacks in migraineurs (1). This was concomitant with an increase in plasma CGRP levels suggesting enhanced CGRP release (2), which was absent in healthy volunteers after infusion of GTN (47). We have recently found that infusion of an NO donor causes also delayed upregulation of putatively NO-producing neurons in the spinal trigeminal nucleus (29). An increase in NO production after infusion of NO donors has not yet directly been shown, but elevated plasma levels of NO metabolites and increased exhaled NO gas have been found in spontaneous migraine attacks (13) and migraine-like headaches induced by NO donor administration (48,49). NO is known as a “retrograde transmitter” to facilitate spinal transmitter and neuropeptide release and enhance synaptic transmission throughout the central nervous system (50–52). CGRP may also enhance spinal release of excitatory neurotransmitters (53) from spinal terminals of primary afferents, which have been shown to carry abundant CGRP receptors (24,54). CGRP released from spinal terminals of primary afferents is thought to facilitate nociceptive transmission (55) and may contribute to the increase in spinal trigeminal activity, as inhibition of CGRP receptors blocks this activity (56). Taken these findings together, we suggest in this study that the enhanced CGRP and nNOS immunoreactivity of trigeminal ganglion neurons may reflect the delayed functional changes towards an increased nociceptive activity obviously induced by NO in animal experiments as well as in migraine patients.

Implications for vascular versus neuronal hypotheses of migraine pain

GTN was infused at a very low rate to avoid vasodilatation and lowering of the blood pressure, which could have influenced the metabolism in the trigeminal ganglion. Nevertheless, the question may arise as to whether direct vascular effects of NO in the trigeminal ganglion or trigeminal tissues may have been supportive for the changes observed after GTN infusion, as NO and CGRP are potent vasodilators of intra- and extracranial arteries involved in neurogenic processes (10,57–59). This issue is interesting with regard to the vascular and neuronal hypotheses of migraine.

The original vascular hypothesis of migraine, which held that vascular motility was responsible for migraine pain, was later modified and extended to the neurogenic inflammation hypothesis (reviewed in 60). The model of meningeal neurogenic inflammation in rodents, which

was valuable in predicting the effectiveness of anti-migraine drugs (61), includes the dilatation of meningeal blood vessels through neuropeptides released from trigeminal afferents and mediators, such as histamine released from degranulated mast cells (62,63). An increase in CGRP-releasing and nNOS-producing trigeminal neurons could in principle facilitate neurogenic vasodilatation in the meninges, provided that these peptides are carried by axonal transport to the peripheral terminals, where CGRP and NO can be released (64). However, this process probably does not occur rapidly enough to explain the induction of migraine attacks within few hours after NO donor infusion (1).

Another question is if the infused NO donors have similar effects in the cranial dura mater as observed in the trigeminal ganglion, that is, if they induce an increase in NOS producing cells and changes in CGRP-releasing nerve fibres. NO donors, though at much higher doses than used in the present study, released CGRP from the rat cranial dura, promoting increases in meningeal blood flow (20). In a rat model of intravital microscopy, neurogenic vasodilatation of meningeal blood vessels was attenuated by inhibition of nNOS, while CGRP-induced vasodilatation was attenuated by eNOS inhibition (65). Specific inhibition of iNOS was not effective in this model. Meningeal vasodilatation caused by CGRP, however, does not excite trigeminal afferents innervating the rat cranial dura mater (66). Therefore, it is unlikely that vascular effects in the cranial dura induced by NO or CGRP cause nociceptive inputs to the trigeminal ganglion, which can be responsible for the increase in CGRP- and nNOS-ir neurons. We assume, rather, that there is a direct impact of the NO donor on trigeminal ganglion cells, which is also evidenced by the expression experiments performed on cultured trigeminal ganglion neurons (21). These data, if transferred to humans, are supportive for a neuronal rather than a vascular hypothesis of migraine generation.

Clinical trials have shown that unspecific inhibition of NO synthases can relieve migraine pain (67) and decrease tension-type headache (68). With regard to the above preclinical findings and the present study, it seems likely that it is the inhibition of nNOS and eNOS, rather than iNOS, which causes this therapeutic effect. Specific iNOS inhibitors such as GW274150, which has been shown to be analgesic in rat models of inflammatory and neuropathic pain (69), may thus be less suited to treat migraine pain. Using immunohistochemistry, we have seen co-localisation of the CGRP-receptor proteins CLR and RAMP1 indicating functional CGRP receptors in the rat trigeminal ganglion on a large subgroup of neurons (24), and another subgroup of neurons showed immunoreactivity for soluble guanylate cyclase (sGC), the intracellular receptor for

NO (unpublished results). An intraganglionic, possibly reciprocal, signalling between nNOS and CGRP-expressing neurons could therefore play a role in trigeminal nociception. The NO production within the ganglion may build up and increase the activity of spinal trigeminal neurons (4), a process likely to cause headaches in patients suffering from primary headaches (70).

Acknowledgements

We acknowledge Karin Löschner, Birgit Vogler, Jana Schramm and Maria Schulte for their excellent technical assistance. The present study was supported by the BMBF (German Headache Consortium).

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