

# Calcitonin gene-related peptide (CGRP) modulates nociceptive trigeminovascular transmission in the cat

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**1** Calcitonin gene-related peptide (CGRP) is released into the cranial circulation of humans during acute migraine. To determine whether CGRP is involved in neurotransmission in craniovascular nociceptive pathways, we microiontophoresed onto neurons in the trigeminocervical complex and intravenously administered the CGRP receptor antagonists  $\alpha$ -CGRP-(8–37) and BIBN4096BS.

**2** Cats were anaesthetised with  $\alpha$ -chloralose, and using halothane during surgical preparation. A craniotomy and C<sub>1</sub>/C<sub>2</sub> laminectomy allowed access to the superior sagittal sinus (SSS) and recording site. Recordings of activity in the trigeminocervical complex evoked by electrical stimulation of the SSS were made. Multibarrelled micropipettes incorporating a recording electrode were used for microiontophoresis of test substances.

**3** Cells recorded received wide dynamic range (WDR) or nociceptive specific (NS) input from cutaneous receptive fields on the face or forepaws. Cell firing was increased to 25–30 Hz by microiontophoresis of L-glutamate ( $n = 43$  cells).

**4** Microiontophoresis of  $\alpha$ -CGRP excited seven of 17 tested neurons.

**5** BIBN4096BS inhibited the majority of units (26 of 38 cells) activated by L-glutamate, demonstrating a non-presynaptic site of action for CGRP.  $\alpha$ -CGRP-(8–37) inhibited a similar proportion of units (five of nine cells).

**6** Intravenous BIBN4096BS resulted in a dose-dependent inhibition of trigeminocervical SSS-evoked activity (ED<sub>50</sub> 31  $\mu\text{g kg}^{-1}$ ). The maximal effect observed within 30 min of administration.

**7** The data suggest that there are non-presynaptic CGRP receptors in the trigeminocervical complex that can be inhibited by CGRP receptor blockade and that a CGRP receptor antagonist would be effective in the acute treatment of migraine and cluster headache.

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**Keywords:** Headache; migraine; cluster headache; CGRP antagonist; trigeminocervical complex

**Abbreviations:** AMPA,  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid; ANOVA, analysis of variance; BIBN4096BS, 1-piperidinecarboxamide, *N*-[2-[[5-amino-1-[[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl)]; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; 5-HT, serotonin; i.v., intravenous; LTM, low threshold mechanoreceptor; NMDA, *N*-methyl-D-aspartate; NS, nociceptive specific; RAMP, receptor activity-modifying protein; SSS, superior sagittal sinus; WDR, wide dynamic range

## Introduction

The neurobiology of migraine essentially involves three components (Goadsby *et al.*, 2002): first, the inherited migrainous diathesis, currently best characterized in familial hemiplegic migraine by mis-sense mutations in channel genes (Ophoff *et al.*, 1996; De Fusco *et al.*, 2003); secondly, migraine involves activation of brainstem regions (Weiller *et al.*, 1995; Bahra *et al.*, 2001; Matharu *et al.*, 2003) that uniquely mark the condition when compared to other forms of primary headache, such as cluster headache (May *et al.*, 1998); thirdly, the pain component of migraine seems to involve activation (Goadsby *et al.*, 1990), or at least the perception of activation, of the trigeminal innervation of pain-producing intracranial components (Wolff, 1948). The trigeminal innervation of the cranial circulation contains a number of neuropeptides, including calcitonin gene-related peptide (CGRP) (Edvinsson *et al.*, 1987). During acute

migraine and cluster headache, CGRP levels are elevated in both adults and adolescents (Edvinsson & Goadsby, 1998), so that the effect of CGRP antagonists on models of trigeminovascular nociception may aid in understanding the potential role of such compounds in these disorders.

Stimulation of the trigeminal ganglion in cat and humans results in elevations in CGRP and substance P levels in the cranial circulation (Goadsby *et al.*, 1988). However, during acute attacks of migraine (Goadsby *et al.*, 1990; Gallai *et al.*, 1995) and cluster headache (Goadsby & Edvinsson, 1994a; Fanciullacci *et al.*, 1995) CGRP is elevated but substance P is not. Triptans, serotonin (5-HT<sub>1B/1D</sub>) receptor agonists (Goadsby, 2000), which are effective in the treatment of acute migraine (Ferrari *et al.*, 2001) and cluster headache (Ekbom & The Sumatriptan Cluster Headache Study Group, 1991), inhibit release of CGRP into the cranial circulation of experimental animals when it is evoked by trigeminal ganglion activation (Goadsby & Edvinsson, 1993; 1994b). Moreover, successful treatment of acute migraine (Goadsby & Edvinsson,

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1993) or cluster headache (Goadsby & Edvinsson, 1994a; Fanciullacci *et al.*, 1995) with sumatriptan normalises cranial CGRP levels.

Stimulation of the superior sagittal sinus (SSS) in humans produces pain that is substantially referred to the first (ophthalmic) division of the trigeminal nerve (Feindel *et al.*, 1960). During stimulation of the superior sagittal sinus (SSS), neurons can be studied using population-based anatomical techniques, such as measurement of Fos with immunohistochemistry (Kaube *et al.*, 1993b), or metabolic activity with 2-deoxyglucose (Goadsby & Zagami, 1991), or single neurons can be more closely tracked using electrophysiological techniques (Hoskin *et al.*, 1996; Cumberbatch *et al.*, 1997). Electrophysiological methods incorporating microiontophoresis facilitate characterization of the pharmacology of neurons of interest by repeated local application of appropriate agonist and antagonist combinations (Bloom, 1974). It has been shown that neurons in the trigeminocervical complex of the cat or rat are inhibited by administration of triptans intravenously or by microiontophoresis, or both (Goadsby, 2000).

In this study, we determined whether the peripheral release of CGRP in the cranial circulation during acute migraine was mirrored in the trigeminocervical complex. The development of a potent specific CGRP receptor antagonist in the form of BIBN4096BS facilitates addressing this issue (Doods *et al.*, 2000). By combining intravenous and microiontophoretic application of CGRP receptor antagonists, we determine here that there is a non-presynaptic CGRP receptor in the trigeminal nucleus that is a potential therapeutic target in migraine and cluster headache.

## Methods

All studies reported were conducted and terminated under general anaesthesia in accordance with a project license issued by the Home Office of the United Kingdom under the Animals (Scientific Procedures) Act, 1986. Cats weighing  $3.62 \pm 0.30$  kg (mean  $\pm$  s.d.) were anaesthetised with  $\alpha$ -chloralose ( $60 \text{ mg kg}^{-1}$  i.p.; Sigma, St Louis, MO, U.S.A.) and prepared for physiological monitoring. Halothane (Rhone Merieux, Essex, U.K.) (0.5–3% in a 40% oxygen:air mixture) was administered from an anaesthetic machine during surgical procedures and then discontinued during experimental protocols. For each cat, a catheter was placed in the femoral artery for arterial blood sampling and continuous measurement of blood pressure (DTXplus transducer, Ohmeda, Madison, WI, U.S.A.; PM-1000 amplifier, CWE Instruments, Ardmore, PA, U.S.A.). A second catheter placed in the femoral vein allowed for fluid and drug administration. Supplementary doses of  $\alpha$ -chloralose in 2-hydroxypropyl- $\beta$ -cyclodextrin (RBI, Natick, MA, U.S.A.) were given i.v. at a rate of  $5\text{--}10 \text{ mg kg}^{-1} \text{ h}^{-1}$  (Storer *et al.*, 1997). The cats were intubated after local anaesthesia with lidocaine hydrochloride (Intubeaze, Arnolds, Shrewsbury, U.K.) and fixed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, U.S.A.).

A Jackson/Foley urethral catheter was inserted to drain the bladder, providing more even temperature regulation, more stable control of blood pressure through control of bladder distension, and monitoring of urine output. Core temperature was monitored and maintained between

37 and 39°C using a rectal thermistor probe and a low-electromagnetic-noise-emitting homeothermic heater blanket system (Harvard Apparatus, Holliston, MA, U.S.A.). The cat was ventilated with a 40% oxygen:air mixture (Harvard Apparatus), end-tidal CO<sub>2</sub> was maintained between 2 and 4%, and expired oxygen continuously monitored (Datex-Ohmeda, Helsinki, Finland). Heart rate was monitored by electrocardiogram (CT-1000; CWE Instruments). The depth of anaesthesia was monitored periodically throughout the experiment by testing for sympathetic (pupillary and cardiovascular) responses to noxious stimulation and withdrawal reflexes in the absence of neuromuscular blockade.

## Surgery

A midline craniotomy (20-mm diameter) and C<sub>1</sub>–C<sub>2</sub> laminectomy were performed, allowing access to the superior sagittal sinus and the recording site in the spinal cord. The sinus was isolated by dissecting the dura and falx cerebri adjacent to the sinus over approximately 15 mm. A small polyethylene sheet was inserted under the isolated sinus and laid over the outlying dura mater, then tucked under the edges of the craniotomy to hold it in position. To prevent dehydration and to provide electrical insulation to the cortex, a cylindrical polypropylene dam was sealed to the bone around the craniotomy with dental acrylic (Vertex, Zeist, Netherlands) and filled with liquid paraffin (BDH Laboratory Supplies, Poole, England). Possible artefacts from arterial pulsation and respiratory movement were reduced by: bilateral pneumothoraces, held patent with polypropylene tubes; immobilization of the spine by clamping a thoracic spinous process to the stereotaxic frame; clamping the C<sub>1</sub> transverse processes to auxiliary ear bar holders on the frame, and clamping the remaining caudal portion of the dorsal C<sub>2</sub> spinous process to the frame.

## Stimulation and recording

The isolated SSS was gently lifted onto a pair of bipolar platinum hook electrodes connected to a stimulus isolation unit (SIU5A; Grass Instruments, West Warwick, RI, U.S.A.). To activate primary trigeminal afferents, the SSS was supramaximally stimulated with stimulus-isolated (Grass SIU) square wave pulses from a Grass S88 stimulator ( $250 \mu\text{s}$ , 110–150 V, 0.3–0.5 Hz) after neuromuscular blockade with gallamine triethiodide (Concord, Essex, U.K.), initially  $10\text{--}15 \text{ mg kg}^{-1}$  i.v. and maintained at  $5\text{--}10 \text{ mg kg}^{-1} \text{ h}^{-1}$ . Extracellular recordings were made using microiontophoretic combination electrodes consisting of seven- or nine-barrelled glass pipettes, incorporating a central tungsten recording electrode with an exposed recording tip length of approximately  $12 \mu\text{m}$  (Hellier *et al.*, 1990). Recording electrode impedances were typically  $400 \text{ k}\Omega$  when measured at 1 kHz in 0.9% saline. The dura mater above the recording regions on the surface of the spinal cord was reflected and held to the edges of the laminectomy with *N*-butyl-cyanoacrylate. After local removal of the pia mater, the electrode was lowered into the cord substance caudal to the C<sub>2</sub> roots in the area of the dorsal root entry zone. The electrode was advanced or retracted in  $5 \mu\text{m}$  steps using a microelectrode positioner consisting of a piezoelectric motor (IW-711, Burleigh Instruments, Harpenden, U.K.) and ultra-low-noise controller (6000ULN) attached to a micromanipulator.

lator (Kopf 1760-61). Tissue culture grade agar (3% ( $wv^{-1}$ ) in saline; Sigma, St Louis, MO, U.S.A.) was set over the exposed cord after electrode insertion to further reduce cardiovascularly related movements. Signal from the recording electrode attached to a high-impedance headstage preamplifier (NL100AK; Neurolog, Digitimer, Herts, U.K.) was fed *via* an AC preamplifier (Neurolog NL104, gain  $\times 1000$ ) through filters (Neurolog NL125; bandwidth approximately 300 Hz to 20 kHz) and a 50 Hz noise eliminator (Humbug, Quest Scientific, North Vancouver, BC, Canada) to a second-stage amplifier (Neurolog NL106) providing variable gain ( $\times 20 - \times 90$ ). This signal (total gain approximately  $\times 20,000 - \times 90,000$ ) was fed to a gated amplitude discriminator (Neurolog NL201) and analogue-to-digital converter (Labmaster DMA, Scientific Solutions, Mentor, OH, U.S.A.) in a personal computer, where the signal was processed and stored. Filtered and amplified action potentials were fed to loudspeaker *via* a power amplifier (Neurolog NL120) for audio monitoring and displayed on an oscilloscope to assist the isolation of single-unit activity from adjacent cell activity and noise.

In order to record the response of single units to stimulation, post-stimulus histograms were constructed on-line and saved to disk. Free-running neuronal activity, such as stimulated by local L-glutamate microiontophoresis, was analysed as cumulative rate histograms, where activity gated through the amplitude discriminator was collected into successive 1-s bins. Averaged action potentials were constructed using an averaging routine and an analogue signal delay unit (NL202), to assist the discrimination between somatic and axonal recordings, setting the NL125 filter bandwidth from d.c. to approximately 30 kHz. During experiments electrophysiological data, blood pressure, heart rate and end-tidal  $CO_2$  were processed and recorded on a video home system (VHS) magnetic tape (Pulse Code Modulator; Vetter, Rebersburgh, PA, U.S.A.) for documentation and latter review.

The position of the recording electrodes was controlled by use of a stereotaxic micropositioner (Kopf 1760-61) with reference to the mid-point of the  $C_2$  dorsal roots. Together with the depth of the recording electrode tip with respect to the surface of the spinal cord at the dorsal root entry zone, as determined by the distance travelled display on the ULN6000 piezoelectric motor controller (Burleigh Instruments), this provided the coordinates of the recording sites. The location of selected recording sites were marked with Pontamine sky blue dye ('Gurr' 6BX dye (C.I.24410), BDH Laboratory Supplies, Poole, U.K.; 2.5% in 100 mM sodium acetate) using a  $-2.00 \mu A$  current for 5-10 min. Animals were euthanased with sodium pentobarbital (400 mg), followed by KCl (10%  $wv^{-1}$ ; 5 ml). After termination of experiments, the sections of spinal cord containing the recording sites were resected, fixed with neutral buffered 10% formalin, and sectioned (40  $\mu m$ ). Pontamine sky blue marks were counterstained with neutral red, a Nissl procedure that allowed identification of the laminae of the grey matter. The position of the recording sites within the cord were determined from histologically identified dye marks, and unmarked recording sites were located by reference to other dye marks, for example marked at the end of recording tracks, and the stereotaxic coordinates of the recording electrode tip.

### *Receptive fields*

Cells responding to superior sagittal sinus (SSS) stimulation were characterised as receiving low threshold mechanoreceptor (LTM) input if they responded to non-noxious input such as brush or stroke on cutaneous receptive fields on the face or forepaws. They were characterized as nociceptive specific (NS) if they responded to noxious mechanical stimuli, such as pinch or pricking with a needle, or wide dynamic range (WDR) if they responded to both. These cells usually had increased firing in response to noxious stimuli (Hu *et al.*, 1981).

### *Test compounds*

Micropipette barrels used for microiontophoresis were filled with 1.0 M L-glutamate, monosodium, pH 8.0 (Sigma); saline (for controls); 20 mM BIBN4096BS in helium sparged water for injection and titrated with an equimolar amount of 0.1 M HCl, pH 5.4-5.8; 1.0 mM  $\alpha$ -CGRP in helium sparged saline (150 mM NaCl), pH 4.0-6.5; and 1.0 mM  $\alpha$ -CGRP-(8-37) in helium sparged 160 mM NaCl, pH 4.0-6.5; 2.5% Pontamine sky blue in 100 mM sodium acetate. L-Glutamate and Pontamine sky blue were ionized as anions and retained with small positive currents ( $\sim 3-5$  nA). BIBN4096BS (Boehringer Ingelheim GmbH, Biberach, Germany), human  $\alpha$ -CGRP (Sigma) and human  $\alpha$ -CGRP-(8-37) (Sigma) were ionized as cations and were retained in the pipette barrels with small negative retaining currents ( $\sim -3$  to  $-5$  nA). Ejection currents (2-200 nA) in directions opposite to the retaining currents were used. Chloride or sodium ions ejected from the barrel containing saline were used as controls. Current balancing was provided through a barrel containing 1 M NaCl. For intravenous injections BIBN4096BS was made up to 17.4 mg  $ml^{-1}$  and diluted in saline for injection (BP).

### *Microiontophoresis*

After filling, the iontophoretic electrode micropipette barrels had resistances 60-150 M $\Omega$ . A microiontophoresis current generator (Dagan 6400, Dagan Corporation, Minneapolis, MN, U.S.A.) provided the current for ejecting test substances from the barrels. Retaining and balancing currents were used routinely (Bloom, 1974). The L-Glu ejection current was adjusted so cells had free running activity at a rate of around 20 Hz, such that inhibition of the cell activity could be distinguished from random firing. Where L-Glu was applied in pulses the evoked firing rate was often higher. When examining its effect on SSS-evoked firing control, post-stimulus histograms were constructed from the response to 50 supramaximal electrical stimuli to the SSS. BIBN4096BS was applied microiontophoretically for 300 s and the response of units to 50 supramaximal electrical stimuli to the SSS immediately recorded.

### *Intravenous BIBN4096BS administration*

BIBN4096BS was administered intravenously through a catheterized femoral vein in saline as a vehicle after recording control post-stimulus histograms during 50 supramaximal electrical stimuli to the SSS. Post-stimulus histograms were again recorded at 5 min intervals for up to 45 min after BIBN4096BS administration.

### Statistical analysis

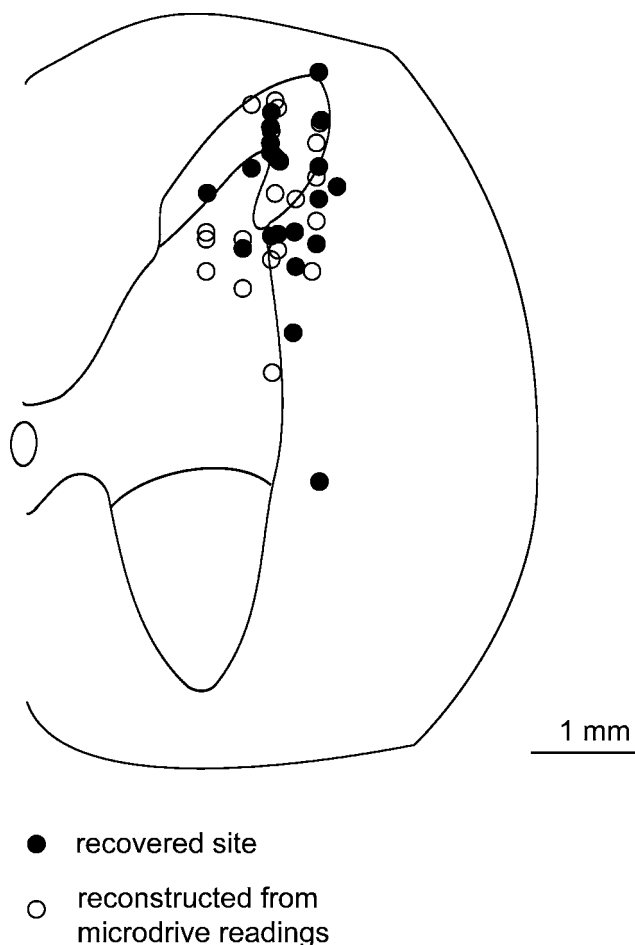
Neuronal firing was distinguished from noise using an amplitude discriminator. Statistical evaluations were made using the average rate of firing in Hz evoked during each epoch of microiontophoretic application of L-glutamate. The background neuronal discharge was calculated by averaging the period of ongoing activity immediately preceding each epoch of excitation and subtracting this value from the evoked responses. For each unit a minimum of five-paired baseline-response data were collected. For the control responses to L-glutamate, an analysis of variance (ANOVA) with repeated measures was carried out. When this was not significant, the reliability of the measurements was tested using Cronbach's alpha. The baseline data were then pooled and used to further analyse the responses to drugs using either again pooling if the drug effect was reliable (Cronbach's alpha) and doing a *t*-test for independent samples, or an ANOVA with repeated-measures design, if not. Mauchly's test of sphericity was evaluated and when appropriate the Greenhouse–Geisser correction applied. The *P*-values were assessed at the 0.05 level and the Bonferroni test applied to comparisons between drugs (SPSS v.10, Chicago, IL, U.S.A.). Where we considered individual cells, we applied the *critical ratio* test (Nagler *et al.*, 1973; Mandelbrod *et al.*, 1974; 1983) that is based on the count data having a Poisson distribution, and employs the standard normal deviate (Armitage & Berry, 1994) to determine at the 5% level whether two firing rates differ. In practice, this very closely approximates a 30% change from baseline, which we considered significant change. We classified change between 10 and 30% as unclear. Summary data are presented as the mean  $\pm$  standard error of the mean.

## Results

Animals ( $n = 23$ ), whose data contributed to these results had cardio-respiratory parameters that were normal for the anaesthetised cat. Blood gas parameters were measured at intervals throughout the experiment and were within normal limits: arterial blood pH  $7.39 \pm 0.04$  and  $p\text{CO}_2$   $3.39 \pm 0.41$  kPa.

### Localisation and neuronal characteristics

Extracellular recordings were made and data collected from 43 neurons in the trigeminocervical complex (Kaube *et al.*, 1993b). Cells were located +4 mm rostral to –4 mm caudal to the midpoint of the C<sub>2</sub> rootlets, 0–150  $\mu\text{m}$  lateral to the dorsal root entry zone at a depth of approximately –700  $\mu\text{m}$  to around –2000  $\mu\text{m}$  below the (dorsal) cord surface (Figure 1). Cells responded to electrical sagittal sinus stimulation with latencies consistent with A- $\delta$  fibres (typically 8–10 ms). Recordings were made from cell bodies and were characterized by their unfiltered biphasic action potential morphology (Fussey *et al.*, 1970) and the reversible excitatory effect of L-glutamate on cell firing. Cells received wide dynamic range or nociceptive specific mechanoreceptor input from cutaneous receptive fields on the face and forepaws. The receptive fields were all ipsilateral and at least involved the ophthalmic (first) division of the trigeminal nerve.



**Figure 1** Localisation of recording sites: A transverse section through the spinal cord at the level of C<sub>2</sub> is represented by this schematic diagram. Microiontophoretically ejected Pontamine sky blue dye (6BX (C.I.24410)) was used to mark recording sites. Solid circles indicate sites where recording sites were identified histologically. The positions of unmarked recording sites, or sites where marks could not be recovered, were identified by reference to the position of dye marks at other recording sites and, or at the end of electrode tracts, and electrode tip coordinates, and are indicated by open circles. Although the recorded units are only mapped to one side of the cord in the schematic, they represent the data obtained from both the left-hand side and right-hand side of the spinal cord. The scale bar represents a distance of 1 mm in both directions.

### Control ejections

Cell firing in response to control ejection of sodium ions from a barrel containing saline at similar currents (–30 to –80 nA;  $n = 13$ ) to those used for ejection of active compounds produced similar negligible effects across five applications of L-glutamate ( $F_{2,4,28} = 0.57$ ,  $P = 0.69$ ).

### Baseline L-glutamate firing

Neurons identified as linked to stimulation of the superior sagittal sinus were tested for the stability of their baseline response to L-glutamate application. All SSS-linked neurons responded to L-glutamate. The firing rate for trigeminal nucleus caudalis neurons was  $30.5 \pm 3.0$ ,  $30.0 \pm 3.0$ ,  $26.9 \pm 2.6$ ,  $24.7 \pm 2.2$ , and  $25.1 \pm 2.2$  Hz for each of the five consecutive

applications of L-glutamate ( $-30$  to  $-80$  nA;  $n=22$ ). There was no difference across these responses ( $F_{4,84}=2.03$ ,  $P=0.098$ ). The reliability of these responses across time was excellent, with an alpha of 0.98. We thus pooled these data for further comparison with other drug treatments. Higher rates of firing could be achieved by greater ejection currents up to  $-120$  nA.

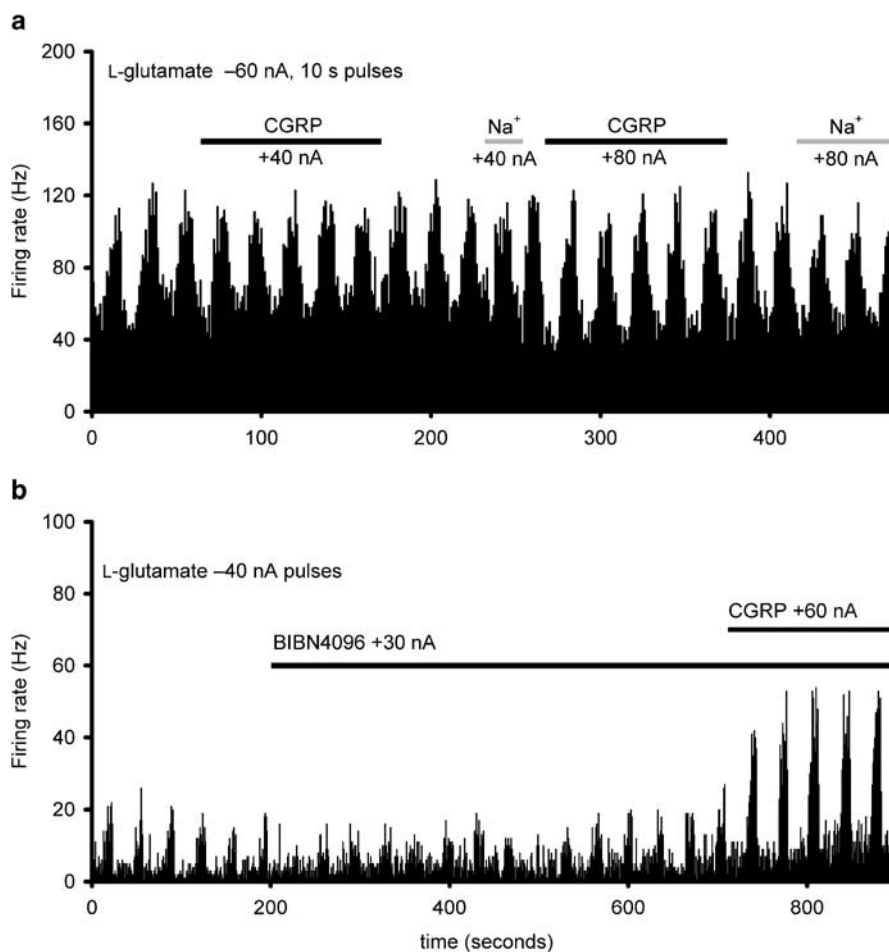
### Effect of $\alpha$ -CGRP

The firing rate for trigeminal nucleus caudalis neurons during ejection of  $\alpha$ -CGRP was  $16.2\pm 8.5$ ,  $15.5\pm 7.0$ ,  $19.1\pm 7.6$ ,  $15.8\pm 7.2$ , and  $17.9\pm 7.8$  Hz for each of the five consecutive applications of  $\alpha$ -CGRP (60 nA;  $n=6$ ). There was no difference across these responses ( $F_{4,16}=2.2$ ,  $P=0.12$ ). The reliability of these responses across time was excellent, with an alpha of 0.99. When  $\alpha$ -CGRP was iontophoresed in the presence of L-glutamate ( $-30$  to  $-80$  nA), there was no significant added effect ( $n=17$ ;  $t_{16}=0.55$ ; Figure 2), for the group as a whole. Seven of the 17 neurons tested increased their firing rate by more than 30%.

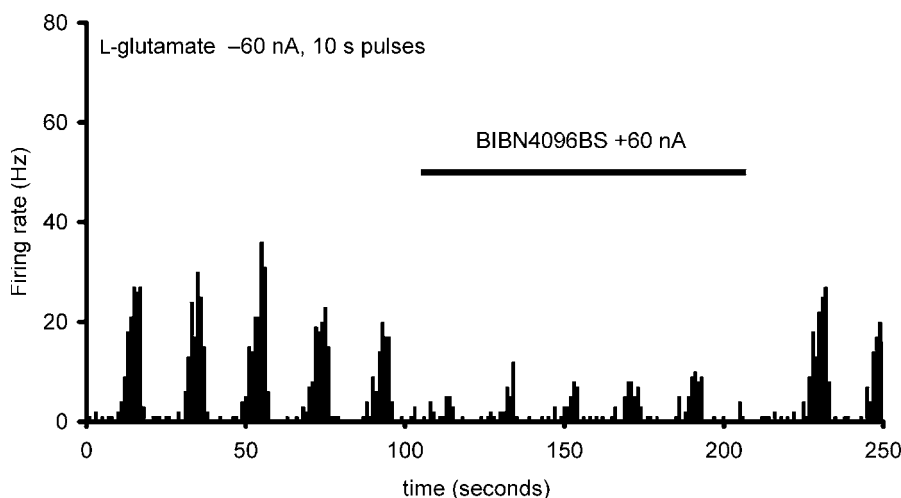
### Effect of BIBN4096BS

**Microiontophoresis** The firing rate for trigeminal nucleus caudalis neurons during ejection of L-glutamate across five consecutive applications was  $8.4\pm 1.5$ ,  $7.4\pm 1.9$ ,  $7.4\pm 1.8$ ,  $8.3\pm 1.8$ , and  $7.9\pm 1.7$  Hz during BIBN4096BS administration (60 nA;  $n=16$ ). There was no difference across these responses ( $F_{2,0,29,6}=0.51$ ,  $P=0.61$ ). The reliability of these responses across time was excellent, with an alpha of 0.95. When compared with the effect of L-glutamate alone, there was a significant inhibition of trigeminal neuronal firing ( $t_{37}=2.3$ ,  $P< 0.03$ ; Figure 3). The effect of BIBN4096BS on evoked trigeminal neuronal activity was dose-dependent across the range of currents from 10 to 120 nA. BIBN4096BS inhibited trigemino-cervical neuronal activity evoked by stimulation of the superior sagittal sinus after 300 s application (Figure 4). BIBN4096BS also inhibited the spontaneous firing of neurons linked to SSS stimulation (Figure 5).

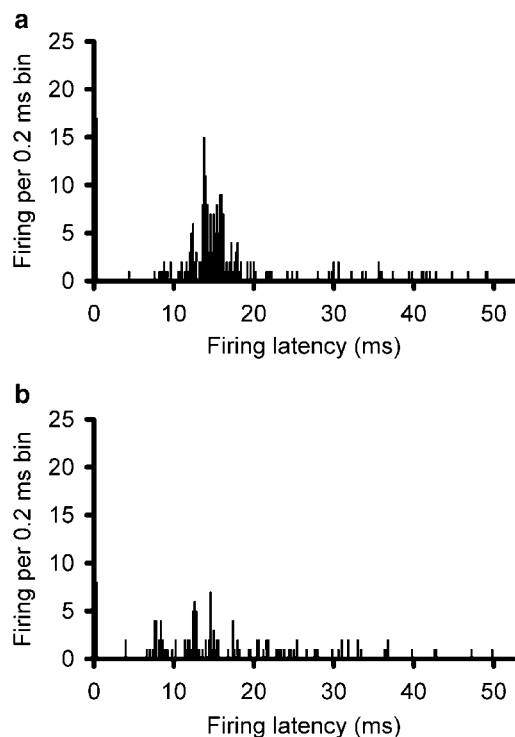
**Intravenous administration** Intravenous administration of BIBN4096BS at cumulative doses of 1, 3, 10, 30,



**Figure 2** Effect of  $\alpha$ -CGRP and L-glutamate: L-glutamate (1.0 M, pH 8.0) was applied microiontophoretically ( $-60$  nA) and increased the firing of trigemino-cervical neurons linked to stimulation of the superior sagittal sinus. When the firing had reached a steady state over five epochs,  $\alpha$ -CGRP (1 mM in 150 mM NaCl, pH 4.5) was co-microiontophoresed (solid line) with no added effect from sodium ( $+40$  and  $+80$  nA) in 10/17 units (panel a). In 7/17 units, trigeminal activity was increased by CGRP, albeit in the presence of BIBN4096BS (panel b). There was no significant effect of microiontophoresis of sodium. The histogram indicates the rate of firing seen in one second bins.



**Figure 3** *Effect of BIBN4096BS*: Microiontophoresis of BIBN4096BS (20 mM, pH 5.7; +60 nA, solid bar) produces a robust reduction in L-glutamate-evoked (1.0 M, pH 8.0; 60 nA) firing in the trigeminocervical complex. The histogram indicates the rate of firing seen in one second bins.



**Figure 4** *Effect of BIBN4096BS microiontophoresis on SSS-evoked firing*: Post-stimulus histograms showing that supramaximal electrical stimulation ( $50 \times 250 \mu\text{s}$ ) of the SSS *via* bipolar platinum hook electrodes recruits units in the trigeminocervical complex responding to the stimulus with a latency peak of 12 ms (panel a) that is inhibited immediately after microiontophoresis of BIBN4096BS (20 mM, pH 5.8; +60 nA for 300 s) (panel b). The apparent response within the first 0.2 ms is part of the stimulus artefact.

$100 \mu\text{g kg}^{-1}$  resulted in a dose-dependent inhibition of superior sagittal sinus evoked trigeminocervical nucleus activity ( $n=4$ ; Figure 6). Maximal effects were seen within 30 min of drug administration with a calculated  $\text{ED}_{50}$  of  $31 \mu\text{g kg}^{-1}$  (Goadsby & Lambert, 1986).

#### *Neuronal characteristics and BIBN4096BS*

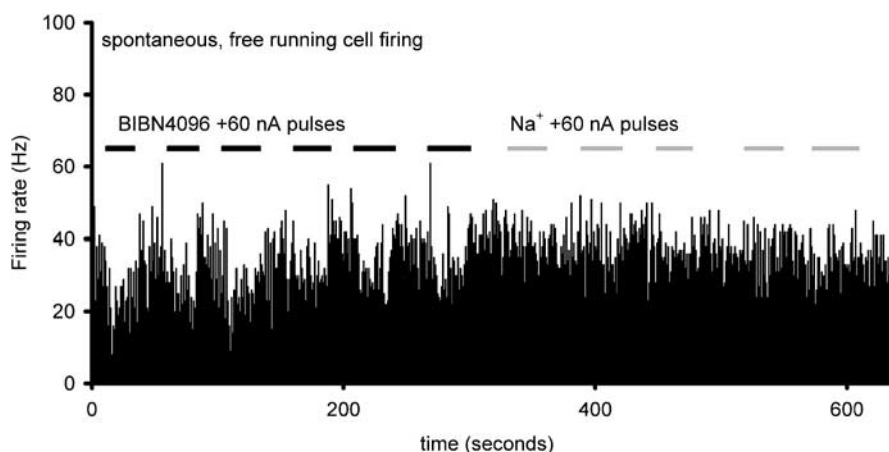
Four cells were characterized as nociceptive specific and had receptive fields on ipsilateral forepaws, face, or bridge of the nose. One was inhibited by microiontophoretically applied BIBN4096BS, there was no effect on one cell and the effect on two cells was not clear. In all, 13 cells were characterized as wide dynamic range, with receptive fields on ipsilateral forearms, forepaws, or face. Eight cells were inhibited by BIBN4096BS, and the effect of BIBN4096BS was unclear in three, two cells were not tested with BIBN4096BS. Three cells were classified as LTM and had receptive fields on the ipsilateral face, two of these were inhibited by BIBN4096BS and the effect on the other was not clear. Cutaneous receptive fields were not found for seven tested cells, five were inhibited by BIBN4096BS and two were not tested. In all, 18 cells were not characterized, eight of these were inhibited by BIBN4096BS, four were not inhibited by BIBN4096BS and the effect of BIBN4096BS on four cells was not clear.

#### *Effect of $\alpha\text{-CGRP-(8-37)}$*

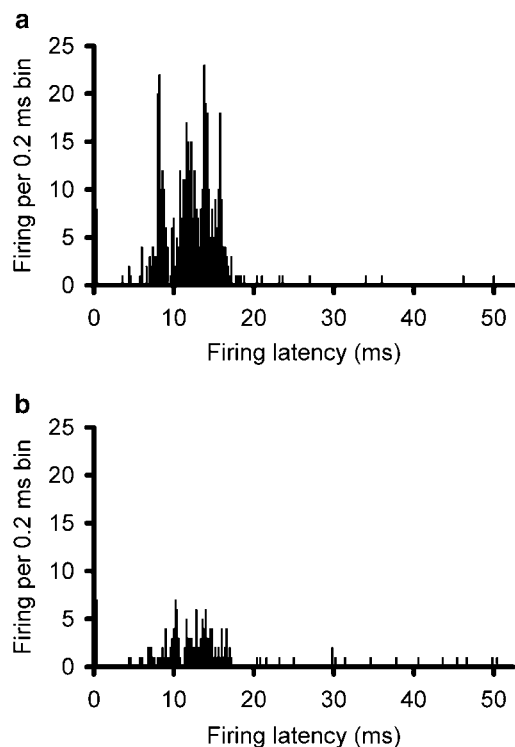
The firing rate for trigeminal nucleus caudalis neurons during ejection of L-glutamate was  $9.1 \pm 1.8$ ,  $9.6 \pm 2.1$ ,  $9.3 \pm 1.8$ ,  $9.5 \pm 1.6$ , and  $9.6 \pm 1.9$  Hz for each of the five consecutive applications of  $\alpha\text{-CGRP-(8-37)}$  (50 nA;  $n=6$ ). There was no difference across these responses ( $F_{4,20}=0.24$ ,  $P=0.91$ ). The reliability of these responses across time was excellent with an alpha of 0.99. When compared with the effect of L-glutamate alone, there was a significant inhibition of trigeminal neuronal firing ( $t_{17}=2.5$ ,  $P=0.03$ ). The effect of  $\alpha\text{-CGRP-(8-37)}$  on evoked trigeminal neuronal activity was dose-dependent across a range of currents from 40 to 120 nA, but apparently not as potent as that of BIBN4096BS under these experimental conditions (Figure 7).

## Discussion

These studies demonstrate that the CGRP receptor antagonists BIBN4096BS and CGRP-(8-37), can inhibit neurons in the



**Figure 5** Effect of BIBN4096BS on spontaneous trigeminal neurons: Neurons firing spontaneously at a rate indicated in the histogram as firing per 1 s bin have a reduced firing frequency when BIBN4096BS is microiontophoretically ejected (20 mM, pH 5.7; +60 nA; black bars), while sodium (150 mM, pH 7.0) has no apparent effect at similar currents (grey bars).



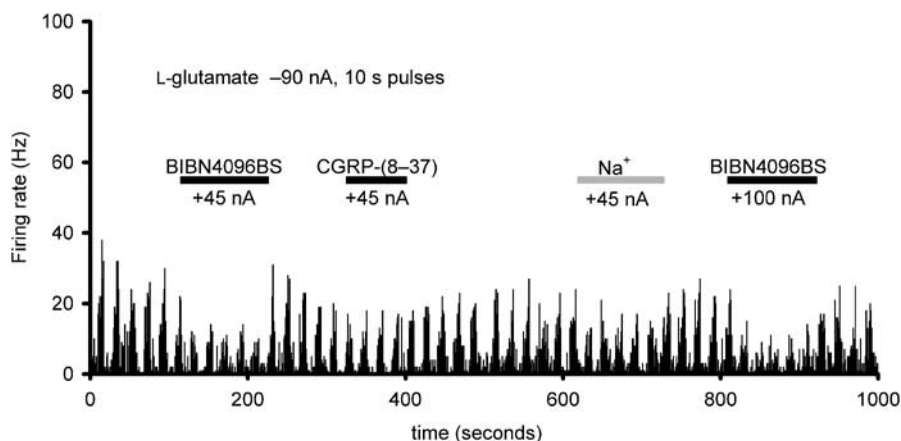
**Figure 6** Effect of intravenously administered BIBN4096BS on SSS-evoked firing: Post-stimulus histograms showing that supra-maximal electrical stimulation ( $50 \times 250 \mu\text{s}$ ) of the SSS via bipolar platinum hook electrodes recruits units in the trigeminocervical complex (panel a) that are substantially inhibited within 30 min of intravenous administration of BIBN4096BS ( $30 \mu\text{g kg}^{-1}$ ; panel b). The apparent response within the first 0.2 ms is part of the stimulus artefact.

trigemincervical complex that are linked to stimulation of the superior sagittal sinus. That microiontophoresis of BIBN4096BS and CGRP-(8–37) is effective demonstrates that one group of trigeminovascular CGRP receptors involved must be local to the trigeminocervical complex. Furthermore, since local application of the CGRP antagonists blocks the activation seen with iontophoretic application of L-glutamate, this CGRP receptor must be non-presynaptic, probably post-

synaptic, in location (Goadsby *et al.*, 2001). Moreover, at least in this model, BIBN4096BS was capable of reducing resting spontaneous firing, suggesting some ongoing trigeminovascular activity independent of SSS stimulation, perhaps due to surgical trauma (Hoskin & Goadsby, 1999). The data are consistent with the recent preliminary report that BIBN4096BS is an effective treatment of acute migraine (Olesen *et al.*, 2003).

Why study trigeminocervical neurons? The trigeminocervical complex is pivotal in the transmission of nociceptive input from pain-producing intracranial structures (De Vries *et al.*, 1999). Stimulation of both intracranial structures, such as dural afferents by chemical irritation (Cutrer *et al.*, 1995), superior sagittal sinus (Kaube *et al.*, 1993b; Goadsby & Hoskin, 1997) or middle meningeal artery (Hoskin *et al.*, 1999) by electrical stimulation, or cutaneous structures, such as cornea (Panneton & Burton, 1981), result in activation of neurons in the trigeminal nucleus caudalis and for intracranial structures its extension into the dorsal horn of C<sub>1</sub> and C<sub>2</sub>. It is possible to study trigeminocervical complex neurons using electrophysiological methods, and these experiments produce comparable pharmacological results to population-based methods, such as Fos immunohistochemistry (Goadsby & Hoskin, 1996; Hoskin *et al.*, 1996; Hoskin & Goadsby, 1998). We have chosen electrophysiological methods here so that we could determine in the same animal, and recording from the same unit in the trigeminocervical complex, whether CGRP-mediated transmission made a significant contribution at this pivotal synapse. This method has the advantage of temporal resolution, although it cannot provide an assessment of the population implications of blocking CGRP-mediated transmission, that question would require a method such as Fos immunohistochemistry.

In this study, we employed both peripheral activation with superior sagittal sinus stimulation and direct activation of trigeminal neurons using L-glutamate. Glutamate is an important transmitter in the trigeminal nucleus. *N*-Methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate and metabotropic glutamate receptors have been identified in the superficial laminae of the trigeminal nucleus caudalis of the rat (Tallaksen-Greene *et al.*,



**Figure 7** Comparison of the effect of BIBN4096BS (20 mM, pH 5.8; +45 and +100 nA) and  $\alpha$ -CGRP-(8–37) (1 mM in 160 mM NaCl, pH 4.5; +45 nA) in inhibiting the firing evoked by 10 s pulses of microiontophoretically applied L-glutamate (1.0 M, pH 8.0; –90 nA) onto a trigemino-cervical complex unit linked to stimulation of the SSS, where sodium (150 mM; pH 7.0) has no apparent effect (+45 nA, grey bar). The firing rate indicated on the ordinate is indicated for 1 s bins by the rate histogram.

1992). It has been shown that microiontophoretically applied L-glutamate excites neurons in the trigeminal nucleus caudalis (Hill & Salt, 1982; Salt & Hill, 1982). It can be shown that blockade of NMDA and AMPA receptors attenuates trigemino-vascular nociceptive transmission (Mitsikostas *et al.*, 1998; 1999; Storer & Goadsby, 1999). Similarly, in the cornea, a substantially first (ophthalmic) division of trigeminal innervated structure, there is a significant glutamatergic component to transmission (Bereiter *et al.*, 1996). Microiontophoresis of D,L-homocysteic acid (Storer & Goadsby, 1997) or L-glutamate (Storer *et al.*, 2001a) produces a rapid and tightly stimulus-linked increase in trigeminal neuronal activity well suited to pharmacological exploration, while co-microiontophoresis of other agonists and antagonists offers clear characterization of trigeminal synaptic pharmacology (Storer *et al.*, 2001b). We have used a minimal repetition of five epochs to ensure stable baseline measurements, and saline controls to test whether there is an ordering effect of activation prior to antagonist administration. No such effect was seen. In some experiments (data not shown), more than five epochs were used and neurons continued to respond. In addition, we observed that local microiontophoresis of  $\alpha$ -CGRP following L-glutamate still produced significant neuronal activation. Taken together, tachyphylaxis is an unlikely explanation for the inhibitory effects we have seen. Moreover, although presynaptic glutamate receptors have been identified in dorsal horn (Carlton *et al.*, 2001), even if they are activated and involved in the release of CGRP, it remains likely that the major action of CGRP itself is post-synaptic, and thus the effect we observed is probably post-synaptic. The question of localization will be best finalised with electron microscopy. Although excellent in terms of localization of effect, microiontophoresis cannot precisely predict dosing of compounds. While the ejection current is indicative of the amount of substance delivered, physicochemical issues, such as compound valency, molecular weight, and solubility, make absolute comparisons based on current inappropriate. The relative molecular mass of BIBN4096BS is approximately one-quarter that of the peptides, so its flux, electrophoretic mobility, and diffusional properties will be greater than for the peptides. BIBN4096BS's valency is estimated at +3 which

is the same as that expected at the pH range used for the CGRP fragment 8–37, but higher than that expected for CGRP (+2). Thus, while the effect and localization of the effect of BIBN4096BS is clear, its relative potency cannot be determined from these studies.

CGRP receptors have been difficult to characterize pharmacologically, probably due to the recently described presence of the receptor activity-modifying proteins (RAMPs) (McLatchie *et al.*, 1998). There are two classes of CGRP receptor, CGRP<sub>1</sub> and CGRP<sub>2</sub>, broadly defined by the ability of CGRP-(8–37) to inhibit the actions of CGRP at one receptor (Dennis *et al.*, 1990). These receptors are made up of combinations of two distinct seven transmembrane receptors, the calcitonin receptor and the calcitonin receptor-like receptor (CRLR) (Choksi *et al.*, 2002), along with the appropriate RAMP. CGRP release into the cranial circulation during acute headache has been described in both migraine and cluster headache (Edvinsson & Goadsby, 1998). CGRP has a pivotal role in vasodilation due to trigemino-vascular activation in the cerebral (Goadsby, 1993) and meningeal circulation (Williamson *et al.*, 1997), and nitric oxide generation mediates some component of that effect in some settings (Wahl *et al.*, 1994; Akerman *et al.*, 2002). CGRP release is under opioid influence in the spinal cord (Collin *et al.*, 1993), has been implicated in some pain mechanisms (Yu *et al.*, 1996), and may be involved in morphine tolerance (Powell *et al.*, 2000). CGRP immuno-reactivity is found near trigeminal neurons that have 5HT<sub>1B</sub> and 5HT<sub>1D</sub> receptors in neurons in rat (Ma *et al.*, 2001) and human (Hou *et al.*, 2001) trigeminal ganglia. Given that 5HT<sub>1B/1D</sub> receptor agonists are effective in the treatment (Ferrari *et al.*, 2001), the role of CGRP receptors in the trigemino-vascular system is an important question for understanding the pathophysiology of these disorders.

The new data are consistent with an important role for CGRP in the transmission of trigemino-vascular nociceptive information, and thus for a pivotal role of CGRP in acute attacks of migraine and cluster headache. The emerging data, buttressed by the clinical evidence that BIBN4096BS is effective in acute migraine (Olesen *et al.*, 2003), invite some consideration of the role of CGRP in migraine and how and where blockade of its effect alters the acute attack.



BIBN4096BS is apparently devoid of direct vascular actions in animals (Moreno *et al.*, 2002) or humans (Petersen *et al.*, 2003b). It also has no apparent effect on brain blood flow (Petersen *et al.*, 2003a), which is consistent with the lack of effect of triptans on cerebral blood flow (Weiller *et al.*, 1995). Given that there are no consistent changes in brain blood flow in migraine without aura (Olesen, 1991), and no relationship to the pain in terms of timing (Olesen *et al.*, 1990), or effects of acute anti-migraine compounds (Friberg *et al.*, 1991; Limmroth *et al.*, 1996), evidence has been accumulating that a vascular effect may be unnecessary for acute anti-migraine compounds. It seems more likely that acute anti-migraine compounds work by blocking trigeminal nociceptive traffic either at the vessel/nerve interface (Moskowitz & Cutrer, 1993), or in the trigeminocervical complex (Kaube *et al.*, 1993a), or both. The new data establish that for CGRP antagonists the trigeminocervical complex is one possible target, and further suggest that newer compounds should actively target central structures.

This study has shown that local microiontophoretic application of the CGRP receptor antagonists BIBN4096BS

and CGRP-(8–37) blocks nociceptive trigeminovascular transmission in the trigeminocervical complex of the cat. This effect was seen with both peripheral activation of the pathway by stimulation of the superior sagittal sinus and by local trigeminal nucleus activation with microiontophoresis of L-glutamate. The effect was present during L-glutamate and BIBN4096BS co-microiontophoresis, where pre-synaptic mechanisms are by-passed, suggesting a non-presynaptic, probably post-synaptic effect. The data establish a role for CGRP-mediated transmission of trigeminovascular nociceptive inputs in the trigeminocervical complex, and argue strongly for further clinical development of CGRP antagonists for the acute treatment of both migraine and cluster headache.

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