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Effects of eletriptan on the peptidergic innervation of the cerebral dura mater and trigeminal ganglion, and on the expression of *c-fos* and *c-jun* in the trigeminal complex of the rat in an experimental migraine model

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Abstract

Nociceptive axons and terminals in the supratentorial cerebral dura mater display an intense calcitonin gene-related peptide (CGRP) immunoreactivity. In an experimental migraine model, it has been shown that electrical stimulation of the rat trigeminal ganglion induced an increase in the lengths of CGRP-immunoreactive axons, increased size and number of pleomorphic axonal varicosities in the dura mater, and an increased number of *c-jun* and *c-fos* protein-expressing nerve cells in the trigeminal complex. We demonstrate the effect of the highly specific and moderately lipophilic serotonin agonist eletriptan (Pfizer) which prevents the effects of electrical stimulation in the dura mater. Eletriptan also affected the caudal trigeminal complex; it markedly reduced the numbers of the oncoprotein-expressing cells, mainly after stimulation and to some extent also in nonstimulated animals. Eletriptan also affected expression of CGRP in perikarya of trigeminal ganglion cells, insofar as the number of small nerve cells exhibiting a compact CGRP immunoreaction was decreased to one quarter of the original value. In all these respects, eletriptan acted in a similar way to sumatriptan, with the notable exception that eletriptan also blocked the stimulation-induced effects in the nucleus caudalis trigemini and the upper cervical spinal cord (trigeminal complex), whereas sumatriptan did not. It is concluded that eletriptan, acting on perikarya and both the peripheral and the central axon terminals of primary sensory neurons, exerts its antimigraine effect by an agonist action on 5-$HT_{1B/1D}$ receptors throughout the entire trigeminal system, probably by passing the blood–brain-barrier because of its lipophilic character.

Introduction

The trigemino-vascular system is known to play an important role in the pathomechanism of migraine headache. Far from being an inert tissue of collageneous fibers, histochemical studies (Andres *et al*., 1987; Keller & Marfurt, 1991) have revealed that the supratentorial dura mater of the rat is equipped with a very rich innervation apparatus. The pain-sensitive intracranial structures such as the blood vessels and the supratentorial dura mater are innervated by branches of the trigeminal nerve arizing from cell bodies located in the trigeminal ganglion (Penfield, 1934). The vascular supply of the dura is intimately correlated both to vasomotor axons and to numerous sensory fibers which derive mainly from the ophthalmic (and, to a smaller extent, from the maxillary and mandibular) divisions of the trigeminal nerve, and, partly also from the glossophrayngeal and vagus nerves. Sensory axons are known to display calcitonin gene related peptide (CGRP) and substance P immunoreactivity (Uddman *et al*., 1985) and they proceed with parasympathetic and sympathetic axons together in common strands ensheathed by the cytoplasms of Schwann cells (Keller & Marfurt, 1991).

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Neuropeptides are known to be released from the perivascular peripheral nerve terminals; CGRP induces vasodilatation of arterioles, while SP induces vasodilatation of postcapillary venules and plasma extravasation. Neuropeptides released from peripheral nerve endings cause neurogenic inflammation in the cerebral dura (Buzzi *et al*., 1991; Buzzi & Moskowitz, 1992); sensitization of these nerve terminals induces release of pain-related neuropeptides from central terminals of the affected primary sensory neurons, impinging upon second-order sensory cells in the caudal trigeminal nucleus in the medulla and upper spinal cord. Consequently, trigeminal brainstem neurons will be activated and neural impulses are sent to the thalamus via the trigeminal lemniscus pathway (Knyihár-Csillik *et al*., 1997).

Eletriptan, a moderately lipophilic serotonin agonist acting on 5-$HT_{1B/1D}$ receptors (Saxena, 1999) is a novel antimigraine drug. The objective of the present investigation was to show, by means of light microscopic immunohistochemical methods, the cytological effects which ensue in an experimental migraine model, after intravenous application of eletriptan. Using morphometrical analysis of the trigeminal system we tried to establish the immunocytochemical correlates characterizing the migraine attack (Goadsby *et al*., 1988, 1990; Kovács *et al*., 1991) and the alterations induced by eletriptan which may account for its therapeutic effect. Our results were briefly published in abstract form (Knyihár-Csillik *et al*., 1999).

Materials and methods

Investigations were performed on 24 young adult albino rats (Rattus norvegicus albus) of both sexes (12 males and 12 females), **W**istar strain, 200–250 gram body weight. Care of the animals complied with the guidelines of the Hungarian Ministry of Welfare; experiments were carried out in accordance with the European Communities Council Directive (November 24, 1986; 86/609/EEC) and the Albert Szent-Gyögyi Medical University Guidelines for Ethics in Animal Experiments. The animals were divided into six groups, each group consisting of four rats. Group I was treated with isotonic saline. Group II was treated with isotonic saline and subjected to 30 min electrical stimulation of the trigeminal ganglion. Group III was treated with eletriptan (0.1 mg/kg i.v., according to the proposal of the manufacturer). Group IV was treated with eletriptan (0.1 mg/kg i.v.), and 30 min later subjected to electrical stimulation of the trigeminal ganglion. Group V was treated with eletriptan (0.1 mg/kg i.v.), and simultaneously subjected to electrical stimulation of the trigeminal ganglion. Group VI was subjected to electrical stimulation of the trigeminal ganglion and, 5 mins after the commencement of the stimulation, treated with eletriptan (0.1 mg/kg i.v.). For i.v. injections in males, the superficial dorsal penis vein or the tail vein, and in females, the femoral vein or the tail vein was used. Prior to electrical stimulation, rats were deeply anaesthetized with chloral hydrate (i.p. 0.4 g/kg body weight). Stimulation of the trigeminal ganglion was performed on animals placed in the horizontal plane in a stereotactic apparatus. The concentric bipolar electrode (FHC, Brunswick, ME, USA, cat. No: 17–75–2, centre pole connected to cathode) was placed stereotaxically into the left trigeminal ganglion, by insertion through a hole bored on the skull 3.2–3.4 mm posteriorly from the bregma and 2.8–3.2 mm laterally. The tip of the stimulating electrode was 9.3 mm below the dural surface. These values correspond to those described for the trigeminal ganglion in the stereotactic atlas of Schneider *et al.* (1981). Stimulation was performed with square pulses of 5 ms duration, 5 Hz frequency, 0.1–1.0 mA for 30 min. Sixty minutes after stimulation, rats were subjected to transcardial fixation with 500 mL cold 4% paraformaldehyde, preceded by a brief flush of 125 mL 0.1 M phosphate buffered saline, pH = 7.4 at room temperature. After perfusion, the cerebral dura, the trigeminal ganglia, the medulla and the cervical spinal cord were dissected. The location of the tip of the electrode in the trigeminal ganglion was checked in autopsy using frozen sections stained with methylene blue.

The cerebral dura and the trigeminal ganglia were removed *in toto,* postfixed in the same solution at 4 °C for 12 h. The cerebral dura was used as a whole-mount preparation. While the entire extent of the dura mater was subjected to the immunohistochemical reaction and investigated, the precise location of the area where photomicrographs were taken and the determination of the nerve fibre density and the sizes of varicosities were estimated was in the square area located 2 mm laterally from the sagittal sinus, extending in the lateral direction by 2 mm, and 3 mm posteriorly from the frontal pole, extending in the posterior direction by 2 mm.

After cryoprotection, 40 µm cryostat serial sections were obtained from the trigeminal ganglia. Samples were pretreated in 2% H_2O_2 and incubated in a blocking serum consisting of 0.1 M phosphate buffered saline, 2% normal goat serum (NGS), 1% bovine serum albumin (BSA, United States Biochemical Corp., Cleveland, OH, USA) and 0.3% Triton X-100 (Reanal Rt. Hungary), on a shaker plate at room temperature for 1 h. Samples were transferred into the primary antibody (rabbit-anti-CGRP, Sigma RBI, 1 : 4000) at 4 °C for 36 h, or at room temperature for 12 h. The anti-CGRP serum used in these studies recognizes both and CGRP but does not cross-react with calcitonin. After incubation in the primary antibody, a 90-min incubation in biotinylated antirabbit secondary antibody, raised in goat (Vector Laboratories, Burlingame, CA, USA) followed and the sections were processed according to the avidin-biotin system (ABC, Vectastain Elite, Vector Laboratories). The reaction was visualized by diamino-benzidine (DAB, Polysciences Inc., Warrington, PA, USA) to which hydrogen peroxide was added (3 L of 30% H_2O_2–10 mL of 1% DAB). Whole mounts of the cerebral dura and the sections from the trigeminal ganglia were either mounted on silane-pretreated slides, dehydrated in a graded series of ethanol and processed through carbol-xylene or treated according to the free-floating technique. Slides were coverslipped with Permount.

Specificity of the immunohistochemical reaction was assessed either by incubating slides in nonimmune normal rabbit serum lacking the primary antibody, or by preabsorption of the CGRP antiserum with the commercially available synthetic rat CGRP, or by omitting steps to visualize the antigen-antibody reaction. Absence of any immunohistochemical staining in the control experiments proves validity of the CGRP immunoreaction in the experimental material.

Immunohistochemical localization of *c-fos* and *c-jun* was performed in the medulla and in the two uppermost cervical segments of the spinal cord (C_1 and C_2), using 40 µm cryostat sections obtained from the 4% paraformaldehyde fixed brains of the same animals. Samples to be stained for *c-fos* were postfixed in the same fixative overnight, and those for *c-jun* for 3 h. After cryoprotection, serial sections were incubated with the polyclonal primary antibody for *c-fos* protein (sheep), obtained from Genosys Biotechnologies (Cambridge, UK) in 1 : 1000 dilution in PBS with 0.3% Triton X-100 and 2% normal rabbit serum (NRS, Vector Laboratories), at 4 °C overnight, followed by 90 min incubation in biotinylated sheep secondary antibody (Vector Laboratories, Burlingame, CA, USA) at room temperature on a shaker plate. For the demonstration of *c-jun* protein, the polyclonal antibody (rabbit), obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) in 1 : 200 dilution in PBS with 5% NGS, 1% BSA, 0.1% Triton X-100 at 1 h at room temperature was used. This was followed by 90 min incubation in biotinylated antirabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) at room temperature on a shaker plate. In both cases, the further procedure followed the avidin-biotin technique; visualization of the reaction product in the *c-fos*- and *c-jun*-expressing cells, resp., was performed with nickel-DAB according to the technique used in earlier studies (Knyihár-Csillik *et al.*, 1997; Csillik *et al.*, 1998; Hoskin & Goadsby, 1998).

Morphometry

Density of the innervation of the supratentorial cerebral dura mater (the ratio of axons exerting light microscopically visible CGRP immunopositivity vs. surface area) was determined by measuring the length of the CGRP-reactive axons with the Neurolucida three dimensional microscope program. Density was expressed as the ratio of axon length vs. surface area.

Dynamics of CGRP expression in nerve cells of the trigeminal ganglion was estimated by counting nerve cells of different sizes and different structural characteristics. Counting was performed in a blind manner, by a person unaware of the treatment of the animals, in serial longitudinal sections of the trigeminal ganglion, obtained from three normal control animals and three eletriptan-treated rats (0.1 mg/kg body weigth). The counts were analysed according to the dissector technique (West, 1999). The numbers in the five main classes, i.e. (i) small compactly stained cells, diameter 173 µm; (ii) small granular cells, diameter 175 µm; (iii) medium-sized granular cells, diameter 223 µm; (iv) large granular cells, diameter 408 µm and (v)

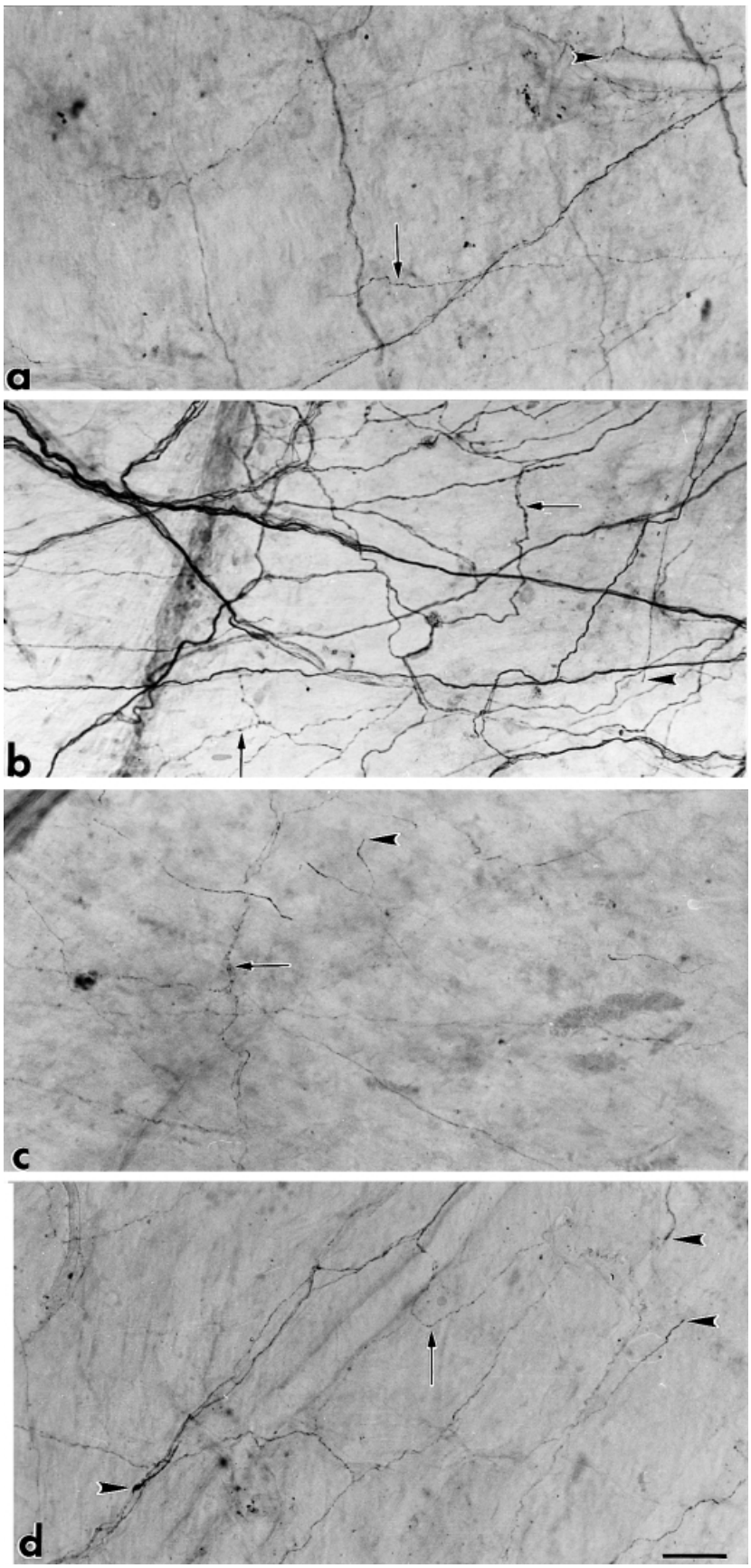

FIG. 1. CGRP-immunoreactive axons in the supratentorial cerebral dura mater of the rat. Arrows point at varicose nerve fibers; arrowheads indicate nerve endings. (a) Normal dura. (b) Thirty minute electrical stimulation of the ipsilateral trigeminal ganglion. (c) Eletriptan-treated (0.1 mg/kg i.v.). (d) Thirty minute electrical stimulation, preceded by eletriptan (0.1 mg/kg i.v.). Scale bar, 50 µm.

nonreacting cells were determined. In the largest sections, the total number of nerve cells was 600–650. Serial sections of trigeminal ganglia of nontreated and eletriptan-treated animals were studied and mean ± SEM values determined. Summative percentage of each class was documented as a segment of a pie-chart.

Sections immunostained for *c-jun* and *c-fos* were viewed on a Nikon ECLIPS 600 photo microscope. Microscopic fields were converted into digital images by means of an MTI CCD 72 video camera and amplifier and transferred to a Macintosh Quadra 700 computer running the Image Pro Plus v4.0 program analysis software, in order to determine the number of *c-fos*- and *c-jun*-expressing cells, using a threshold to differentiate strongly and medium reacting cells from weakly reacting ones.

Statistical comparisons of *c-fos*- and *c-jun*-expressing cells in control and treated groups were made in identical areas of the trigeminal complex separately. The number of immunoreactive cells was determined in the middle area of the caudal trigeminal nucleus and in the medial half of the superficial dorsal horn of the cervical spinal cord ($675 \times 300\,\mu m$ in both cases). Considering the noncontinuous distribution of data, these are reported as a median with interquartile ranges. The resulting values have been compared with the Mann–Whitney *U*-test. The Friedman non-parametric ANOVA yielded nonsignificant differences between six and six slices of the same cohort. In addition, a two-way repeated mesurements ANOVA was also performed, with one between-subject factor (control vs. treated group) and one within-subject factor (slices). The resulting *P*-values proved significant differences between control vs. treated group but not between slices of the same origin.

Results

Supratentorial cerebral dura mater

Under normal conditions, the cerebral dura displays a fair number of CGRP immunopositive nerve fibers which proceed together with with nonreacting fibers alongside blood vessels, partly in larger or smaller nerve trunks or in the shape of single fibers, terminating either perivascularly or freely in the connective tissue. These fibers establish a network throughout the dura (Fig. 1a), in close topographical relation to blood vessels. The length of CGRP-immunoreactive axons in the supratentorial dura mater was 51.8 mm per mm^2 (Fig. 2). Terminal portions of these fibers, devoid of a Schwann envelope, are beaded (Fig. 3a); the varicosities are fairly regular (Fig. 4).

Electrical stimulation of the trigeminal ganglion resulted in peculiar alterations of CGRP-immunopositive axons (Fig. 1b) and their terminals (Figs 3b and 4). The length of CGRP-expressing fibers was increased to 94.8 mm per mm^2 (Fig. 2), evidently as a result of increased CGRP in those axons in which, under normal conditions, it did not reach the level of immunohistochemical detectability.

After eletriptan treatment (0.1 mg/kg i.v.) the axonal network of the dura mater consisted mainly of tiny beaded axons; the length of the CGRP-immunoreactive axons decreased considerably to 8.7 (Figs 1c and 2). The varicosities were characterized by irregular sizes (Figs 3c and 4)

Eletriptan, administered i.v. (0.1 mg/kg) 30 min prior to 30 min electrical stimulation, induced a series of structural alterations, including accumulation of CGRP immunoreactivity in axon terminals (Fig. 1d). CGRP-reactive axons seem to be thinner, most of them beaded and their number is decreased as compared to normal and stimulated samples, to 10.8 mm per mm^2 (Fig. 2). The varicosities of the preterminal portion became smaller and pleomorphic; their number was increased (Figs 3d and 4).

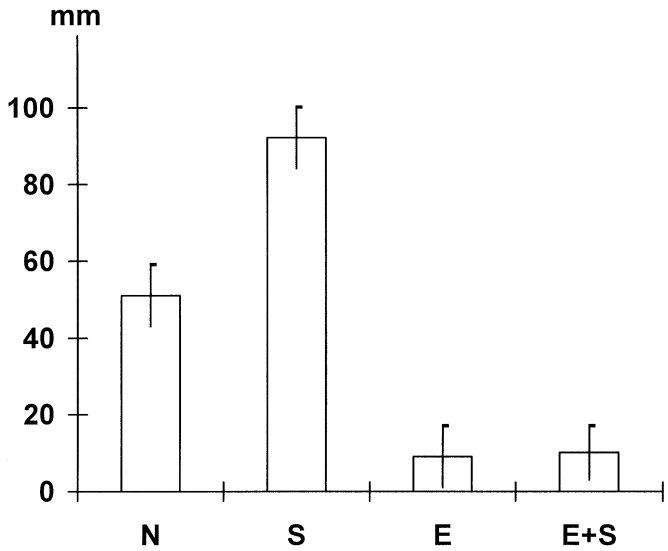

FIG. 2. Length of CGRP-immunoreactive axons in the cerebral dura mater is 51 ± 8 mm/mm^2 under normal conditions (N). After stimulation of the trigeminal ganglion, the value is increased to 92 ± 8 mm/mm^2 (S). With eletriptan treatment of nonstimulated animals, the value decreased to 8 ± 6 mm/mm^2. With stimulation of the trigeminal ganglion, preceded by 30 mins eletriptan treatment (0.1 mg/kg i.v) the length of CGRP-immunoreactive axons is decreased to 10 ± 7 mm/mm^2 (E+S). The changes are statistically significant, $P < 0.01$

Medulla

Under normal conditions, *c-jun* immunoreactivity is present in a restricted number of second-order sensory cells in the caudal subnucleus (Fig. 5, *c-jun*; Fig. 6a). The number of *c-fos*-expressing nerve cells is smaller (Fig. 5, *c-fos*; Fig. 7a).

Electrical stimulation of the trigeminal ganglion (30 min) greatly enhanced expression of both oncoproteins at the side of stimulation. While the number of the number of *c-jun*-expressing nerve cells was increased by four times after stimulation (Fig. 5, *c-jun,* Fig. 6b), the number of *c-fos*-expressing cells was increased by six times per section under similar conditions, i.e. after 30 min stimulation of the trigeminal ganglion (Fig. 5, *c-fos*; Fig. 7b).

Eletriptan treatment (alone) induced a slight decrease in the number of oncoprotein-expressing cells (*c-jun*, Figs 5, and 6a and c; *c-fos*, Figs 5, and 7a and c).

Eletriptan treatment prior to electrical stimulation of the trigeminal ganglion (30 min) induced a significant decrease in the number of oncoprotein-expressing cells. The number of *c-jun*-expressing cells decreased to one-fourth compared with the untreated and stimulated samples (Figs 5, and 6b and d) while that of *c-fos*-expressing nerve cells decreased to half of the original value (Figs 5, and 7b and d).

Eletriptan administered simultaneously or slightly (5 mins) after beginning of the stimulation resulted in marked reduction in the number of *c-jun*- and *c-fos*-expressing cells in the caudal trigeminal nucleus (to less than half of the original value).

Spinal cord

Under normal conditions, *c-fos* immunoreactivity was present in several second-order sensory cells scattered in lamina I and II (substantia gelatinosa), both in C1 and C2 (Fig. 5, *c-fos*). The number of *c-jun*-expressing nerve cells was higher (Fig. 5, *c-jun*).

Electrical stimulation of the trigeminal ganglion (30 min) greatly enhanced expression of both oncoproteins at the side ipsilateral to stimulation. The number of *c-fos*-expressing cells was increased to 5

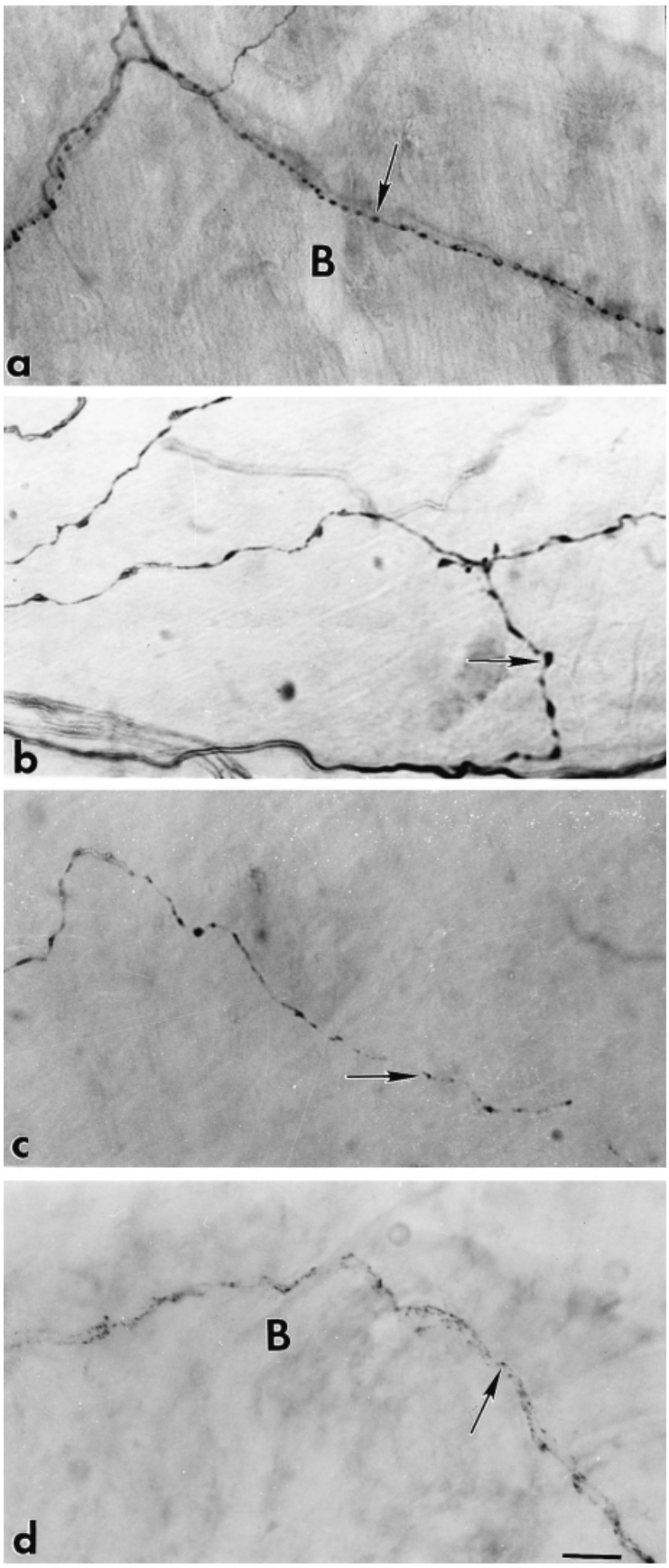

FIG. 3. Varicosities (arrows) of CGRP-immunoreactive axons in the dura mater (B, blood vessels). Arrows point at varicosities. (a) Normal dura. (b) Dura mater, after 30 mins electrical stimulation of the trigeminal ganglion. Note diminished number and enlargement of varicosities. (c) Dura mater from eletriptan-treated animal (0.1 mg/kg i.v., 30 mins prior to fixation), without stimulation. Note size and heterogeneity of varicosities. (d) Dura mater after 30 min electrical stimulation of the trigeminal ganglion, preceded by eletriptan treatment (0.1 mg/kg). Note diminution of the sizes of varicosities. Scale bar, 5 μm.

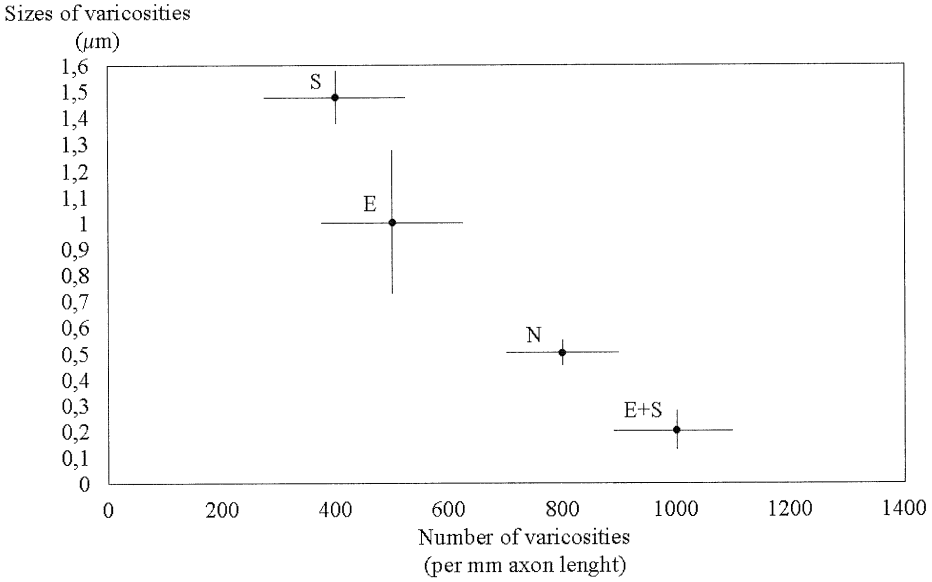

FIG. 4. Sizes of varicosities and their number in normal (N), in eletriptan-treated (E), in stimulated (S) and in eletriptan-treated and stimulated (E+S) samples ($n = 8$), in the CGRP-stained supretentorial dura mater. Number of varicosities analysed was 1450.

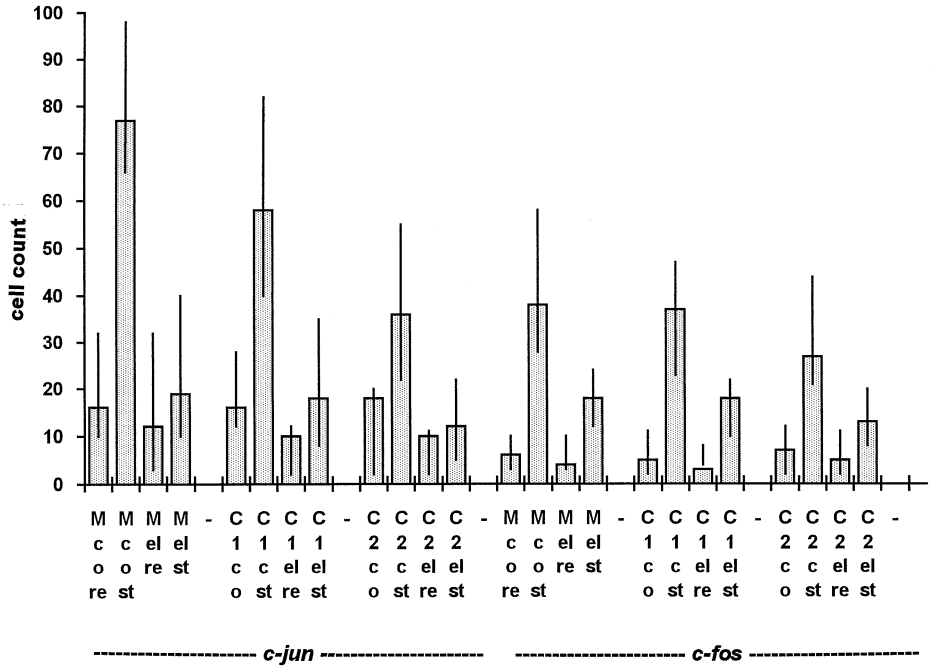

FIG. 5. Effect of electrical stimulation of the trigeminal ganglion (30 min) and eletriptan treatment (0.1 mg/kg, i.v.) of stimulated and nonstimulated rats on the expression of *c-jun* (columns 1–12) and *c-fos* (columns 13–24) in the trigeminocervical complex (M, medulla; C1 and C2, spinal segments). Abbreviations: co, control; re, resting; st, stimulated; el, eletriptan treated. Numbers of immunoreactive cells counted in the middle area of the caudal trigeminal nucleus and in the medial half of the superficial dorsal horn of the cervical spinal cord (675 × 300 μm in both cases) are shown. Data are presented as median with interquartile range.

times in C1 and to 4 times in C2 (per section), after 30 min stimulation of the ipsilateral trigeminal ganglion (Fig. 5, *c-fos*). Under similar conditions, the number of *c-jun*-expressing nerve cells was increased to 3 times in C1 and was doubled in C2 (Fig. 5, *c-jun*).

Eletriptan treatment alone (0.1 mg/kg i.v.) induced a slight decrease in the number of oncoprotein-expressing cells in the spinal cord. The number of *c-fos*-expressing cells remained nearly unchanged in C1 and C2 in the eletriptan-treated spinal cord, compared with the normal ones (Fig. 5, *c-fos*) while that of *c-jun*-expressing nerve cells decreased by one-third in C1 and in C2 (Fig. 5, *c-jun*).

Eletriptan treatment (0.1 mg/kg i.v.) prior to electrical stimulation of the trigeminal ganglion (30 min) induced a significant decrease in the number of oncoprotein-expressing cells in the spinal cord. The number of *c-fos*-expressing cells was one-half of the stimulated sample in C1 and C2 in the eletriptan-treated and stimulated spinal cord (Fig. 5, *c-fos*) while that of *c-jun*-expressing nerve cells was one third of the stimulated sample in C1 and in C2 (Fig. 5, *c-jun*).

According to the statistical analysis, the differences between resting and stimulated samples are highly significant ($P < 0.001$) and the differences between stimulated and eletriptan + stimulated samples are also significant ($P < 0.01$). The differences between normal and eletriptan-treated samples, though similarly decreased, were not significant.

Trigeminal ganglion

At the light microscopic level, five types or classes of nerve cells can be distinguished in the rat trigeminal ganglion after CGRP immunostaining (Fig. 8a). Class 1 consists of small nerve cells (diameter, 173 μm) in which the CGRP immunoreactivity is very strong and the cytoplasm is complete with a homogeneous reaction product (Fig. 8c). Class 2 neurons are of a similar size but the CGRP immunoreaction is granular, outlining Nissl bodies (Fig. 8b). Class 3 neurons are medium-sized (diameter, 223 μm), the CGRP immunoreaction is granular, outlining Nissl bodies (Fig. 8a). The largest cells belong to Class 4

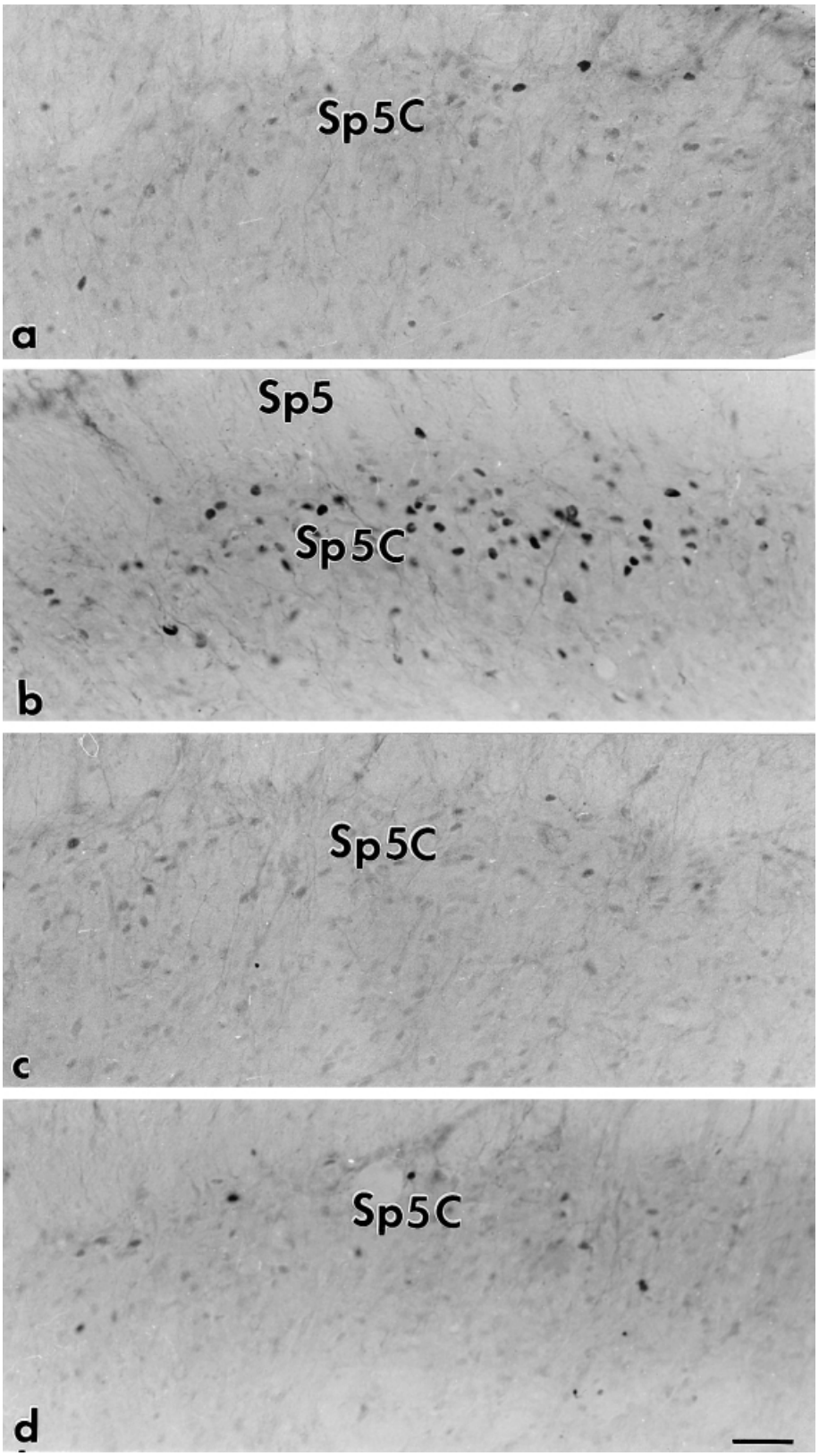

FIG. 6. Expression of c-jun in the caudal trigeminal nucleus. Sp5, descending trigeminal tract; Sp5C, caudal trigeminal nucleus. (a) Normal. (b) After 30 min stimulation of the ipsilateral trigeminal ganglion. (c) Eletriptan-treated animal (0.1 mg/kg i.v., 30 mins prior to fixation) without stimulation. (d) After 30 min stimulation of the ipsilateral trigeminal ganglion preceded by eletriptan treatment (0.1 mg/kg, i.v.). Scale bar, 50 µm.

(diameter, 40.8 µm) with a granular CGRP immunoreaction (Fig. 8c) which outlines Nissl bodies, just as in the smaller Class 2 and Class 3 cells. Finally, the majority of the ganglion cells belong to Class 5; these are characterized by the absence of CGRP immunoreaction in the perikaryon, irrespective of their sizes (Fig. 8a and b).

Cells were counted in serial longitudinal sections of the trigeminal ganglion. Under normal conditions, Class 1 neurons make up $12.7 \pm 2{,}3\%$, Class 2 cells $1.1 \pm 0.5\%$, Class 3 cells $1.9\% \pm 0.5\%$, Class 4 cells $3.8 \pm 0.9\%$ and Class 5 cells $80.4 \pm 6.1\%$ of the nerve cell population in the ganglion. (Fig. 10a). Thirty minutes after

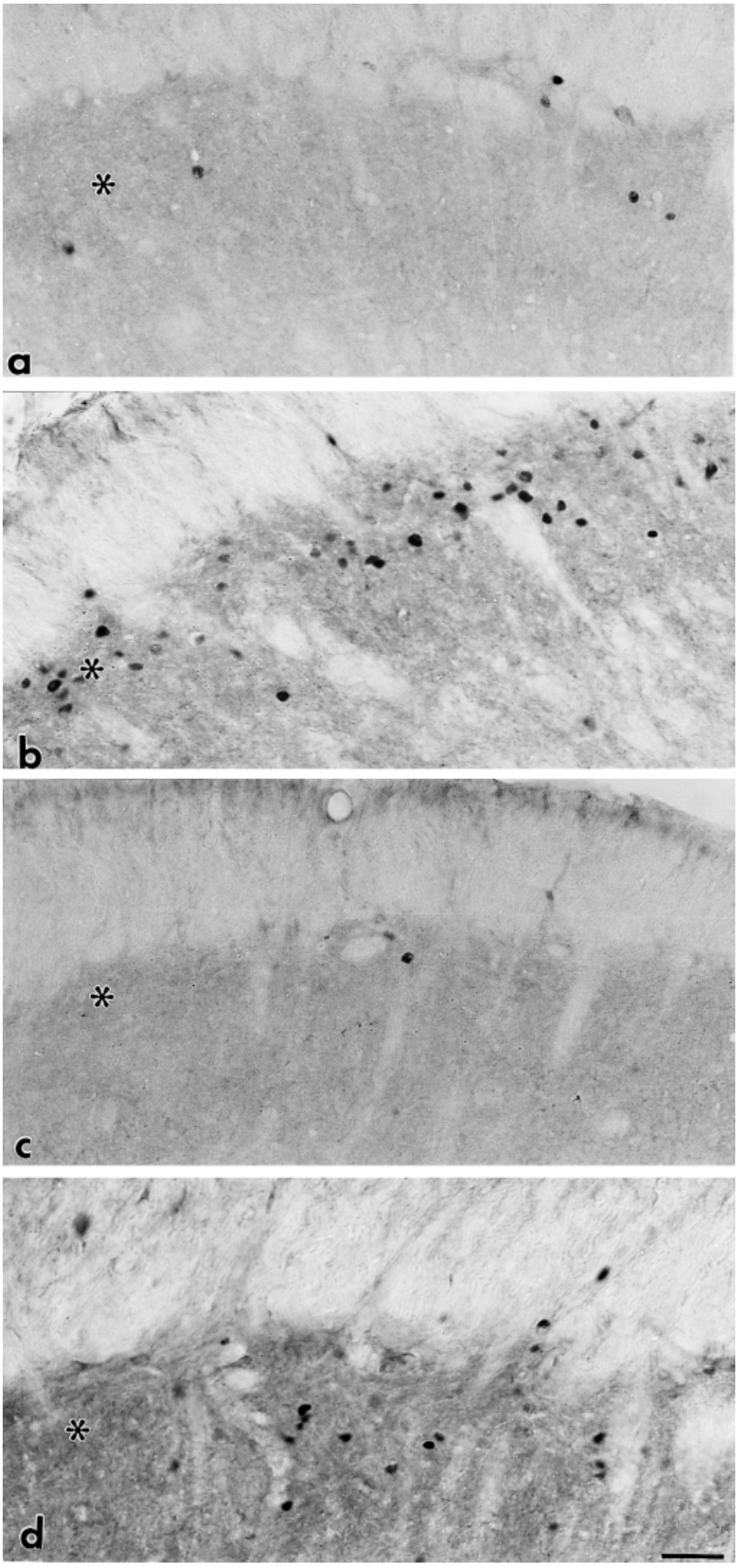

FIG. 7. Expression of *c-fos* in the caudal trigeminal nucleus. Asterisk indicates caudal trigeminal nucleus. (a) Normal. (b) After 30 min stimulation of the ipsilateral trigeminal ganglion. (c) Eletriptan-treated animal (0.1 mg/kg i.v., 30 mins prior to fixation), without stimulation. (d) After 30 min stimulation of the ipsilateral trigeminal ganglion preceded by eletriptan treatment (0.1 mg/kg, i.v.). Scale bar, 50 μm.

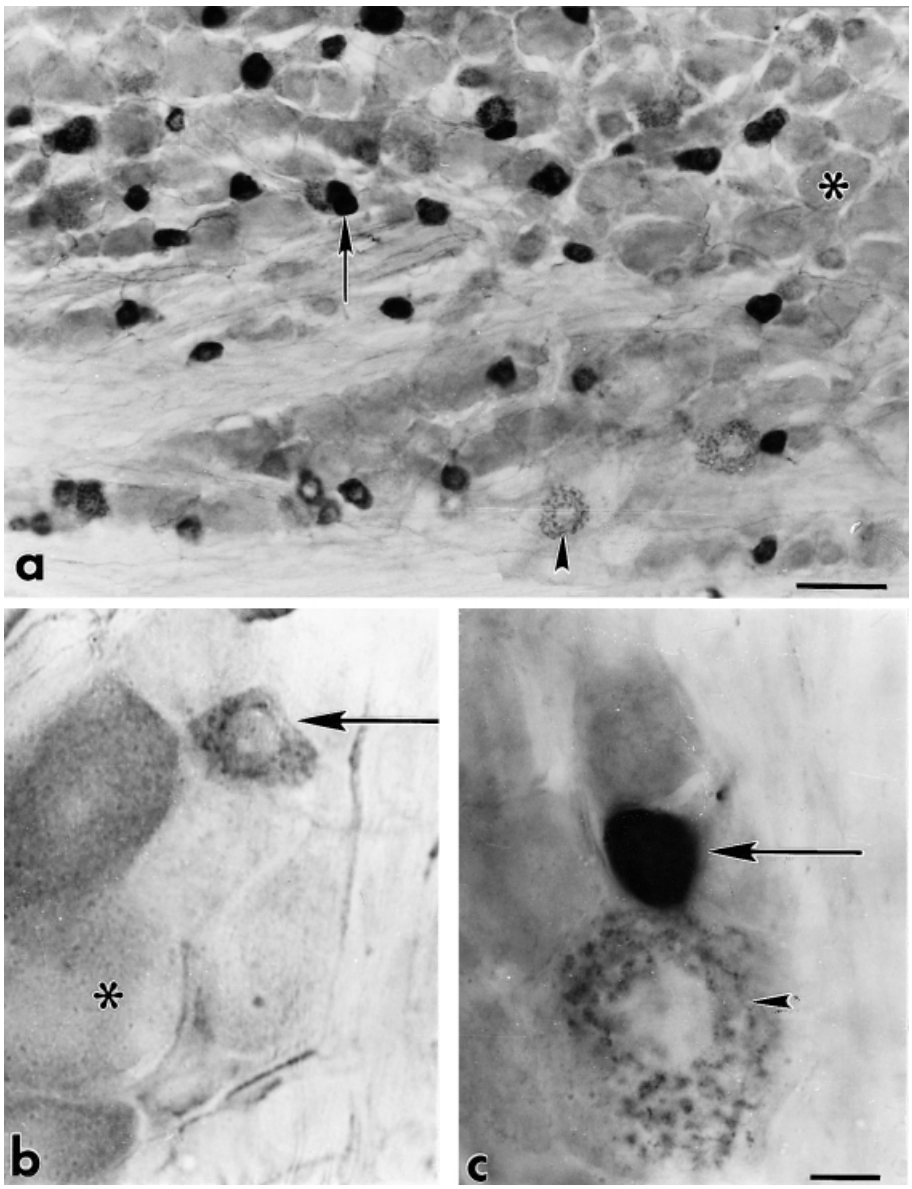

FIG. 8. CGRP immunohistochemistry in the normal trigeminal ganglion. (a) Non-reactive nerve cell (asterisk), small cells displaying a compact reaction (arrow), cell with a granular reaction (arrowhead). (b) Granular reaction in a small nerve cell (arrow); nonreacting cell (asterisk). (c) Compact CGRP immunoreaction in a small nerve cell (arrow); granular reaction in a large ganglion cell (arrowhead). Scale bars, 100 µm (a) and 5 µm (c).

eletriptan treatment (0.1 mg/kg body weight i.v.), most of the small nerve cells which exhibited a homogenous CGRP reaction under normal conditions, became granular (Fig. 9a and b). Accordingly, the percentage of Class 1 neurons decreased to $2.5 \pm 0.6\%$ at the same time, and that of Class 2 neurons increased to $10.5 \pm 3.1\%$. Also, the percentage of Class 3 neurons increased to $4.1 \pm 0.8\%$, whereas that of Class 4 neurons decreased to $2.2 \pm 0.4\%$. The number of Class 5, i.e. the CGRP-immuno-negative, cells remained virtually unchanged ($80.4 \pm 6.1\%$ in normal animals and $80.7 \pm 5.9\%$ after eletriptan treatment; Fig. 10b).

Discussion

It is known that electrical stimulation of the trigeminal ganglion induces release of CGRP from nerve fibers of the cerebral dura mater (Buzzi *et al*., 1991). Since this is similar to the alterations reported during migraine headache (Moskowitz, 1984, 1992; Goadsby *et al*., 1988; Goadsby & Edvinsson, 1993), stimulation of the trigeminal ganglion has been regarded as one of the experimental migraine models (Buzzi *et al*., 1991; Goadsby *et al*., 1990; Knyihár-Csillik *et al*., 1995, 1997, 1998). CGRP, the most effective vasodilator among neuropeptides, is released from the perivascular peripheral nerve terminals of the trigeminal ganglion (Buzzi & Moskowitz, 1992).

The density of the innervation apparatus may give information as to the possibilities of CGRP release. By measuring axonal length, those CGRP-reactive fibers are determined in which the amount of CGRP reached the threshold of detectability. Undoubtedly, there might be, and in fact, there are many more fibers in a given sample which are invisible at the level of light microscopic immunohistochemistry since they contain subthreshold amounts of CGRP. However, such fibers obviously do not take part in neuropeptide secretion. On the other hand, the fibers in which the amount of CGRP reached the threshold of detectability, can be supposed to take part in impulse transmission; the length of the immunoreactive axons is proportional with the dural area supplied by them.

On the basis of such an immunohistochemical methodological approach, we investigated the effect of eletriptan, one of the newly developed triptans in the migraine model outlined above. According to these investigations, eletriptan, a serotonin agonist acting on 5-

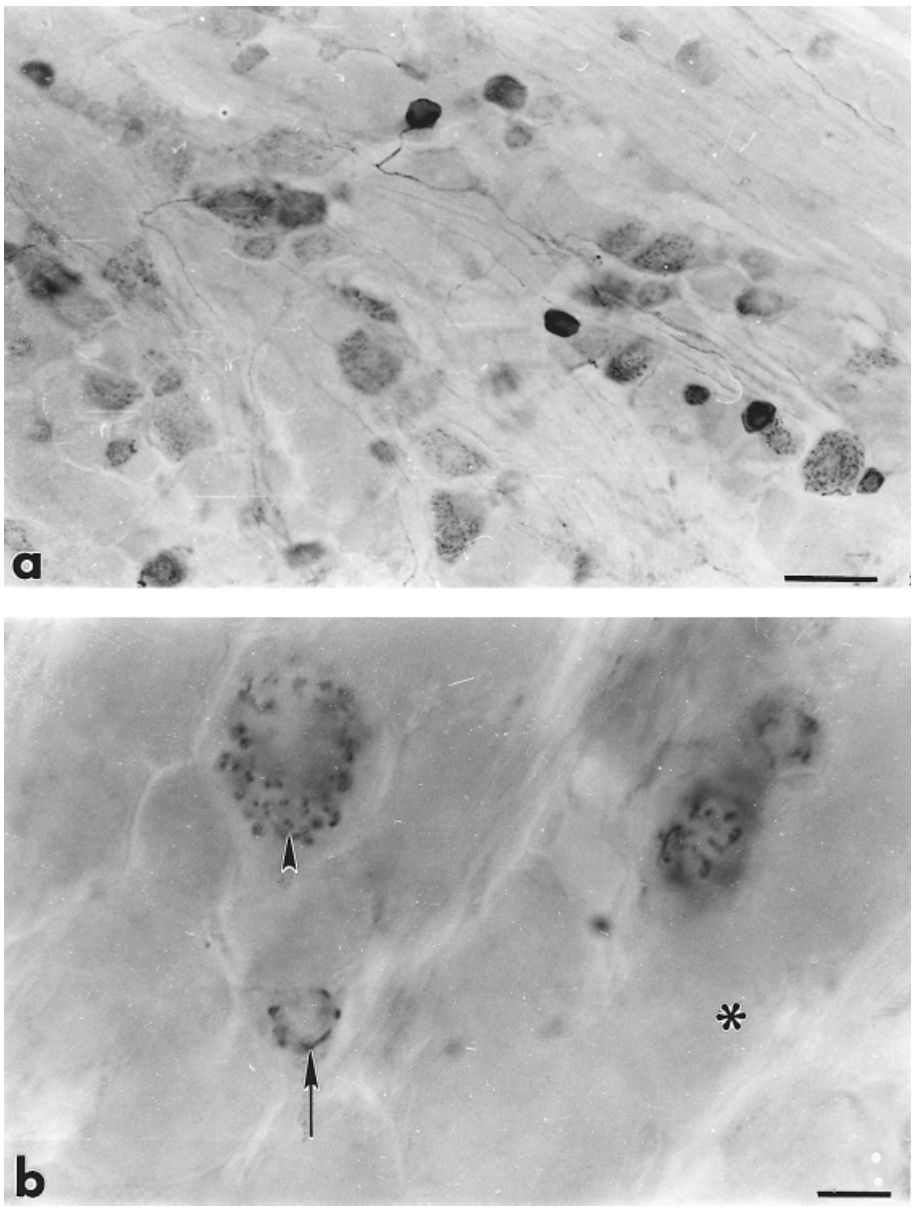

FIG. 9. CGRP immunohistochemistry in the trigeminal ganglion after 30 min eletriptan treatment (0.1 mg/kg i.v.). (a) General distribution of nonreactive and slightly reactive nerve cells in the ganglion. (b) Non-reactive nerve cells (asterisk); small cell (arrow) and large cell (arrowhead) with a granular reaction. Scale bars, 100 μm (a) and 5 μm (b).

$HT_{1B/1D}$ receptors, induced significant alterations in the immunohistochemical pattern of CGRP in the supratentorial dura mater, if injected to normal animals or if injected 30 mins prior to, simultaneously with or slightly after, electrical stimulation of the trigeminal ganglion. Therefore, the immunohistochemical patterns might help to explain the mechanism of the microstructural events which, in an acute migraine treatment, mitigate or abolish the migraine attack. Recent studies (Ashina *et al*., 1999) suggest that CGRP blood levels of migraineurs are significantly higher, even in interictal periods, than those of nonmigraineurs. While the question whether or not this means a tonic action of CGRP cannot be answered with certainty, this observation is in accord with the notion that CGRP levels are not reflections of the migraine.

The microstructural alterations which ensue in the supratentorial cerebral dura mater as a consequence of eletriptan administration prior to electrical stimulation can be regarded as immunohistochemical consequences of eletriptan binding to 5-$HT_{1B/1D}$ receptors in the dura mater and to a diminished CGRP expression in pseudounipolar primary sensory ganglion cells (Durham *et al*., 1997). This is in accord with our observations regarding the parent nerve cells in the trigeminal ganglion; we have shown that the number of small ganglion cells exerting cytological signs of intense CGRP expression (compact immunoreaction) was significantly decreased after eletriptan treatment, while the rest of the small cells exerted a CGRP immunoreaction only in a granular form. Accordingly, though the total number of CGRP-positive cells was not changed, a considerable qualitative redistribution of the CGRP-positive cell types took place (Fig. 10). 5-HT receptor activation by serotonin agonists leads to an intracellular Ca^{2+} increase which blocks the CGRP promoter and the mitogen-activated protein kinase controlling the CGRP-enhancer (Durham & Russo, 1998, 1999). This way, CGRP gene transcription is inhibited. Since eletriptan is known to bind to 5-$HT_{1B/1D}$ receptors, it can be assumed that a similar effect might be responsible for the immunohistochemical alterations seen in the trigeminal ganglia of eletriptan-treated animals. Durham's (1999) observations exclude the theoretical possibility that the CGRP depletion caused by eletriptan would be elicited by a massive CGRP release.

Diminished CGRP production is reflected also by the immunohistochemical appearance of axons. In consequence of eletriptan treatment, be it followed by stimulation or not, an apparent

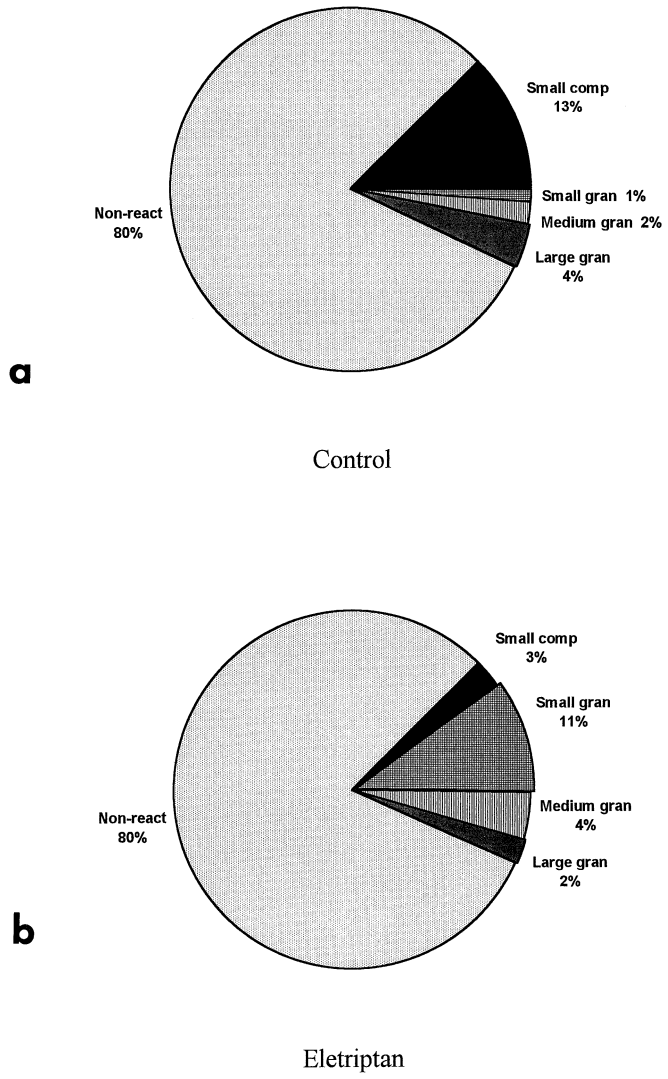

FIG. 10. Percentages of various types of CGRP-positive nerve cells in the trigeminal ganglion (a) in control samples and (b) after eletriptan treatment (0.1 mg/kg i.v).

attenuation of axons ensues, most probably because of their diminished immunohistochemically detectable CGRP content. The other alteration concerns axonal varicosities. Varicosities in eletriptan-treated samples are usually smaller and more heterogenous than under normal conditions. The pleomorphy of varicosities suggests stagnation of axoplasmic material, as indicated by Ochs *et al*. (1996). The subaxolemmal proteins of the membrane skeleton (spectrin, fodrin, actin and ankyrin) taking part in beading constrictions appear to be responsible for the pleomorphy of beads (Ochs *et al*., 1997). The fact that, in eletriptan-treated samples, mainly varicose axons are present, may suggest that eletriptan prevented the release of CGRP from dural axon terminals by an action at 5-$HT_{1B/1D}$ receptors. In other words, these alterations can be regarded as immunohistochemical equivalents of accumulation and stagnation of CGRP positive axoplasmic material in the terminal portions of CGRP immunopositive sensory nerve fibres.

According to our studies, eletriptan acts not only peripherally but also on 5-$HT_{1B/1D}$ receptors in the central nervous system. Such an action was evident from the expression of oncoproteins in second-order neurons of the caudal trigeminal nucleus, in the medulla and in C1–C2 of the spinal cord.

Immunohistochemical identification of *c-fos* protein has been used as a marker for neuronal activity after peripheral stimulation throughout the central nervous system (Hunt *et al*., 1987; Menetrey *et al*., 1989; Mugniani *et al*., 1989; Bullitt, 1990; Molander *et al*., 1992; Persson *et al*., 1993). The rationale of this approach is that *c-fos* is expressed within several types of neurons following voltage-gated calcium entry into the cell (Morgan & Curran, 1986), i.e. depolarization. From the point of view of the present investigations it is noteworthy that electrical stimulation of the superior sagittal sinus (Kaube *et al*., 1993) or mechanical stimulation of dural blood vessels (Strassman *et al*., 1994) induced numerical increase of *c-fos*-expressing neurons in the upper cervical dorsal horn and in the medulla.

Another approach to visualize neurocellular activity is the demonstration of *c-jun* expression after electrical stimulation (Herdegen *et al*., 1991; Morgan & Curran, 1991; Gass *et al*., 1997). Electrical stimulation greatly enhanced expression of *c-jun and c-fos* in the caudal trigeminal nucleus, both in the medulla and in C1–C2 segments of the spinal cord, at the side of stimulation.

Eletriptan treatment significantly decreased the number of oncoprotein-expressing cells in the caudal trigeminal nucleus, mainly if injected 30 min prior to simulation but also, to a lesser extent, if injected simultaneously or slightly after the beginning of electrical stimulation. On the other hand, the differences between eletriptan-treated and nontreated normal (resting) samples were not significant, although the trend was similar.

According to our previous results (Knyihár-Csillik *et al*., 1997) pretreatment of the animals with sumatriptan did not prevent *c-fos* expression in second-order sensory neurons after electrical stimulation of the trigeminal ganglion. The reason for this might have been that one of the characteristic features of sumatriptan is that it passes the blood–brain barrier only poorly (Shepheard *et al*., 1995). In contrast, eletriptan, a highly specific serotonin agonist, virtually prevented stimulation-induced activation of both *c-fos* and *c-jun* protein expression in the trigeminocervical system. These effects may be attributed to the more lipophilic nature of eletriptan (Saxena, 1999) which may have resulted in penetration of the central nervous system.

It follows from our experiments that in the therapy of migraine headache the 5-$HT_{1B/1D}$ receptor agonists which can be used successfully act both peripherally and centrally; of these, eletriptan seems to be a promising candidate. (Schoenen, 1997; Pitman, 1999; Wells, 1999). Recent clinical studies suggest that subjects receiving eletriptan experience less time loss than those on sumatriptan (Wells, 1999). In another study, Poole & Haughie (1999) concluded that a second dose of eletriptan is highly effective in treating headache recurrence. Compared with sumatriptan in a randomized, placebo-controlled trial, orally administered eletriptan was more effective than oral sumatriptan at providing relief of functional disability, nausea, photophobia and phonophobia (Pryse-Phillips, 1999). Therefore, though distinction between peripheral and central mechanisms of serotonin agonists might seem to be a purely theoretical question, in reality it may be of major practical importance.

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Abbreviations

CGRP, calcitonin gene-related peptide; DAB, diamino-benzidine; 5-HT, 5-hydroxy-tryptamine.

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