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# Sensory nitrergic meningeal vasodilatation and non-nitrergic plasma extravasation in anaesthesized rats

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**Abstract**

The aim of the present study was to evaluate the role of nitric oxide (NO) of sensory neural origin in neurogenic inflammatory response in the trigeminovascular system. Antidromic vasodilatation and plasma extravasation in response to electrical stimulation (15 V, 5 Hz, 0.5 ms, 100 impulses) of the trigeminal ganglion were investigated in the dura mater and nasal mucosa/upper eyelid by laser Doppler flowmetry and [$^{125}$I]-labelled bovine serum albumin, respectively. Electrical stimulation of the trigeminal ganglion of rats elicited a reproducible ipsilateral enhancement of both meningeal and nasal mucosal blood flow. $N^{\omega}$-nitro-L-arginine (L-NNA; 4, 8, and 16 mg/kg, i.v.), a nonselective inhibitor of nitric oxide synthase (NOS), inhibited antidromic vasodilatation both in the dura mater (15.86±2.05%, 22.82±2.51%, and 36.28±4.37%) and nasal mucosa (35.46±8.57%, 58.72±9.2%, and 89.99±8.94%) in a dose-dependent manner. Specific inhibitors of neuronal NOS, 7-nitroindazole (7-NI; 20 mg/kg, i.v.) and 3-bromo-7-nitroindazole (3Br-7NI; 10 mg/kg, i.v.) were administered to assess the possible role of NO released from the trigeminal sensory fibres. The meningeal vasodilatation was inhibited by both 3Br-7NI and 7-NI (63.36±7.7% and 49±6.5%, respectively). The nasal hyperaemic response was also reduced by 3Br-7NI (78.26±8.7%). Plasma extravasation in the dura mater and upper eyelid evoked by electrical stimulation of the trigeminal ganglion (25 V, 5 Hz, 0,5 ms, 5 min), expressed as extravasation ratios (ERs) of the stimulated vs. nonstimulated sides, was 1.80±0.8 and 4.63±1.24, respectively. This neurogenic oedema formation was not inhibited by neither L-NNA nor 3Br-7NI. It is concluded that neural nitrergic mechanisms are involved in the meningeal vasodilatation evoked by electrical stimulation of the trigeminal ganglion.


*Keywords:* Migraine; Nitric oxide; Trigeminovascular system; Middle meningeal artery; Laser Doppler

## 1. Introduction

Antidromic stimulation of the trigeminal ganglion causes a neurogenic inflammatory response in the innervated area including the dura mater encephali, which is one of the intracranial structures that has a sensory innervation and thus is capable of nociception. It is assumed that neurogenic inflammation plays a role in the pathogenic mechanisms of migraine headache (Moskowitz, 1984, 1993; Goadsby and Edvinsson, 1993). The dura mater encephali is innervated by a dense network of peptidergic trigeminal afferent fibres, and the excitation of these sensory fibres leads to the release of their contents surrounding meningeal arteries. This response can be induced either by electrical (i.e., antidromic stimulation of the trigeminal ganglion) or chemical (i.e., capsaicin administration) excitation. While substance P (SP) and neurokinin A (NKA) seem to be primarily responsible for plasma extravasation from postcapillary venules of the dura mater, calcitonin gene-related peptide (CGRP) is the main mediator of meningeal vasodilatation, but has no effect on oedema formation. The primary sensory neurons of the trigeminal nerve synthesize and store several neuropeptides, such as CGRP, SP, and NKA. CGRP is considered a powerful vasodilator both in vitro and in vivo in several

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vascular beds, including facial skin and dura mater, since its selective inhibitor, $hCGRP_{8-37}$, can block the vasodilator response in exteroceptive (Escott et al., 1995) and interoceptive areas (Messlinger et al., 1995; Kurosawa et al., 1995) evoked by electrical stimulation of the trigeminal ganglion.

Experimental and clinical data indicate that nitric oxide (NO) has a role in migraine and other types of vascular headache. It is known that workers in factories making explosives often suffer from headaches (Laws, 1898; Trainor and Jones, 1966). Other investigators showed that intravenous administration of NO donors, such as glyceryl trinitrate or nitroglycerin, causes attacks in migraineurs (Thomsen et al., 1993) and nonspecific vascular headache in healthy subjects (Iversen et al., 1989) via stimulation of the NO–cGMP system (Kruuse et al., 2003; Ignarro et al., 1981; Axelsson et al., 1979). NO is synthesized from L-arginine by nitric oxide synthase (NOS) (Palmer et al., 1987), and, to date, three isoforms of NOS have been identified. The inducible form of the enzyme (Forstermann and Kleinert, 1995; Ogden and Moore, 1995) is produced de novo in a calcium-independent manner in a number of different cell types, including macrophages, following exposure to bacterial endotoxins or a range of cytokines (Knowles and Moncada, 1994). The constitutive form of the enzyme is activated in a calcium-dependent manner. It has two subtypes, called endothelial (eNOS) and neuronal (nNOS) isoforms, according to the main localisation (i.e., endothelial cells and neurons, respectively). The nonspecific inhibitors of NOS, such as $N^{\omega}$-nitro-L-arginine (L-NNA), block all of the three isoforms of NOS enzyme. However, indazole derivates, including 7-nitroindazole (7-NI) and 3-bromo-7-nitroindazole (3Br-7NI), almost selectively inhibit the activation of neuronal NOS. The NADPH-diaphorase reaction can be used to demonstrate NOS in neurons (Dawson et al., 1991), and a number of immunohistochemical studies have revealed the presence of NADPH-diaphorase-positive (i.e., NOS-positive) neurons between the muscular layers of arteries (Toda et al., 1995), including meningeal arteries (Berger et al., 1994).

Infusion of NO donors in control subjects or migraineurs causes an immediate headache followed by a delayed headache 4–6 h after the infusion (Olesen et al., 1993), fulfilling the criteria of migraine headache according to the guidelines of the International Headache Society. In this delayed inflammatory response to infusion of NO donors, inducible NOS participates in the development of neurogenic plasma protein extravasation within the dura mater (Reuter et al., 2001). However, our knowledge about the role of NO in oedema formation induced by the activation of the trigeminovascular system is limited.

The present work investigated the role of NO in neurogenic vasodilatation of the middle meningeal artery and plasma extravasation in the dura mater of the rat evoked by electrical stimulation of the trigeminal ganglion. Furthermore, in this experimental set-up, attempts were made to establish the possible origin of NO responsible for the evoked antidromic meningeal vasodilatation, using the nonspecific NOS inhibitor, L-NNA, and specific nNOS inhibitors, 7-NI and 3Br-7NI. Finally, we studied the role of NO in neurogenic meningeal plasma protein extravasation elicited by electrical stimulation of the trigeminal ganglion.

## 2. Materials and Methods

### 2.1. Ethics

The present experiments conform to the European community guiding principles for the care and use of laboratory animals. The experimental protocol has been approved by the Ethics Committee for Animal Research, University of Debrecen (13/2003 DEMAB).

### 2.2. General procedures

The experiments were carried out on male Wistar rats weighing 250–350 g. The animals were housed at the Laboratory Animal Centre of the University Medical School of Pécs under pathogen-free conditions at 24–25 °C and provided with standard rat chow and water ad libitum. All procedures used in this study are in agreement with the rules of the Ethics Committee on Animal Research of the University Medical School of Pécs. The animals were anaesthesized with thiopental sodium (Trapanal, 50 mg/kg, i.p.). The right femoral artery was cannulated (Portex) to measure arterial blood pressure with a Statham pressure transducer (P23Db). The responses were recorded on a polygraph (type RM; Beckman). A fine polyethylene cannula introduced into the right femoral vein was used for drug administration. The cannula inserted into the trachea was connected to a small animal ventilator (SAR-830/P; IITC/Life Science Instruments), by which the animals were artificially ventilated after skeletal muscle paralysis with pipecuronium bromide (Arduan, 0.3 mg/kg, i.v.; additional doses were given when necessary). In the first experiments using nonparalysed animals, the level of anaesthesia evoked by 50 mg/kg thiopental sodium was checked ($n$=5) for up to 6 h by painfully pinching the extremities. No movements or increases in blood pressure indicative of inadequate anaesthesia were observed in these animals. In experiments in which muscle paralysis was used, the muscle relaxant was administered only if the animal was unresponsive to such test stimuli. These experiments lasted for no more than 4 h. Rectal temperature was kept at 37±0.5 °C by a controlled infrared lamp (Experimetria). At the end of the experiments, the animals were killed with an overdose of thiopental sodium (100 mg/kg, i.v.).

### 2.3. Electrical stimulation of the trigeminal ganglion and measurement of meningeal and nasal mucosal blood flow

The animal's head was fixed in a stereotaxic frame and the skull was exposed by a midline incision. The skin, periosteum, and dorsal part of the masseter muscle were removed. A burr hole (2 mm diameter) was drilled on the left side of the top of the skull at 3.2 mm lateral to the sagittal suture and 3.7 mm posterior to the bregma for bipolar stimulating electrodes (Peitl et al., 1999). The electrodes were lowered into the left trigeminal ganglion to a depth of 9.5 mm from the dura mater overlying the dorsal surface of the brain. It is unlikely that this procedure would have evoked cortical-spreading, depression-dependent, long-lasting dural hyperaemia, since in most cases, the blood flow in the middle meningeal artery remained unaltered. In a few cases, lowering of the electrode was followed by a transient increase in meningeal blood flow (a possible sign of cortical-spreading depression), but in these cases, the blood flow returned to the basal level within 5 min. The left trigeminal ganglion was electrically stimulated (15 V, 0.5 ms, 5 Hz, 100 impulses). In order to diminish sympathetic pressor reflexes evoked by trigeminal ganglion stimulation, the animals were treated with an adrenergic neuron-blocking agent, guanethidine (8 mg/kg, i.v.), at the beginning of each experiment. Blood flow was monitored in two areas innervated by the trigeminal nerve: in the meninges as described above and in the nasal mucosa. In the latter case, another rectangular needle-type probe of the Moor laser Doppler flowmeter was gently touched to the mucosa of the nasal septum close to the aperture in the left nasal cavity. In some cases, meningeal and mucosal blood flows were measured contralateral to the side of stimulation (i.e., on the right side). Meningeal and mucosal blood flow values expressed in arbitrary units were recorded on the polygraph and stored on a personal computer using a Haemosys software (Experimetria).

### 2.4. Plasma extravasation evoked by electrical stimulation of the trigeminal ganglion

After intravenous administration of [$^{125}$I]-labelled bovine serum albumin (70 μCi/kg), the electrodes were lowered into the left trigeminal ganglion as described above. Ten minutes later, the trigeminal ganglion was electrically stimulated (25 V, 0.5 ms, 5 Hz, 5 min) and, after a further 10 min, the animals were exsanguinated. The dura was dissected free on both sides of the skull, and the surrounding area where the electrode penetrated was discarded. Correct electrode position was confirmed by the presence of electrode pin prick marks in the trigeminal ganglion. Upper eyelids from both sides were harvested as being innervated by the extracranial trigeminal nerve. Samples were rinsed, dried overnight, and weighed, and radioactivity was counted to quantify extravasation. The amount of extravasation in the stimulated and nonstimulated sides was calculated and expressed as an extravasation ratio (ER) of stimulated/nonstimulated sides. The percentage of inhibition of extravasation in drug-treated animals was calculated with respect to a vehicle-treated group.

### 2.5. Drugs and chemicals

Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril), guanethidine, and $N^{\omega}$-nitro-L-arginine were purchased from Sigma; bovine serum albumin was from BHD; Na[$^{125}$I] was from the Institute of Isotopes of the Hungarian Academy of Sciences; NaCl was from Reanal; pipecuronium was from Richter Gedeon; Sephadex G-100 was from Pharmacia; 3Br-7NI and 7-NI were from ICN; and thiopental sodium (Trapanal) was from BYK. All drugs were dissolved and diluted in saline, except for 3Br-7NI and 7-NI (dimethyl sulfoxide and saline 4:1 vol/vol in 0.1 ml/kg). [$^{125}$I]-labelled bovine serum albumin was prepared in our laboratory (Nemeth et al., 2002).

### 2.6. Data analysis

All blood flow data measured by laser Doppler (Moor Instruments) and blood pressure changes were recorded on a Beckmann Dynograph type RM, and all output signals were fed into and stored in a personal computer equipped with an A/D card (Experimetria). Data collection and offline analysis were preformed with the Haemosys software (Experimetria). All values presented in the text give the mean and its standard error (S.E.M.). One-way analysis of variance and post-hoc two-tailed $t$ test were used for statistical analysis with a significance limit of $P<0.05$.

## 3. Results

### 3.1. Electrical stimulation of the trigeminal ganglion

Before each experiment, animals were treated with guanethidine (8 mg/kg, i.v.) to avoid a systemic pressor response caused by sympathetic nervous system activation (not shown). Electrical stimulation of the trigeminal ganglion evoked an ipsilateral enhancement of both meningeal and nasal mucosal blood flows (control recordings of Fig. 1). The blood flow both in the meninges and nasal mucosa remained unaltered ($n$=5) on the contralateral side. For sham trigeminal stimulation, the stimulating electrode was lowered 3–4 mm near the trigeminal ganglion. Electrical stimulation in this position failed to evoke blood flow changes either in the meninges or in the nasal mucosa ($n$=5). The latency to and the recovery from the increase in

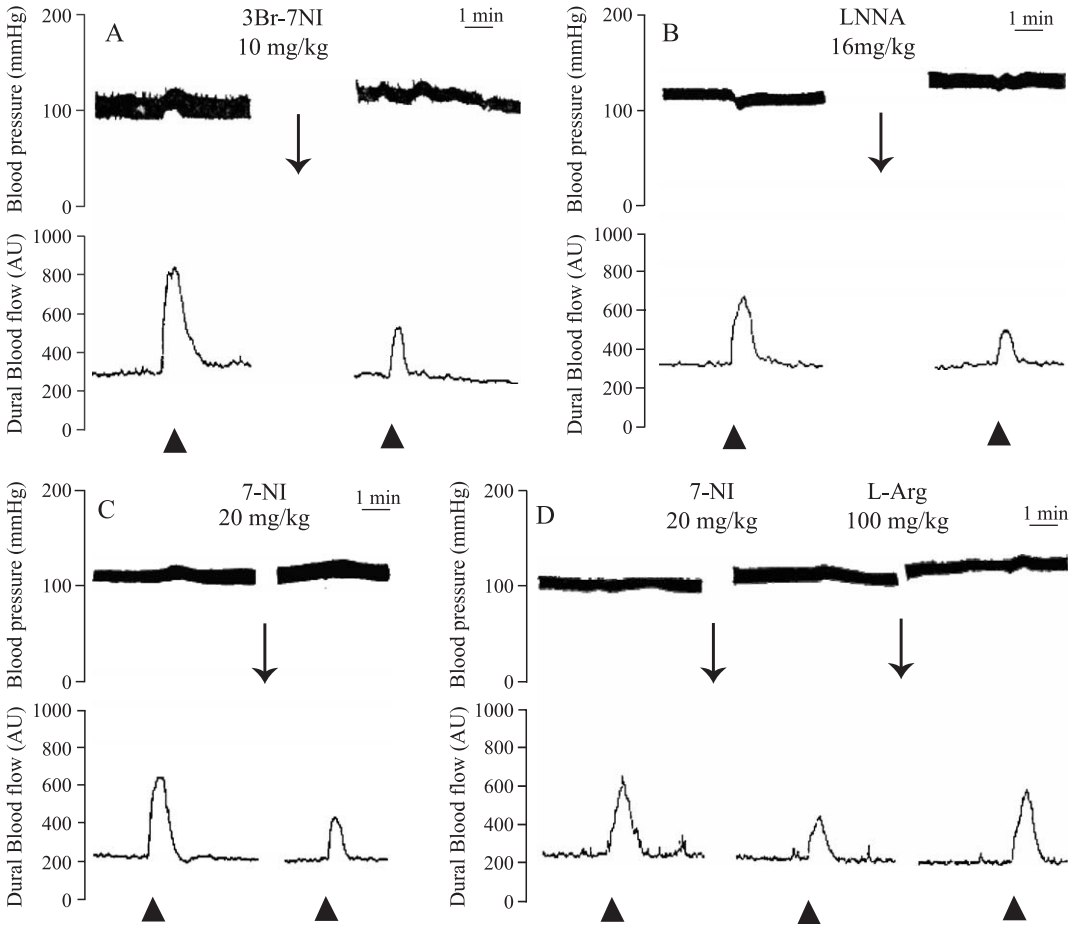


Fig. 1. (A) The effect of 3Br-7NI (10 mg/kg, i.v.) on dural vasodilatation evoked by electrical stimulation (15 V, 5 Hz, 0.5 ms, 20 s) of the trigeminal ganglion. (B) The effect of L-NNA (16 mg/kg, i.v.) on dural vasodilatation evoked by electrical stimulation (15 V, 5 Hz, 0.5 ms, 20 s) of the trigeminal ganglion. (C) The effect of 7-NI (20 mg/kg, i.v.) on dural vasodilatation evoked by electrical stimulation (15 V, 5 Hz, 0.5 ms, 20 s) of the trigeminal ganglion. (D) The effect of 7-NI (20 mg/kg, i.v.) and L-arginine (100 mg/kg, i.v.) on dural vasodilatation evoked by electrical stimulation (15 V, 5 Hz, 0.5 ms, 20 s) of the trigeminal ganglion. AU: arbitrary unit; arrowheads: electrical stimulations,; arrows: injection of drugs.

blood flow was shorter in the meninges than in the nasal mucosa.

### *3.2. Effect of L-NNA, 7-NI, and 3Br-7NI on meningeal and nasal vasodilatation evoked by electrical stimulation of the trigeminal ganglion*

The role of NO in the vasodilatation elicited by electrical stimulation of the trigeminal ganglion was investigated by using NOS inhibitors (3Br-7NI, L-NNA, and 7-NI) differing in their selectivity for various isoforms of NOS. In the first group of animals, L-NNA (4, 8, and 16 mg/kg, i.v.) caused an elevation in the resting mean arterial blood pressure (105±7 mm Hg) in a dose-dependent manner (115±7, 128±11, and 135±14 mm Hg, respectively). This rise in blood pressure was accompanied by a transient increase in meningeal as well as mucosal blood flow, lasting about 10 min, but the blood pressure remained elevated. The evoked hyperaemic response in the dura mater was reduced nonsignificantly by 4 mg/kg, i.v., L-NNA (15.8±2.05%), but higher doses (8 and 16 mg/kg, i.v.) significantly reduced the response (22.82±2.51%, $P<0.05$ and 36.28±4.37%, $P<0.01$, respectively; Fig. 2A). The nasal hyperaemic response was significantly inhibited by all three doses of L-NNA (4, 8, and 16 mg/kg, i.v.; 35.46±8.57%, $P<0.05$; 58.72±9.2%, $P<0.01$; and 89.99±8.94%, $P<0.01$, respectively; Fig. 2B).

Neither 7-NI nor 3Br-7NI caused significant changes in the resting mean arterial blood pressure, although a transient decrease, lasting for about 5–10 min, occurred. In the second group, 7-NI (20 mg/kg, i.v.) was given intravenously to determine the contribution of NO to the meningeal vasodilatation originating from sensory nerve stimulation. In these animals, meningeal vasodilatation was reduced by 49.85±6.53%, $P<0.01$ (Fig. 2A and B). In the third group, the meningeal and nasal hyperaemic response was reduced by 3Br-7NI (10 mg/kg, i.v.), a more potent inhibitor of neural NOS than 7-NI, causing a marked reduction in the meningeal and nasal blood flows (63.36±7.70%, $P<0.01$ and 78.28±8.75%, $P<0.01$, respectively) (Fig. 2A and B).

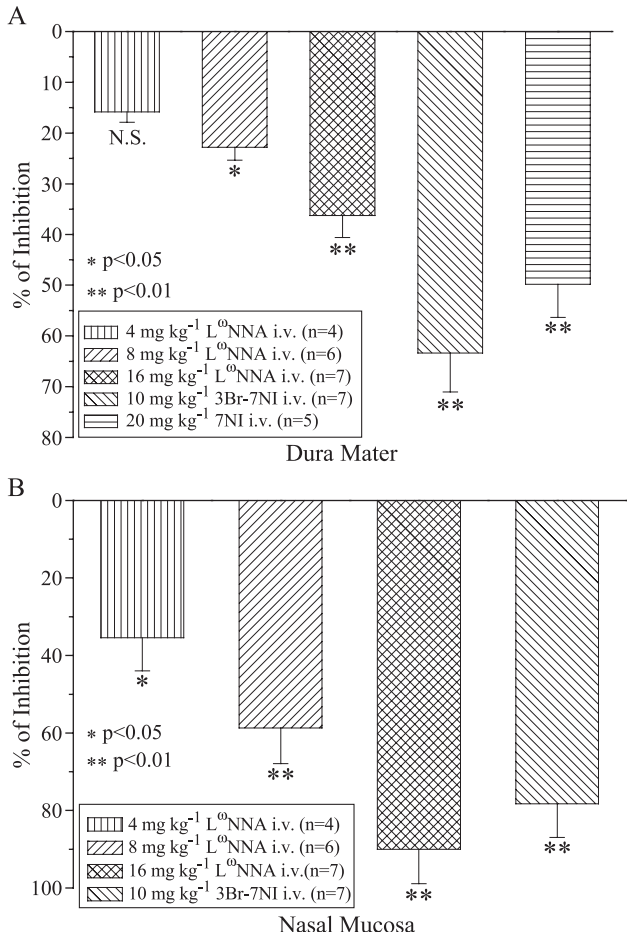


Fig. 2. Quantitative evaluation of the effect of different NOS inhibitors on (A) dural and (B) nasal vasodilatation evoked by electrical stimulation (15 V, 5 Hz, 0.5 ms, 20 s) of the trigeminal ganglion. Responses are expressed as percentage deviation from control values (mean±S.E.M.). *$P$<0.05, **$P$<0.01. N.S.: nonsignificant.

### *3.3. Effect of NOS inhibitors on meningeal plasma extravasation evoked by electrical stimulation of the trigeminal ganglion*

Electrical stimulation (25 V, 0.5 ms, 5 Hz, 5 min) of the trigeminal ganglion in the control animals induced ipsilateral plasma extravasation in the dura mater (Fig. 3A) and the upper eyelid (Fig. 3B). The enhancement, expressed as the ER, of the stimulated/nonstimulated sides was 1.80±0.8 and 4.63±1.24. In rats where the electrodes were lowered into the trigeminal ganglion without nerve stimulation, no significant extravasation was seen in either the dura mater or the upper eyelid (ER: 1.1±0.25 and 1.03±0.18, respectively).

Experiments were carried out to investigate the role of NO reflected in changes in the electrically evoked plasma extravasation. Neither NOS inhibitor (L-NNA: 16 mg/kg, i.v.; 3Br-7NI: 10 mg/kg, i.v.) reduced plasma extravasation evoked by electrical stimulation of the trigeminal ganglion (ER: 4.64±1.35 and 5.29±1.41, respectively).

## 4. Discussion

The role of NO in the pathogenesis of migraine and other types of vascular headache is well described. Workers in munitions factories may develop nitroglycerin-evoked headache or have migraine triggered (Laws, 1898; Trainor and Jones, 1966). Infusion of nitroglycerin can provoke headache in healthy volunteers, or migraine attack in migraineurs (Iversen et al., 1989; Thomsen et al., 1993). There is evidence that the release of neuropeptides from trigeminal sensory fibres is modulated by endogenous NO during neurogenic inflammation (Kajekar et al., 1995). There are a number of clinical and experimental studies showing that the NO donor sodium nitroprusside or glyceryl trinitrate activates the NO–cGMP pathway, leading to vasodilatation in the meningeal and/or cerebral arteries accompanied by migraine headache (Thomsen, 1997; Olesen et al., 1995; Iversen et al., 1989; Thomsen and Olesen, 2001).

In the present study, we demonstrated that neurogenic vasodilatation evoked by the electrical stimulation of the trigeminal ganglion in rats is mediated by NO. Furthermore, we have proven that NO is generated by nNOS, which does not play a role in the neurogenic plasma protein extravasation evoked by electrical stimulation of the trigeminal ganglion.

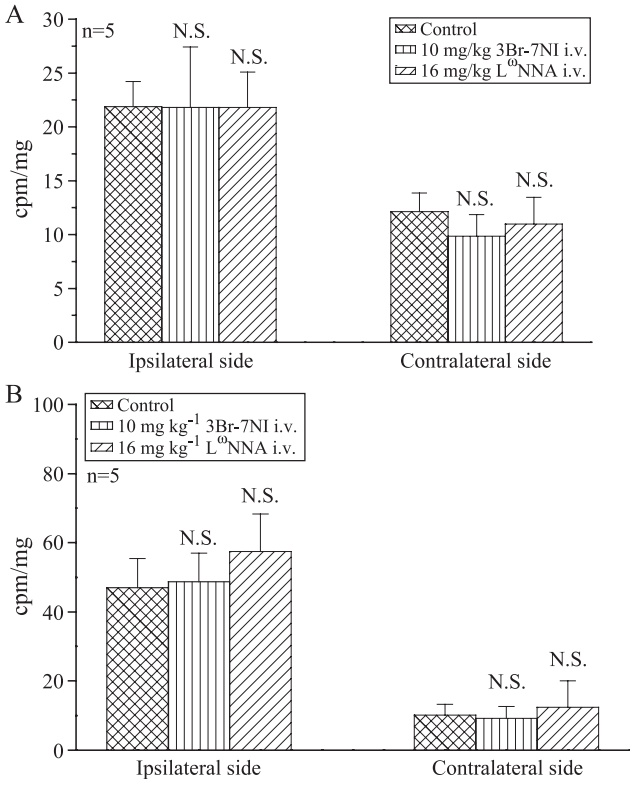


Fig. 3. Quantitative evaluation of the effect of 3Br-7NI (10 mg/kg, i.v.) and L-NNA (16 mg/kg, i.v.) on the plasma extravasation of the (A) dura mater and (B) the upper eyelid evoked by electrical stimulation of the trigeminal ganglion (15 V, 5 Hz, 0.5 ms, 20 s). Responses are expressed as percentage deviation from control values (mean±S.E.M.). *$P$<0.05, **$P$<0.01. N.S.: nonsignificant; cpm: count per minute.

To demonstrate the vasodilator effect of NO on the electrically evoked antidromic vasodilatation of the middle meningeal artery, we used nonspecific (L-NNA) and two neuronal NOS inhibitors (7-NI and 3Br-7NI). The autoregulation of intracranial blood flow follows the rule of Moore. Accordingly, elevation of blood pressure is accompanied by vasoconstriction to maintain the intracranial pressure. Nevertheless, the vasoconstriction evoked by the nonspecific NOS inhibitor induces a blood pressure elevation, which does not seem to play a role in the depressed vasorelaxant response since the specific neural NOS inhibitors 7-NI and 3Br-7NI also altered the evoked vasodilation, but they have an effect opposite to that of L-NNA on blood pressure and vessel diameter. Intravital microscopic study also demonstrated that the nonselective NOS inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) induced a transient decrease in the vessel diameter, which then returned to the preinjection level (Akerman et al., 2002). The existence of eNOS and nNOS raises the possibility that both of them are responsible for the production of NO. The observed inhibitory effect of 7-NI and 3Br-7NI on antidromic vasodilatation of the middle meningeal artery in our experimental set-up suggests that only the neuronal form of NOS is involved in this mechanism, since nNOS inhibitors were as effective as the nonspecific NOS inhibitor, L-NNA, in reducing the evoked vasodilator response to electrical stimulation of the trigeminal ganglion. This result is in accordance with the recent findings of Akerman et al. (2002); thus, our results support the hypothesis that NO participates in the neurogenically induced vasodilatation in the trigeminovascular system.

Previous studies (Rees et al., 1990; Palmer et al., 1988; Kajekar et al., 1995; Messlinger et al., 2000) demonstrated that intravenous administration of L-NAME— a nonselective inhibitor of NOS (Rees et al., 1990)—causes an elevation of resting arterial blood pressure, which is accompanied by a transient increase (lasting about 10 min) in meningeal as well as mucosal blood flow, whereas the blood pressure remains elevated for a long time. The ability of L-NAME to reduce the evoked vasodilatation of the middle meningeal artery and of L-arginine to restore the response indicates the role of NO in this vasorelaxant response. Our experiments suggest that the source of NO is the perivascular sensory nerve fibres. It has been shown earlier that 7-NI shows selectivity for the neural isoform of the enzyme (Silva et al., 1995). Moreover, 3Br-7NI was found to be more selective than 7-NI in rats (Bland-Ward and Moore, 1995). Recently, Dawson et al. (1991) have demonstrated that nNOS and neuronal NADPH-diaphorase are identical in brain and peripheral tissues, and numerous immunohistochemical studies carried out under physiological and pathophysiological circumstances have shown the existence of NADPH-diaphorase-positive nerve fibres along the axis of the wall of arteries in various vascular beds in several species, including the rat. Furthermore, NADPH-diaphorase-containing neurons are also present in the trigeminal ganglia (Rodella et al., 2000) and nerve fibres along the meningeal arteries of the rat (Berger et al., 1994). Santizo et al. (2000) have investigated the relative contribution of nNOS and eNOS to the modulation of intraischemic cerebral blood flow changes, using laser Doppler flowmetry in rats. They have found that nNOS, rather than eNOS, is the predominant producer of the NO liberated, especially during intraischemic vasodilatation in the cortex. In classic migraine (migraine with aura), intracranial blood flow is reduced during aura and initial headache is followed by vasodilatation (Olesen et al., 1990; Olesen, 1991). These observations and our results support the view that NO is involved in the pathogenesis of migraine headache and that NO is of trigeminal sensory neuron origin.

In the delayed phase of the migraine headache, starting 4–6 h after the acute attack, considerable plasma protein extravasation occurs within the meningeal tissues (Reuter et al., 2001). This dural plasma leakage at the postcapillary venules is due to activation of $NK_1$ tachykinin receptors (Shepheard et al., 1993), and SP is the main mediator of the neurogenic plasma extravasation in meningeal tissues (Markowitz et al., 1987). Infusion of the NO donor, nitroglycerin, can provoke not only acute (Iversen et al., 1989) but also delayed headache (Thomsen et al., 1993; Thomsen, 1997) and an inflammatory response in the dura mater (Reuter et al., 2001) accompanying the enhanced expression of iNOS in resident macrophages. Nonetheless, there are no available data supporting the relative contribution of NOS to the neurogenically evoked plasma protein leakage. Since, in our experiments, neither L-NNA nor 3Br-7NI was able to reduce the plasma protein extravasation in the meningeal tissues and in the upper eyelid in response to electrical stimulation of trigeminal sensory fibres, we assume that constitutive NOS does not participate in neurogenic oedema formation in these tissues; however, inducible NOS may play a role in the delayed inflammatory phase (Reuter et al., 2001).

Taken together, our results demonstrate that NO is involved in meningeal vasodilatation and that trigeminal sensory neurons are the site of NO production. Neural NOS has no role in neurogenic oedema formation in the meningeal tissues.

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