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Systemic administration of nitric oxide donor nitroglycerin as an experimental model for migraine headache in rats

Ph.D. Thesis

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List of abbreviations

5-HT - serotonin

CGRP- calcitonine gene-related peptide

COX-2 – cyclo-oxigenase 2

CSD – cortical spreading depression

Ir – immunoreactive

Lys-ASA – lysine-acetylsalicylate

NOS – nitric oxide synthase

nNOS – neuronal nitric oxide synthase

NO – nitric oxide

NOS – nitric oxide synthase

NSAID – non steroid anti-inflammatory drug

NTG - nitroglycerin

PAG –periaqueductal gray

PBS – phosphate buffered saline

PFA – paraformaldehide

s.c. - subcutaneous

SP – substance P

TNC – spinal portion of the caudal trigeminal nucleus

Original publications related to the PhD thesis

I. Párdutz Á, Krizbai I, Multon S, Vécsei L, Schoenen J (2000) Systemic nitroglycerin increases nNOS levels in rat trigeminal nucleus caudalis, *Neuroreport* **11**, 3071-5.
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II. Párdutz Á, Multon S, Malgrange B, Párducz Á, Vécsei L, Schoenen J (2002) Effect of systemic nitroglycerin on CGRP- and 5-HT-afferents to rat caudal spinal trigeminal nucleus and its modulation by estrogen, *Eur J Neurosci.* **15**, 1803-9.
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III. Párdutz Á, Szatmári E, Vécsei L, Schoenen J (2004) Nitroglycerin-induced nNOS increase in rat trigeminal nucleus caudalis is inhibited by systemic administration of lysine acetylsalicylate but not of sumatriptan, *Cephalalgia* (in press).
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Abstracts related to the PhD thesis

I. Párdutz Á, Vécsei L, Schoenen J Systemic nitroglycerin administration increases nitric oxide synthase-immunoreactive cells in rat spinal trigeminal nucleus: towards a better understanding of the possible role of nitric oxide in migraine?
Headache World, London, UK, 2000, *Cephalalgia* **20**: 281.

II. Párdutz Á,Vécsei L, Schoenen J Systemic nitroglycerin administration as an animal model for migraine headache
8th Congress of the Hungarian Headache Society, 2001, *Cephalalgia Hungarica* **8**:14.

III. Párdutz Á, Vécsei L, Schoenen J The effect of systemic nitroglycerin administration on 5-HT and CGRP innervation of the rat spinal trigeminal nucleus
IHC, New York, US, 2001, Cephalalgia **21**:400.

IV. Párdutz Á, Vécsei L, Schoenen J Nitric oxide donor nitroglicerin modifies the 5-HT and CGRP innervation in the rat spinal trigeminal nucleus
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V. Párdutz Á, Vécsei L, Schoenen J The possible role of nitric oxide in the pathogenesis of migraine.
9th Congress of the Hungarian Headache Society, 2002, *Cephalalgia Hungarica* **9**:13.

VI. Párdutz Á, Párducz Á, Vécsei L, Schoenen J Systemic nitroglycerin decreases CGRP-afferents to rat caudal spinal trigeminal nucleus, an effect modulated by estrogen
6th Congress of EFNS, Vienna 2002, *Eur J of Neurology* **9**:10.

VII. Párdutz Á, Párdutz Á, Vécsei L, Schoenen Systemic nitroglycerin increases 5-HT-afferents to rat caudal trigeminal nucleus , an effect modulated by estradiol
7th Congress of EFNS, Helsinki 2003, *Eur J of Neurology* **9**:10.

VIII. Párdutz Á, Párducz Á, Vécsei L, Schoenen Systemic nitroglycerin increases 5-HT-afferents to rat caudal trigeminal nucleus , an effect modulated by estradiol
7th Congress of EFNS, Helsinki 2003, *Eur J of Neurology* **9**:10.

IX. Párdutz Á, Vécsei L, Schoenen J Administration of lysine acetylsalicylate but not of sumatriptan inhibits nitroglycerin-induced nNOS increase in rat trigeminal nucleus caudalis
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Summary

Migraine is the most common neurological disorder which affects up to 14% of the total population. Despite recent and continuous advance in its research, the precise pathomechanism remains unclear.

One of the human models of migraine is the systemic administration of nitroglycerin, a nitric oxide donor. Nitroglycerin produces a rapid vasodilatation, which is probably responsible for an immediate headache that may occur in healthy subjects, but more often so in migraineurs. After a delay of several hours it also triggers typical attacks without aura in many migraine patients, but not in healthy volunteers.

Nitric oxide is for med from L-arginine by nitric oxide synthase and as an unconventional transmitter substance it can cross cell membranes rapidly without any specialized release machinery. The presence of one of its isoforms, the neuronal nitric oxide synthase in the superficial layers of the dorsal horn of the spinal cord suggests that nitric oxide has functions in sensory and pain processing. There is evidence that nitric oxide has a key role in hyperalgesia and sensitization of sensory neurons.

The cellular and molecular mechanisms of the delayed nitoglycerin-triggered migraine attacks are not known. Because of the involvement of the trigeminovascular system in vascular head pain, it has been suggested that this is the most likely target for nitric oxide in migraineurs. It has not been determined, however, whether nitric oxide would trigger a migraine attack via an effect on the pre- or on the postsynaptic arm of this system. The delay between nitroglycerin administration and the attack offers room for a number of central and peripheral actions of nitric oxide, including sensitization of peripheral trigeminal afferents or central sensitization similar to that shown for second order nociceptive neurons in the spinal cord. In any case, changes in nitric oxide activity may play a pivotal role in these processes and in a pilot trial it has been reported that a nitric oxide inhibitor was effective in the treatment of migraine attacks.

Serotonin$_{1D}$ receptors are located presynaptically on the distal and proximal portions of these afferents and their activation is thought to contribute to the anti-migraine effect of serotonin$_{1B/D}$ agonists, also called triptans. Non-steroidal anti-inflammatory drugs, such as acetylsalicylic acid are also effective in the treatment of acute migraine headache and tension-type headache.

Gonadal steroids, in particular estradiol, modulate the clinical expression of migraine. After puberty women are three times more affected. Abrupt falls in estrogen plasma levels can trigger the attacks, e.g. in the premenstrual phase, and they may disappear during pregnancy or after menopause, when plasma level of estrogen is stable. The neurobiological mechanisms which underlie these modulatory effects of estrogen on migraine remain speculative.

Calcitonin gene-related peptide, a key transmitter in primary nociceptive afferents, can be released in animals by nitric oxide-mediated mechanism and is increased in jugular blood during migraine attacks. After electrical stimulation of the Gasserian ganglion in rats, an animal model for migraine producing meningeal plasma extravasation, calcitonin gene-related peptide-immunoreactive fibers in dura mater undergo morphological changes suggestive of transmitter release. These calcitonin gene-related peptide changes in migraineurs and in rats are both reversed or prevented by serotonin$_{1B/D}$ agonists, the most effective acute anti-migraine drugs. Serotonergic mechanisms are pivotal in the control of nociception and in migraine pathogenesis.

There is experimental evidence for an effect of estrogen both on calcitonin gene-related peptide and serotonin neurotransmission.

In our study we found that systemic administration of the nitric oxide donor nitroglycerin after a delay of 4 hours increased the expression neuronal nitric oxide synthase and c-fos oncoprotein in the caudal trigeminal nucleus of the rat, an effect also seen after nociceptive stimulus, a subcutaneous injection of formalin. The most probable explanation for this phenomenon is the direct action of nitric oxide on the peripheral afferents of the trigeminal system. This may lead to a self amplifying process which may explain the central sensitisation recently discovered in migraine patients.

Contrary to lysine-acetylsalicylate the serotonin$_{1B/D}$ agonist sumatriptan pretreatment was not able to suppress the nitric oxide induced nitric oxide synthase activation. The lack of effect of sumatriptan in our study may therefore suggest that activation of nociceptive afferents has no role in the nitroglycerin-induced neuronal nitric oxide synthase increase or that serotonin$_{B/D}$ receptors are not capable to oppose such a chemical activation. Earlier studies on migraine headache have emphasized a possible role for serotonin$_{2B/C}$, or more likely for serotonin$_{2B}$ receptors, which mediate the release of nitric oxide from the endothelium. Recent experiments showed that serotonin$_{2A}$ receptor activation was able to enhance neuronal nitric oxide synthase expression in trigeminovascular neurons and

upregulation of platelet serotonin$_2$ receptors was reported by the same group in transformed migraine. Taken together these findings underline that, besides serotonin $_{1B/D}$, other receptor subtypes play a role in trigeminovascular pain, which could be an explanation for the lack of effect of sumatriptan in our model. An alternative explanation for the ineffectiveness of sumatriptan must be taken into account: its hydrophilicity and reduced ability to cross the blood-brain barrier. Lys-acetylsalicylate may also exert an anti-nociceptive effect by acting directly upon the periaqueductal gray matter. Taken together, these data and our present results suggest that the stimulating effect of nitroglycerin, and thus of nitric oxide, on neuronal nitric oxide synthase expression in secondary trigeminal nociceptors might be mediated by prostanoids and in particular by cyclo-oxygenase-2 expressing interneurons in the caudal trigeminal nucleus.

Four hours after the systemic nitroglycerin administration we found the reduction of calcitonin gene-related peptide in the superficial layers of the caudal trigeminal nucleus. The decrease of the area occupied by calcitonin gene-related peptide-immunoreactive afferents found here is thus likely a consequence of an increased release of calcitonin gene-related peptide due to the nitric oxide-mediated stimulation of trigeminal nociceptive Aδ and C afferents. We could also demonstrate a decrease in the size of calcitonin gene-related peptide-immunoreactive boutons which would support the hypothesis of an increased release of the peptide due to the nitroglycerin administration.

By the same token, the nitroglycerin-induced increase in serotonin-immunoreactivity in the same spinal gray areas may indicate a reduced release of serotonin from supraspinal afferents. It is not known if nitroglycerin would reduce serotonin release in laminae I-II because of a local interaction with serotonergic terminals or via a secondary modulation of the serotonergic descending pain control pathway. Whatever the mechanism might be, in both cases it seems to be selective for spinal trigeminal nucleus caudalis, as we found no detectable changes in superficial dorsal horn of the thoracic spinal cord.

Contrary to the acute changes in immunoreactivities occurring after nitroglycerin injection, those found after the experimental modulation of estradiol levels are on a different time scale and reflect slower plastic adaptations of neurotransmitter expression. Our results suggest that ovarian hormones, which greatly influence the course of migraine, have indeed the capacity to modify the expression of pivotal transmitters in the trigeminovascular nociceptive pathway. In animals with high estradiol levels, i.e. ovariectomized females treated

with 17ß-estradiol, calcitonin gene-related peptide innervation was clearly reduced and nitroglycerin produced no significant changes in the superficial layers of the caudal trigeminal nucleus, compared to animals with low estradiol levels, i.e. males and ovariectomized non-treated females.

17ß-estradiol treatment increased the serotonin expression in the above mentioned area compared to the male and ovariectomized female rats. In this case as well nitroglycerin produced no changes in the innervation area. It has been shown earlier that ovarian steroids increase the expression of tryptophane hydroxylase, the key enzyme for serotonin synthesis and decreases the re-uptake transporter of this transmitter.

It is not known if these data may be relevant for the hormonal influences in migraine. It is interesting, however, to draw a parallel between our immunocytochemical data in rats showing that estradiol is able to suppress activation of trigeminal afferents by nitroglycerin, a well known trigger of migraine attacks and the fact that the clinical picture of migraine improves during pregnancy when the estradiol levels are high and stable.

In our paper we established a reproducible animal model for trigeminal activation. Our finding could be relevant for understanding the pathomechanisms of migraine headache and the role of nitric oxide and ovarian steroids in the pathogenesis of headaches.

I. Introduction

Migraine headache is the most common neurological disorder, affecting up to 14% of the population (Raskin 1993, Fozard *et al.*, 1994, Saxena 1994, Moskowitz *et al.*, 1995). Despite recent advances, the exact pathogenesis of migraine is not fully understood (Saxena 1994). There is evidence that migraine is a neurovascular disorder with a complex interrelationship between neuronal and vascular mechanisms. Hence, although a number of highly effective symptomatic treatments have been developed, a succesful prevention remains to be achieved.

Numerous factors are putatively involved in the etiology of migraine such as susceptibility to particular stimuli (stress, nitrated foods etc) or changes within the central nervous system (Moskowitz *et al.*, 1995, Lance 1991). Several hypothesis have been put forward to account for the pain that accompany migraine, its maintenance and transmission from extracerebral tissues. Symptoms of central origin such as the abnormal sensory phenomena (experienced in migraine with aura), nausea and hypersensitivity to sensory stimuli are also typical features of migraine but their mechanisms remain unclear.

Gonadal steroids, in particular estradiol, modulate the clinical expression of migraine. After puberty women are three times more affected. Abrupt falls in estrogen plasma levels can trigger the attacks, e.g. in the premenstrual phase, (Somerville 1975) and they may disappear during pregnancy or after menopause, when plasma level of estrogen is stable (Marcus 1995; Silberstein & Merriam 2000). The neurobiological mechanisms which underlie these modulatory effects of estrogen on migraine remain speculative.

Recently, a decisive progress in the pharmacology of migraine has been achieved with the discovery of the involvement of nitric oxide (NO). It appears that the short-lived and ubiquitous molecule rediscovered as a major neurotransmission and pain regulator, may have a crucial role in migraine. Systemic administration of nitroglycerin (NTG), an NO donor, in migraineurs can trigger attacks without aura after a delay of several hours (Sicuteri *et al,.* 1987, Olesen *et al.*, 1993). Similarly to spontaneous migraine attacks, the NTG-induced attacks can be interrupted by antimigraine drug sumatriptan (Iversen *et al.*, 1996), but their precise neurobiological mechanisms are still unknown.

The throbbing pain experienced in migraine was first attributed to the pulsations of abnormally dilated vessels (Blau & Dexter 1981; Friedberg *et al.*, 1991). Nociceptive inputs generated from the pain-sensitive extracerebral vessels are then sent via the trigeminal ganglion and subsequently the spinal trigeminal nucleus caudalis (TNC) to higher centers involved in pain processing (Saxena 1994). A vascular origin of pain was also congruent with the possible release of vasoactive substances from the blood (such as 5-hydroxytryptamine (5-HT)) or the vessel wall (such as endothelial NO) (Fozard *et al.*, 1994).

Nevertheless vasodilatation per se could not account for a number of observations in migraine sufferers (Friberg *et al.*, 1991, Humphrey 1994) and a neuronal origin of the pain was suggested (Moskowitz 1993). According to this hypothesis vasoactive peptides such as calcitonine gene-related peptide (CGRP) and substance P (SP) released from the trigeminal afferents to the meningeal vessels produce not only vasodilation but a painful state of local inflammation which in turn, further stimulates perivascular nociceptive trigeminal fibers.

However administration of CGRP and SP alone is reportedly not able to trigger pain (Pedersen-Bjerregard *et al.*, 1991, Shekar *et al.*, 1991) and the possibility of involvement of other substances with a broader spectrum of actions such as NO, was suggested (Fozard *et al.*, 1994, 1989, Olesen *et al.*, 1995). In this respect a recent study demonstrated that NO, additionally to its vasodilatory effects, could elicit a direct activation of nociceptive trigeminal fibers (Wei *et al.*, 1992). Moreover a strong correlation between the ability of a number of drugs to induce migraine and their affinity at 5-HT_2 receptors putatively associated with endothelial NO release was also demonstrated (Fozard *et al.*, 1989, 1994). Hence NO appeared as a vascular substance with potent nociceptive effects on both vascular and neural tissues. Moreover studies proved that NO and the related cGMP system is capable to inhibit the activity of spinothalamic pathways via glycine and GABAergic mechanisms (Lin *et al.*, 1999).

However, it is noteworthy that the vascular wall is not the only possible site of release of NO within the meningeal tissues since the Gasserian ganglion contains NO neurons (Gulbenkian *et al.*, 1999). Neuronally produced NO has also been evidenced in cerebral cortex during cortical spreading depression (CSD) (Goadsby *et al.*, 1992, Wahl *et al.*, 1994, Read *et al.*, 1996), a mechanism that might underly the aura experienced by some migraine patients (Avioli *et al.*, 1991, Lauritzen 1994). CSD is a slow wave electrophysiological activity that occurs in response to physical cortical stimulation and propagates along the

cerebral cortex triggering the release of numerous neurochemicals and changes in the tone of extracerebral vessels (Lauritzen 1994) in which NO has been directly implicated (Wahl *et al.*, 1994). Taken together, these studies show that NO can be released in multiple structures directly involved in migraine and affect both vascular and neural tissues. It is therefore in a position to play a critical role in the sequential development of the migraine crisis, first being released during CSD and later on during the events associated with migraine pain.

The strongest evidence for the key-role of NO in the etiology of migraine stems from observations in migraine sufferers and led to the "NO hypothesis of migraine" put forward by Olesen and colleagues (1995). The administration of the NO donor NTG consistently elicited migraine in susceptible suspects (Sicuteri *et al.*, 1987, Thomsen *et al.*, 1994, Olesen *et al.*, 1993, 1995). This effect is due to the release of NO which is the only known biological effect of NTG. The role of NO is also supported by the fact that other drugs that are able to induce migraine such histamine, reserpine or the serotoninergic antagonist mCPP all have in common to be associated with the release of endogenous nitric oxide (Olesen *et al.*, 1995, Fozard *et al.*, 1994).

The infusion of NTG in human elicits an immediate short lasting headache that can be attributed to the rapid vasodilatory effect of NO on extracerebral arteries (Iversen *et al.*, 1989, Thomsen *et al.*, 1994, Olesen *et al.*, 1995). Contrary to healthy subjects, in migraine patients and subjects with family history of migraine, the initial headache is followed by the incidence of a genuine migraine, mimicking the features of a spontaneous attack except the aura (Olesen *et al.*, 1993, Thomsen *et al.*, 1994, Olesen *et al.*, 1995). It is characteristically accompanied by symptoms of central origin such as nausea and photophobia. Surprisingly in the view of short half-lives of NTG (Murad 1990) and NO (Kelm 1999) in vivo, the migraine occurs several hours after NTG infusion (Thomsen *et al.*, 1994). Thus it appears that NO is a cause of migraine through mechanisms that develop over a long period of time. This is consistent with the possibility of a delayed and sustained production of NO by nitric oxide synthases (NOS) in a large number of tissues (Moncada *et al.*, 1991, Forstermann *et al.*, 1995). The importance of endogenous NO production during the headache phase of migraine has been evidenced in a recent study where the inhibition of NOS relieved the symptoms of spontaneous migraine with high efficacy (Lassen *et al.*, 1997). Overall these studies suggest that NO has not only a rapid effect in vasodilation and pain but is also critically involved in the long-term development of genuine migraine crisis.

Due to its high lipophilicity, NO can readily cross the blood brain barrier and its action is primary limited by its very short half-life (Kelm 1999). NO-induced vasodilation is primarily attributed to its local release from the vascular endothelium to the adjacent smooth muscle where it triggers the production of cGMP and muscular relaxation (Monacada *et al.*, 1991). NO has also been shown to elicit a direct and immediate activation of trigeminal nerve terminals afferent to extracerebral vessels (Wei *et al.*, 1992). NO has also been involved in longer lasting effects. A large number of studies show that NO can elicit long term changes in nociception in particular in the spinal cord where it plays an important role in the development of hyperalgesia (Urban *et al.*, 1999). Interestingly sensitisation of primary afferents –which is an important trigger of the development of hyperalgesia- has been demonstrated in trigeminal meningeal afferents (Strassmann *et al.*, 1996). Further, cellular activation within the caudal trigeminal nucleus has been shown to be mediated by trigeminal fibers after CSD (Mostkowitz *et al.*, 1993) or noxious stimulation (Nozaki *et al.*, 1992). Moreover a recent study demonstrated that the activation of the trigeminovascular system in response to pain can be markedly reduced by a NOS inhibitor (Hoskin *et al.*, 1999). It thus appears possible that NO might mediate the development of a supersensitivity to pain within the trigeminal ganglion and the TNC. In support to this view, NTG administration in the rat was also shown to elicit the activation of pain-mediating TNC neurons (Tassorelli *et al.*, 1995, 1997). This effect was maximal after a delay of 4 hours, congruent with the delay of NTG induction of migraine in human (Thomsen *et al.*, 1994, Olesen *et al.*, 1995). Noticeably in the animal model a large number of the activated neurons also exhibited NOS immunoreactivity (Tassorelli *et al.*, 1995).

There is no doubt that NO and one of the three isoforms of NOS, the neuronal nitric oxide synthase (nNOS) play an important role in the sensory system especially in nociception (Saito *et al.*, 1994, Lin *et al.*, 1999). nNOS is also present in the trigeminal system suggesting involvement in pain processing (Dohrn *et al.*, 1994). NOS inhibitors reduce c-fos activation by nociceptive stimuli in the dorsal horn in rats (Wang *et al.*, 1999, Wu *et al.*, 2000) and have produced an anti-migraine effect in a preliminary study (Lassen et al. 1998). Activation of the trigeminovascular sytem in response to pain can be markedly reduced by the administration of a NOS inhibitor (Ackerman *et al.*, 2002). Thus the study of NO long-term effects within the structures relevant to migraine may provide critical information for the understanding of the

neurovascular events related to the initiation of the crisis and the developement of migraine headache.

Anti-migraine drugs can also play an important role in the action of NO on the trigeminal system. The so called triptans are 5-$HT_{1B/D}$ receptor agonists which are the most effective treatment available. NO may act on peripheral trigeminal afferents thus these drugs may have a modulatory effect on its action. Non-steroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (Aspirin®), are also effective in the treatment of acute migraine headache (Lange *et al.*, 2000) and tension-type headache (Schoenen *et al.*, 2000). This effect could be due to their inhibitory action on cyclo-oxygenase 2 (COX-2) and prostaglandins in the spinal trigeminal complex (Yaksh *et al.*, 2001), but they could also inhibit NOS activation by reducing the induction of transcription factor NF kappa ß (Ma *et al.*, 1998). Concordantly, it was shown that pretreatment with indomethacin reduces the NTG induced c-fos activation in the caudal trigeminal nucleus (Tassorelli *et al.*, 1997).

CGRP, a key transmitter in primary nociceptive afferents, can be released in animals by NO-mediated mechanism (Garry *et al.*, 2000) and is increased in jugular blood during migraine attacks (Goadsby *et al.*, 1990). After electrical stimulation of the Gasserian ganglion in rats, an animal model for migraine producing meningeal plasma extravasation (Buzzi & Moskowitz, 1992), CGRP-immunoreactive (Ir) fibers in dura mater undergo morphological changes suggestive of transmitter release (Knyihar-Csillik *et al.*, 1995, 2000). These CGRP changes in migraineurs and in rats are both reversed or prevented by serotonin $(5\text{-HT})_{1B/D}$ agonists, the most effective acute anti-migraine drugs. Serotonergic mechanisms are pivotal in the control of nociception (Roberts 1984; Fashmer *et al.*, 1985) and in migraine pathogenesis (Sicuteri 1972; Ferrari *et al.*, 1989).There is experimental evidence for an effect of estrogen both on CGRP and 5-HT neurotransmission. Estradiol reduces the CGRP content in cervical spinal cord (Moussaoul *et al.*, 1996) and the number of CGRP-Ir neurons in sensory ganglia (Yang *et al.*, 1998). It also increases the expression of tryptophan hydroxylase, the rate-limiting enzyme of 5-HT synthesis, and of its mRNA in the raphe nuclei (Pecins-Thompson *et al.*, 1996; Lu *et al.*, 1999; Bethea *et al.*, 2000), while it decreases that of the serotonin re-uptake transporter (Pecins-Thompson *et al.*, 1998; Rehavi *et al.*, 1998).

As CGRP and 5-HT are involved in migraine pathogenesis and both are influenced by estrogen, they could be pivotal for a better understanding of the hormonal influences and other trigger factors in this disorder.

II. Aims

The aims of our studies were to

i.) determine the effect of systemic NTG administration on the nNOS expression of the caudal trigeminal nucleus and compare the effect to a experimental somatic nociceptive stimulus, s.c. formaline administration.

ii.) examine the possible modulatory effects of 5-$HT_{1B/D}$ agonist sumatriptan and lysine-acetylsalicylate (Lys-ASA) in the above process.

iii.) study the effect of NO on the CGRP and 5-HT innervation of the caudal trigeminal nucleus and check the possible effects of gonadal steroids.

III. Materials and methods

All experimental procedures described in this paper followed the guidelines of the International Association for the Study of Pain and the European Communities Council s (86/609/EEC). They were approved by the Ethics Committee of the Faculty of Medicine, University of Liège. The animals were raised and maintained in standard laboratory conditions with tap water and regular rat chow available ad libitum on a 12h:12h dark-light cycle.

III.1. nNOS

Animals: Thirty adult male Wistar rats (weight 250-350 g) were used. In the first group 8 animals received a single subcutaneous (s.c.) injection of NTG (prepared from Nitrolingual® Pumpspray, Pohl-Boskamp Co GmbH, Hohenlockstedt, Germany) at a dose of 10 mg/kg b.w.; 8 animals received a s.c. injection of the vehicle of the drug (gift from Pohl-Boskamp). Four hours later, the rats were deeply anesthetized with sodium-pentobarbital (Nembutal®, 80 mg/kg b.w) and transcardially perfused with 100 ml of physiological saline followed by 500 ml of 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The

cervical (C1-C2) and thoracic (Th1) spinal cord was removed. The tissue blocks were postfixed overnight for immunohistochemistry.

The second group of animals consisted of 6 rats which received NTG (n=3) or placebo (n=3) according to the same procedures. Four hours later, they were deeply anaesthetized and transcardially perfused with 200 ml of physiological saline. Cervical (C1-C2) and thoracic (Th1) spinal cords were removed and freezed in liquid nitrogen for western blotting.

Eight animals received s.c. injection of formalin (50 µl, 4%) in the right upper lip. Four hours later the animals were anaesthetized and transcardially perfused like the rats in the first group. The brainstem and upper cervical cord were removed, postfixed overnight and processed for nNOS and c-fos immunohistochemistry.

Immunohistochemistry: After cryoprotection (30% sucrose overnight), 30µm thick cryostat sections were cut and serially collected in 16 wells containing cold PBS. Each well received sections at a 0.5 mm distance throughout the rostrocaudal extent of the C1-C2 and Th1 segments and the caudal brain stem. After a pretreatment with 0.3% H_2O_2, the free floating sections were rinsed several times in 0.1 M PBS containing 1% Triton X-100. Then samples from cervical and thoracic spinal cord and the brain stem were kept for 2 nights at 4°C in polyclonal nNOS (Euro-Diagnostica AB, Malmö, Sweden n°.: B 220-1) antibody, at a dilution of 1:20000. Sections from the cervical spinal cord and the brain stem were incubated with c-fos antibody (Santa-Cruz Biotechnology, n°.: sc-52-G) at a dilution of 1:3000 for 3 nights at 4°C. The immunocytochemical reaction was visualized using the avidin-biotin kit (ABC) of Vectastain (Vector Laboratories Inc. Burlingame, Ca, USA, n°.: PK-6101) and staining with nickel-ammonium-sulfate-intensified 3′,3′-diaminobenzidine. Specificity of the immune reactions was controlled by omitting of the primary antiserum.

Western blotting: Dorsal part of the spinal cord segments (C1-C2 and Th1) were homogenised in cold Tris-HCl buffer (50 mM , pH=7.4). Protein concentration was measured according to Bradford using BSA as a standard (Bradford 1976). Equal amounts of protein samples (20 µg/lane) were separated by standard SDS-PAGE procedures at 200V for 1 hour and transferred to immobilon P membrane (Millipore, Billerica, Mass, USA). Following the transfer and blocking in 5% non-fat dry milk, membranes were incubated with the nNOS antibody (Transduction Laboratories n°.: N31020), diluted to 1:1000. Protein bands were visualized using the ECL Western blotting analysis kit (Amersham Biosciences AB, Uppsala,

Sweden). The quantitative analysis was performed using a laser densitometer (Pharmacia LKB, Uppsala Sweden).

Data analysis: nNOS- and c-fos-positive cells were counted by an observer blinded to the procedures in the laminae I-III of the cervical spinal cord, in three different series of sections in each animal. The individual sections in these series were taken at 0.5 mm distance along the rostrocaudal axis. nNOS-positive neurons were scored, if they contained cytoplasmic and dendritic staining and a nucleus. In case of c-fos the intensively stained nuclei were counted. In the thoracic segment, nNOS-Ir cells were counted in laminae I-III in ten sections, in each animal. In each experimental group normal distribution was checked by means of the Kolmogorov test. Since the hypothesis of a normal distribution was not rejected, Student's t test was used to determine the significance of differences between the mean values of data groups. In the animals, which received s.c. formalin, c-fos and nNOS positive cells were also counted on the ipsi- and contralateral sides of the caudal trigeminal nucleus in the brainstem (three different series per animal) of sections being 0.5 mm apart. The difference between the injected and control side for each section was calculated and analyzed with the paired Student's t-test. The relative optical densities from western blotting, were compared with the Student's t test.

III.2. Sumatriptan and Lys-ASA pretreatment

Animals: Seventy-eight male Wistar rats (250-350 g) were used. For immunohistochemistry, 48 animals were divided into three groups of 16. In the first group the rats did not receive any pretreatment. In the second group the rats received a subcutaneous (s.c.) injection of sumatriptan (Imitrex®, Glaxo Smith Kline, Research Triangle Park, NC, USA) at a dose of 0.6 mg/kg. The animals of the third group were injected intramuscularly with lys-ASA (Aspegic®, Sanofi-Synthelabo, Paris, France) at a dose of 50 mg/kg. Sumatriptan was diluted in physiological saline, lys-ASA in its commercially available solvent (distilled water). Ten minutes later in each group 8 animals received a s.c. injection of NTG (prepared from Nitrolingual®) at a dose of 10 mg/kg and 8 animals received a s.c. injection of the vehicle).

Four hours after the NTG or placebo injections the rats were deeply anaesthetised by pentobarbital (Nembutal® 80 mg/kg) and transcardially perfused with 100 ml physiological saline followed by 500 ml 4% PFA in PBS. The portions of cervical spinal cords comprised between –5 and –11 mm from the obex were removed and postfixed overnight for immunohistochemistry.

For Western blotting 30 rats were divided in three groups of 10 and received pretreatment and NTG or placebo injections as above. Four hours later, they were deeply anaesthetized and transcardially perfused with 200 ml of physiological saline. Cervical spinal cords between –5 and –11 mm from the obex were removed and frozen in liquid nitrogen for Western blotting.

Immunohistochemistry: The protocol was the same as described above for nNOS staining.

Western Blotting: The dorsal portions of spinal cord segments were homogenized in cold Tris-HCl buffer (50 mM, pH=7.4). Protein concentration was measured according to Bradford (1976) using BSA as a standard. Equal amounts of protein samples (20 µg/lane) were separated by standard SDS-PAGE procedures at 200 V for 1 hour and transferred to immobilon P membrane (Millipore, Billerica, Mass, USA). Following the transfer and blocking in 5% non-fat dry milk, membranes were incubated with a nNOS antibody (Transduction Laboratories, N31020), diluted to 1:1000. After the detection of nNOS protein the membranes were stripped and reprobed for ß-actin antibody (Sigma Chemical Co., St. Louis, Mo, USA A5441) diluted to 1:4000, which was used as an internal control. Protein bands were visualized using the ECL Western blotting analysis kit (Amersham Biosciences AB, Uppsala, Sweden). They were quantitatively analysed using a laser densitometer (Pharmacia LKB, Uppsala, Sweden). Optical densities of specific bands were quantified by densitometry and corrected for protein loading by dividing by the ß-actin signal of the same sample.

Data analysis: nNOS-positive cells were counted by an observer blinded to the procedures in laminae I-III of the cervical spinal cord, in three different series of sections in each animal. The individual sections in these series were taken at 0.5 mm distances along the rostrocaudal axis. nNOS-positive neurons were scored, if they contained cytoplasmic and dendritic staining and a nucleus. The cell counts of nNOS and of relative Western blot optical densities were analysed with multiple variance analysis (ANOVA) and post hoc test Scheffe.

The statistical tests were performed by StatWiew (Version 4.57 for Windows, Abacus Concepts Inc., Berkley, NC, USA). Significance level was set at $p<0.05$.

III.3. CGRP and 5-HT

Animals : Sixteen male and 32 female Wistar rats (250-350 g) were used At the age of 2 months, the female animals (n=32) were ovariectomized under Nembutal° anaesthesia and half of them had a 5 mm long Silastic capsule (3.18 mm OD and 1.57 mm ID; Dow Corning, Midland, Mich, USA) filled with a 1:1 mixture of cholesterol (Sigma Chemical Co., St. Louis, Mo, USA) and 17ß-estradiol (Fluka, Buchs, Switzerland) inserted subcutaneously in the interscapular region. The capsules maintain estradiol plasma levels in a range that is typical of those found in female rats at early proestrus. (Smith *et al.*, 1977) .

At the age of 3 months, half of the animals in all three groups (8 males, 8 ovariectomized females - ovx, 8 ovariectomized females treated with estradiol - ovx+E_2) received a subcutaneous injection of NTG (prepared from Nitrolingual° spray, Pohl-Boskamp GmbH, Hohenlockstedt, Germany) at a dose of 10 mg/kg. The other half received an injection of the vehicle (gift from Pohl-Boskamp GmbH) in the same location. Four hours after NTG or vehicle injections, the rats were deeply anaesthetised with pentobarbital (Nembutal° 80 mg/kg; Sanofi-Synthélabo, Paris, France) and transcardially perfused with 100 ml physiological saline followed by 500 ml 4% paraformaldehyde in phosphate-buffered saline. The cervical (C1-C2) spinal cords from all animals, as well as the thoracic (Th1) cord from males, were removed and postfixed overnight for immunohistochemistry.

Immunohistochemistry: The sections of the C1-C2 and Th1 segments were obtained as described earlier. After pretreatment with 0.3% H_2O_2, the free-floating sections were rinsed several times in 0.1 M phosphate-buffered saline containing 1% Triton X-100 and then kept for 2 nights at 4°C in polyclonal anti-CGRP (Amersham Biosciences AB, Uppsala, Sweden, RPN. 1842) or anti-5-HT (DiaSorin Inc., Stillwater, Mn., USA, 20080) primary antisera at respective dilutions of 1:20000 and 1:200000. The immunocytochemical reaction was visualized using the Vectastain° (Vector Laboratories Inc., Burlingame, Ca, USA, PK-6101) avidin-biotin kit (ABC) with nickel-ammonium-sulfate intensified 3′,3′-diaminobenzidine

(Sigma Chemical Co., St. Louis, Mo, USA). Specificity of the immune reactions was controlled by omitting the primary antisera.

Data analysis: The area covered by CGRP- and 5-HT-Ir fibres in laminae I-II of the cervical and thoracic dorsal horns was determined by video imaging using an Image Pro Plus 4.0 image analysis software (Media Cybernetics, Silver Spring, Md, USA). Stained sections were examined under bright field with an Olympus microscope and a 10x objective. Images were recorded with a SONY 950-P CCD camera (Sony Corp., Japan) and transmitted to the frame grabber (Flashpoint 128; Integral Technologies, Inc., Indianapolis, Ind, USA) which converts the image into a digital matrix of 1600 X 1200 pixels. After image acquisition, a threshold gray level was established in order to detect Ir fibers in the digitized microscopic image, the so-called discrimination step. To avoid the subjective bias of manual thresholding, the threshold was determined on the basis of the density histogram displayed by the program. It was set on the point where the flat part of the histogram (pixels with high densities) started to raise steeply. The program expressed the area innervated by the Ir fibers as number of pixels having densities above the threshold. For the calibration we measured known areas of different shapes. Using sections from the thoracic spinal cord processed in parallel we tested reproducibility of measurements in homologous areas of the dorsal horns. Measurements were taken in a blinded fashion from at least 16 sections for each staining in each animal group and averaged.

The size of immunoreactive boutons was measured by the same digital system using a 40x objective. At this high magnification different optical planes of the same section could be examined. For the determination of the cross-sectional areas we selected boutons which were in focus and were recognized and measured by the program as single objects. In each experimental group 450-500 boutons were analyzed.

Generalized linear models were used to compare the data in function of group and treatment. Within each of the 3 groups of animals (males, ovx and ovx+E_2) differences between NTG and vehicle treatment were analyzed with Student's t test. The analyses were performed by the software SAS (Version 6.12 for Windows, SAS Institute, Cary, NC, USA). Significance level was set at $p<0.05$.

IV. Results

IV.1. nNOS

Transverse sections of the cervical spinal cord demonstrated prominent nNOS-IR neurons in the dorsal horn Laminae I-III and some cells in the Lamina X. In the superficial dorsal horn, small to medium size neurons (8-15 μm diameter) with few dendrites were most common. The nNOS-Ir neurons located around the central canal were multipolar and of medium to large size (15-30 μm). There was no significant difference in the number of NOS positive cells at different levels of the C1-C2 region. In animals sacrificed four hours after s.c. NTG injection we found a significant increase ($p<0.001$) in the number of nNOS positive neurons as compared to vehicle treated animals (Figure 1A, 1B). By contrast, we found no difference in the number of nNOS-Ir neurons between the two groups of animals in the thoracic spinal cord. The results of the statistical analysis are shown in Figure 2.

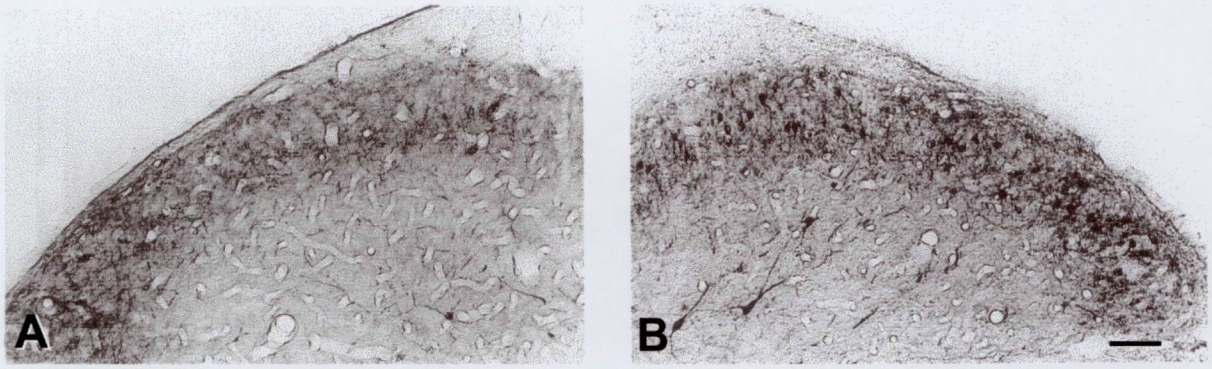

Figure 1. nNOS-immunoreactivity on transverse sections of the upper cervical spinal cord in placebo- (A) and NTG-treated (B) rats. The number of immunoreactive cells is increased 4 hr after s.c. NTG (10 mg/kg). Scale bar = 50μm.

Western blot analysis of the C1-C2 region confirmed the results obtained by immunohistochemistry. We could identify a band at 155 kDa characteristic for the nNOS protein. In animals, which had received NTG 4 hours before, the density of the nNOS protein band was higher in C1-C2 segments, but not in the Th1 segment (Figure 3). Densitometric analyses confirmed that the nNOS band on western blots was significantly enhanced after NTG administration in cervical (control: 103.7±2, NTG-treated: 149.7±5.8, $p<0.01$), but not in thoracic segments (control: 107±4.3, NTG-treated: 116.7±4.4, n.s.).

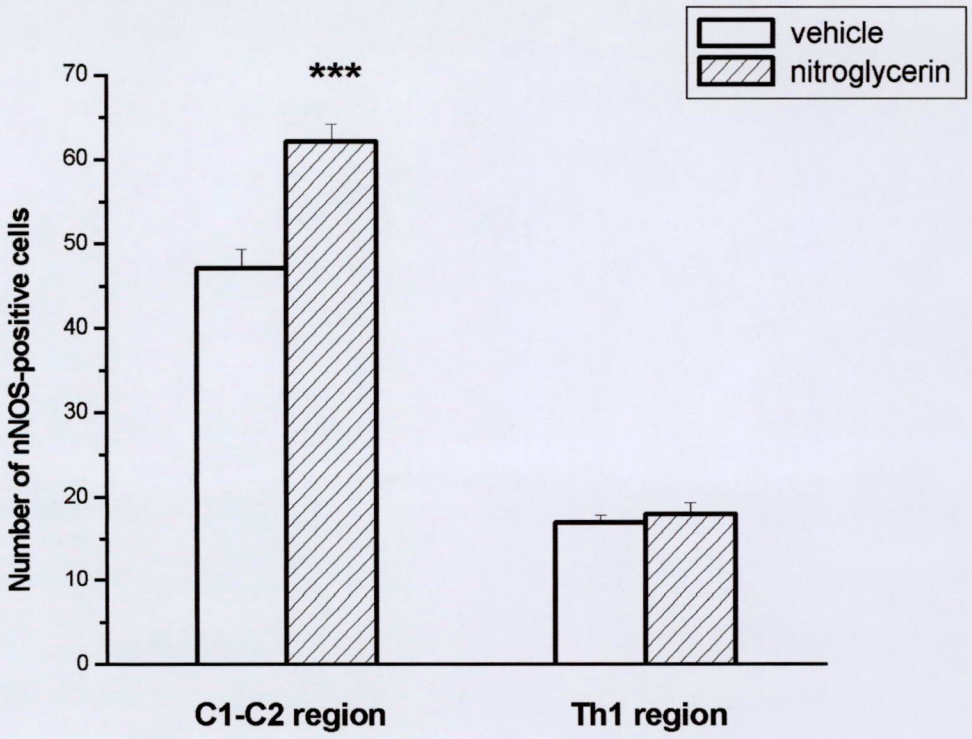

Figure 2. Histogram illustrating the number of nNOS immunoreactive neurons in the cervical and thoracal segment of the rat spinal cord. (mean ± SEM; n=8) *** $p < 0{,}001$

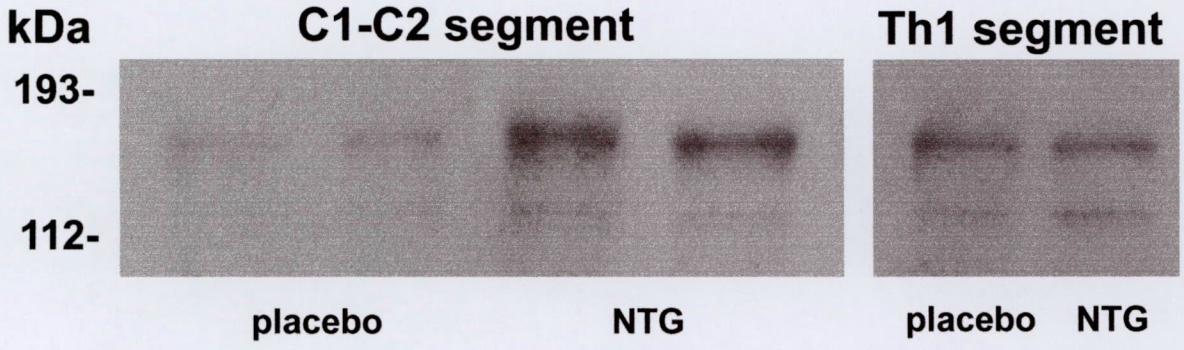

Figure 3. Western blotting of nNOS in the cervical and thoracic regions of rat spinal cord. After s.c. NTG (10 mg/kg) treatment nNOS levels (155 kDa) are increased in the C1-C2 segment, but not in the Th1 segment.

In parallel with the nNOS detection we have also performed c-fos immunostaining in the C1-C2 segment of the spinal cord. c-fos immunopositive cells were identified in dorsal horns; their number was significantly higher in NTG-treated than in placebo-treated animals (Figure 4A, 4B, Figure 5).

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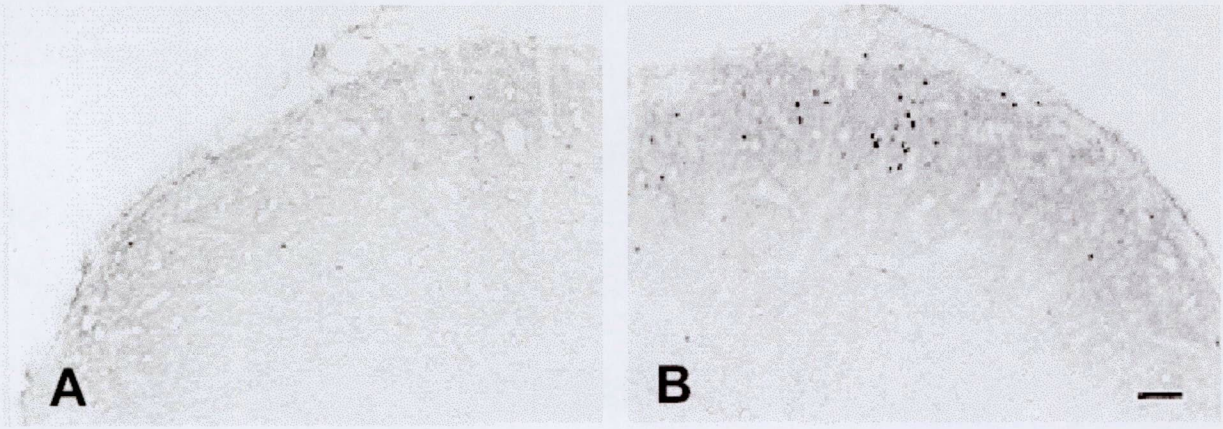

Figure 4. c-fos immunorectivity in transverse section of the upper cervical dorsal horn. The number of immunoreactive cells is increased 4 hours after s.c. NTG (10 mg/kg) (B), compared to placebo (A). Scale bar: 50 μm.

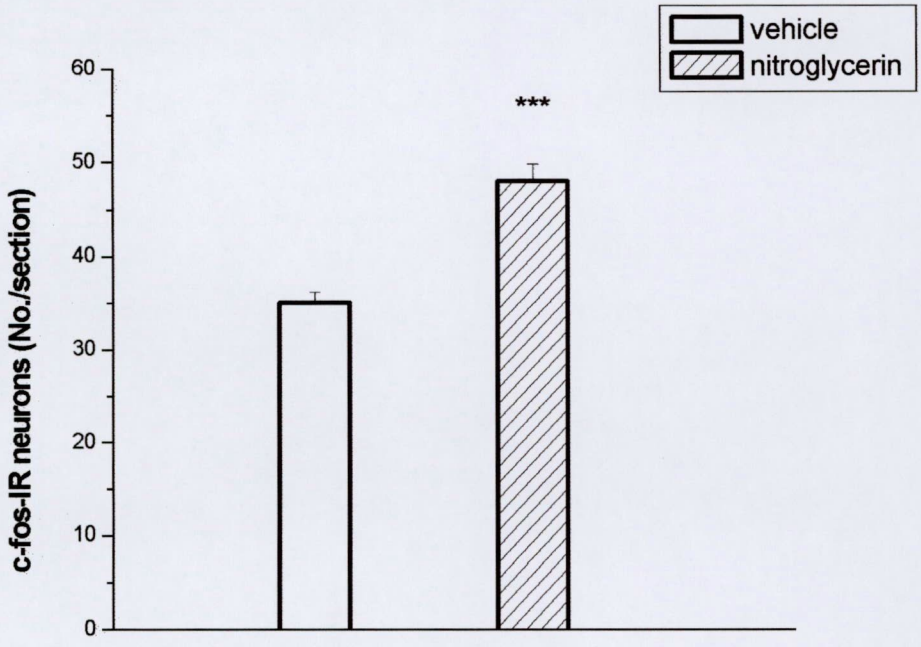

Figure 5. Histogram illustrating the significant difference between NTG and placebo treated rats in number of c-fos imunoreactive neurons per 30 μm transverse sections in the cervical dorsal horn. (Mean ± SEM; n=8), *** p<0,001

In animals which were given a s.c. formalin injection in the lip, the number of both nNOS (Figure 6A) and c-fos (Figure 6B) positive cells in the caudal trigeminal nucleus increased ipsilaterally to the injection in the appropriate segment of the cervico-medullary junction.

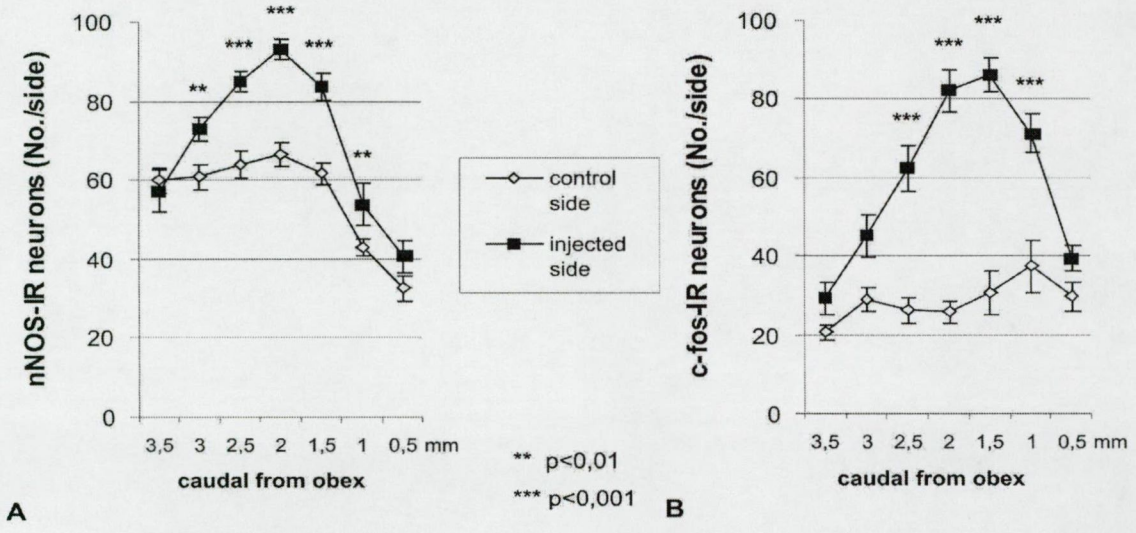

Figure 6. Histogram showing the number of nNOS (A) and c-fos (B) immunoreactive neurons in the caudal trigeminal nucleus in 30 μm transverse sections at increasing caudal distances from obex, ipsi- and contralaterally to a s.c. formalin injection in the lip. (Mean ± SEM; n=8)

IV.2. Sumatriptan and Lys-ASA pre-treatment

A similar increase in the number of nNOS immunoreactive neurons was observed in the sumatriptan-pretreated group of animals after NTG treatment compared to the placebo. Lys-ASA pretreatment, on the contrary, abolished the NTG effect on nNOS-positive neurons (Figure 7C,D and E,F, Figure 8).

Western blot results of the spinal cord segments are in line with the data of the immunohistochemistry. In animals, which had received NTG 4 hours before, the density of the C1-C2 nNOS protein band was increased compared to vehicle-injected rats. This increase was similar in control and sumatriptan pretreated groups. After pretreatment with lys-ASA, the nNOS band was comparable after NTG or vehicle injection (Figure 9). Densitometric analyses of the bands (corrected for protein loading) confirmed the results of the visual inspection of the Western blots: relative to vehicle injections, the optical density of the nNOS band was significantly enhanced after NTG administration with or without sumatriptan pretreatment, but not when the NTG injection was preceded by a pretreatment with lys-ASA (Figure 10).

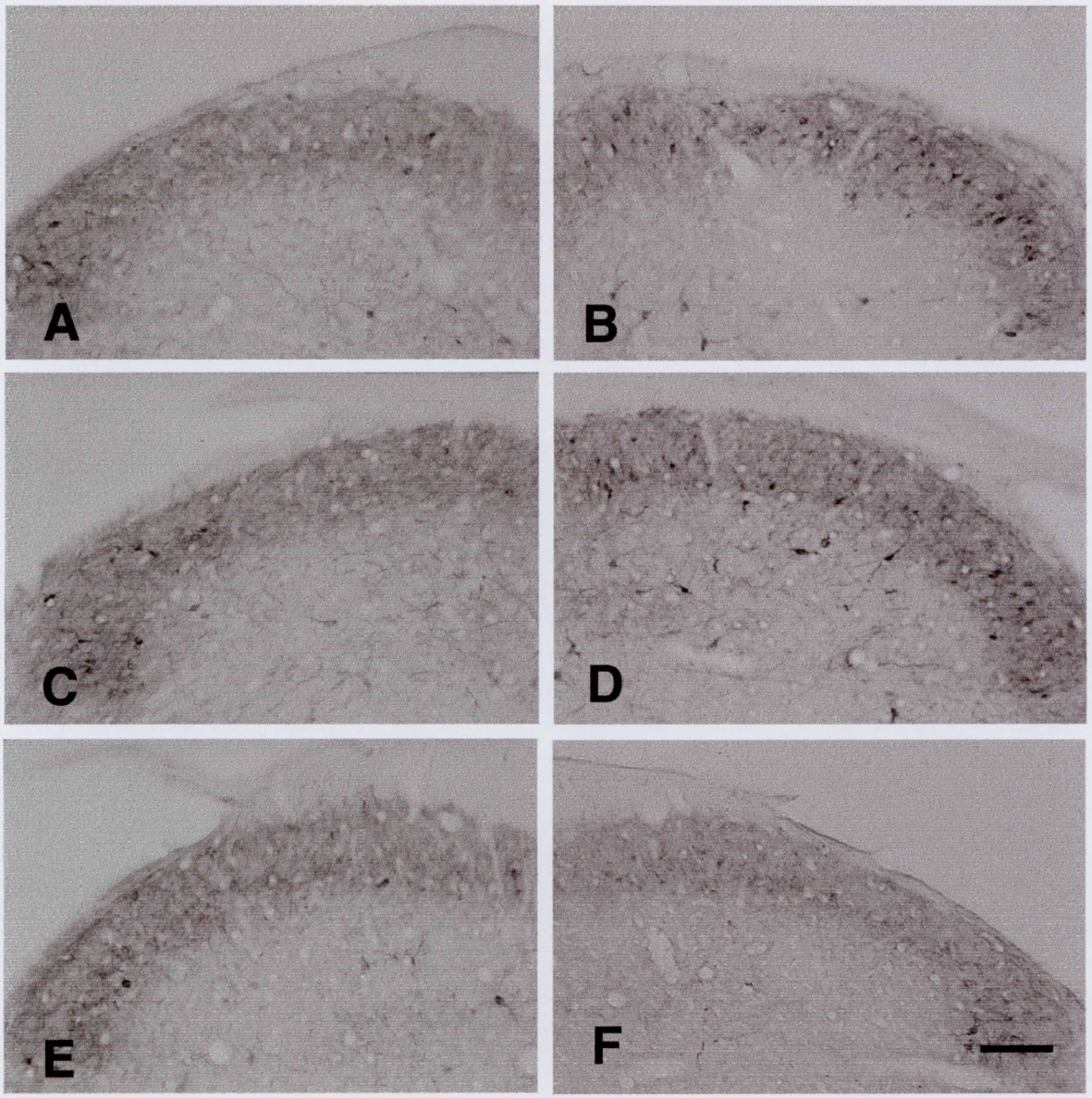

Figure 7. nNOS-immunoreactivity on transverse sections of the upper cervical spinal cord in non pretreated (control) (A,B), sumatriptan-pretreated (C,D) and lysine-acetylsalicylate-pretreated (E,F) rats. NTG administration (B,D,F) compared to vehicle (A,B,C) increases the number of nNOS-immunoreactive cells in controls and after sumatriptan pretreatement (B,D) but not after lysine-acetylsalicylate-pretreatment (E,F). Scale bar = 50μm.

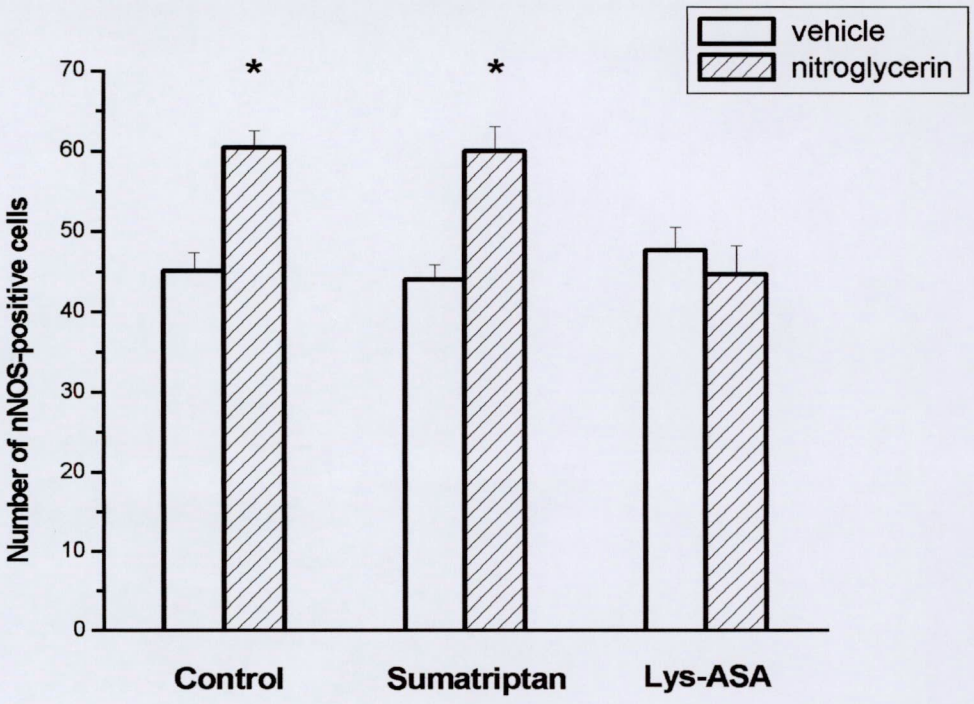

Figure 8. Histogram showing the mean number of nNOS-immunoreactive cells in the superficial layers of the C1-C2 dorsal horns in the 3 animal groups 4 hours after subcutaneous injection of vehicle (light bars) or NTG (hatched bars) (mean+S.E.M, n=8 per group). In control and sumatriptan-pretreated animals there is a significant increase of nNOS-immunoreactive cells (* $p<0.05$) but not in lysine-acetylsalicylate pretreated rats.

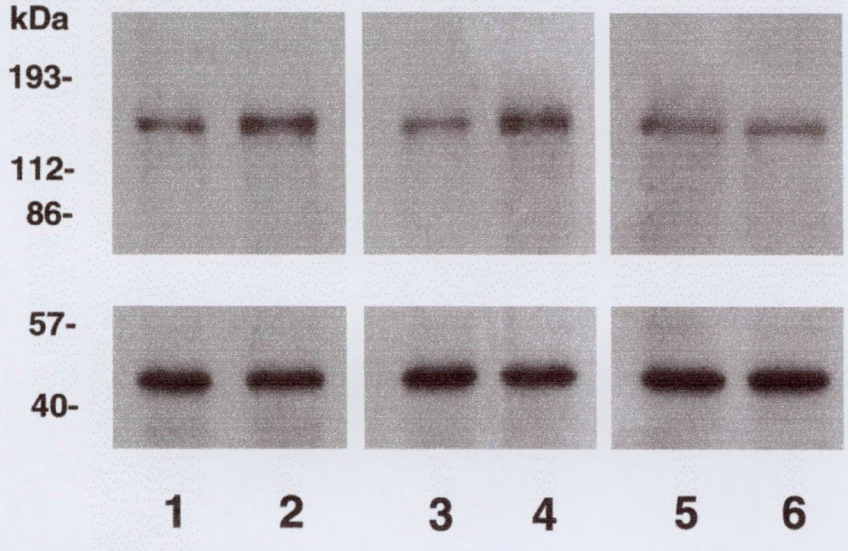

Figure 9. Western blotting of nNOS in C1-C2 spinal cord in control (lanes 1,2), sumatriptan- (lanes 3,4) and lysine-acetylsalicylate pretreated (lanes 5,6) animals. Compared to the vehicle (1,3,5), NTG administration (2,4,6) enhances the nNOS band (155 kDa) in the control (2) and sumatriptan-pretreated group (4), but not in the lysine-acetylsalicylate-pretreated group (6). The corresponding ß-actin bands are shown below for each animal group.

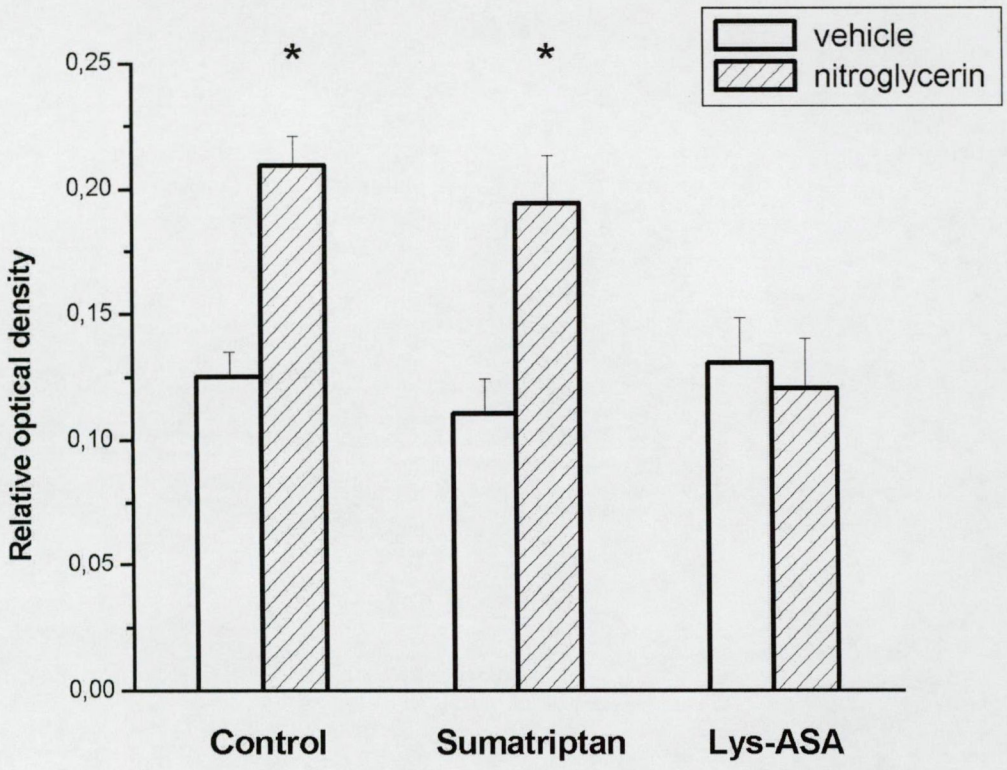

Figure 10. Histogram showing the optical densities of nNOS Western blots in the C1-C2 segments of the 3 animal groups 4 hours after subcutaneous injection of vehicle (light bars) or NTG (hatched bars) (mean+S.E.M, n=5 per group). Data are expressed as proportion of ß-actin. In controls and after sumatriptan pretreatment there is a significant increase of optical density in the 155kDa nNOS band (* $p<0.05$), while this increase is absent after lysine-acetylsalicylate pretreatment.

IV.3. CGRP and 5-HT

On transverse sections of the C1-C2 spinal segments there were abundant CGRP-Ir fibers in the superficial layers of the caudal spinal trigeminal nucleus. The area covered by these fibers was not significantly different between the various rostro-caudal levels, nor between sides of the C1-C2 segments.

As far as the total area covered by CGRP-Ir in the superficial laminae I-II of sTNC after vehicle injection was concerned, we found no significant difference between male rats and ovariectomized females. By contrast, the CGRP-innervated area in the estradiol-treated ovariectomized group was significantly smaller than in any of the two other groups (Figures 9A, 9C, 9E).

Four hours after subcutaneous NTG administration there was a significant decrease of the area covered by CGRP-Ir fibres in males (Figures 9A, 9B) and in ovariectomized females (Figures 9C, 9D) compared to vehicle injections, but no change was found in estradiol-treated ovariectomized rats (Figures 9E, 9F).

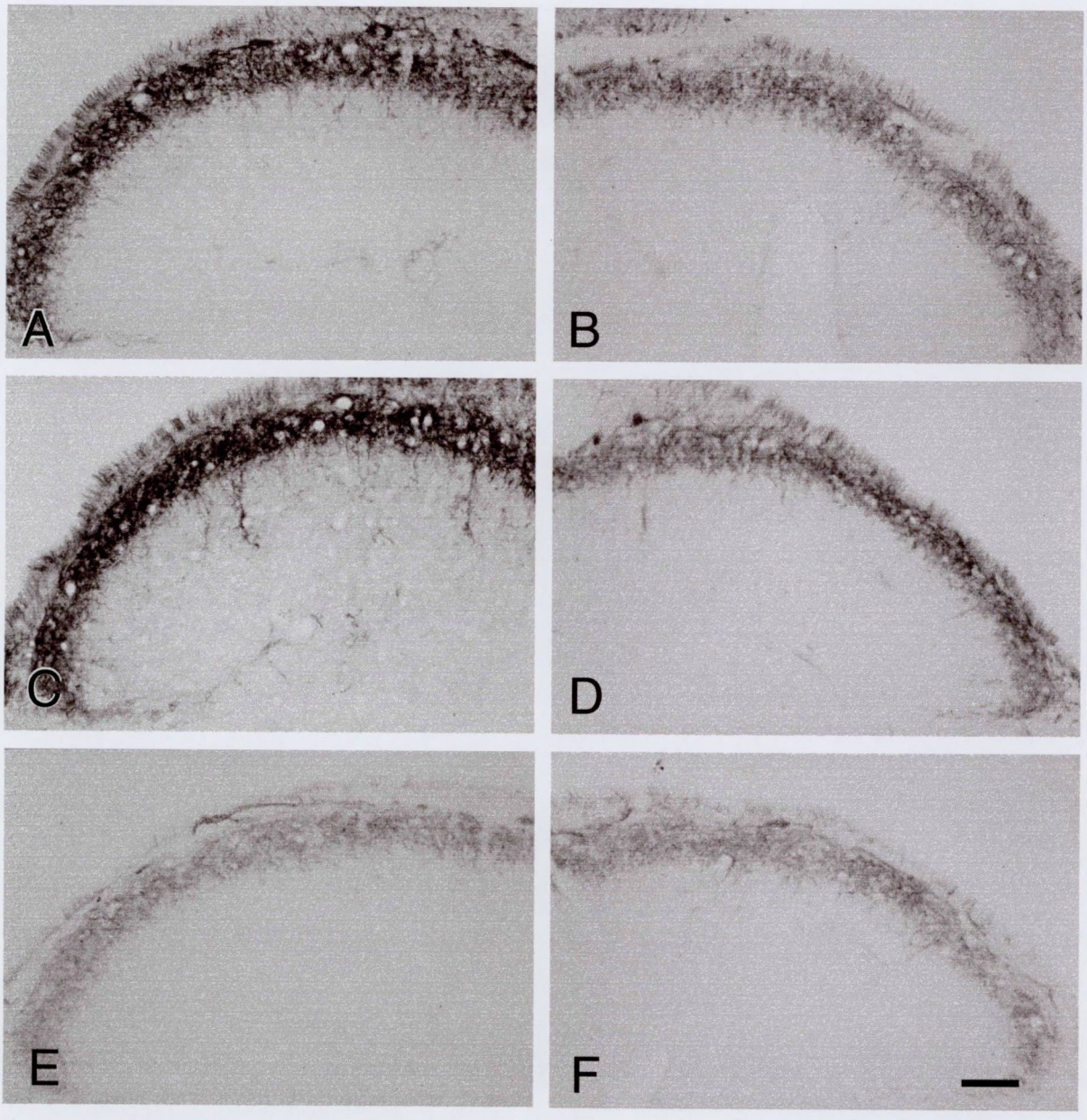

Figure 9. CGRP-immunoreactivity on transverse sections of the upper cervical spinal cord in males (A,B), ovx (C,D) and ovx+E_2 females (E,F). NTG administration (B,D,F) compared to vehicle (A,B,C) reduces the immunoreactivity in males and ovx females (B,D) but not in ovx+E_2 rats where the baseline immunoreactivity is lower. Scale bar = 50μm.

At the Th1 level in male rats, where the superficial dorsal horn area innervated by CGRP-fibers is much smaller, there was no significant difference between NTG- and vehicle-injected animals (data not illustrated).

These results of the CGRP innervation are synoptically presented in the histogram of Figure 10 and in Table 1.

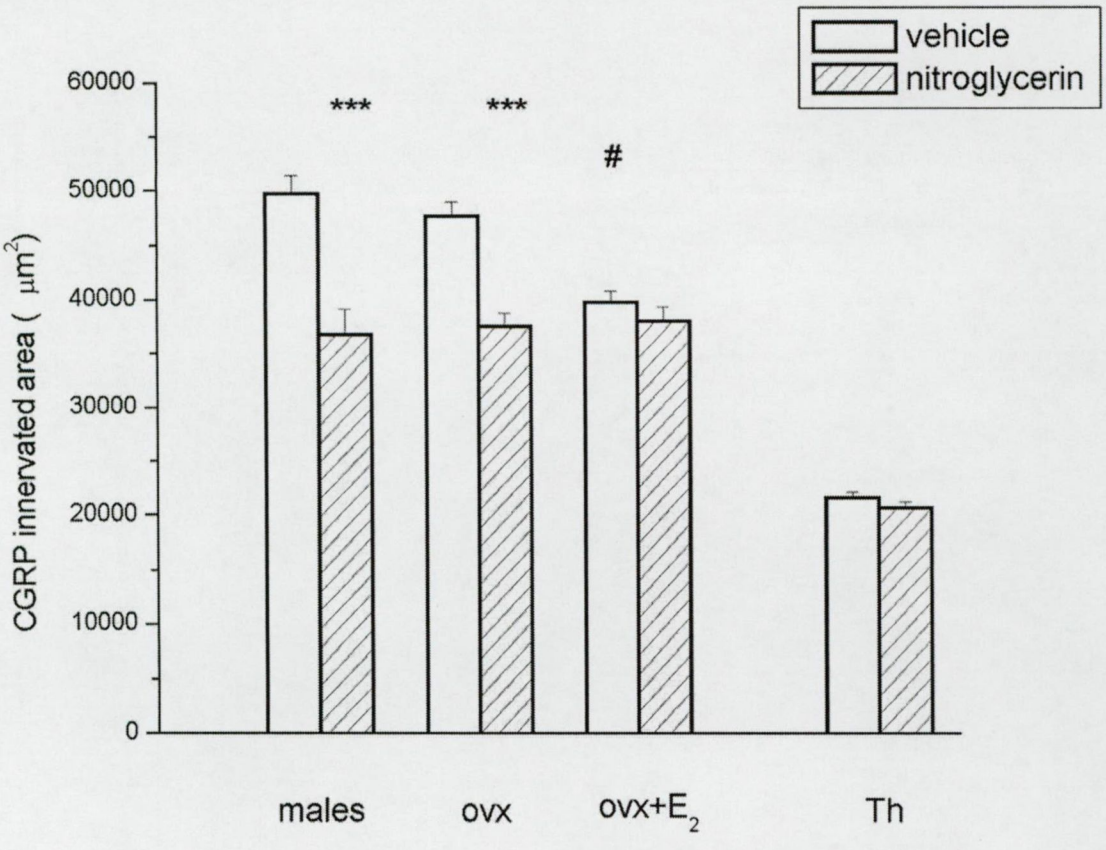

Figure 10. Histogram showing the area in μm^2 covered by CGRP-Ir fibers in superficial laminae I-II of sTNC at C1-C2 in the 3 animal groups and of segment Th1 (th) in males 4 hours after subcutaneous injection of vehicle (light bars) or NTG (hatched bars) (mean+S.E.M, n=8 per group). In males and ovx animals there is a significant decrease in the area innervated by CGRP-Ir fibers (*** $p<0.001$). The level after vehicle injection is significantly lower in ovx+E_2 animals as compared to the male or ovx rats ($^{\#}$ $p<0.05$).

At higher magnifications the size of the CGRP-Ir boutons in laminae I-II of TNC was not different between males and ovariectomized females but was significantly lower in the estradiol treated group. After NTG injections, the bouton size decreased significantly in males (Figure 11 and Table 2) and ovariectomized animals, but not in the ovariectomized + estradiol treated rats. We found no changes in bouton sizes at the Th1 level (Figure 12).

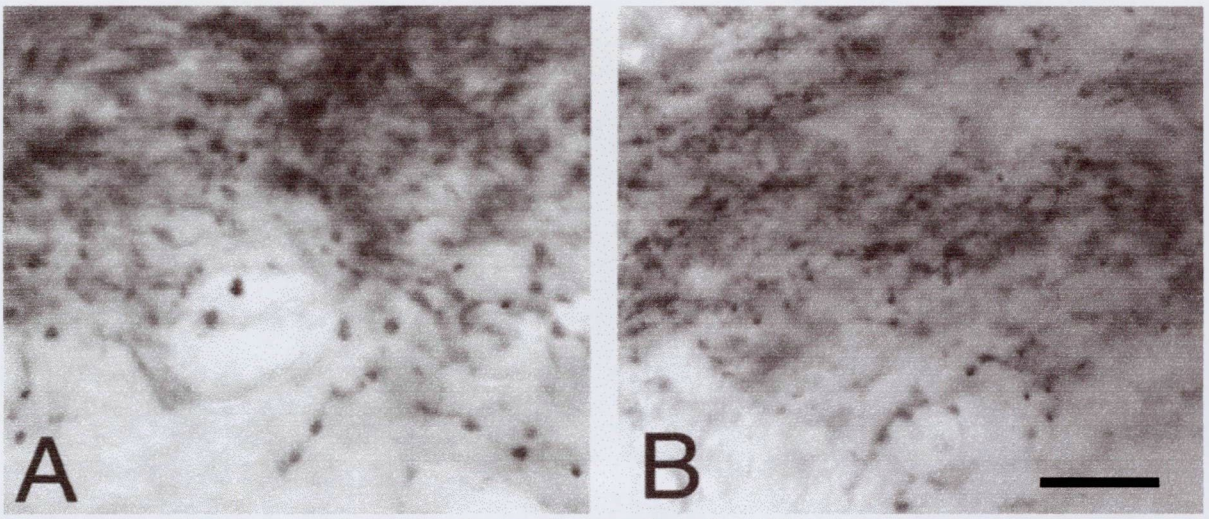

Figure 11. High magnification photomicrograph of CGRP immunoreactive boutons in laminae I-II of sTNC of vehicle (A) and nitroglycerin (B) treated male rats. After NTG administration the bouton size is smaller. Scale bar = 10 μm

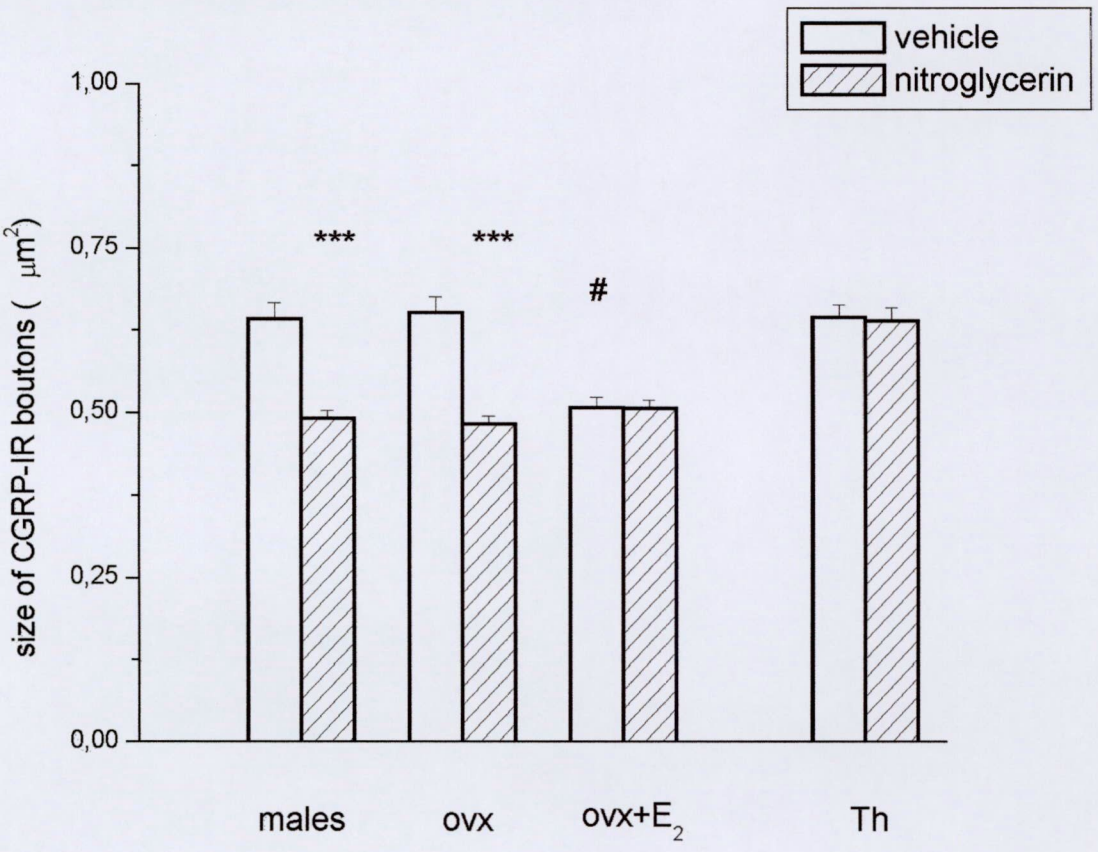

Figure 12. Histogram showing the CGRP-Ir bouton size in superficial laminae I-II of sTNC at C1-C2 in the 3 groups of animals and in segment Th1 (th) of males 4 hours after subcutaneous injection of vehicle (light bars) or NTG (hatched bars) (mean+S.E.M, n=8 per group). In males and ovx animals there is a significant decrease in the bouton size of CGRP-Ir fibers (*** $p<0.001$). The bouton size after vehicle injection is significantly lower in ovx+E_2 animals as compared to the male or ovx rats ($^{\#}$ $p<0.05$).

Serotonergic fibers project to the entire gray matter of the spinal cord, but the densest innervation by 5-HT-Ir fibers is found in superficial laminae I-II of the dorsal horn. This is also the case at the level of sTNC. Similarly to the results obtained with CGRP, the area in sTNC laminae I-II innervated by 5-HT-Ir fibers was not significantly different in male rats compared to ovariectomized females. By contrast with CGRP-fibers, however, the 5-HT-Ir area was significantly greater in the estradiol-treated ovariectomized animals compared to the two other groups (Figures 13A and 13C compared to 13E).

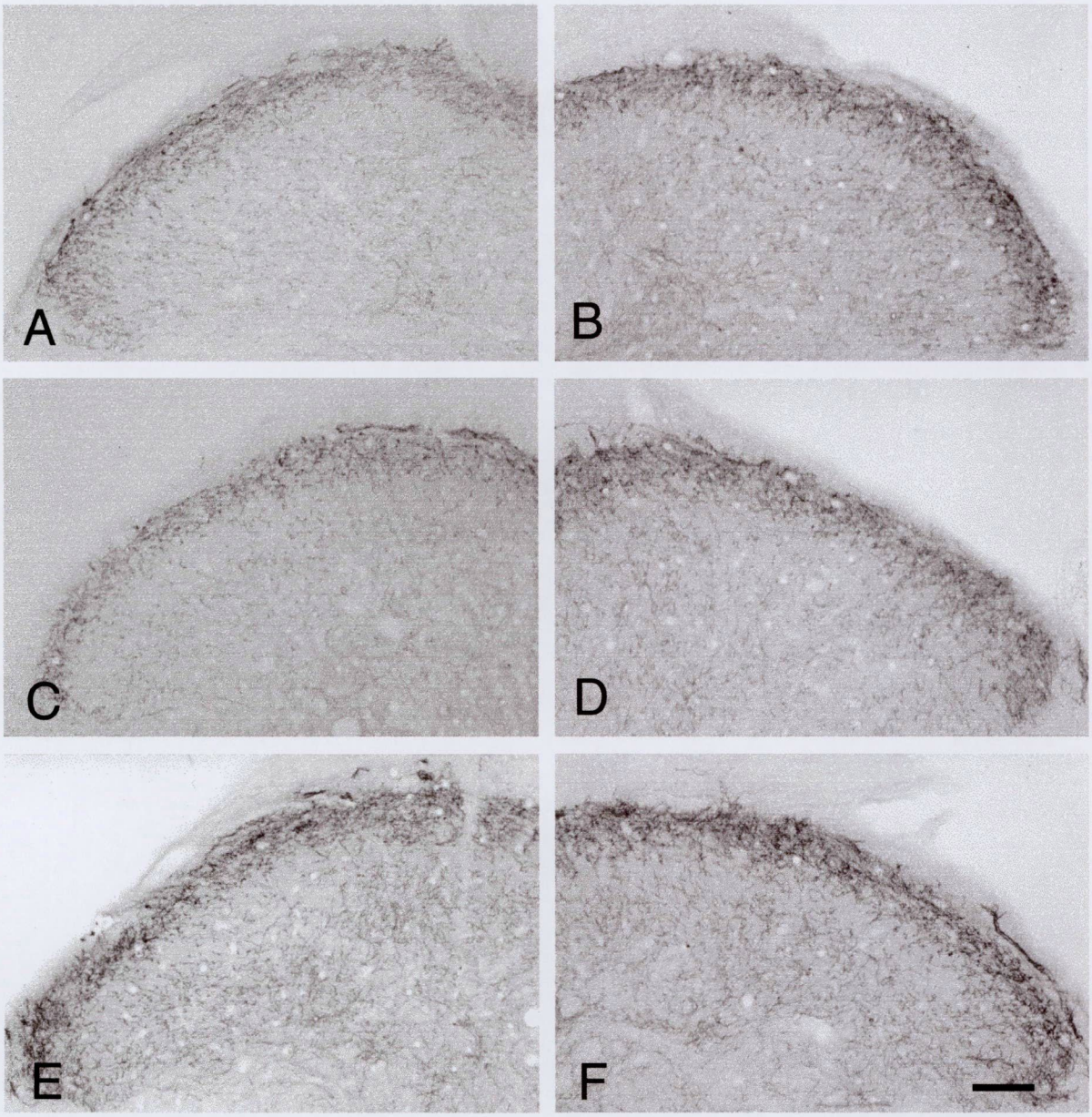

Figure 13. 5-HT-immunoreactivity on transverse sections of the upper cervical spinal cord in males (A,B), ovx (C,D) and ovx+E_2 females (E,F). NTG administration (B,D,F) compared to vehicle (A,B,C) increases the immunoreactivity in males and ovx females (B,D) but not in ovx+E_2 rats where the baseline immunoreactivity is higher. Scale bar = 50μm.

While the area occupied by 5-HT-Ir fibres in the superficial dorsal laminae at C1-C2 segmental levels significantly increased after NTG injections in male (Figures 13A,13B) and ovariectomized rats (Figures 13C, 13D), these injections had no effect on the 5-HT innervation in estradiol-treated ovariectomized animals (Figures 13E, 13F).

At segmental level Th1, the area covered by 5-HT-Ir fibers in laminae I-II of the dorsal horns was much smaller (not illustrated) than the one measured at C1-C2 and it remained unchanged after NTG administration (see Figure 14).

The results for 5-HT immunoreactive areas are summarized in the histogram of Figure 14 and in Table 2. At higher magnification there was no detectable size difference of serotoninergic boutons in the superficial layers of sTNC between animal groups neither before nor after NTG administration (Table 2). The numerical data for CGRP- and 5-HT-Ir area and bouton size are summarized in Table 1 and 2 respectively.

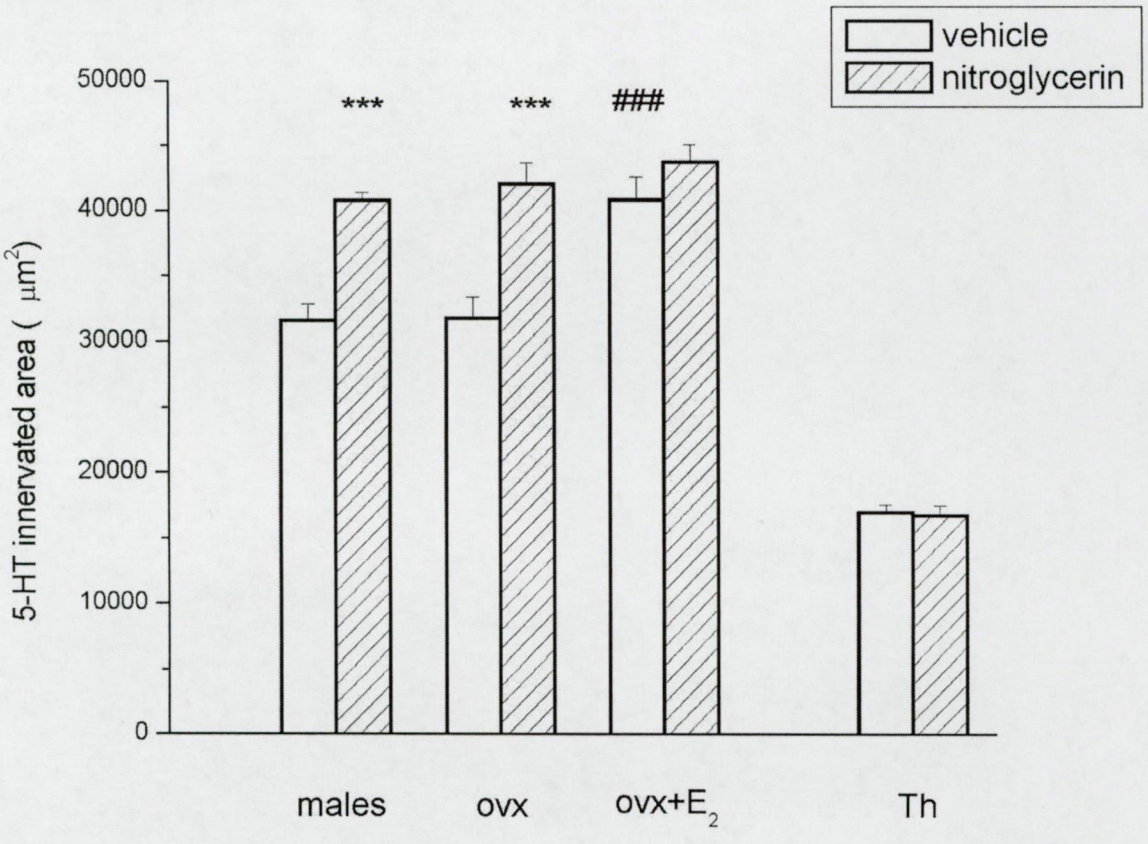

Figure 14. Histogram showing the area in μm^2 covered by 5-HT-Ir fibers in superficial laminae I-II of sTNC at C1-C2 in the 3 animal groups and of segment Th1 (th) in males 4 hours after subcutaneous injection of vehicle (light bars) or NTG (hatched bars) (mean+S.E.M, n=8 per group) In males and ovx animals there is a significant increase in the area innervated by 5-HT-Ir fibers (*** $p<0.001$). The level after vehicle administration is significantly higher in ovx+E_2 animals as compared to the male or ovx rats ($^{###}$ $p<0.001$).

	CGRP		5-HT	
	Vehicle	Nitroglycerin	Vehicle	Nitroglycerin
Males	4.97 ± 0,16	3.67 ± 0.23***	3.16 ± 0.12	4.07 ± 0.06***
Ovx	4.76 ± 0.12	3.75 ± 0.13***	3.17 ± 0.17	4.2 ± 0.16***
Ovx+E_2	3.98 ± 0.1 #	3.79 ± 0.14	4.08 ± 0.17 ###	4.37 ± 0.14
Th	2.15 ± 0.05	2.06 ± 0.06	1.69 ± 0.06	1.67 ± 0.07

Table 1. Areas innervated by CGRP and 5-HT fibers in the superficial laminae of sTNC and the thoracic (Th) dorsal horns. The values are expressed as means ± S.E.M. in 10^4 μm^2. There are significant differences
(*** $p<0,001$) between vehicle and nitroglycerin in the male and ovx group as well as after vehicle between males and ovx+E_2 animals (# $p<0,05$, ### $p<0,001$).

	CGRP		5-HT	
	Vehicle	Nitroglycerin	Vehicle	Nitroglycerin
Males	0.64 ± 0.03	0.49 ± 0.01***	0.56 ± 0.04	0.61 ± 0.05
Ovx	0.65 ± 0.02	0.48 ± 0.01***	0.56 ± 0.03	0.59 ± 0.03
Ovx+E_2	0.51 ± 0.02 #	0.51 ± 0.01	0.58 ± 0.03	0.59 ± 0.04
Th	0.64 ± 0.02	0.63 ± 0.02	0.6 ± 0.03	0.61 ± 0.04

Table 2. Size of CGRP and 5-HT immunoreactive boutons expressed as the cross sectional area in μm^2 (mean ± S.E.M.) There is a significant reduction in the size of CGRP-Ir boutons (*** $p < 0,001$) between vehicle and nitroglycerin-treated male and ovx animals. After vehicle administration the boutons are significantly smaller (# $p<0,05$) in ovx+E_2 rats compared to the males.

V. Discussion

V.1. nNOS and c-fos experiments

Our data demonstrate that systemic NTG administration increases significantly nNOS immunoreactivity in the superficial dorsal horn of the upper cervical spinal cord, in addition to the effect already described for c-fos immunoreactivity (Tassorelli *et al.*, 1995). To understand the mechanisms responsible for the nNOS increase and its possible relevance for migraine it is important to note that NTG-induced c-fos and nNOS-positive neurons appear in a comparable anatomic territory, which receives C and Aδ afferents from peripheral somatic trigeminal territories and meningeal vessels (Kaube *et al.*, 1993). This suggests that both immunoreactivities are interdependent and related to activation of second order nociceptive neurons and interneurons. Such a relationship was demonstrated in the spinal cord after intradermal capsaicin injections (Wu *et al.*, 2000) and more recently in trigeminal nucleus caudalis after formalin injections into the face which resulted in colocalization of c-fos and nNOS in 14% of neurons (Leong *et al.*, 2000).

Increased nNOS immunoreactivity may be due to increased synthesis or to reduced utilisation and cytoplasmic accumulation. Because of the concomitant c-fos activation and the fact that NTG or nociceptive stimuli cause activation of second order neurons, it is more likely that increased synthesis is causing this phenomenon.

The nNOS increase in second order trigeminal nociceptors could be due to i.) a direct effect of NO on these neurons, ii.) to a modulation by NO of descending brain stem inputs or iii.) to an indirect activation via NO stimulation of peripheral nociceptive afferents. Since NTG produced no detectable changes in nNOS content of the thoracic dorsal horn, a direct effect of NO on second order sensory neurons containing nNOS is most unlikely. There is some evidence that local microinjections of NO donors in the rat are able to inhibit periaqueductal gray (PAG) matter neurons (Lovick *et al.*, 1996). The ventrolateral part of PAG can inhibit neurons in trigeminal nucleus caudalis, including those that receive afferents from intracranial vessels (Li *et al.*, 1996). Although the studies on local injections of NO donors were performed on the dorsolateral, i.e. the "autonomic", portion of PAG, we cannot rule out that the nNOS activation in trigeminal nucleus caudalis is secondary to disinhibition

because of reduced descending inputs from PAG neurons. The most likely explanation for the increased nNOS immunoreactivity, however, is the secondary activation of second order nociceptive neurons and/or interneurons because of excitation of their peripheral afferents. NTG is indeed able to activate meningeal nociceptive fibers (Knyihar-Csillik *et al.*, 1999) and we have confirmed in the present paper that a somatic nociceptive stimulus (s.c. formalin) is able to activate both c-fos and nNOS in the trigeminal system. It has been shown, moreover, that c-fos activation in trigeminal nucleus caudalis neurons by s.c. NTG is reduced after the destruction of unmyelinated fibers by capsaicin (Tassorelli *et al.*, 1997) and that c-fos activation by NO donors in the spinal cord is abolished by pretreatment with a NOS inhibitor (Wu *et al.*, 2000).

Our findings could be relevant for the pathophysiology of the trigeminovascular system and thus for migraine pathogenesis and its NO hypothesis. Increased nNOS activity in superficial trigeminal nucleus caudalis suggests that the local availability of NO is enhanced. This may be responsible for a central sensitization, possibly via activation of c-fos which can induce a number of excitatory or inhibitory transmitters related to pain mechanisms; such a relationship between NO and c-fos was recently demonstrated in the spinal cord after a peripheral nociceptive stimulus (Lin *et al.*, 1999, Wu *et al.*, 2000). The effects of NO on nNOS activity in second order nociceptive neurons appear to be specific to the trigeminal system, as we did not observe them in the thoracic spinal cord. The neurobiology of trigeminal and peripheral nociception may thus be different. Whether this difference could be related to the different presynaptic receptor population (e.g. 5-$HT_{1B/D}$ receptors) on nociceptive afferents, which is underscored by the selective efficacy of triptans in migrainous headache (Buzzi *et al.*, 1991), or to other factors remains to be proven. In any case the data presented here suggest that NO donors are able to initiate a feedback process in which their direct effect on nociceptive trigeminovascular afferents may be amplified and prolonged by the nNOS activation in trigeminal nucleus caudalis. Such a process might be important for central sensitization and, possibly, for migraine headache. It may be related to the clinical signs of central trigeminal nociceptor sensitization which were recently shown in migraine patients (Burstein *et al.*, 2000). It might also explain why NTG causes an attack in migraineurs only after a delay of several hours, which corresponds roughly to the time it takes to induce the increased nNOS activity in the rat.

V.2. Sumatriptan and Lys-ASA

In our study we examined the effect of anti-migraine drugs on the above described NTG-induced increase of nNOS expression in the superficial layers of trigeminal nucleus caudalis. Pretreatment with sumatriptan failed to influence the increase in nNOS expression induced by the NO donor NTG, while the NSAID lys-ASA attenuated the NTG-induced nNOS activation.

It is not known for sure how NTG modifies nNOS in TNC. There are several possible ways for NO to induce nNOS expression in secondary trigeminal nociceptors, but we hypothesized nonetheless from our first study that the most likely explanation was a secondary activation of second order nociceptive neurons and/or interneurons because of excitation by NO of their peripheral afferents.

The NTG effect on nNOS seemed to be specific for TNC as it was not found in upper thoracic segments, we cannot rule out the involvement of other neuronal subpopulations. It has been shown indeed that NO can exert a dual effect on nociception depending on the dose used (Sousa *et al.*, 2001, Colasanti *et al.*, 2000).

If the latter hypothesis is correct, one would expect that an agonist of presynaptic 5-$HT_{1B/D}$ receptors which inhibit the release of transmitters in primary nociceptive afferents (Arvieu *et al.*, 1993), is able to attenuate the NO effect on nNOS expression. Sumatriptan was indeed shown to reduce the CGRP release from meningeal trigeminal fibers after electric stimulation of the Gasserian ganglion in the rat, while lys-ASA was not (Limmroth *et al.*, 2001). It was also reported that sumatriptan prevents the increase of dural perivascular CGRP-Ir nerve endings after electric stimulation of the Gasserian ganglion (Knyihar-Csillik *et al.*, 1997). The lack of effect of sumatriptan in our study may therefore suggest that activation of nociceptive afferents has no role in the NTG-induced nNOS increase or that 5-$HT1_{B/D}$ receptors are not capable to oppose such a chemical activation. Earlier studies on migraine headache have emphasized a possible role for 5-$HT_{2B/C}$ (Fozard *et al.*, 1994), or more likely for 5-HT_{2B} receptors, which mediate the release of NO from the endothelium (Schmuck *et al.*, 1996). Recent experiments showed that 5-HT_{2A} receptor activation was able to enhance nNOS expression in trigeminovascular neurons (Srikiatkhachorn *et al.*, 2002) and upregulation of platelet 5-HT_2 receptors was reported by the same group in transformed migraine

(Srikiatkhachorn *et al.*, 1994). Taken together these findings underline that, besides 5-$HT_{1B/D}$, other 5-HT receptor subtypes play a role in trigeminovascular pain, which could be an explanation for the lack of effect of sumatriptan in our model.

An alternative explanation for the ineffectiveness of sumatriptan must be taken into account: its hydrophilicity and reduced ability to cross the blood-brain barrier (Humphrey *et al.*, 1991). For instance, systemic administration of sumatriptan in cat is able to inhibit the firing of central trigeminal neurons induced by sagittal sinus stimulation only after disruption of the blood-brain barrier by mannitol (Kaube *et al.*, 1993), which contrasts with more lipophilic triptans like zolmitriptan and naratriptan (Goadsby *et al.*, 1996, 1997). Interestingly, in acute migraine treatment sumatriptan has little efficacy, if it is taken at the time of the aura, i.e. before the headache phase (Banerjee *et al.*, 1992), whereas aspirin will not lose efficacy on the headache if given during the aura (Ross-Lee *et al.*, 1983).

There is no doubt that sumatriptan is effective in NTG-induced attacks in migraineurs (Iversen *et al.*, 1996), but such attacks are triggered by microgram-range doses of NTG administration, which seems to produce sensitisation, but not c-fos activation of trigeminal neurons (Jones *et al.*, 2001). It may thus be possible that higher doses of NTG, such as those used here, induce a more robust activation of central trigeminal nociceptors.

Inducible cyclo-oxygenase 2 (COX-2) is expressed in the superficial dorsal horn of the rat spinal cord (Beiche *et al.*, 1998) and plays a role in central sensitisation (Samad et al., 2001). It mediates most of the analgesic effects of NSAIDs (Yaksh *et al.*, 2001). After hindpaw inflammation in mice some of the COX-2 expressing cells in laminae I-II are also positive for nNOS (Maihofner *et al.*, 2001) suggesting an interaction between the two enzymes. Lys-ASA, like all NSAIDs, inhibits cyclo-oxygenase 1 and COX-2. It is effective in migraine, penetrates easily the blood-brain barrier and has a long–lasting effect as well as a long half-life (Gatti *et al.*, 1989). Lys-ASA may also exert an anti-nociceptive effect by acting directly upon the periaqueductal gray matter (Tortorici *et al.*, 1995). Taken together, these data and our present results suggest that the stimulating effect of NTG, and thus of NO, on nNOS expression in secondary trigeminal nociceptors might be mediated by prostanoids and in particular by COX-2 expressing interneurons in TNC superficial laminae.

V.3. CGRP and 5-HT

To our knowledge this study demonstrates for the first time that systemic administration of NTG is able to change CGRP and 5-HT immunoreactivities in laminae I-II of the spinal portion of trigeminal nucleus caudalis. Up to now, NTG-induced c-Fos (Tassorelli *et al.*, 1995) and the above mentionned nNOS activations were reported in the same area of the upper cervical cord. Release or increased turnover of CGRP in spinal dorsal horns of appropriate segments was reported after localised peripheral inflammation (Sluka *et al.*, 1992), capsaicin administration (Garry *et al.*, 2000) or subcutaneous formalin injections (Zhang *et al.*, 1994). The decrease of the area occupied by CGRP-Ir afferents found here is thus likely a consequence of an increased release of CGRP due to the NO-mediated stimulation of trigeminal nociceptive Aδ and C afferents. Morphological changes suggestive of transmitter release were also found in the distal portions of trigeminal CGRP-Ir afferents in dura mater after electrical stimulation of the Gasserian ganglion (Knyihar-Csillik *et al.*, 2000). We could also demonstrate a decrease in the size of CGRP immunoreactive boutons which would support the hypothesis of an increased release of the peptide due to the NTG administration. By the same token, the NTG-induced increase in 5-HT immunoreactivity in the same spinal gray areas may indicate a reduced release of serotonin from supraspinal afferents. The molecular and functional relation between 5-HT afferents to the superficial spinal dorsal horn and spinal nociceptors is complex. Multiple 5-HT receptors (such as 5-HT_{1B} , 5-HT_{1D} and 5-HT_{1F}) are found in laminae I-II (Castro *et al.*, 1997). When activated, all of them may decrease c-fos expression in trigeminal nucleus caudalis induced by nociceptive stimulation (Mitsikostas & Sanchez del Rio 2001; Hoskin *et al.*, 1996). By contrast, spinal nociceptive transmission after peripheral inflammation can be enhanced by the action of serotonin on 5-HT_3 receptors (Green *et al.*, 2000). It is not known if NTG would reduce 5-HT release in laminae I-II because of a local interaction with serotonergic terminals or via a secondary modulation of the serotonergic descending pain control pathway. Increased 5-HT-metabolism was demonstrated in nucleus raphe magnus and in the spinal dorsal horn after subcutaneous injection of formalin (Puig *et al.*, 1992). Carrageenan-induced inflammation in the paw also increased the 5-HT concentration in periaqueductal gray and the lumbar spinal dorsal horn of the rat (Zhang *et al.*, 2000). Whatever the mechanism might be, it seems to be selective for spinal trigeminal nucleus caudalis, as we found no detectable change in

superficial dorsal horn of the thoracic spinal cord, a selectivity already pointed out for NTG-induced NOS activation in 2nd order nociceptors (Pardutz *et al.*, 2000). The selectivity of the NTG effect for the projection site of trigeminovascular afferents underlines the relevance of the NTG model for migraine. The reason for this selectivity has to be determined, but it might be related to the presence on visceral trigeminal Aδ and C afferents of specific receptor populations, such as the 5-$HT_{1B/D}$ receptors, or to other regional differences in neuronal metabolism. Interestingly, the specific 5-$HT_{1B/D}$ agonist eletriptan, which is highly effective in migraine treatment, prevents the morphological changes of CGRP-Ir peripheral dural afferents induced by trigeminal ganglion stimulation (Knyihar-Csillik *et al.*, 2000).

Contrary to the acute changes in immunoreactivities occurring after NTG injection, those found after the experimental modulation of estradiol levels are on a different time scale and reflect slower plastic adaptations of neurotransmitter expression. Our results suggest that ovarian hormones, which greatly influence the course of migraine, have indeed the capacity to modify the expression of pivotal transmitters in the trigeminovascular nociceptive pathway. In animals with high estradiol levels, i.e. ovariectomized females treated with 17ß-estradiol, CGRP innervation was clearly reduced in the superficial sTNC layers, compared to animals with low estradiol levels, i.e. males and ovariectomized non-treated females. This is in line with findings by Moussaoul *et al.* (1996). Estrogen receptors are present on spinal sensory ganglion neurons (Yang *et al.*, 1998, Taleghany *et al.*, 1999) and in spinal gray matter (Shugrhue *et al.* 1997). Estradiol may thus act at the genomic level, which would modify the phenotypic expression of CGRP and annihilate any detectable change in immunoreactivity after NTG.

Contrasting with CGRP, the area covered by 5-HT-Ir fibers in sTNC laminae I-II was higher in estradiol-treated ovariectomized rats. This finding has to be discussed in the light of the well documented effect of estrogen on various aspects of serotonin transmission and metabolism in other brain areas. It has been shown that ovarian steroids increase tryptophan hydroxylase in species such as macaques or guinea pigs (Pecins-Thompson *et al.*, 1996; Bethea *et al.*, 2000; Lu *et al.*, 1999), while they decrease mRNA expression of the serotonin re-uptake transporter in monkeys and rats (Pecins-Thompson *et al.*, 1998; Rehavi *et al.*, 1998). Estrogen is also able to desensitize 5-HT_{1A} receptors in hypothalamic neurons (Raap *et al.*, 2000), which may activate serotonergic neurons by decreasing auto-inhibition. Among these various and possibly interrelated effects, an estrogen-dependent increase in activity of

the synthesizing enzyme tryptophan hydroxylase in descending raphe-spinal serotonergic neurons would probably best account for our finding of increased 5-HT immunoreactivity in sTNC after estradiol treatment. Estrogen receptors are known to be localized on serotonergic neurons in raphe nuclei (Leranth *et al.*, 1999) and may mediate some of these effects. Like for CGRP, there was no significant change in the sTNC 5-HT innervation after NTG in estradiol-treated ovariectomized rats, probably because there was no possibility for further 5-HT increase after stimulation of its anabolism by the estrogen. On the other hand, it has been shown that estradiol induces structural synaptic remodeling in certain sex steroid-responsive brain areas (Garcia-Segura *et al.* 1994), so that the changes in the CGRP and 5-HT-Ir innervation pattern observed here in estradiol-treated animals could be the result of synaptic rearrangements in the superficial laminae of trigeminal nucleus caudalis. Whether they may be relevant for the hormonal influences in migraine remains speculative. It is interesting, however, to draw a parallel between our immunocytochemical data in rats showing that estradiol is able to suppress activation of trigeminal afferents by nitroglycerin, a well known trigger of migraine attacks (Olesen *et al.* 1993), and the clinical observation that migraine markedly improves in most women during pregnancy, i.e. when sex hormone levels are high and stable (Marcus 1995; Silberstein & Merriam., 2000).

In summary, NTG, a NO donor, is able to decrease the area covered by peripheral CGRP-Ir afferents in superficial laminae of rat spinal trigeminal nucleus caudalis and to decrease the area occupied in the same laminae by descending 5-HT-Ir afferents. These effects are annihilated in animals in which the baseline activity of CGRP is decreased or that of 5-HT is increased because of chronically high levels of estradiol. Whatever the underlying molecular mechanisms of the acute NTG-induced and the chronic estradiol-dependent change might be, both seem to be selective for the trigeminal sytem and may shed some light on the role of NO donors and ovarian steroids in trigeminovascular pain syndromes, such as migraine. If, as deduced from our immunocytochemical observations, the release of CGRP is indeed enhanced and that of 5-HT reduced by NO at the level of the spinal trigeminal nucleus, one may expect increased nociception. On the other hand, the opposite changes in animals with high estradiol levels would lead to decreased trigeminal nociceptive transmission. This might be at least part of the neurobiological explanation for the attack-triggering effect of NTG and other NO donors in migraine patients and for the protective action on this disorder of high and steady levels of ovarian hormones, as for instance during pregnancy.

VI. Conclusions

In our paper we established a reproductive animal model for migraine headache the systemic administration of NTG in rats.

i. The NO donor NTG causes a delayed induction of nNOS in the caudal trigeminal nucleus, a similar effect witnessed after nociceptive stimulus. This phenomenon may cause a self amplified process explaining the central sensitisation discovered in migraine patients (Burstein et al. 2000).

ii. Lys-ASA but not the 5-$HT_{1B/D}$ agonist sumatriptan is able to modulate the nNOS inducing effect of NTG suggesting the involvement of prostanoids in the process.

iii. We also examined the modulatory effect of estradiol, a gonadal steroid which influences the clinical picture of migraine headache on two relevant transmitters CGRP and 5-HT. NTG was able to decrease the CGRP and increase the 5-HT content of the TNC in male and ovariectiomised rats but not in estradiol treated females.

These data may contribute to a better understanding of the pathomechanisms of migraine headache and the relevance of the modulating effect of estradiol in these conditions.

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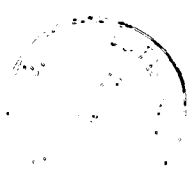

APPENDIX