Modulation of the nitroglycerin-induced activation of second order trigeminal neurons in the rat

Ph.D. Thesis

HEDVIG VARGA

Department of Neurology
University of Szeged
Albert Szent-Györgyi Medical and Pharmaceutical Center
Faculty of Medicine

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List of abbreviations

5-HT	serotonin
AMPA	α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid
CamKII	calmodulin dependent protein kinase II
cGMP	cyclic guanosine monophosphate
CGRP	calcitonine gene-related peptide
COX	cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
COX-3	cyclooxygenase-3
CSD	cortical spreading depression
eNOS	endothelial nitric oxide synthase
FHM	familial hemiplegic migraine
iNOS	inducible nitric oxide synthase
Ir	immunoreactive
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NSAID	non-steroid anti-inflammatory drug
NTG	nitroglycerin
PBS	phosphate buffered saline
s.c.	subcutaneous
TNC	spinal portion of the caudal trigeminal nucleus

Table of contents

Summary 4

I. Introduction 6

II. Aims 15

III. Materials and methods 15
III.1. CamKII 15
III.1.1. Estradiol pretreatment 15
III.1.2. Selective COX inhibitor pretreatment 17
III.2. nNOS 17

IV. Results 19
IV. 1. CamKII 19
IV.1.1. Estradiol pretreatment 19
IV.1.2. Selective COX inhibitor pretreatment 25
IV.2. nNOS 28

V. Discussion 31
V.1. NTG-induced CamKII and its modulation by estradiol 31
V.2. Effect of COX inhibitors 34

VI. Conclusions 36

VII. Acknowledgement 36

References 38

Appendix 51

Summary

Migraine headache is the most common neurological disorder where the activation and the sensitization of the trigeminovascular system are crucial. It affects up to 12% of the population and after puberty women are three to four times more frequently affected by this headache disorder. 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 nitric oxide (NO) donor, nitroglycerin (NTG) can trigger a migraine attack, after a delay of several hours in migraineurs, but not in healthy people. This long delay does not favor a pure vasodilatatory action. Due to the similarity to the normal migraine attacks, NO may cause trigeminal activation and sensitization. NTG administration in the rat was also shown to elicit the activation of pain-mediating trigeminal nucleus caudalis (TNC) neurons. This effect was maximal after a delay of 4 hours, congruent with the delay of NTG induction of migraine in humans. In rats, subcutaneous administration of NTG (10 mg/kg) increases significantly the number of neuronal nitric oxide synthase (nNOS) -immunoreactive neurons in the TNC after 4 hours, which could be attenuated by acetyl-salicylate (Aspirin), a nonselective cyclooxygenase (COX) -inhibitor. In the rat NTG model estradiol pretreatment blunts calcitonin gene related peptide (CGRP) and serotonin (5-HT) changes in the superficial laminae of the TNC, which suggest that ovarian hormones have the capacity to modify the expression of pivotal transmitters in the trigeminovascular nociceptive pathway. Calmodulin-dependent protein kinase II (CamKII) present in superficial dorsal horns is thought to play a role in sensitization of central nociceptors, a phenomen present in migraineurs.

The aims of our studies were to 1. study the effect of systemic NTG administration on the CamKII expression of the most caudal part of the TNC of the rat; 2. examine the possible modulatory effects of estradiol on this phenomenon. 3. determine which isoform of the COX enzyme plays a role in the nNOS and CamKII activation caused by NTG in the same region.

In male rats and in ovariectomized females, after 4 h NTG increased significantly CamKII expression in the superficial layers of TNC, but not in the upper thoracic spinal cord. NTG had no effect on CamKII expression in estradiol-treated ovariectomized animals. Thus NTG, i.e. NO, selectively enhances CamKII in the rat TNC and estradiol blocks this effect.

We also demonstrated that pretreatment with NS398, the selective COX-2 inhibitor attenuated the NTG-induced nNOS and CamKII expression in the TNC at doses of 3 and 5mg/kg. In contrast, SC560, a selective COX-1 inhibitor failed to modulate this phenomenon in any of the dosages used (1, 5 and 10 mg/kg).

These findings suggest that metabolites deriving from COX-2 (but not COX-1) may be the most important factors in the NTG-induced CamKII and nNOS and chronic estradiol administration can modulate the NO mediated CamKII activation in the trigeminal system. This may help us to understand the mechanisms by which NO triggers migraine attacks and estrogens influence migraine severity.

I. Introduction

Migraine headache is the most common neurological disorder, affecting up to 12% of the population (Raskin 1993; Fozard & Kalkmann, 1994; Saxena 1994; Moskowitz *et al.*, 1995). The cost of migraine to European society is 27.000 million (27 billion) euros per year, primarily because of decreased working efficiency and workdays lost (Andlin-Sobocki *et al.*, 2005). Among others gonadal hormones are important modulators of migraine (MacGregor 2004). After puberty women are three to four times more frequently affected by this headache disorder (Rasmussen *et al.*, 1991).

Migraine is commonly known as a throbbing unilateral head pain that is readily aggravated by routine physical activities. The clinical definition of migraine includes a host of transient neurological symptoms other than pain, namely, nausea, photophobia, phonophobia, osmophobia, fatigue, and numerous disturbances in autonomic, mental, sensory and motor functions. 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; Moskowitz 2007). The neural mechanisms underlying the development of migraine attacks are not yet fully understood (Saxena 1994).

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). Nevertheless vasodilatation per se could not account for a number of observations in migraine sufferers (Friedberg *et al.*, 1991; Humphrey & Goadsby, 1994) and a neuronal origin of the pain was suggested (Moskowitz 1993). According to this hypothesis activation of meningeal nociceptors could cause the sensory fibers to secrete from their peripheral branches calcitonin gene-related peptide (CGRP) (Ebersberger *et al.*, 1999), a neuropeptide capable of initiating neurogenic inflammation in the dura (Goadsby & Edvinsson, 1993; Moskowitz & Macfarlane, 1993) and the introduction of inflammatory agents such as histamine, serotonin (5-HT), bradykinin and prostaglandins to their environment. The local inflammation produced by vasoactive peptides in turn, further

stimulates perivascular nociceptive trigeminal fibers. However administration of vasoactive peptides is not able to trigger pain (Pedersen-Bjerregard *et al.,* 1991; Shekar *et al.,* 1991) – except pituitary adenylate cyclase activating peptide-38 which infusion caused headache and vasodilatation in both healthy subjects and migraine patients (Schytz *et al.,* 2009) - the possibility of involvement of other substances with a broader spectrum of actions such as NO, was suggested (Fozard & Gray, 1989; Fozard & Kalkmann, 1994; Olesen *et al.,* 1995). NO may have a crucial role in this process as an important mediator in the initiation or the propagation of a neurogenic cranial vessel inflammatory response that might eventually result in a migraine attack (Shukla *et al.,* 2001), and in the changes in cerebral blood flow during migraine (Fidan *et al.,* 2006). In this respect a study demonstrated that NO, additionally to its vasodilatory effects, could elicit a direct activation of nociceptive trigeminal fibers (Wei *et al.,* 1992). Moreover studies proved that NO and the related cyclic guanosine monophosphate (cGMP) system is capable to inhibit the activity of spinothalamic pathways via glycine and GABAergic mechanisms (Lin *et al*., 1999).

However these peripheral mechanisms are not able to explain some associated symptoms of migraine, such nausea, vomiting, sensitivity to light, sound, or movement and allodynia, and the most recent neuronal theories of migraine have been suggested. Using a positron emission tomography study it was shown that in migraine without aura certain areas in the brain stem, such as periaqueductal gray, raphe nuclei and locus coeruleus, were activated during the headache state, but not in the headache free interval (Weiller *et al.,* 1995). It was suggested that this brain stem activation is inherent to the migraine attack itself and represents the so called 'migraine generator'. Further studies also postulated that the brainstem plays a pivotal role in migraine (Goadsby *et al.,* 1991; Welch *et al.,* 2001). These results support and emphasize the role of certain brain stem areas as possible "generators" of migraine attacks, potentially by dysfunctional control of the trigeminovascular nociceptive system.

Cortical spreading depression (CSD) might be the biological basis for the neurologic aura that precedes headache in 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 was first described by Leao (1944). It triggers the release of numerous neurochemicals and changes in the tone of extracerebral

vessels (Lauritzen 1994). In animal studies, CSD can activate the meningeal trigeminal nociceptive afferents and evoke alterations in the meninges and brainstem consistent with the development of headache (Bolay *et al.,* 2002). These studies support the idea that CSD may initiate migraine attacks (Pietrobon 2005; Thomsen *et al.,* 2002). Neuronally produced NO has also been evidenced in cerebral cortex during CSD (Goadsby *et al.,* 1992; Wahl *et al.,* 1994; Read *et al.*, 1996; Shen & Gundlach, 1999; Read & Parsons, 2000).

Familial hemiplegic migraine (FHM) is a rare autosomal dominant subtype of migraine with aura, whose aura symptoms include motor weakness or paralysis (often, but not always, unilateral). Mutations in the genes *CACNA1A* and *SCNA1A*, encoding the α1 subunits of the neuronal voltage-gated Ca^{2+} channels and Na^{+} channels are responsible for FHM1 and FHM3, respectively, whereas mutations in *ATP1A2*, encoding the α2 subunit of the Na^{+}, K^{+} ATPase, are responsible for FHM2 (Pietrobon 2007). Studies showed that, FHM1, FHM2, and FHM3 mutations share the ability of rendering the brain more susceptible to CSD by causing either excessive synaptic glutamate release (FHM1) or decreased removal of K^{+} and glutamate from the synaptic cleft (FHM2) or excessive extracellular K^{+} (FHM3) (Pietrobon 2005; van den Maagdenberg *et al.,* 2004; Sanchez-Del-Rio *et al.,* 2006; Moskowitz *et al.,* 2004).

Another important asset of migraine pathology is the presence of sensitization process during the attack. Strassman et al. (1996) showed that meningeal primary afferent neurons can become mechanically hypersensitive upon exposure of their dural receptive field to inflammatory agents. Mechanically insensitive neurons became mechanosensitive after the chemical stimulation of the dura, and the mechano-sensitivity of neurons that showed only minimal response prior to the chemical stimulation of the dura increased significantly minutes after the chemical stimulation. In humans, such mechanical hypersensitivity could mediate the throbbing pain of migraine and its worsening during coughing, bending over, or other physical activities that increase intracranial pressure (Blau & Dexter, 1981; Anthony & Rasmussen, 1993).

The sensitization process is not only restricted to the peripheral nerves but involves the central nervous system. The central sensization hypothesis, which proposes that altered processing of sensory input in the brainstem, principally the trigeminal nucleus caudalis, could account for many of the temporal and symptomatic features of migraine (Dodick & Silberstein, 2006).

Burstein et al. (1998) showed that the sustained firing of sensitized meningeal nociceptors eventually leads to activation and subsequent sensitization of central trigeminovascular neurons, which process sensory signals that originate not only from the dura, but also from the periorbital skin, resulting in increased responsiveness not only to mild changes in intracranial pressure but also to innocuous skin stimulation. This central sensitization, which occurs during migraine in many patients (Burstein *et al.*, 2000) is manifested as cutaneous allodynia. This concept has important clinical implications. The triptans, because of their apparent lack of central activity, are only effective in providing total relief of migraine pain if they are administered prior to the onset of the sensitization process (Dodick & Silberstein, 2006).

Central sensitization involves the TNC, as it is associated with abnormal neuronal excitability in this nucleus (Silberstein 2004). Several studies suggest that the NO donor nitroglycerin (NTG) may have a hyperalgesic effect (Sandrini *et al.*, 2002; Tassorelli *et al.*, 2003) and that sensitization of pain pathways in the spinal cord may be caused by – or associated with – the generation of NO (Wu *et al.*, 2000). High doses of NTG reduced tail flick latency in rats and it seems that prolonged elevation of NO in the spinal cord is necessary to maintain central sensitization after it has been established (Lin *et al.*, 1999; Luo & Cizkova, 2000; Costa *et al.*, 2005). In several models of neuropathic and inflammatory pain, neuronal nitric oxid synthase (nNOS) inhibition reduces central sensitization and, indeed, pain responses in these models are increased by NO donors (Coderre & Yashpal, 1994; Mao *et al.*, 1997). During the spontaneous and the NTG-induced migraine attack, cutaneous pain thresholds to heat laser stimulus were found to be significantly reduced on both the symptomatic and nonsymptomatic sides in comparison with the headache-free phase (de Tommaso *et al.*, 2002, 2004), confirming the role of central sensitization in migraine (Burstein *et al.*, 2000).

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). Systemic administration of NTG, a NO donor, induces an immediate headache in humans (Iversen *et al.*, 1989). In migraineurs, this headache is more severe, lasts longer, and can continue to or be followed by a specific attack of migraine without aura (Sicuteri *et al.*, 1987; Thomsen *et al.*, 1994; Olesen *et al.*,1993, 1995). A

biphasic headache can also be triggered by NTG in patients suffering from chronic tension-type headache (Ashina *et al.,* 2000). This effect is very likely to be related to NTG-derived NO. 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 NO (Olesen *et al.,* 1995; Fozard *et al.,* 1994). The infusion of NTG in human induces an immediate throbbing headache in healthy subjects, while most migraine patients experience a more severe delayed headache, that is identical to spontaneous migraine attacks except the aura (Iversen *et al.,* 1989; Sicuteri *et al.,* 1987; Olesen *et al.,* 1993, 1995; Thomsen *et al.,* 1994). Due to the vasodilating effect of NO, the immediate headache is a consequence of the selective action of NTG on extra- and intracranial blood vessels, principally the dural arteries and large penetrating cerebral arteries, while the delayed headache might be mainly due to an effect of NO on neuronal function (Tassorelli *et al.,* 1999). Although the half-lives of NTG and NO are very short in vivo (Murad 1990; Kelm 1999), the migraine attack 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 (NOSs) in a large number of tissues (Moncada *et al.,* 1991; Forstermann & Kleinert, 1995).

NO is formed from L-arginine by nitric oxide synthase (NOS) and as an unconventional transmitter substance it can cross cell membranes rapidly without any specialized release machinery. The synthetizing enzyme has three isoforms: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). The presence of nNOS in the superficial layers of the dorsal horn of the spinal cord suggests that NO has functions in sensory and pain processing (Saito *et al.,* 1994). After s.c. formalin injection in the paw, the number of c-fos- and nNOS positive neurons increases in the ipsilateral dorsal horn of the appropriate lumbar spinal segment (Hunt *et al.,* 1987; Lam *et al.,* 1996). Dural mastocytes and trigeminal nerve endings are nNOS positive (Berger *et al.,*1994) and the enzyme can also be detected in the caudal trigeminal nucleus (Dohrn *et al.,* 1994) and in 5% of the Gasserian ganglion cells (Zhang *et al.,* 1996). NOS inhibitors reduce c-fos activation by nociceptive stimuli in the dorsal horn in rats (Wang *et al.,* 1999; Wu *et al.,* 2000). Activation of the trigeminovascular sytem in response to pain can be markedly reduced by the administration of a NOS inhibitor

(Hoskin *et al.,* 1999; Ackerman *et al.,* 2002). The importance of endogenous NO production during the headache phase of migraine has been evidenced in the study of Lassen et al. (1997) where the inhibition of NOS relieved the symptoms of spontaneous migraine with high efficacy. Taken together, this suggests that nNOS is an important factor in trigeminal pain processing, and that 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.

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 (Moncada *et al.,* 1991). In the guinea-pig dura mater NO induces extravasation and other changes similar to those induced by neurogenic inflammation (Johnson *et al.,* 2003). NO has also been shown to elicit a direct and immediate activation of trigeminal nerve terminals afferent to extracerebral vessels (Wei *et al.,* 1992). Furthermore a recent study demonstrated that NO can induce delayed, slowly developing activation of central trigeminal neurons and that endogenous release of NO may contribute to the ongoing activity of these neurons (Koulchitsky *et al.,* 2004). Several studies have suggested that NO is strongly involved in the development and maintenance of hyperalgesia in the spinal cord (Urban & Gebhart, 1999). Furthermore in an open pilot study hydroxycobalamin, a NO scavenger, reduced migraine attacks by more than 50% in half of the patients (van der Kuy *et al.,* 2002). Overall these studies suggest that NO might mediate the development of a supersensitivity to pain within the TNC and 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.

Results from animal experiments supports that NTG activates the pain-mediating TNC neurons, as the administration of NTG in the rat significantly enhanced the number of Fos-immunoreactive neurons in brain areas involved in sensory nociceptive perception, including the TNC (Tassorelli & Joseph, 1995; Tassorelli *et al.,* 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). Furthermore in the animal model a large number of the activated neurons also exhibited NOS immunoreactivity (Tassorelli & Joseph, 1995). In

neurons of the lower TNC (including the upper cervical spinal cord), where most of the trigeminal nociceptors project, NTG also increases the expression of nNOS (Pardutz *et al.,* 2000). The most likely explanation for this increased nNOS immunoreactivity is the secondary activation of second order nociceptive neurons and/or interneurons because of excitation of their peripheral afferents. The increased nNOS level in the second order trigeminal neurons can be associated with a central sensitization phenomenon, which is characteristic in migraineurs (Burstein *et al.,* 2000; Kaube *et al.,* 2002). This effect is inhibited by pretreatment with acetyl-salicylate, but not with sumatriptan, indicating that prostanoids are involved in this process (Pardutz *et al.,* 2004).

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 central sensitization of pain pathways, which leads migraine pain. In the rat systemic NTG administration is a valuable tool for trigeminovascular activation and for investigation the modulatory effects of certain drugs on the trigeminovascular system.

Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) is a major protein kinase that is capable of regulating the activities of many ion channels and receptors. It is found throughout the central nervous system and it regulates calcium signalling in synaptic transmission (Hudmon & Schulman, 2002). CamKII is capable of autophosphorylation, which increases its activity (Yang & Schulman, 1999) and is necessary for long-term potentiation induction in the hippocampus (Havik *et al.,* 2003). It binds to N-methyl-D-aspartate (NMDA) receptors in rats and its a subunit increases ion currents through a-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) and NMDA receptors (Kolaj *et al.,* 1994). There is strong evidence that CamKII plays a key role in nociceptive processing and sensitization of central sensory neurons (Choi *et al.,* 2006; Luo *et al.,* 2008). In the superficial layers of the spinal dorsal horns it is abundant both in neuronal perikarya and in the neuropil (Fang *et al.,* 2002). The latter is explained by the fact that more than half of dorsal root ganglion cells are CamKII+, especially in the trigeminal ganglion (Ichikawa *et al.,* 2004). CamKII immunoreactivity in laminae I–II of the spinal cord is increased after subcutaneous injections of formalin (Liang *et al.,* 2004) or capsaicin (Fang *et al.,* 2002), or after intrathecal injections of substance P (Choi

et al., 2005), and this increase can be blocked by CamKII inhibitors (Fang *et al.*, 2002). After acute noxious stimulation CamKII expression is upregulated in dorsal horn synapses formed by peptidergic primary afferents (Larsson & Broman, 2006), which are also crucial in the pathogenesis of headaches.

Taken together CamKII is a pivotal agent in nociceptive processing and central sensitization suggesting an important role in the pathomechanism of migraine.

Non-steroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (Aspirin®), are effective in the treatment of acute migraine headache (Lange *et al.*, 2000) and tension-type headache (Schoenen 2000). This effect could be due to their inhibitory action on cyclooxygenase-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). Prostaglandins are thought to play role in many neurological functions including the nociceptive processing. Several isoforms of their synthetizing enzyme are known: cyclooxygenase-1 (COX-1) and -2 and the most recently discovered and described cyclooxygenase-3 (COX-3) (Willoughby *et al.*, 2000; Kis *et al.*, 2004). COX-1 is expressed constitutively in most cells of the brain and it participates in various homeostatic processes. Constitutive COX-2 expression is rare or even absent in most organs of the body, except in the brain. Recent data suggest that COX-2 may play a critical role in brain development and synaptic signaling and COX-2 expression is upregulated under several pathological conditions (Institoris *et al.*, 2009). Mainly COX-2 is thought to be responsible for nociceptive processing, but there are also reports suggesting that COX-1 may become over-expressed during inflammation (Wallace *et al.*, 1998). Constitutively expressed COX-2 is found in the superficial dorsal horn of the rat spinal cord (Beiche *et al.*, 1998). It has been proposed that prostaglandins synthesized by COX-2 assist in synaptic transmission (Adams *et al.*, 1996) and enhance postsynaptic activity of both excitatory and inhibitory neurotransmitters (Kimura *et al.*, 1985). These data may explain the fact that COX-2 plays an important role in central sensitization (Samad *et al.*, 2001) and it is thought to mediate most of the analgesic effects of NSAIDs (Yaksh *et al.*, 2001). In rats, infusion of COX-1/COX-2 inhibitors blocked sensitization of meningeal nociceptors and suppressed ongoing sensitization in spinal trigeminovascular neurons (Jakubowski *et al.*, 2005). A dual-acting

COX-2 inhibitor was effective in a rat model of capsaicin-induced central sensitization (Bingham *et al.,* 2005).

Gonadal steroids, in particular estradiol, modulate nociception (Craft *et al.,* 2004) and 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). In the study of Magos et al. (1983) a subcutaneous implant of 17β-estradiol decreased headache intensity of more than 80% of women with menstrual migraine. Moreover, in women undergoing in vitro fertilization, where an analogue of gonadotropin-releasing hormone was administered to downregulate estrogen levels, the low levels of 17β-estradiol correlated with an increased headache prevalence (Amir *et al.,* 2005). The neurobiological mechanisms which underlie these modulatory effects of estrogen on migraine remain speculative. Estrogen receptors are present on spinal sensory ganglion neurons (Yang *et al*., 1998; Taleghany *et al*., 1999) and in spinal gray matter (Shughrue & Merchenthaler, 2001). In rats, estrogens are reported to decrease myogenic tone through a NO-dependent mechanism in rat cerebral arteries (Geary *et al.,* 1998). In mice it has been shown that estrogens decrease nociception, notably in the trigeminal system (Multon *et al.,* 2005). In the rat nitroglycerin model estradiol pretreatment blunts CGRP and 5-HT changes in the superficial laminae of the TNC (Pardutz *et al.,* 2002), which 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.

On the basis of these data we decided to investigate the possible modulatory effects of COX-inhibition and hormonal influences in the NTG model of migraine.

II. Aims

The aims of our studies were to

i.) study the effect of systemic NTG administration on the CamKII expression of the most caudal part of the TNC of the rat

ii.) examine the possible modulatory effects of estradiol and selective COX inhibitors in the above process.

iii.) determine which isoform of the COX enzyme plays a role in the nNOS activation caused by NTG in the same region.

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 (86/609/EEC). They were approved by the Ethics Committee of the Faculty of Medicine, University of Liège and University of Szeged. 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. CamKII

III.1.1. Estradiol pretreatment

Animals: Twenty-four male and 48 female Wistar rats (weighing between 250 and 350 g) were used. At the age of 2 months, the female animals (n = 48) were ovariectomized under Nembutal anaesthesia and half of them had a 5 mm long Silastic capsule (3.18 mm o.d. and 1.57 mm i.d.; Dow Corning, Midland, MI, USA) filled with a 1 : 1 mixture of cholesterol (Sigma Chemical Co., St Louis, MO, USA) and 17β-estradiol (Fluka, Buchs, Switzerland) inserted subcutaneously in the interscapular region. The capsules maintain estradiol plasma levels in a range 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 [12 males, 12 ovariectomized females (ovx), 12 ovariectomized females treated with estradiol (ovx + E2)] received a subcutaneous (s.c.) 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 injection, the rats for the immunohistochemistry (eight in each group) were deeply anaesthetized 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 (PBS). The cervical (C1–C2) spinal cords, as well as the thoracic (Th1) cord from all animals, were removed and postfixed overnight for immunohistochemistry. The animals for Western blotting (four in each group) were perfused only with physiological saline and the spinal cords were removed as above and processed for Western blotting.

Immunohistochemistry: After cryoprotection (30% sucrose overnight), 30 µm thick transverse cryostat sections were serially cut and collected in 16 wells containing cold PBS. Each well received sections from sequential 500 µm long tissue portions over the entire rostrocaudal extent of the C1–C2 and the Th1 spinal segments. After 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 and then kept for three nights at 4°C in monoclonal anti-CamKII primary antisera (Sigma; C265) at a dilution of 1 : 2000. The immunocytochemical reaction was visualized using the Vectastain (Vector Laboratories Inc., Burlingame, CA, USA; PK-6101) avidin–biotin kit (ABC) with nickel–ammonium–sulphate-intensified 3′,3′-diaminobenzidine (Sigma). The specificity of the immune reactions was controlled by omitting the primary antisera.

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 (Bradford 1976) using bovine serum albumin as a standard. Equal amounts of protein samples (20 mg/lane) were separated by standard SDS–PAGE procedures at 200 V for 1 h and transferred to immobilon P membrane (Millipore). Following the transfer and blocking in 5% non-fat dry milk, membranes were incubated with the CamKII antibody (Sigma; C265), diluted to 1 : 1000. After the detection of CamKII protein, the membranes were stripped and reprobed for a β-actin antibody (Sigma; A5441) diluted to 1 : 4000, which was used as an internal control. Protein bands were visualized using the ECL Western blotting analysis kit (Amersham, Piscataway, NJ, USA). They were quantitatively analysed using a laser

densitometer (Pharmacia LKB, Piscataway, NJ, USA). Optical densities of specific bands were quantified by densitometry and corrected for protein loading by dividing by the β-actin signal of the same sample.

Data analysis: An observer blinded to the experimental procedures counted CamKII-immunoreactive (Ir) cells in laminae I–III of the C1–C2 and Th1 segments of the spinal cord, on three different series of immunostained sections in each animal. In each series the distance between the individual sections was approximately 500 µm along the rostrocaudal axis. The cell counts and relative Western blot optical densities were analysed with ANOVA and post hoc Scheffe's test. Statistical analyses were performed by SPSS (Version 11.0 for Windows; SPSS Inc., Chicago, IL, USA). The significance level was set at $P < 0.05$.

III.1.2. Selective COX inhibitor pretreatment

Animals: Eighty-four adult male Sprague-Dawley rats (weighing between 200 and 250 g) were used. The rats were divided into three groups. Group 1 (n=12): the animals did not receive any pretreatment. Group 2 (n=36): the rats received a s.c. injection of the selective COX-2 inhibitor, NS398 at a dose of 1 mg/kg (n=12), 3 mg/kg (n=12) or 5 mg/kg (n=12). Group 3 (n=36): the rats were injected subcutaneously with the selective COX-1 inhibitor, SC560 at a dose of 1 mg/kg (n=12), 5 mg/kg (n=12) or 10 mg/kg (n=12). Thirty minutes later half of the animals received a s.c. injection of NTG (Nitrolingual®; Pohl-Boskamp GmbH, Hohenlockstedt, Germany), at a dose of 10 mg/kg and the other half of the rats received a s.c. injection of the vehicle of NTG (gift from Pohl-Boskamp GmbH). Four hours after the NTG and the placebo injections the rats were deeply anaesthetized by chloral hydrate (0.4 g/kg i.p.) and transcardially perfused with 100 ml PBS followed by 500 ml 4% paraformaldehyde in PB. Portions of the cervical spinal cords, representing the lowest part of the TNC, between 5 and 11 mm caudal to the obex were removed and postfixed overnight for immunohistochemistry.

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

Data analysis: The protocol was the same as described above for CamKII-Ir cells analysis.

III.2. nNOS

Animals: Eighty-six adult male Sprague-Dawley rats (weighing between 200 and 250 g) were used. The rats were divided into 3 groups. In the first group (n = 12), the animals did not receive any pretreatment. In the second group, the rats received a s.c. injection of the selective COX-2 inhibitor NS398 at a dose of 1 mg/kg (n = 12), 3 mg/kg (n = 12), or 5 mg/kg (n = 18). The animals of the third group were injected subcutaneously with the selective COX-1 inhibitor SC560 at a dose of 1 mg/kg (n = 12), 5 mg/kg (n = 12), or 10 mg/kg (n = 8). Thirty minutes after the drug administration, half of the animals belonging to each group and dosage received a s.c. injection of NTG (Nitrolingual®; Pohl-Boskamp GmbH, Hohenlockstedt, Germany) at a dose of 10 mg/kg and the other half of the animals received a s.c. injection of the vehicle (gift from Pohl-Boskamp GmbH). Four hours after the NTG or the placebo injections, the rats were deeply anesthetized by chloral hydrate (0.4 g/kg) and transcardially perfused as in the CamKII experiments. The portions of cervical spinal cords were removed and postfixed overnight for immunohistochemistry.

Immunohistochemistry: After cryoprotection (30% sucrose overnight) 30 µm cryostat sections were cut and serially collected as in the CamKII experiments. After several rinses in PBS containing 1% Triton X-100, sections were kept for 2 nights at 4°C in anti-nNOS antibody (Euro-Diagnostica, B220-1) at a dilution of 1 : 20000. The immunocytochemical reaction was visualized using the avidin-biotin kit (ABC) of Vectastain (Vector Laboratories Inc., PK-6101) and stained with nickel ammonium sulfate-intensified 3'-3'-diaminobenzidine. Specificity of the immune reaction was controlled by omitting the primary antiserum.

Data analysis: nNOS-IR cells were counted by an observer blinded to the procedures in laminae I-III of the cervical spinal cord, in 3 different series of sections in each animal. The individual sections in these series were taken at 0.5-mm intervals along the rostrocaudal axis. nNOS positive neurons were scored, if they contained a cytoplasmic and dendritic staining and a nucleus. Group values are stated as mean ±SEM. Statistical comparisons among the control and NTG-treated groups at each pretreatment dose were made using analysis of variance (ANOVA) followed by Scheffe' test. Both analyses were implemented in SPSS (Version 11.0 for Windows, SPSS Inc.), with $P < 0.05$ considered statistically significant.

IV. Results

IV.1. CamKII

IV.1.1. Estradiol pretreatment

On microscopic examination of immunostained transverse sections, CamKII immunoreactivity was found in neurons of the TNC and in the neuropil of lamina II. As mentioned in the methods, we focused on immunoreactive neurons. CamKII-Ir cells were abundant in the superficial layers of the caudal spinal trigeminal nucleus. The number of cells was not significantly different between the various rostro-caudal levels, nor between sides of the TNC. After vehicle injection there was no significant difference in the number of CamKII-Ir cells in the TNC superficial laminae I-III between male rats, ovariectomized (ovx) and ovariectomized-estradiol treated females (ovx+E2) (Fig. 1A, 1C, 1E and 2). In contrast, 4 h after subcutaneous NTG administration there was a significant increase in the number of CamKII-Ir cells in males (Fig. 1B vs. 1A) and in ovx animals (Fig. 1D vs. 1C) compared with vehicle injections, but no change was found in ovx + E2 rats (Fig. 1E, 1F).

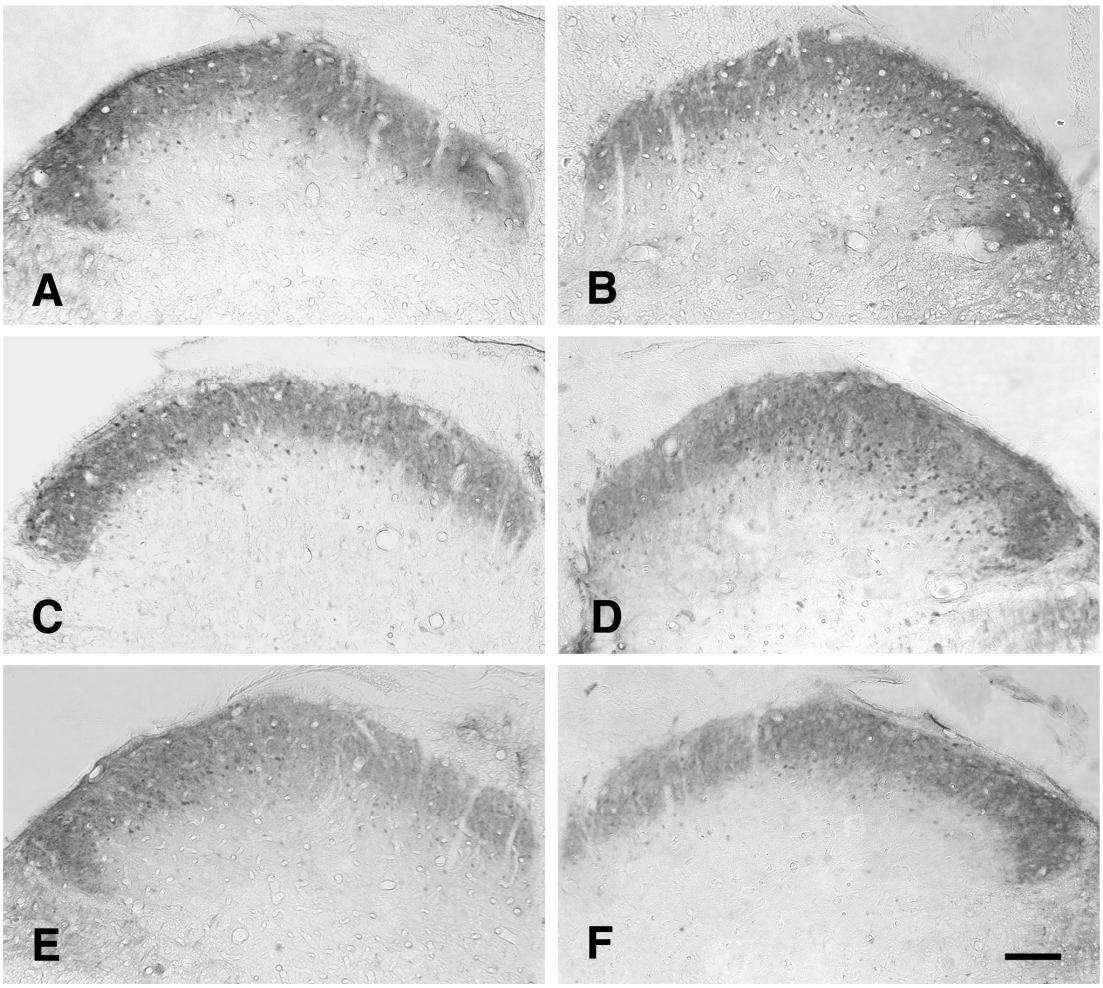

Figure 1 Calmodulin-dependent protein kinase II immunoreactivity on transverse sections of the upper cervical spinal cord in males (A, B), ovariectomized (ovx) (C, D) and ovariectomized and treated with oestradiol (ovx + E2) females (E, F). Nitroglycerin administration (B, D, F) compared with vehicle (A, C, E) increases the number of immunoreactive cells in the superficial layers in males and ovx females (B, D) but not in ovx + E2 rats (F). Scale bar = 50 µm.

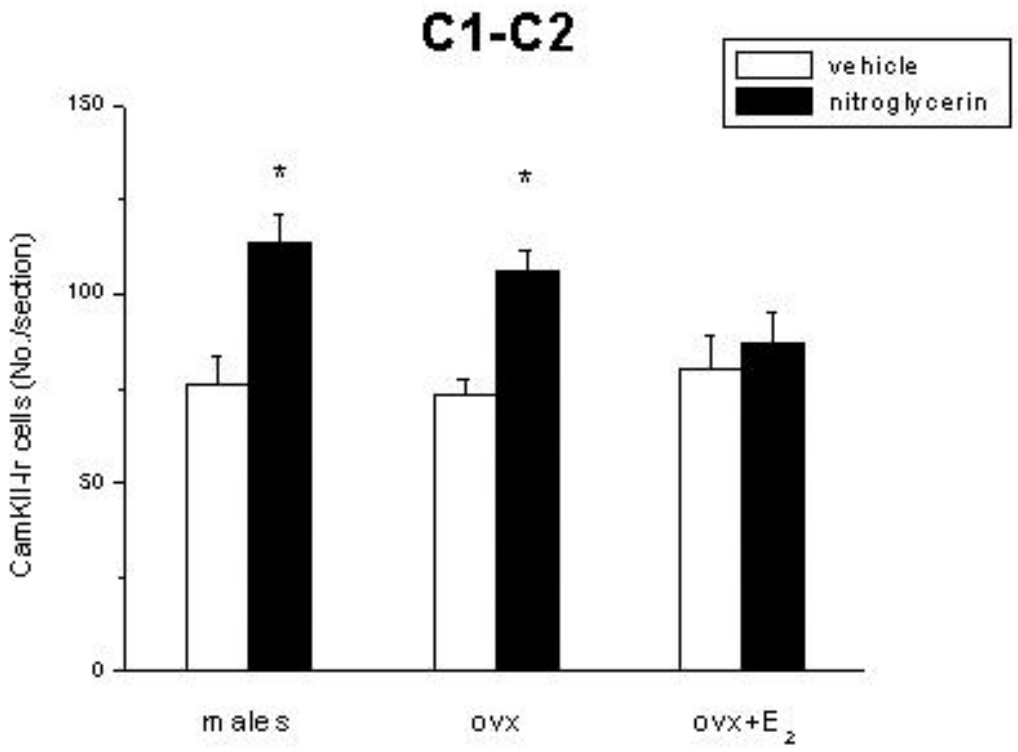

Figure 2 Histogram showing the number of CamKII-Ir cells in superficial laminae I–III of C1–C2 segments in the three animal groups 4 h after subcutaneous injection of vehicle (light bar) or nitroglycerin (dark bar) (mean + SEM, $n = 8$ per group). In males and ovariectomized (ovx) animals, but not in ovariectomized and treated with oestradiol (ovx + E2) animals, there is a significant increase in the number of CamKII-Ir cells (*$P < 0.05$).

At the Th1 level the number of CamKII-Ir cells was overall smaller (sections not shown) and there was no significant difference between animal groups or between treatment conditions (Fig. 3).

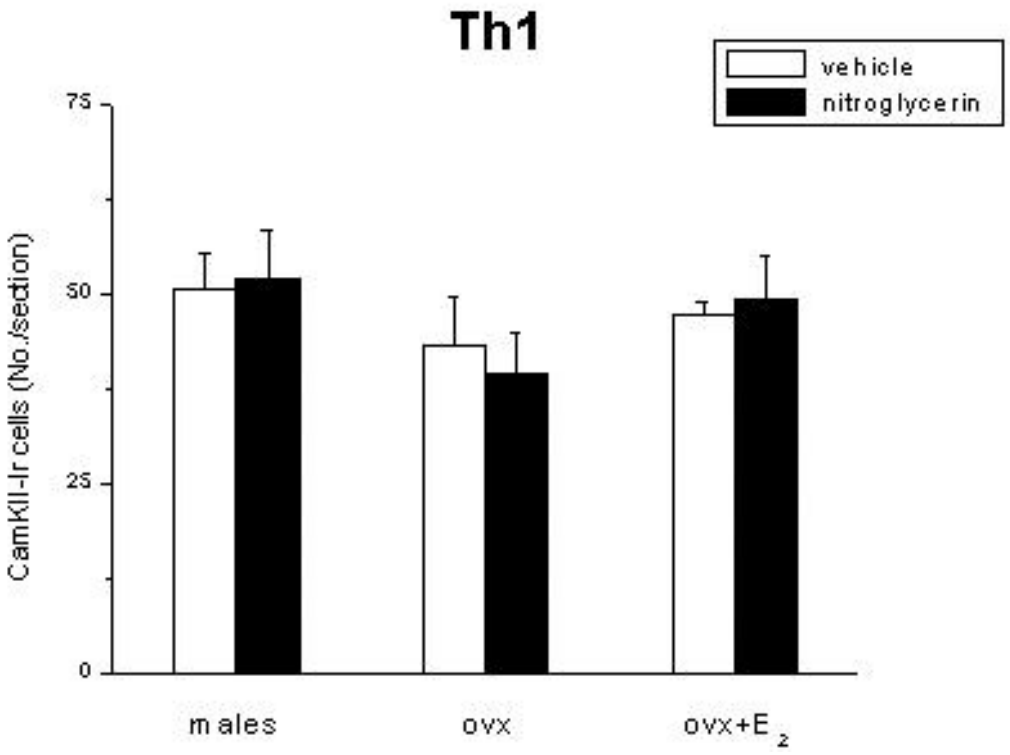

Figure 3 Histogram showing the number of CamKII-Ir cells in superficial laminae I–III of the Th1 spinal cord segment in the three animal groups 4 h after subcutaneous injection of vehicle (light bar) or nitroglycerin (dark bar) (mean + SEM, *n* = 8 per group). There is no significant difference in either of the groups.

The results of Western blotting were in line with those of immunohistochemistry. We identified the bands representing the CamKII protein. In male and ovx animals, which had received NTG 4 h before (Fig. 4, lanes 2 and 4), the density of the CamKII protein band in the

dorsal portion of the C1–C2 segments was increased compared with vehicle-injected rats (Fig. 4, lanes 1 and 3). In the ovx + E2 group the CamKII band was comparable after NTG (Fig. 4, lane 6) or vehicle injection (Fig. 4, lane 5). In the Th1 segments there was no apparent difference in either of the groups (blots not shown).

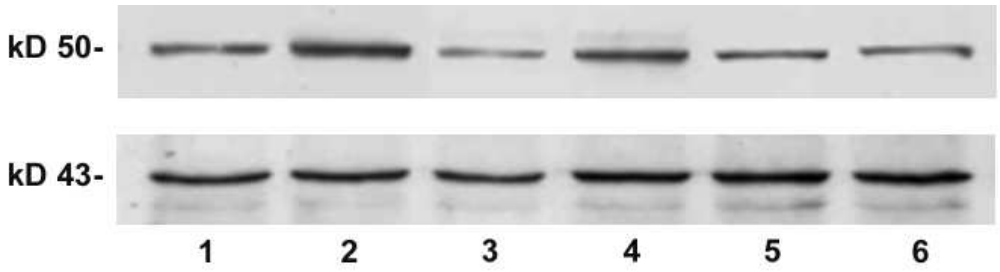

Figure 4 Western blotting of CamKII (50 kDa) and β-actin (43 kDa) in dorsal portions of C1–C2 spinal segments in males (lanes 1, 2), ovariectomized (ovx) (lanes 3, 4) and ovariectomized and treated with oestradiol (ovx + E2) (lanes 5, 6) animals. Compared with the vehicle (1, 3, 5), nitroglycerin administration (2, 4, 6) enhances the CamKII band in the male (2) and ovx groups (4), but not in the ovx + E2 group (6).

Densitometric analyses of the blots confirmed that CamKII expression in the dorsal C1–C2 segments was significantly enhanced after NTG administration in male and ovx rats, but not in ovx + E2 animals (Fig. 5) and that there was no difference between NTG and vehicle injections in the dorsal part of the Th1 spinal segments in either group (Fig. 6).

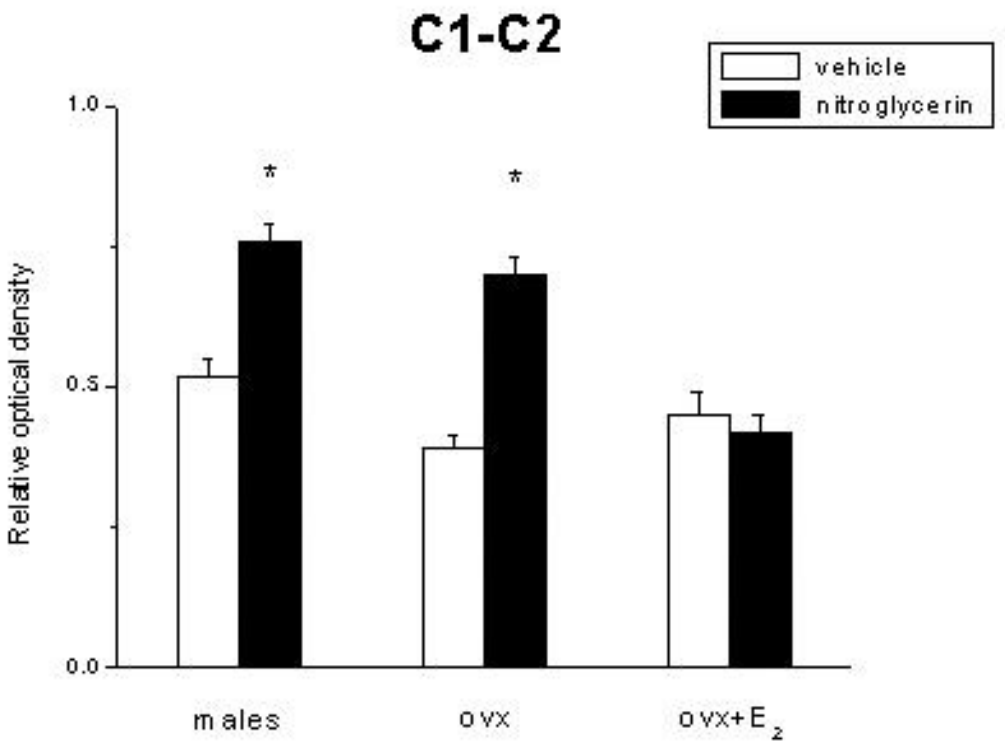

Figure 5 Histogram showing the optical densities of CamKII Western blots in the C1–C2 segments of the three animal groups 4 h after subcutaneous injection of vehicle (light bar) or nitroglycerin (NTG) (dark bar) (mean + SEM, n = 4 per group). Data are expressed as proportions of β-actin band densities. In male and ovariectomized (ovx) rats there is a significant increase in optical density in the CamKII band after NTG (*P < 0.05), whereas this increase is absent in ovariectomized and treated with oestradiol (ovx + E2) rats.

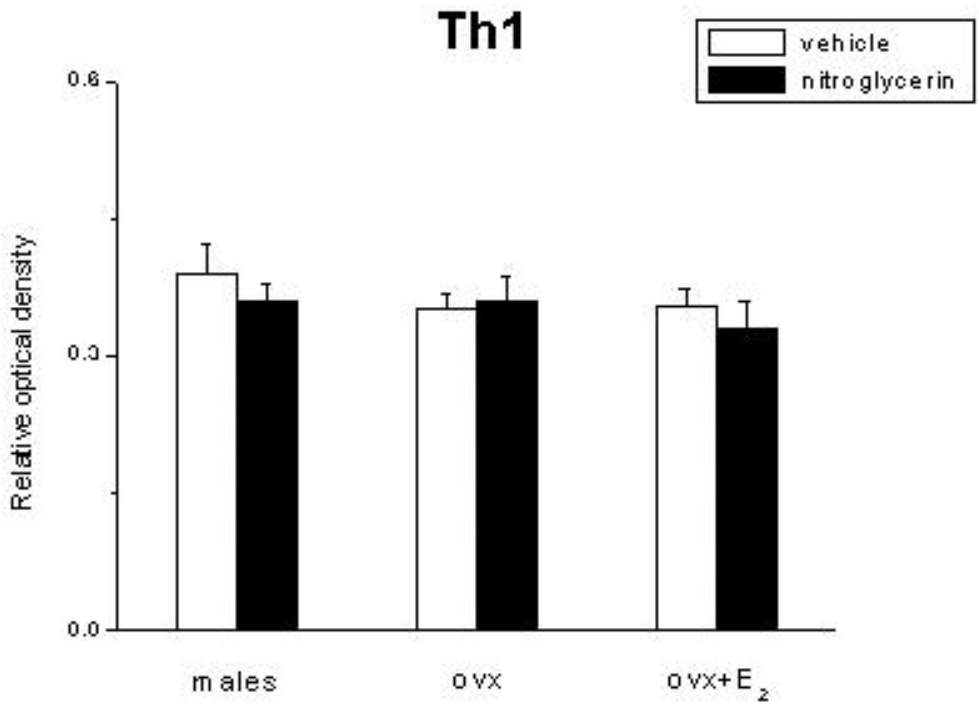

Figure 6 Histogram showing the optical densities of CamKII Western blots in the Th1 segments of the three animal groups 4 h after subcutaneous injection of vehicle (light bar) or nitroglycerin (dark bar) (mean + SEM, *n* = 4 per group). Data are expressed as proportions of β-actin band densities. There is no significant difference between the two animal groups.

IV.1.2. Selective COX inhibitor pretreatment

During microscopic examinations CamKII-Ir cells were numerous in the superficial layers of the caudal trigeminal nucleus. NTG produced a significant increase of CamKII-positive cells in the superficial layers of the caudal trigeminal nucleus in the non-pretreated rats ($P < 0.05$) (Fig. 7a, 7b). This phenomenon was similar when rats received the COX-1 inhibitor, SC560 in various doses (1 mg/kg, 5 mg/kg or 10 mg/kg) before NTG ($P < 0.05$, $P < 0.05$, $P < 0.05$ respectively) (Fig. 7c, 7d and 8).

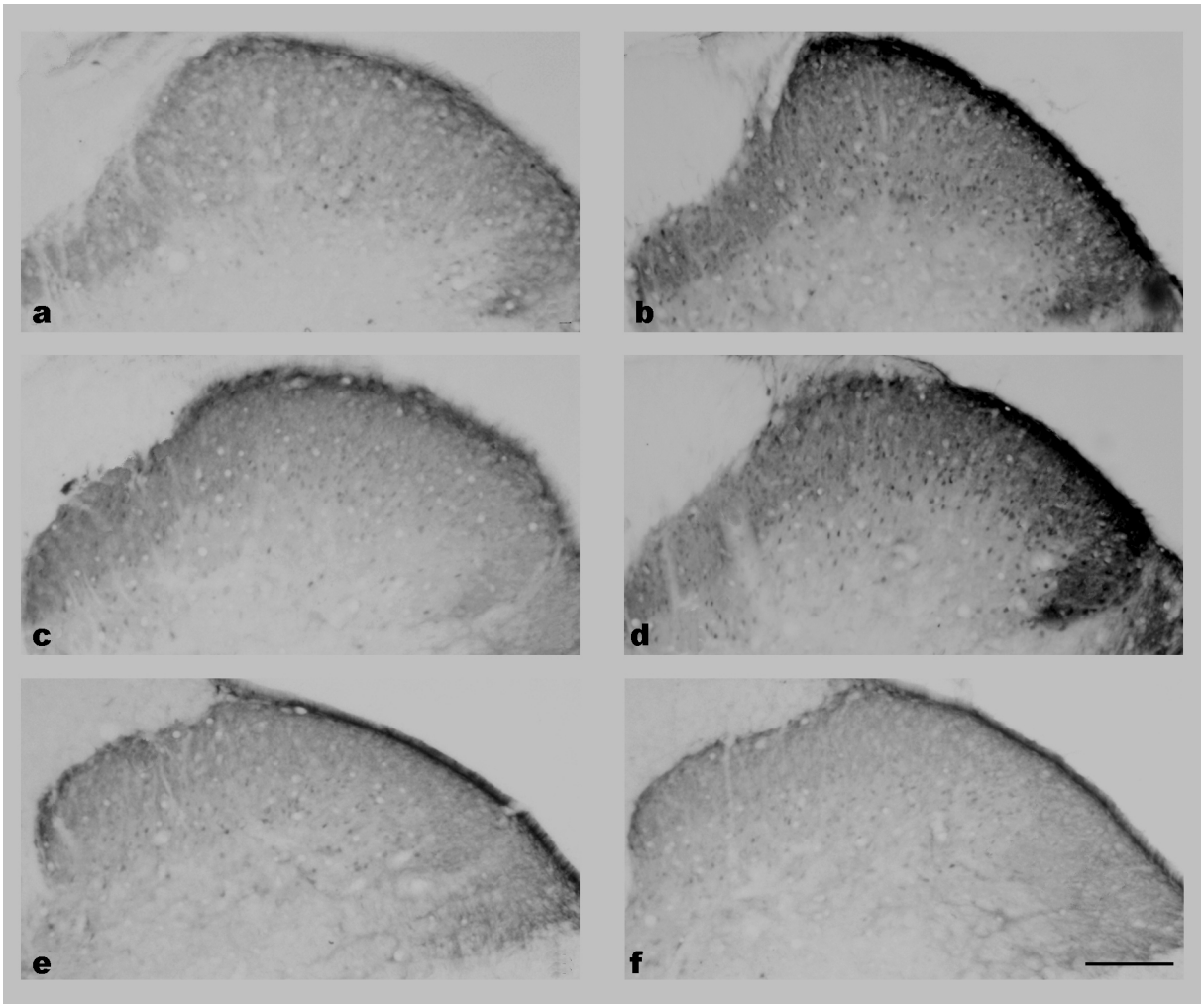

Figure 7 CamKII-immunoreactivity in the upper cervical spinal cord in control (a, b), SC560- (c, d), and NS398-pretreated (e, f) rats after placebo (a, c, e) or NTG (b, d, f) injections. NS398 pretreatment attenuated the NTG-induced CamKII-activation. Scale bar = 50 μm.

Pretreatment with COX-1 inhibitor in any dosages failed to modulate the NTG-induced CamKII enhancement in the TNC (P = 1 for all doses used) (Fig. 8).

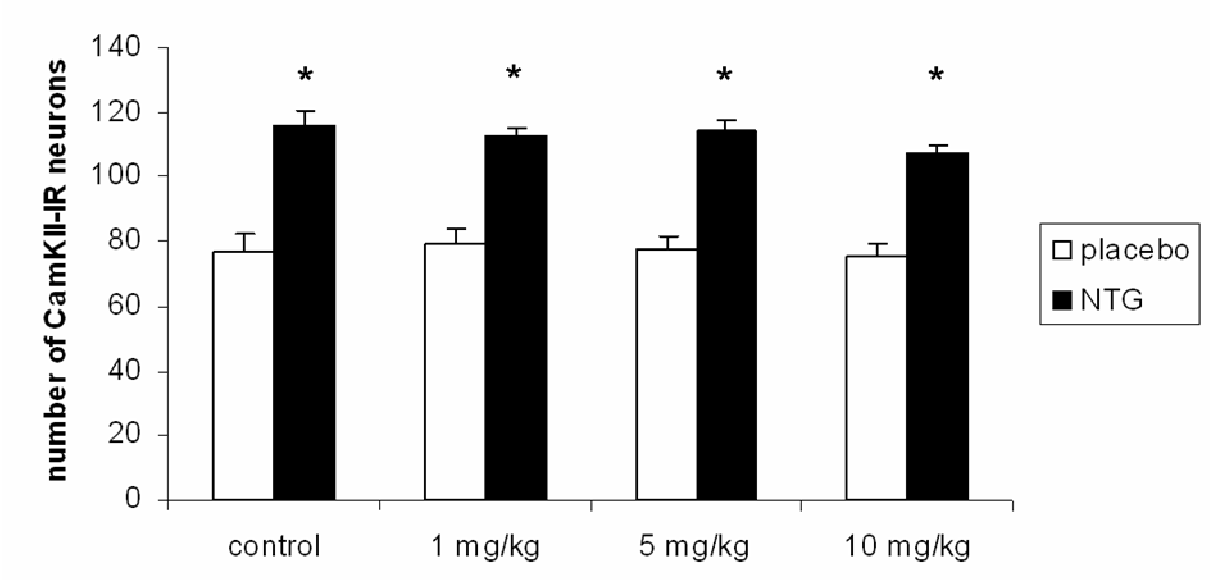

Figure 8 Mean number of CamKII-immunoreactive cells in dorsal horns of SC560-pretreated animals after vehicle (light bar) or NTG (dark bar) injection. There is a significant increase of CamKII-immunoreactive cells after NTG injection in all groups ($^{*}P < 0.05$).

At doses of 3 and 5 mg/kg of the selective COX-2 inhibitor NS398, NTG treatment failed to induce a statistically significant increase in CamKII expression (P = 0.84, P = 0.7, respectively) (Fig. 7e, 7f and 9). The NTG-induced CamKII upregulation was attenuated by 3 mg/kg (P < 0.05) and 5 mg/kg (P < 0.001) of the selective COX-2 inhibitor (Fig. 9).

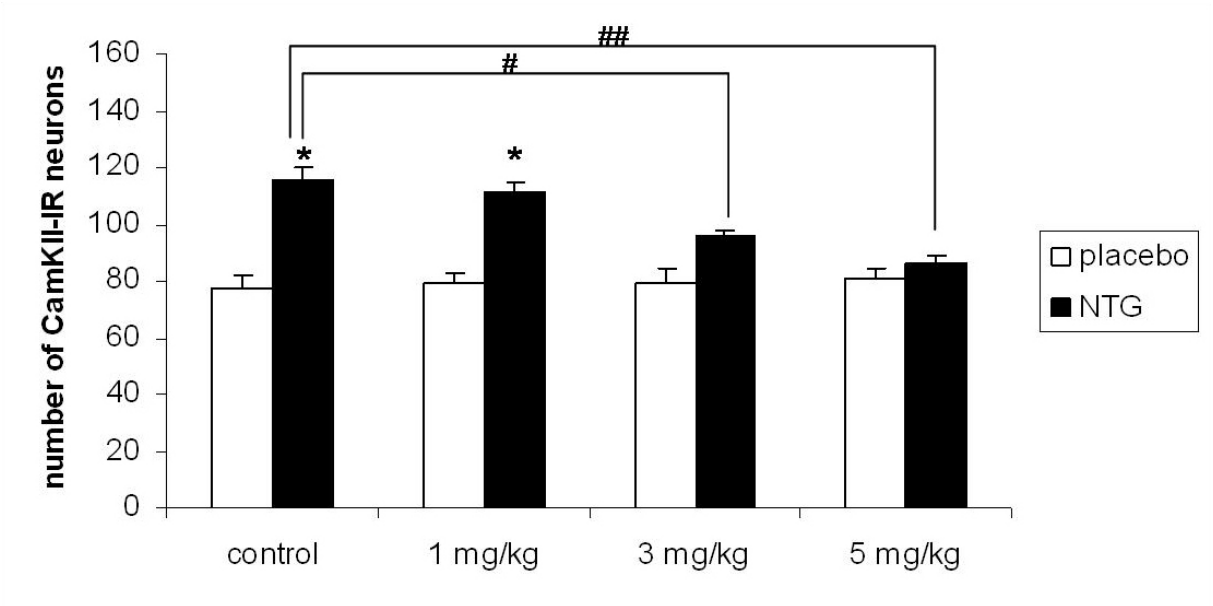

Figure 9 Mean number of CamKII-immunoreactive cells in dorsal horns of NS398-pretreated animals after vehicle (light bar) or NTG (dark bar) injections. 3 and 5 mg/kg NS398 pretreatment attenuated the NTG-induced increase in CamKII-activation, but 1 mg/kg not ($^{*}P < 0{,}05$; $^{\#}P < 0{,}05$; $^{\#\#}P < 0{,}001$).

IV.2. nNOS

Transverse sections of the cervical spinal cord demonstrated many nNOS-Ir neurons in the superficial laminae of the dorsal horns. These cells are mainly small to medium sized neurons (8-15 µm in diameter) with few dentrites. There was no significant difference in the number of Ir cells at different levels of the C1-C2 region. NTG produced significant increase of nNOS-positive cells in the superficial layers of the caudal trigeminal nucleus in the non-pretreated rats ($P < 0.01$) (Fig. 10a, 10b).

This phenomenon was similar when rats received the COX-1 inhibitor, SC560 in various doses (1 mg/kg, 5 mg/kg or 10 mg/kg) before NTG ($P < 0.05$, $P < 0.01$, $P < 0.05$ respectively) (Fig. 10e, 10f and 11).

The COX-2 inhibitor NS398 given at the dose of 3 mg/kg ($P = 0.82$) or 5 mg/kg ($P = 1$), but not of 1 mg/kg ($P < 0.05$) prior to NTG attenuated the NO-induced nNOS increase (Fig. 10c, 10d and 12). Compared to the control group, after NTG injections, the number of nNOS-Ir neurons was significantly lower in the animals, which received the highest dose of NS398 ($P < 0.05$) (Fig. 12).

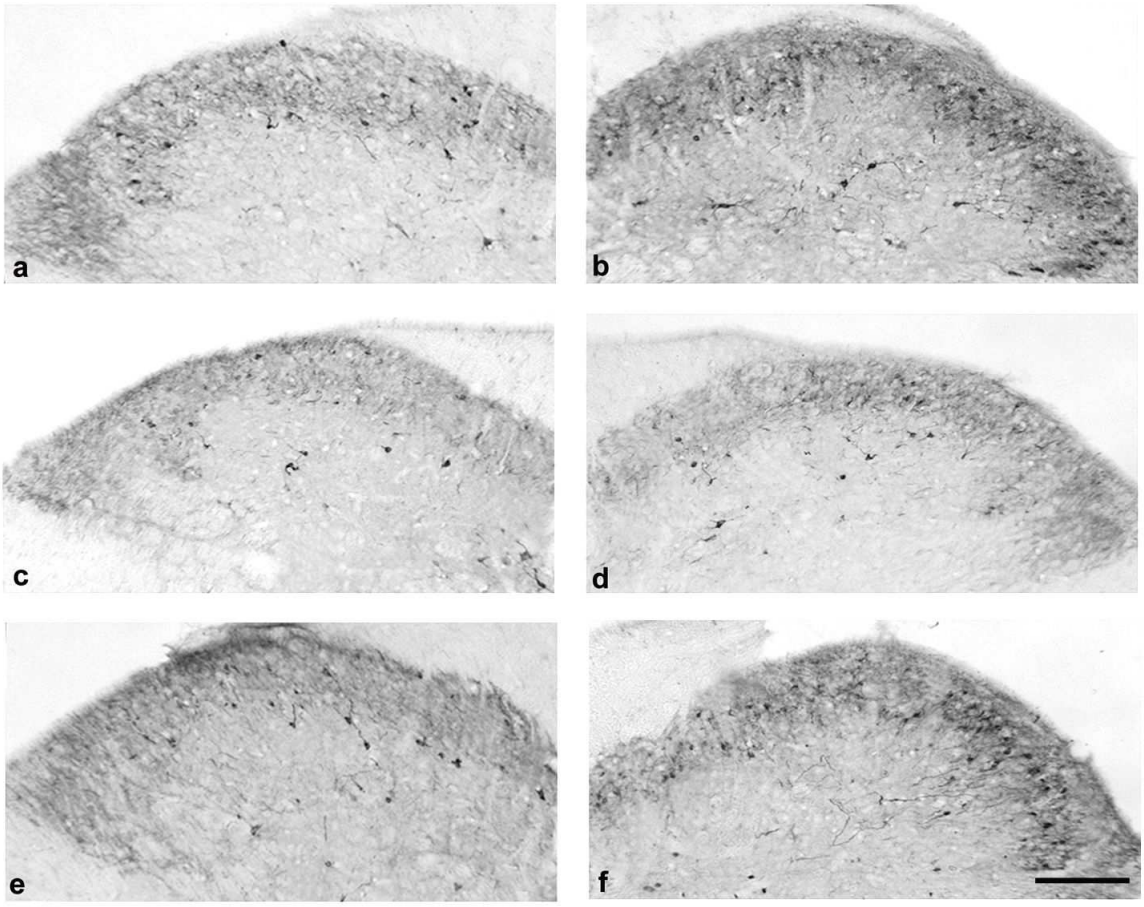

Figure 10 nNOS-immunoreactivity in the upper cervical spinal cord in control (a, b), NS398- (c, d), and SC560-pretreated (e, f) rats after placebo (a, c, e) or NTG (b, d, f) injections. NS398 pretreatment attenuated the NTG-induced nNOS-activation. Scale bar = 50 μm.

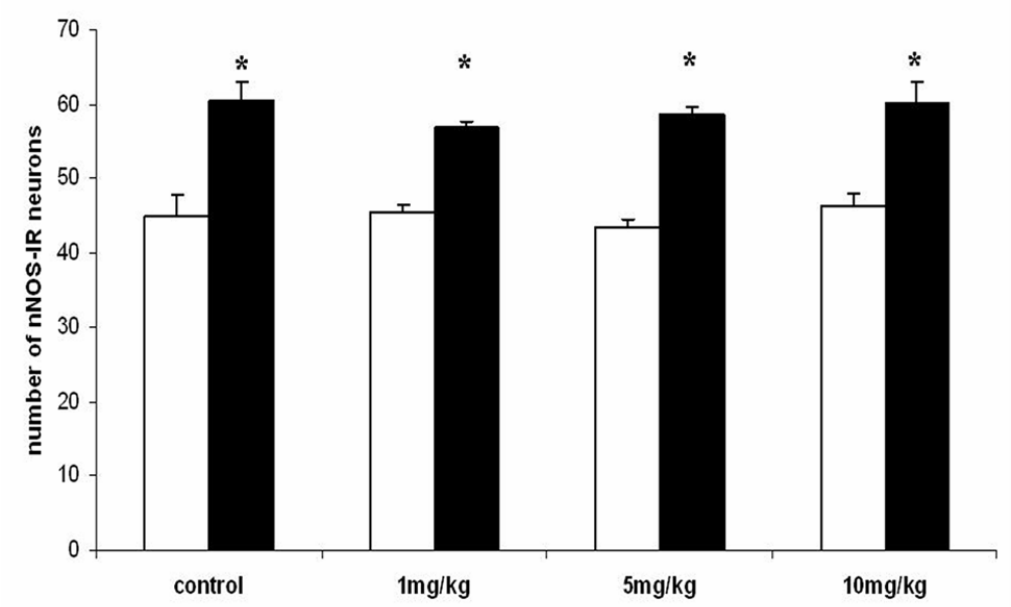

Figure 11 Mean number of nNOS-immunoreactive cells in dorsal horns of SC560-pretreated animals after vehicle (light bar) or NTG (dark bar) injection. There is a significant increase of nNOS-immunoreactive cells after NTG injection in all groups ($^{*}P < 0.05$).

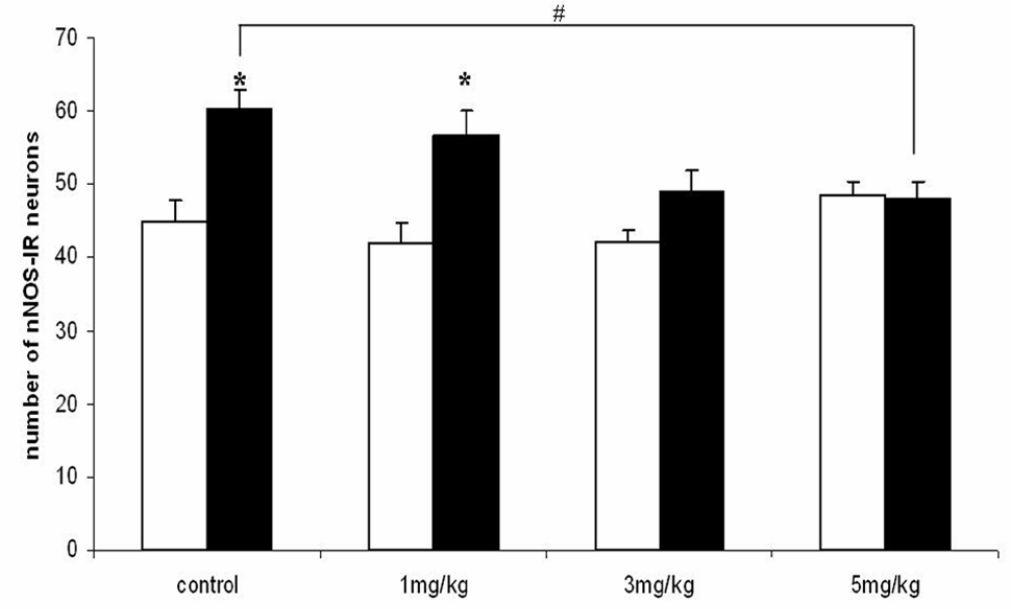

Figure 12 Mean number of nNOS-immunoreactive cells in dorsal horns of NS398-pretreated animals after vehicle (light bar) or NTG (dark bar) injections. 3 and 5 mg/kg NS398 pretreatment attenuated the NTG-induced increase in nNOS-activation, but 1 mg/kg not ($^{\#,*}P < 0{,}05$).

V. Discussion

V.1. NTG-induced CamKII and its modulation by estradiol

Our data demonstrated that systemic administration of NTG enhances CamKII immunoreactivity in laminae I–III of the spinal portion of the TNC. Previous studies have demonstrated increases of c-fos (Tassorelli & Joseph, 1995) and nNOS expression (Pardutz *et al.*, 2000) in TNC after NTG administration at a dose of 10 mg/kg. The latter may be a molecular basis for a self-amplifying process and sensitization, as described clinically during migraine attacks (Burstein *et al.*, 2000). The effect of the NO donor on nNOS expression seemed to be selective for the trigeminal system, as no effect was detected in upper thoracic segments (Pardutz *et al.*, 2000). We found the same trigeminal selectivity for the NTG-induced CamKII changes. NO probably activates the trigeminal system via an effect on the peripheral nociceptive afferents, since capsaicin pretreatment, which destroys these small afferent fibres, abolishes the NTG-induced c-fos activation of secondary trigeminal nociceptors (Tassorelli *et al.*, 1997). NTG itself has different effects on nociception depending on the dose administered (Sousa & Prado, 2001; Colasanti & Suzuki, 2000). In the rat, an intravenous infusion of glycerylnitrate at low doses (2–50 mg/kg per min) does not cause c-fos activation in the TNC (Martin & Martin, 2001) or an increase of jugular vein CGRP levels (Offenhauser *et al.*, 2005), despite its capability to enhance stimulus sensitivity in the trigeminal system (Lambert *et al.*, 2002). In humans, microgram doses are used intravenously or sublingually to trigger migraine attacks (Olesen *et al.*, 1993). There may thus be species differences in the dose– response relationship of NTG. As far as the time course of the NTG-induced effects is concerned, its brain concentrations and those of cGMP rise significantly 2 h after subcutaneous administration. Fos expression peaks at 1 h in neurons belonging to vasoregulatory areas, but only at 4 h in TNC neurons (Tassorelli *et al.*, 1999).

There is strong evidence that CamKII plays a key role in nociceptive processing and sensitization of central sensory neurons (Choi *et al.*, 2006; Luo *et al.*, 2008). It was shown that CamKII located in the hippocampus is important regulator during the nociceptive processes induced by formalin, glutamate, pro-inflammatory cytokines, and acetic acid injection (Seo *et al.*, 2008). Autophosphorylation-deficient CamKII mutant mice display

deficiencies in ongoing nociceptive responses in the formalin model, leading to the hypothesis that CamKII is primarily involved in spontaneous nociception (Zeitz *et al.,* 2004). CamKII is abundant in the superficial layers of the dorsal horns, where it is increased during the noxious stimuli induced by capsaicin (Fang *et al.,* 2002) or by formalin (Liang *et al.,* 2004). Intratechal injections of substance P also increases CamKII immunoreactivity in laminae I–II of the spinal cord (Choi *et al.*, 2005), and this increase can be blocked by CamKII inhibitors (Fang *et al.,* 2002). CamKII protein is increased in the medullary dorsal horn following nerve injury to the inferior alveolar nerve wherein mechanical allodynia is alleviated by CamKII inhibition (Ogawa *et al.,* 2005). Peripheral CamKII immunoreactivity is also increased following complete Freund's adjuvant -induced inflammation (Carlton 2002). Price et al. (2005) provided evidence that capsaicin stimulates autophosphorylation of CamKII in sensory neurons and that pharmacological inhibition of CamKII reduces vanilloid receptor 1-mediated CGRP release. It was shown that more than half of dorsal root ganglion cells are CamKII-positive, especially in the trigeminal ganglion, where it is expressed in CGRP- and vanilloid receptor 1-immunoreactive neurons (Ichikawa *et al.,* 2004). The hyperexcitability induced by capsaicin in trigeminal ganglion neurons via inactivation of I(A) currents is also mediated in part by CamKII (Liu & Simon, 2003), as well as the activation of the vanilloid receptor 1 by phosphorylation in rat ganglion cells (Jung *et al.,* 2004). It has been recently discovered that nociceptive stimuli up-regulate CamKII in the dorsal horns by peptidergic afferents (Larsson & Broman, 2006), which are also crucial in migraine. In cultured neurons it has been shown that CamKII is able to decrease nNOS activity by phosphorylating this protein (Komeima *et al.,* 2000), raising the remote possibility that CamKII might counteract the NTG-induced nNOS activation. Moreover, in rats and mice calmodulin can activate various adenylyl cyclases which contribute to sensitization in the spinal cord (Wei *et al.,* 2006). In a recent study the CamKII inhibitor, KN93 dose-dependently prevented the inflammation-induced thermal hyperalgesia and mechanical allodynia (Luo *et al.,* 2008). We now show that CamKII can also be activated in secondary trigeminal nociceptors by high doses of the NO donor NTG, which suggests that it may play a role in NTG-induced migraine headaches. Although the involvement of CamKII in migraineurs is not yet proven, these data suggest that this enzyme plays an important role in migraine pathogenesis. In our study we demonstrated that systemic administration of NTG enhances CamKII expression in the superficial layers of the

TNC, which suggest that CamKII may play a role in sensitization of the trigeminal system and in the pain processes during migraine.

Estrogens are known to modulate nociception (Craft *et al.,* 2004), including migraine attacks (Somerville 1975). It was shown that protracted estrogen deprivation tends to have pronociceptive effects in the orofacial formalin model of pain in mice (Multon *et al.,* 2005) and in the rat NTG model, where it blunts CGRP and 5-HT changes in the superficial laminae of the TNC (Pardutz *et al.,* 2002). In both instances the observed abnormalities are reversed by estradiol administration. In the hippocampus, where CamKII is known to be crucial for long-term potentiation (Havik *et al.,* 2003), estradiol can rapidly induce its neuronal expression (Pozzo-Miller *et al.,* 1999; Sawai *et al.,* 2002). Our results contrast with the latter, in so far as chronic administration of estradiol has no detectable effect on baseline expression of CamKII in TNC, but is able to suppress its activation by nitroglycerin. Estrogen receptors are present on spinal sensory ganglion neurons (Yang *et al.,* 1998; Taleghany *et al.,* 1999) and in the spinal grey matter (Shughrue & Merchenthaler, 2001). Estradiol may thus act at the genomic level, which would modulate the expression of CamKII and hence annihilate any detectable change in its immunoreactivity after NTG administration. Whether these findings are relevant to the hormonal influences on migraine remains speculative. However, combined with the clinical observation that female migraineurs are more sensitive to certain trigger factors during the perimenstrual period, they may suggest that the low estrogen levels of the premenstrual and menstrual phases render female migraineurs also more prone to the attack-triggering effects of NTG. By the same token, one could draw a parallel between our data in rats, showing that chronic estradiol treatment suppresses the selective activation of secondary trigeminal nociceptors by nitroglycerin, and the clinical observation that migraine attacks tend to be suppressed when sex hormone levels are high and stable, such as during pregnancy (Marcus 1995, Silberstein & Merriam, 2000).

In summary, NTG, a NO donor, is able to induce CamKII expression in the superficial layers of the TNC in the rat. This effect is annihilated by chronic high concentrations of estradiol. Considering the known biological properties of CamKII, one may expect that its increased expression after NO enhances nociception in the trigeminal system. In contrast, the suppression of this activation by high estradiol levels can be regarded as trigeminal antinociception. The acute NTG-induced and the chronic estradiol-dependent changes both

seem to be selective for the trigeminal sytem. They may thus be relevant to an understanding of the delayed NTG-triggered headache attacks in migraineurs and in patients suffering from chronic tension-type headache, and of the protective effect of stable plasma estrogen levels. They may also give some hints on the molecular effects of NO donors and ovarian steroids in trigeminovascular pain syndromes, such as migraine.

V.2. Effect of COX inhibitors

The major findings of our studies are (1) the selective COX-2 inhibitor NS398 attenuated the NTG-induced nNOS and CamKII activation in the superficial layers of the most caudal portion of the TNC in the rat, (2) in contrast, the pretreatment with a selective COX-1 inhibitor, SC560, failed to modulate this phenomenon.

It is not precisely known how NTG administration modifies the nNOS expression in the TNC. This modification is probably due to a secondary activation of second order nociceptive neurons and/or interneurons by NO excitation of their peripherial afferents, which might initiate a self-amplifying process of NO production possibly leading to central sensitization (Pardutz *et al.,* 2000). Increased prostaglandin E2 release and NO production of monocytes were found in patients with migraine without aura, which indicates that NOS and COX pathways are linked in monocytes (Stirparo *et al.,* 2000). In migraine patients, it has been shown that there is an early activation of the L-arginine/NO pathway and a late rise in the synthesis of prostanoids after the onset of the headache (Sarchielli *et al.,* 2000). Since in earlier experiments the nonspecific COX-inhibitor lysine acetyl-salicylate treatment attenuated the NTG-induced nNOS expression (Pardutz *et al.,* 2004), the prostanoids may have an important role in the signal transduction. Since lysine acetyl-salycilate has COX-2 inhibiting property, these earlier data are concordant with our present observation about the effect of the selective COX-2 inhibitor NS398 on the NTG-induced nNOS activation. Our study provides the first evidence that the elevation in nNOS immunoreactivity can be influenced selectively by COX inhibitors.

Furthermore we have shown that the selective COX-2 inhibitor, NS398 attenuated the NTG-induced increase of CamKII expression in the superficial layers of the lower TNC in rats. In contrast, pre-treatment with a selective COX-1 inhibitor, SC560 was not able to

modulate this phenomenon. COX-1 can be found in the sensory ganglia and the spinal cord and there are results suggesting its involvement in nociception (Dou *et al.,* 2004; Zhu & Eisenach, 2003). Previous studies have demonstrated that oral SC-560 at a dose of 30 mg/kg can reduce carragenan-induced thermal hyperalgesia (Yaksh *et al.,* 2001). It is also known that SC-560 displays low (<15%) bioavailability after given orally (Teng *et al.,* 2003). Comparable parenteral dosage of SC-560 in our study failed to modulate the NTG induced increase of CamKII expression suggesting that COX-1 is not involved in this phenomenon.

It has been shown that systemic NTG increased the expression of COX-2 and prostaglandin E2 in the lower brain stem after 4 h (Tassorelli *et al.,* 2007). COX-2 inhibitors are effective in the treatment of migraine. In animals, constitutively expressed COX-2 is found in the superficial dorsal horn of the rat spinal cord (Beiche *et al.,* 1998). It has been proposed that prostaglandins, synthesized by COX-2, assist in synaptic transmission (Adams *et al.,* 1996) and enhance postsynaptic activity of both excitatory and inhibitory neurotransmitters (Kimura *et al.,* 1985). These data may explain the fact that COX-2- and/or COX-2-derived metabolites play an important role in central sensitization (Samad *et al.,* 2001) and they are thought to mediate most of the analgesic effects of NSAIDs (Yaksh *et al.,* 2001). In rats, nimesulide, a preferential COX-2 inhibitor, showed a significant analgesic effect in tailflick and in formalin tests, and it was also effective in these tests after NTG-induced hyperalgesia (Tassorelli *et al.,* 2003). Contrary to the COX-2 inhibitor celecoxib, the COX-1 inhibitor SC560 failed to reduce edema and hyperalgesia after carrageenan induced inflammation in the rat (Smith *et al.,* 1998). Oral and intrathecal administration of SC560 did not alter the behavior changes in the formalin test where celecoxib and indomethacin were effective (Yamamoto *et al.,* 2002). Taken together these data and our present results suggest that the stimulating effect of NTG and that of NO on nNOS and CamKII expression in secondary trigeminal nociceptors are mediated by COX-2-expressing interneurons in the TNC superficial laminae. Therefore, COX-2 may participate in the activation of the trigeminal system and may be a crucial enzyme in the pathophysiology of headaches. We have demonstrated that NO-induced nNOS and CamKII expression in the TNC of the rat is dose-dependently attenuated by pretreatment with COX-2 but not with COX-1 inhibitors. These data suggest that prostanoids, especially produced via the COX-2 pathway, are involved in NO-mediated activation of the trigeminal system. The fact that COX-2 plays a role in this

self-amplifying process of the trigeminal area may give us further details about headache and migraine pathophysiology.

VI. Conclusions

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

i. In the caudal trigeminal nucleus of the rat the NO donor NTG caused a delayed induction of CamKII, which is thought to play a role in sensitization of central nociceptors, a phenomen present in migraineurs.

ii. We examined the modulatory effect of estradiol, a gonadal steroid which influences the clinical picture of migraine on the NTG-induced CamKII enhancement. NTG had no effect on CamKII expression in estradiol-treated ovariectomized animals. Thus NTG, i.e. NO, selectively enhances CamKII in the rat TNC and estradiol blocks this effect.

iii. We also investigated the effect of selective COX inhibitors on the NTG-induced nNOS and CamKII expression in the TNC. In earlier experiments the nonspecific COX-inhibitor lysine acetyl-salicylate treatment attenuated the NTG-induced nNOS expression (Pardutz et al., 2004), which suggested that the prostanoids may be involved in NO-mediated activation of the trigeminal system. In our study we demonstrated that NO-induced nNOS and CamKII expression in the TNC of the rat is dose-dependently attenuated by pretreatment with COX-2 but not with COX-1 inhibitors.

These data suggest that COX-2 plays a role in the self-amplifying process of the trigeminal area and may contribute to a better understanding of the pathomechanisms of migraine headache and the relevance of the modulating effect of estradiol and selective COX-2 inhibitors in these conditions.

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