Neuronal nitric oxide synthase is involved in the induction of NGF-induced neck muscle nociception

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Promotionszusammenfassung

Einfluss der neuronalen Stickstoffmonoxid-Synthase auf die nozizeptive Signalverarbeitung aus der Nackenmuskulatur ausgelöst durch NGF bei anästhesierten Mäusen.

Fragestellung

Der Spannungskopfschmerz (TTH) tritt im Vergleich zu allen anderen primären Kopfschmerzen am häufigsten auf. Epidemiologische Studien zeigen eine 1-Jahresprävalenz von 42% weltweit und bis zu 80% in Europa mit einer steigenden Tendenz. Betrachtet man die sozioökonomische Bedeutung, so liegt der TTH unter allen Kopfschmerzen an erster Stelle. Hinsichtlich wissenschaftlicher Studien ist es allerdings der am wenigsten untersuchte Kopfschmerz. Die noch weitgehend unklare Pathophysiologie des TTH wird seit wenigen Jahren mittels eines etablierten tierexperimentellen Modelles erforscht. Es basiert auf Erkenntnisse aus humanexperimentellen Befunden, wonach bei TTH- Patienten eine erhöhte Schmerzempfindlichkeit der perikranialen Muskulatur sowie eine Assoziation von noxischem Input aus der Nackenmuskulatur nachgewiesen wurden. TTH-Patienten zeigen zudem deutlich niedrigere Schwellen für Druckschmerzempfindung und -toleranz im Vergleich zu Kontrollgruppen. Dabei korreliert die Stärke der perikranialen Schmerzempfindlichkeit mit der Intensität und Frequenz des Kopfschmerzes. Diskutiert wird eine resultierende Schwellenwertverstellung des nozizeptiven Systems durch noxischen Input aus der Muskulatur. Der extrazelluläre Botenstoff Stickstoffmonoxid (NO) ist an vielen physiologischen Prozessen beteiligt, unter anderem der Schmerzwahrnehmung und -weiterleitung im zentralen und peripheren Nervensystem. NO wird durch drei Isoenzyme der NO- Synthasen (NOS), der neuronalen NOS (nNOS), der endothelialen NOS und der induzierbaren NOS gebildet. Immunhistochemische Untersuchungen des peripheren und zentralen Nervensystems deuten auf eine Schlüsselrolle der nNOS bei der Schmerzverarbeitung hin. Neben der Eigenschaft als Transmitter der Schmerzwahrnehmung wird NO mit der Ausbildung der zentralen Sensibilisierung in Verbindung gebracht. Folgend konnte in tierexperimentellen Schmerzmodellen eine Reduktion der zentralen Sensibilisierung durch NOS-Inhibitoren nachgewiesen werden. Unter Berücksichtigung der Eigenschaften und der vorliegenden Studien könnte die nNOS das wichtigste NOS-Isoenzym hinsichtlich eines therapeutischen Effektes bei Kopfschmerzen sein.

Im Rahmen des kürzlich entwickelten und etablierten Tiermodells werden die elektrophysiologischen Experimente an männlichen Mäusen in Allgemeinanästhesie durchgeführt. Noxischer Input wird durch Injektion algogener Substanzen in beide Musculi semispinales induziert. Änderungen der nozizeptiven Signalverarbeitung im Hirnstamm werden durch Ableitung des Kieferöffnungsreflexes (Jaw-opening-reflex, JOR) registriert. Der JOR wird durch elektrische Stimulation der Zunge ausgelöst und über Elektroden im Musculus digastricus abgeleitet. Als nozizeptiver Stimulus wurde in der vorliegenden Arbeit der rekombinante, humane Nervenwachstumsfaktor (β-nerve growth factor, NGF) eingesetzt. Vorherige Studien zeigten im identischen tierexperimentellen Aufbau nach Applikation von NGF in beide Musculi semispinales eine signifikante Bahnung der Nozizeption im Hirnstamm mit Herabsetzen der Reflexschwelle.

Zur Untersuchung des Einflusses der selektiven nNOS-Inhibition auf die Nozizeption der Nackenmuskulatur bei anästhesierten Mäusen wurde der selektive nNOS-Inhibitor Nω-propyl-L-arginin (NPLA) intraperitoneal appliziert.

Methodik

Die elektrophysiologischen Experimente wurden an männlichen C57BL/6-Mäusen in Allgemeinanästhesie durchgeführt (vgl. Brain Res Protoc 2003; 11: 178-88). Effekte auf die kraniofaziale, nozizeptive Signalverarbeitung wurden durch Ableitung des JOR nach elektrischer Stimulation der Zungenmuskulatur erfasst (Reizdauer 500 µs). Der Reflex wurde in Serien von je acht Reizen ausgelöst (0.1 Hz), die Serien wurden alle 10 Minuten wiederholt. Latenz, Dauer und Integral des Reflexes wurden gemessen. Nach Bestimmung der JOR-Reflexschwelle (IJOR, 0–2 mA) wurde die Stimulusintensität mit 125% der IJOR festgelegt.

Zur Evaluation des Einflusses von nNOS auf das Auslösen der nozizeptiven Bahnung der Nackenmuskulatur wurde zunächst nach zwei stabilen Baseline-Reflexserien der selektive nNOS-Inhibitor NPLA (100µl; 0.096 mg/kg, 0.96 mg/kg und 1.92 mg/kg, je n=5) respektive einer Kochsalzlösung (n=5) als Kontrolle intraperitoneal appliziert. Folgend wurde nach drei Serienmessungen die bilaterale Injektion von NGF (0.8 µmol/l) in die Musculi semispinales als Korrelat für den noxischen Input durchgeführt. Anschliessend erfolgten die Reflexserien für eine weitere Stunde.

In einem zweiten Versuchsaufbau wurde die Rolle von nNOS hinsichtlich der Erhaltung der durch NGF ausgelösten Reflexbahnung untersucht. Hierbei wurde nach zwei stabilen Baseline-Reflexserien zunächst NGF (0.8 µmol/l) in beide Musculi semispinales appliziert. Nach drei Reflexserien erfolgte dann die intraperitoneale Injektion von NPLA (0.96 mg/kg, n=5) mit anschließenden Messungen über 30 Minuten.

Ergebnisse

Bei vorheriger intraperitonealer Injektion einer Kochsalzlösung respektive 0.096 mg/kg des selektiven nNOS-Inhibitors NPLA führte die Applikation von NGF zu einer anhaltenden Reflexbahnung innerhalb der gemessenen 60 Minuten. Vorherige Verabreichung von 0.96 mg/kg oder 1.92 mg/kg NPLA verhinderte den potentiell bahnenden Effekt von NGF vollständig. Die Injektion von 0.96 -mg/kg NPLA 30 Minuten nach Applikation von NGF hatte keinen Einfluss auf die bereits hervorgerufene und etablierte Reflexbahnung.

Die Bahnung der nozizeptiven Verarbeitung im Hirnstamm, die durch myofaszialen, nozizeptiven Input aus der Nackenmuskulatur ausgelöst wird, kann dosisabhängig durch NPLA verhindert werden.

Schlussfolgerungen

Die Resultate dieser tierexperimentellen Studie weisen auf eine Beteiligung von nNOS beim Auslösen jedoch nicht bei der Aufrechterhaltung der NGF-getriggerten Bahnung der Nozizeption im Hirnstamm hin. Das Verständnis der Rolle von NO in der muskulären Nozizeption sowie das dosis- abhängige Wirkmuster des nNOS-Inhibitors NPLA könnten zu einer zukünftigen pharmakologischen Therapie des Spannungskopfschmerzes beitragen. Es besteht ein grosses Interesse selektive und wirkungsvolle Alternativen zur Therapie des Spannungskopfschmerzes zu entwickeln. Diese Studie zeigt, dass NOS und nNOS als potenzielle pharmakologische Ziele bei der Behandlung chronischer Schmerzen anzusehen sind.

Neuronal nitric oxide synthase is involved in the induction of NGF-induced neck muscle nociception

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Abbreviations: jaw-opening reflex, JOR; nerve growth factor, NGF; nitric oxide, NO; nitric oxide synthase, NOS; neuronal NOS, nNOS; Nω-propyl-L-arginine, NPLA; tension-type headache, TTH; tyrosine kinase A, trkA

Abstract

Background: Neck muscle nociception mediated by nitric oxide may play a role in the pathophysiology of tension-type headache.

Objective: The present study addresses the involvement of neuronal nitric oxide synthase (nNOS) in the facilitation of neck muscle nociception after local application of nerve growth factor (NGF).

Methods: After administration of NGF into semispinal neck muscles the impact of neck muscle noxious input on brainstem processing was monitored by the jaw-opening reflex in anesthetized mice. The modulatory effect of preceding and subsequent administration of an inhibitor of neuronal nitric oxide synthase on central facilitation was addressed in a controlled study.

Results: With preceding intraperitoneal application of saline or 0.096 mg/kg of the specific nNOS inhibitor Nω-propyl-L-arginine (NPLA), NGF induced a sustained reflex facilitation within 60 min. Preceding injection of 0.96 mg/kg or 1.92 mg/kg NPLA completely prevented the potentially facilitatory effect of NGF. Subsequent administration of 0.96 mg/kg NPLA did not affect established NGF-evoked reflex facilitation. Thus, NPLA prevents facilitation of brainstem processing by noxious myofascial input from neck muscles in a dose-dependent manner.

Conclusion: These findings suggest that nNOS is involved in the induction but not the maintenance of NGF-evoked facilitation of nociception in the brainstem. These results from an experimental animal model may support the idea of NOS and nNOS as potential targets for pharmacological treatment of TTH.

Introduction

Neck muscle nociception seems to play a significant role in the pathophysiology of tension-type headache (TTH), one of the most common primary headache disorders.[1-3] Increased tenderness of pericranial muscles in patients is reported in numerous studies and positively associated with both the intensity and frequency of TTH.[3,4] Pressure pain detection and tolerance thresholds of head and neck regions are significantly decreased in TTH as compared to healthy control.[5] Recent findings suggest that central sensitization may play an important role for chronic TTH. Most patients with chronic TTH have started with episodic TTH one or two decades earlier. Repetitive headache attacks associated with peripheral sensitization in myofascial tissues may trigger the process of central sensitization and the corresponding shift to chronic TTH. Besides its putative involvement in TTH, neck muscle pain seems to be an associated feature of migraine headache.[6-8]

Nitric oxide (NO), a free diffusible messenger molecule is involved in various biological processes including nociceptive transmission in the central and peripheral nervous system.[9,10] The NO synthase (NOS) family consists of the three isoenzymes neuronal NOS (nNOS), endothelial NOS, and inducible NOS and catalyses the conversion of L-arginine to NO and citrulline.[11,12] Experiments in animals have shown that NO is an important transmitter in pain pathways of the spinal cord and formation of NO may trigger or be associated with central sensitization.[13,14] Moreover, NOS inhibitors reduce central sensitization in animal pain models[15,16] and nociceptive responses in these models are augmented by NO donors.[17,18]

The involvement of NO in the pathophysiology of TTH has been addressed in double-blind and placebo controlled studies in patients.[19-21] Systemic administration of the NO donor glyceryl trinitrate induced an immediate and a delayed headache. The characteristics of

the delayed headache were similar to the primary headache disorder.[19] Intravenous infusion of the unspecific NOS inhibitor L-NMMA in TTH patients reduced pain intensity[20] and muscle hardness[21] significantly more than placebo. These studies suggest significant contribution of NO to the mechanisms of TTH.

The therapeutic effect of unspecific NOS inhibition in TTH might involve all three isoenzymes. The immunoreactivity of nNOS in the peripheral and central nervous system emphasizes its key role in nociception and central sensitization.[22,23] Therefore, nNOS may be the most important NOS isoenzyme mediating the therapeutic effect in headache.

In order to investigate pathophysiological aspects of TTH an animal model has recently been developed.[24,25] This model allows investigation of the interactions between peripheral myofascial factors and central sensitization.[25-27] Local administration of nerve growth factor (NGF) into neck muscles induces strong and sustained facilitation of brainstem nociception as monitored by the sensorimotor jaw-opening reflex (JOR) in anesthetized mice.[25] NGF interacts with tyrosine kinase A (trkA) and p75 receptors in muscle[28] and excites nociceptive input to the spinal cord and the brainstem via group IV fiber afferents.[29]

The aim of the present study was to investigate whether NGF-induced neck muscle nociception is affected by selective inhibition of nNOS in anaesthetized mice.

Methods

The electrophysiological experiments were performed in 25 adult male C57BL/6 mice (approximately 12 weeks old; 22.9 g to 27.3 g; Charles River Laboratories, http://www.criver.com). All procedures received institutional approval from the local ethics committee (ref. no. 50.203.2-AC 15, 16/03). The principles of laboratory animal care and use of laboratory animals (European Council Directive of November 24, 1986 (86/609/EEC)) were followed. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

A detailed description of anesthesia, surgery and electrophysiological recording in mice recently has been published.[30] Mice were anesthetized by an initial intraperitoneal (i.p.) injection of a 0.5% pentobarbital sodium salt solution (Sigma-Aldrich, http://www.sigmaaldrich.com) with a dose of 70 mg/kg. The depth of anesthesia was checked by ensuring that noxious pinch stimulation (blunt forceps) of hindpaw, forepaw, and ear did not evoke any sensorimotor reflexes. When the animal was sufficiently deeply anesthetized the skin of the throat was carefully shaved and lidocaine hydrochloride gel (Xylocaine 2%, AstraZeneca®, http://www.astrazeneca.com) was applied to induce local anesthesia. Dexpanthenol eye ointment (Bepanthen®, Roche, http://www.roche.com) was applied to cornea and conjunctiva of both eyes to protect them from drying. The right external jugular vein was catheterized for continuous administration of a 2% methohexital sodium salt solution (Brevimytal®, Hikma, http://www.hikma.de) with a dose of 60 mg/kg/h corresponding to a flow rate of about 0.07 ml/h for a 23 g mouse. A pair of Teflon-coated stainless steel wires (140 µm diameter) was inserted into the right anterior digastric muscle (Dig) to record electromyographic activity (EMG) and the jaw-opening reflex (JOR) via a differential amplifier. After tracheotomy, animals were placed in a stereotaxic frame and were artificially respired with a stroke volume of approximately 150 µl and about

200 strokes per minute during the complete experiment (MiniVent Model 845, Harvard Apparatus, http://www.harvardapparatus.com). Body core temperature was maintained at 37.5°C with a heating blanket and a fine rectal thermal probe (FMI, http://www.fmigmbh.de). One platinum needle electrode each (300 µm diameter) was subcutaneously inserted into the left forepaw and the right hindpaw to record the electrocardiogram (ECG) via a differential amplifier. Two stainless steel needle electrodes (150 µm diameter) were longitudinally inserted into the tongue musculature (parallel, 2 mm distance) in order to apply electrical stimuli and to evoke the JOR. The oral cavity was filled up with white Vaseline (Riemser, http://www.riemser.de) to protect the oral mucous membrane from drying. Semispinal neck muscles on both sides were carefully exposed. A pair of Teflon-coated stainless steel wires (140 µm diameter) was inserted into the right semispinal neck muscle to record EMG activity from 5 min before to 5 min after intramuscular (i.m.) injections. One injection canula each (0.4 mm diameter) was inserted into muscle belly of both semispinal neck muscles. Each canula was connected via thin and short tubing to a glass microsyringe (1 ml) that was fixed in a microdialysis pump (CMA/102, http://www.microdialysis.se). This procedure allowed bilateral induction of noxious input from neck muscles in order to mimic bilateral neck muscle pain in TTH patients. Recombinant human β-nerve growth factor (NGF, 0.8 µmol/l, http://www.calbiochem.com) was i.m. administered via canulas in semispinal neck muscles with a volume of 20 µl per muscle during a time period of 1 min (corresponding to a flow rate of 20 µl/min). The dose was selected on the basis of an *in-vivo* study that showed a strong and sustained facilitation of the sensorimotor JOR in anesthetized mice.[25]

After surgery and placement of all electrodes the anaesthetized animal had a rest for at least 1 h. During this time period the level of anesthesia and heart rate were routinely checked and documented, and the depth of anesthesia was maintained.

All electrical signals (EMG, ECG) were recorded by bioamplifiers and led into a data collection system (CED Micro1401, http://www.ced.co.uk) and a personal computer using the Signal® and Spike2® software programs (CED, http://www.ced.co.uk).

The JOR was elicited by rectangular electrical pulses of 500 µs duration with a stimulation frequency of 0.1 Hz. The electrical threshold of the JOR was determined by applying increasing and decreasing stimulus intensities from 0 to 2 mA in steps of 100 µA. The lowest stimulus intensity that just evoked a reflex response was defined as the JOR threshold (I_{JOR}). The test stimulus intensity was adjusted to about 125% of the I_{JOR}. The JOR was evoked in series of eight stimuli each. These series were repeated every 10 min.

In the first scheme the involvement of neuronal NOS in the induction of NGF evoked reflex facilitation was addressed (Fig. 1a). After two stable baseline JOR series either the neuronal NOS inhibitor Nω-propyl-L-arginine (NPLA, 100 µl; 0.096 mg/kg, 0.96 mg/kg, 1.92 mg/kg ; http://www.tocris.com) or isotonic saline (Saline, 100 µl, 0.9%) were i.p. administered and the reflex was recorded for three series. Subsequently, NGF (0.8 µmol/l) was i.m. administered and effects on JOR were monitored for 1 h. In the second scheme the role of neuronal NOS in the maintenance of NGF-evoked reflex facilitation was investigated (Fig. 1b). After two stable baseline JOR series NGF (0.8 µmol/l) was i.m. administered and the reflex was recorded for three series. Subsequently, NPLA (0.96 mg/kg) was i.p. administered and effects on reflex facilitation were monitored for 30 min.

Onset latency, duration and integral of JOR were analyzed in each single sweep. In recent studies, reflex integral turned out to be the key parameter of reflex alteration in the present animal model.[24,25,27,29,31] Reflex integral, indeed, partly depends on duration of reflex. For the sake of clarity and readability, the present study focuses on statistical analysis of reflex

integral. Arithmetic mean and standard error were calculated (mean±sem). One-way repeated measures analysis of variance (ANOVA) was applied in order to analyze differences within groups (F, p value). Differences between groups were analyzed by one-way ANOVA (F, p value) or Kruskal-Wallis one-way ANOVA on ranks (H, p value). Multiple comparison procedures within and between groups were performed by Fisher LSD post hoc test (Difference of Means=DM, p value). JOR thresholds in the beginning of the experiment (pre) were compared to thresholds measured in the end of the experiment (post) by paired t-test (t, p value). All statistics were performed by support of the SigmaStat software (http://www.systat.com).

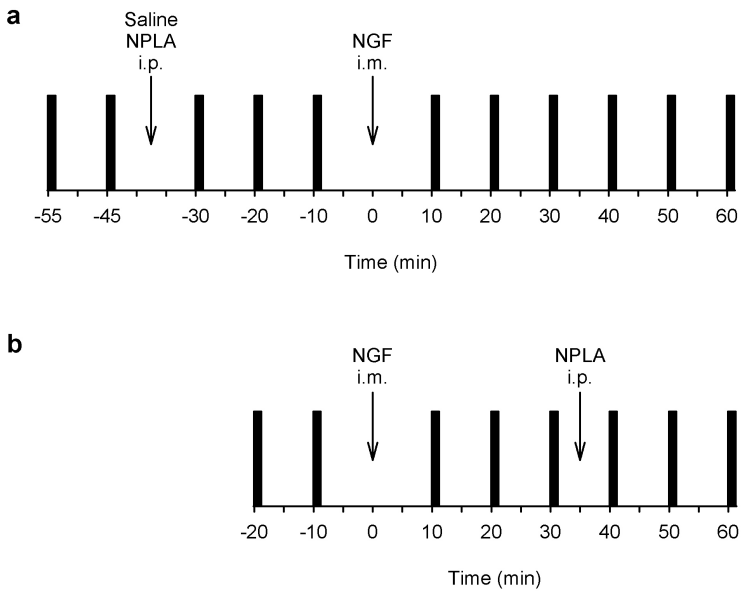

Figure 1. Stimulation protocol.

The jaw-opening reflex was evoked in series (vertical black bars) of 8 electrical stimuli each. The reflex series were repeated every 10 minutes. Saline or the neuronal NOS inhibitor NPLA were applied by i.p. injection. Nerve growth factor (NGF) was applied by i.m. injection into both semispinal neck muscles. (a) After two baseline reflex series, saline or three different doses of NPLA were i.p. administered. After three further reflex series NGF was i.m. injected and the reflex was monitored for one hour. (b) After two baseline reflex series NGF was i.m. administered. After three further reflex series NPLA was i.p. injected and the reflex was monitored for half an hour.

Results

The JOR was elicited in 25 experiments in mice by electrical tongue stimulation with a threshold stimulus intensity of I_{JOR}=608±33 µA (mean±sem). The test stimulus intensity was adjusted to 776± 43 µA corresponding to 127% of the I_{JOR} on average (n=25).

Preceding administration of saline or NPLA (Figs. 2, 3, 4)

After two baseline JOR series, saline or three different doses of NPLA (0.096 mg/kg, 0.96 mg/kg, 1.92 mg/kg) were i.p. administered in five mice each and JOR series were continued for half an hour (Fig. 1a). A Kruskal-Wallis one-way ANOVA revealed no significant differences between possible effects on JOR integral in the four groups within 30 min after i.p. administration (H=2.89, n.s.=not significant) (Fig. 2).

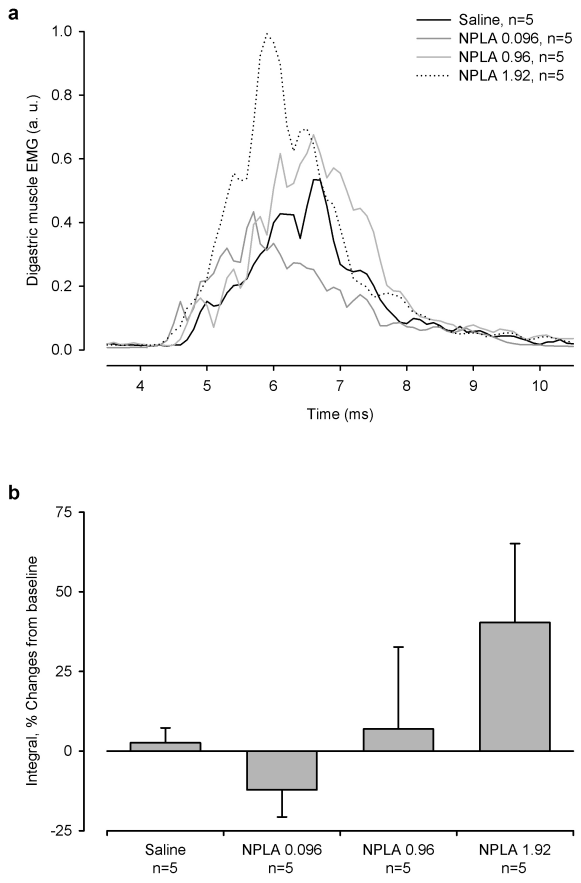

Figure 2. Baseline jaw-opening reflex remains unchanged after i.p. administration of saline or NPLA. (a) Grand averages of reflex responses for five mice each within 30 min after i.p. administration of isotonic saline, NPLA 0.096 mg/kg, NPLA 0.96 mg/kg, or NPLA 1.92 mg/kg. Electromyographic (EMG) activity is given in arbitrary units. (b) Percentage changes of the reflex integral within 30 min after i.p. injection of NPLA (0.096 mg/kg, 0.96 mg/kg, or 1.92 mg/kg, groups of n=5) or isotonic saline (n=5). Changes from baseline are expressed as arithmetic mean and standard error. Percentage changes of reflex integral did not statistically differ.

In five mice i.p. injection of saline was followed by i.m. administration of NGF in both semispinal neck muscles. This group represented the control. Under baseline conditions I_{JOR} was 720±97 µA (mean±sem, n=5). Within one hour after NGF administration, JOR integral significantly increased (one-way repeated measures ANOVA: $F=3.0$, $p<0.05$) (Fig. 3). The I_{JOR} significantly decreased to 600±77 µA after injection (paired t-test: $t=3.21$, $p<0.05$).

In order to investigate the effect of preceding inhibition of neuronal NOS, NPLA was i.p. administered 30 min before i.m. injection of NGF (Fig. 3). Each of three different doses of NPLA were administered to five different mice. With preceding administration of 0.096 mg/kg NPLA and subsequent i.m. NGF the JOR integral significantly increased (one-way repeated measures ANOVA: $F=3.08$, $p<0.05$) within 1 h. I_{JOR} under pre 540±68 µA and post condition 460±75 µA did not statistically differ ($t=2.14$, n.s.). With preceding 0.96 or 1.92 mg/kg NPLA in five mice each, subsequent i.m. administration of NGF had no significant effect on the reflex integral (0.96 mg/kg NPLA: $F=2.2$, n.s., 1.92 mg/kg NPLA: $F=1.99$, n.s.). I_{JOR} under pre conditions were 620±73 µA and 660±51 µA in experiments applying 0.96 mg/kg and 1.92 mg/kg NPLA, respectively. These thresholds remained unchanged as compared to post conditions with 620±58 µA and 660±60 µA ($t=0$, n.s.).

Statistical comparison between groups revealed significantly different effects of preceding i.p. administration of saline or various doses of NPLA on the impact of i.m. NGF (Fig. 4). NGF evoked changes of the reflex integral depended on the dose of preceding NPLA with significant differences between effects of 0.96 mg/kg and 1.92 mg/kg NPLA on the one hand and saline and 0.096 mg/kg NPLA on the other hand (Fisher LSD test, Fig. 4).

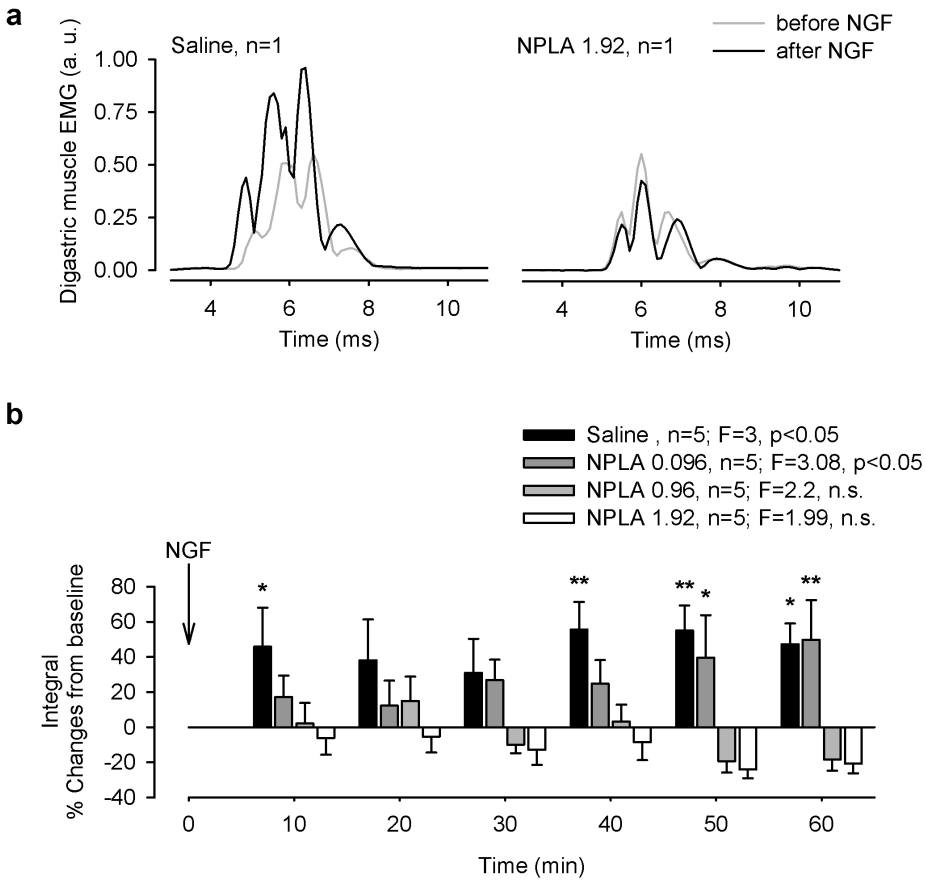

Figure 3. Preceding administration of NPLA prevents NGF-evoked reflex facilitation in a dose-dependent manner. (a) Gray and black line graphs represent averages of eight rectified reflex sweeps 5 min before (gray; before NGF) and 60 min after NGF injection (black: after NGF) into both semispinal neck muscles in one mouse of control group with preceding application of isotonic saline (left) and another mouse after injection of 1.92 mg/kg NPLA (right). Electromyographic (EMG) activity is given in arbitrary units. (b) Reflex integral after NGF injection (marked by arrow) measured for one hour every 10 min with preceding application of isotonic saline (black bar), 0.096 mg/kg NPLA (dark gray bar), 0.96 mg/kg NPLA (light gray bar), or 1.92 mg/kg NPLA (white bar). Each group consists of five mice. The changes from baseline are expressed as arithmetic mean and standard error. The asterisks code level of significance in comparison to the baseline blocks as calculated by Fisher LSD post hoc test (*: $p < 0.05$; **: $p < 0.01$). Results of one-way repeated measures ANOVA are given (F, p value).

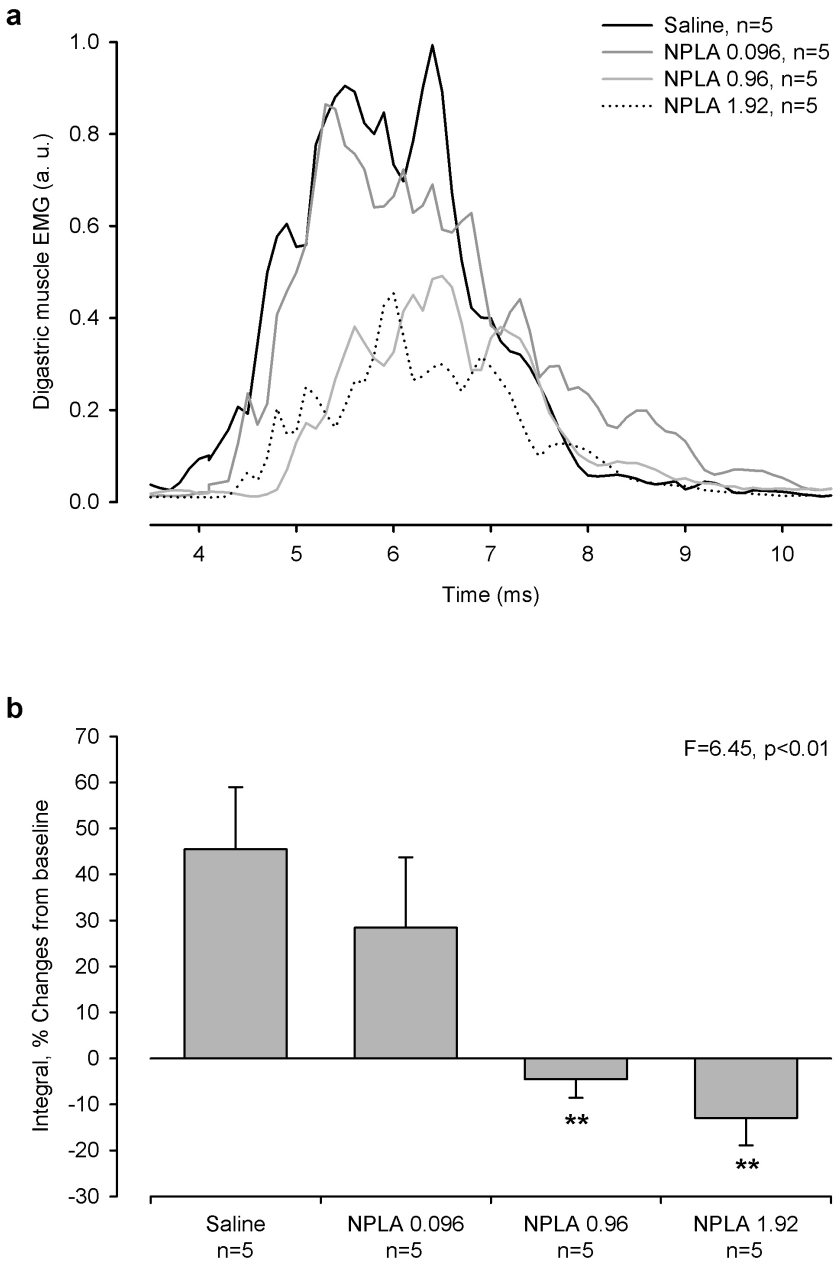

Figure 4. Preceding i.p. injection of NPLA reduces facilitation of jaw-opening reflex evoked by NGF. (a) Black (isotonic saline), dark gray (0.096 mg/kg NPLA), light gray (0.96 mg/kg NPLA) and dotted (1.92 mg/kg NPLA) graphs represent averages of rectified reflex sweeps measured within the time period from 10 to 60 minutes after i.m. NGF injection. Each group consists of five mice. Electromyographic (EMG) activity is given in arbitrary units. (b) Averages of reflex integral for isotonic saline, 0.096 mg/kg, 0.96 mg/kg, and 1.92 mg/kg NPLA groups of each five mice. The changes from baseline are expressed as arithmetic mean and standard error. The asterisks code level of significance in comparison to saline and NPLA 0.096 as calculated by Fisher LSD post hoc test (**: $p < 0.01$). Results of one-way ANOVA are given (F, p value).

Subsequent administration of NPLA (Fig. 5)

In order to characterize the role of NPLA in the maintenance of NGF-evoked reflex facilitation, NPLA (0.96 mg/kg, n= 5) was i.p. administered 35 minutes after local i.m. injection of NGF. At that time point NGF had already induced a clear reflex facilitation. Subsequent administration of NPLA did not evoke any changes of the reflex integral. A comparison between combined application of NGF and NPLA and sole application of NGF did not show any statistical difference.

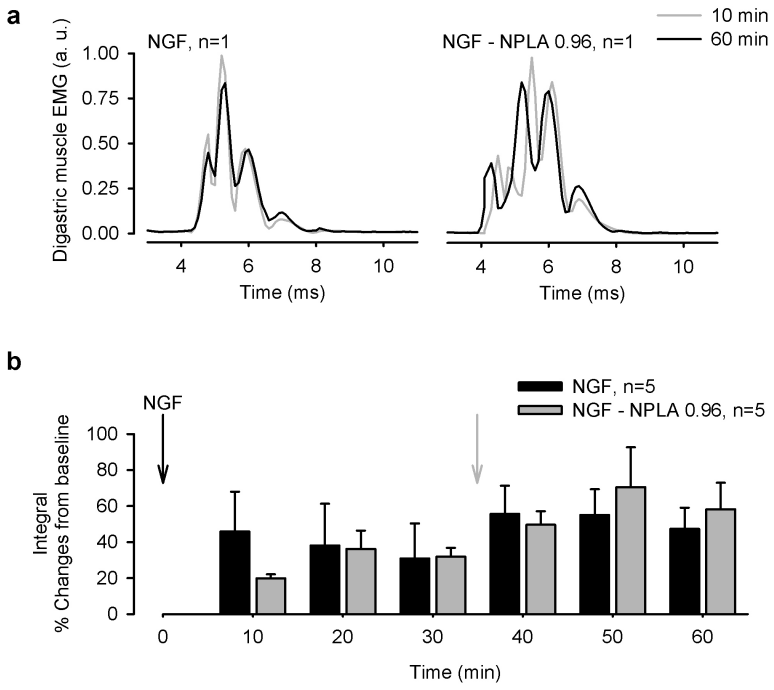

Figure 5. Subsequent administration of NPLA does not affect NGF-evoked reflex facilitation. (a) Gray and black line graphs represent averages of eight rectified reflex sweeps 10 min (gray) and 60 min after NGF injection (black) into both semispinal neck muscles in one mouse with sole i.m. application of NGF (left) and another mouse after combined i.m. injection of NGF and subsequent i.p. application of 0.96 mg/kg NPLA (right). Electromyographic (EMG) activity is given in arbitrary units. (b) Reflex integral after NGF injection (black arrow) measured for one hour every 10 min. Whereas NGF was solely i.m. administered in five mice (black bar), in a second group of five mice 0.96 mg/kg NPLA (gray arrow) was additionally i.p. injected (dark gray bar). The changes from baseline are expressed as arithmetic mean and standard error. Integral changes from baseline were not statistically different between groups.

Discussion

The present data demonstrate that preceding systemic application of the selective nNOS inhibitor, NPLA, prevents NGF-evoked reflex facilitation in a dose-dependent manner in anesthetized mice. However, subsequent administration of NPLA does not affect established NGF-evoked reflex facilitation. These findings indicate an important role of NO in neck muscle nociception and suggest that nNOS is involved in the induction but not the maintenance of NGF-evoked facilitation of nociception in the brainstem.

Prolonged nociceptive input from pericranial muscles evoked by local administration of NGF results in increased excitability of neurons in the brainstem.[25] NGF, a protein of the neurotrophins family, is known to play an important role in the development of the nervous system and increasing evidence exists that functions persist throughout adulthood.[28,32] NGF acts by preferential binding to tyrosine receptor kinase A (TrkA). Local or systemic application of NGF produces hyperalgesia that can last for hours in adult rats.[33] Modulation of neck muscle nociceptive processing by local administration of the sodium channel blocker tetrodotoxin suggests different mechanisms of i.m. NGF in comparison to ATP. A former study suggests preferential excitation of group III muscle afferents by ATP and group IV by NGF.[29]

The JOR is a commonly accepted model to investigate alterations of excitability in sensory brainstem neurons with convergent afferent input from different craniofacial tissues.[34-38] Sensory neurons in the spinal trigeminal nucleus receive convergent impulses from primary afferents (neck muscle, tongue) and project to digastric motoneurons.[39,40] Noxious input from neck muscles leads to sustained reflex potentiation evoked by NGF.[24,25] Brainstem reflex potentiation by additional noxious input from neck muscles is due to heterosynaptic facilitation. Electrical stimulation of afferent nerve fibers in tongue

musculature reliably evokes the JOR via a brainstem reflex network.[30,34] Nociceptive afferents from neck muscles gain access to the same network. Thus, both afferents from tongue musculature and neck muscles converge onto the same central reflex network.

NPLA is a slowly dissociable and cell-permeable NOS inhibitor. It is characterized as a highly selective and potent inhibitor of nNOS (IC_{50}: 57 nM) with 3158-fold and 149-fold selectivity over iNOS and eNOS, respectively.[41] Another study gives an IC_{50} of 85 nM with 2459-fold and 24-fold relative selectivity over iNOS and eNOS, respectively.[42] The reversibility to inhibit nNOS was demonstrated *in-vitro* by application of excess arginine which altered the ratio of arginine to NPLA following a new equilibrium condition with enhanced arginine binding and subsequent partial restoration of NO formation rate.[42,43] However, a recent literature search revealed many studies applying NPLA in different animal models but no pharmacokinetic data on half life under *in-vivo* conditions. NPLA effects in the present study were dose-dependent and did not show attenuation after 90 minutes of recording period. This might indicate a long half-life. Alternatively, nNOS is inhibited by NPLA just in the initial phase of reflex facilitation and is not involved in the maintenance of the effect.

NG-monomethyl-L-arginine acetate (L-NMMA) is a competitive inhibitor of all three NOS isoforms with IC_{50} values of 6.6 µM for inducible NOS, 4.9 µM for neuronal NOS, and 3.5 µM for endothelial NOS.[12] Similar potencies for all three isoenzymes define L-NMMA as an unspecific NOS inhibitor. In a clinical trial chronic TTH patients received an intravenous infusion of 6 mg/kg L-NMMA or placebo on two days. L-NMMA reduced pain intensity significantly more than placebo during 120 min after start of infusion.[20] Muscle hardness in chronic TTH was reduced significantly more following treatment with L-NMMA than with placebo. Compared with baseline, hardness and total tenderness score significantly decreased at 60 and 120 min after L-NMMA treatment.[21] Patient studies

suggest an analgesic effect of NOS inhibition in chronic TTH. However, the unspecific NOS inhibitor L-NMMA inhibits all three isoenzymes. Whereas this broad spectrum effect of L-NMMA on NOS seems to induce significant analgesia in patients, it runs the risk of serious side effects under clinical conditions. Therefore, the present study addresses the role of nNOS in an animal model of neck muscle nociception that has been suggested to be an experimental model for TTH.[26]

Immunohistochemical studies with nNOS antibodies and NADPH-d histochemistry have demonstrated that nNOS is localized in superficial and deep laminae of the spinal trigeminal nucleus and the cervical spinal cord.[44-46] NO is an intracellular and intercellular messenger and involved in nociception and pain. NO release in the spinal dorsal horn contributes to the development of central sensitization and is supposed to contribute to its maintenance.[47] Reduction of central sensitization could be achieved by inhibition of NOS in animal models of persistent pain.[16] Application of NO donors seems to intensify nociceptive responses in these models.[17,18] In contrast to above mentioned studies in the spinal system, NPLA did not reverse established reflex facilitation back to baseline in the present brainstem study. However, our results indicate the involvement of nNOS in induction of NGF-evoked facilitation of nociception in the brainstem using the highly selective nNOS inhibitor NPLA. This finding supports a previous finding that nNOS knockout mice have significantly reduced spinal nerve injury-induced mechanical hypersensitivity.[48] In skeletal muscle of mammals all three NOS isoforms are expressed under normal conditions. nNOS expression and activity are increased by muscle contraction.[49,50] nNOS is expressed about equally in type I (slow-twitch) and type II (fast-twitch) fibers of human skeletal muscles.[51,52] The myotendinous[53] and neuromuscular[54-56] junctions are especially rich in nNOS. Due to systemic administration of NPLA in the present study the exact site of action remains unknown. However, as discussed above,

nNOS activity has been demonstrated in spinal trigeminal nucleus, spinal dorsal horn and in skeletal muscles.[44,46,50,51] Therefore, both areas are candidates for site of action.

Central facilitation manifests within seconds of an appropriate nociceptive conditioning stimulus and can outlast the stimulus for several hours.[57] Long- term effects induced by NGF may be mediated by brain-derived neurotrophic factor (BDNF) which is up-regulated by NGF in TrkA-positive dorsal root ganglion cells.[58] However, the involvement of BDNF in the present mouse model has not been investigated. The time course of reflex facilitation by NGF might indicate other fast, receptor-mediated mechanisms. Once a strong and sustained facilitation of JOR induced by NGF manifests, it is not reversed by subsequent NPLA administration. Intrathecal pretreatment, but not posttreatment with a NOS inhibitor (Nω-nitro-L-arginine) delays the development of thermal hyperesthesia after a nerve constriction injury.[59] This finding seems to be consistent with our results and might be due to different mechanisms of induction and maintenance of long-term facilitation.

There is a strong interest in developing adequate therapies for chronic pain. The present study supports the idea of nNOS as potential target for pharmacological treatment in the future.

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