Title page

Utility of different outcome measures for the nitroglycerin model of migraine in mice

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Abbreviations: 5-HT, serotonin; CGRP, calcitonin gene related peptide; NTG, nitroglycerin; NO, nitrogen monoxide; nNOS, neuronal nitrogen monoxide synthase; TNC, trigeminal nucleus caudalis; TRP, transient receptor potential; TRG, trigeminal ganglion; PBS, phosphate-buffered saline; PBS-T, Triton-X containing PBS; DAB, diaminobenzidine; sGC, soluble guanylate cyclase; cGMP, cyclic guanosine monophosphate; i.p., intraperitoneal; i.v., intravenous; s.c., subcutaneous.

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Abstract

Introduction—Majority of the work for establishing nitroglycerin (NTG)-induced migraine models in animals was done in rats, though recently some studies in mice were also reported. Different special formulations of NTG were investigated in various studies; however, NTG treated groups were often compared to simple saline treated control groups. The aim of the present studies was to critically assess the utility of a panel of potential outcome measures in mice by revisiting previous findings and investigating endpoints that have not been tested in mice yet.

Methods—We investigated two NTG formulations, Nitrolingual and Nitro Pohl, at an intraperitoneal dose of 10 mg/kg, in comparison with relevant vehicle controls, and evaluated the following outcome measures: light aversive behaviour, cranial blood perfusion by laser Doppler imaging, number of c-Fos- and neuronal nitrogen monoxide synthase (nNOS)-immunoreactive neurons in the trigeminal nucleus caudalis (TNC) and trigeminal ganglia, thermal hyperalgesia and tactile allodynia of the hind paw and orofacial pain hypersensitivity.

Results—We could not confirm previous reports of significant NTG-induced changes in light aversion and cranial blood perfusion of mice but we observed considerable effects elicited by the vehicle of Nitrolingual. In contrast, the vehicle of Nitro Pohl was apparently inert. Increased c-Fos expression in the TNC, thermal hyperalgesia, tactile allodynia and orofacial hypersensitivity were apparently good endpoints in mice that were increased by NTG-administration. The NTG-induced increase in c-Fos expression was prevented by topiramate but not by sumatriptan treatment. However, the NTG-induced orofacial hypersensitivity was dose dependently attenuated by sumatriptan.

Discussion—Our results pointed to utilisable NTG formulations and outcome measures for NTG-induced migraine models in mice. Pending further cross-validation with positive and negative control drugs in these mouse models and in the human NTG models of migraine, these tests might be valuable translational research tools for development of new anti-migraine drugs.

Keywords

c-Fos
cranial blood flow
light aversion
methods
migraine
nitroglycerin
nNOS
orofacial allodynia
thermal hyperalgesia
trigeminovascular system

1 Introduction

Migraine is a disabling headache disorder characterized by moderate to severe, intense throbbing or pulsating pain generally occurring on one side of the head and which may be aggravated by routine physical activity; other symptoms may include light or sound sensitivity, nausea and vomiting. Various forms of migraine affect approximately 18% of women and 6% of men (Estemalik & Tepper, 2013). Although acute treatment of migraine is well manageable by triptans, there are many patients who cannot tolerate triptans or would need preventive medication. However, efficacy and tolerability of the preventive armamentarium, including antiepileptics, such as topiramate and valproate, and beta adrenergic receptor blockers is unsatisfactory (Silberstein et al., 2012). Therefore, a great unmet need exists for novel effective, safe and well-tolerated pharmacotherapies of migraine.

Although recent research has revealed numerous details of the mechanisms participating in migraine generation, the primary initiating phenomena, the underlying neural and vascular mechanisms and their interrelationships are not understood and are surrounded by serious debates on various proposed theories, such as the vascular-, the neural- and the neurogenic inflammation theory of migraine (Moskowitz, 1993; Messlinger, Fischer, & Lennerz, 2011; Ashina, 2012; Noseda & Burstein, 2013).

In line with the lack of a well-established theoretical background, numerous models have been proposed as useful tools for testing novel antimigraine drug candidates in animals or in human pharmacodynamic studies. Out of these, the most widely studied and accepted one is the nitroglycerin (NTG)-induced model of migraine (Olesen & Jansen-Olesen, 2012). NTG administration causes an immediate headache, stronger in migraine sufferers than in healthy subjects, and a delayed migraine-like headache only in migraineurs (Thomsen, Kruuse, Iversen, & Olesen, 1994; Olesen, 2008; Ashina, Hansen, & Olesen, 2013). The immediate headache in healthy volunteers was significantly attenuated by sumatriptan (Iversen & Olesen, 1996) and the delayed headache incidence in migraineurs was reduced by valproate (Tvedskov et al., 2004a).

The majority of the work for establishing NTG-induced migraine models in animals was done in rats detecting the effect by various outcome measures, such as increased cerebral- and more controversially meningeal blood flow or blood vessel diameters (Read, Manning, McNeil, Hunter, & Parsons, 1999; Srikiatkhachorn, Suwattanasophon, Ruangpattanatawee, & Phansuwan-Pujito, 2002; Gozalov, Jansen-Olesen, Klaerke, & Olesen, 2008; Greco et al., 2011; Pryazhnikov et al., 2014), increase in c-Fos protein expression in the trigeminal nucleus caudalis (TNC) (Tassorelli & Joseph, 1995; Pardutz, Krizbai, Multon, Vecsei, & Schoenen, 2000; Knyihar-Csillik et al., 2008; Ramachandran et al., 2012), increase in neuronal nitric oxide synthase (nNOS) protein expression in trigeminal ganglia (TRGs) and/or TNC (Pardutz et al., 2000; Srikiatkhachorn et al., 2002; Dieterle, Fischer, Link, Neuhuber, & Messlinger, 2011), electrophysiological detection of increase in neuronal activity of TNC neurons (Jones et al., 2001; Koulchitsky, Fischer, & Messlinger, 2009) and hypersensitivity to pain either on the paw/tail or in the facial (trigeminal) region measured by behavioural responses to chemical, mechanical or thermal stimuli (Tassorelli et al., 2003; Tassorelli, Greco, Wang, Sandrini, & Nappi, 2006; Di et al., 2015; Greco et al., 2015). Whereas the use of rats has

several advantages, there are also reasonable considerations in favour of utilising mice, such as better availability of transgenic animals or the need to use much lower amounts of expensive substances for *in vivo* studies. However, much more limited experience exists concerning the NTG model in mice: Recently reported data indicated that increased c-Fos expression (Bates et al., 2010; Markovics et al., 2012; Goloncser & Sperlagh, 2014), thermal hyperalgesia (Bates et al., 2010; Goloncser & Sperlagh, 2014) and mechanical allodynia of the paw (Bates et al., 2010; Pradhan et al., 2014) are utilisable outcome measures of NTG-induced changes in mice. In addition, our previous study suggested that increased cranial blood flow and light aversive behaviour are also suitable endpoints to detect NTG-induced changes in mice (Markovics et al., 2012). The aim of the present studies was to critically assess a panel of utilisable outcome measures in mice by revisiting previous findings, as well as by adding endpoints that have not been tested in mice yet, e.g. nNOS expression in the TRG and TNC, as well as pain hypersensitivity of the face, which formally might be a more relevant indicator of migraine than paw hyperalgesia.

There was another confounding factor in previous studies, which determined our goals. In various studies different formulations of NTG were used, which were composed either for infusing or for sublingual spray application in patients, and the exact compositions of these often remained elusive. Several different NTG formulations contain propylene glycol and ethanol or propylene glycol and glucose (e.g. Nitrocine - https://www.medicines.org.uk/emc/medicine/1889); others (e.g. Nitrolingual) have even more complex vehicle without description of the exact composition. Some relatively dilute aqueous solutions of NTG (Nitro Pohl and Nitronal) contain only 5% glucose and mild acidification. Some of the above mentioned constituents (e.g. ethanol) may be assumed not to be entirely inert in studies of central nervous system functions. Nevertheless, many previous studies compared the effects of formulated NTG to a saline group instead of using an appropriate vehicle control (Bates et al., 2010; Di et al., 2015; Srikiatkhachorn et al., 2002; Tassorelli et al., 2003; Tassorelli et al., 2006) and in lack of control vehicle we did the same in a previous study (Markovics et al., 2012) using Nitrolingual formulation of NTG. However, in the present studies, to establish well-controlled NTG models, we intended to use appropriate vehicle controls. For this purpose we clarified the composition of Nitrolingual and composed an appropriate vehicle for control experiments. In addition, we started the studies with investigating two different formulations, Nitrolingual and Nitro Pohl, which latter we considered as the possibly most inert one from the assortment.

2 Methods

2.1 Animals

Male CD1 mice (N=48) weighing 22-28 g were used for cranial blood flow and immunohistochemistry studies to preserve comparability to a similar previous study (Markovics et al., 2012). They were bred in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy of the University of Pécs. For the behavioural experiments, male NMRI mice (N=142) weighing 25-35 g were used. In our experience this strain is more tranquil and suitable for behavioural and particularly for reliable pain threshold

studies than the CD1 strain. NMRI mice were obtained from TOXI-COOP Zrt (Budapest, Hungary). The animals were housed under standardised conditions (22 ± 2 °C room temperature) with an artificial 12/12 h light/dark cycle (lights on 06.00–18.00 h, humidity 55±15%). Food (ssniff® R/M-H autoclavable; ssniff Spezialdiäten GmbH, Germany) and tap water were available *ad libitum*. In all behavioural experiments, the animals were acclimatised to the laboratory for at least 30-60 min before testing and were used only once. Experiments were performed in accordance with EU Directive 2010/63/EU for animal experiments. All the procedures involving animals were reviewed and approved either by the Richter Institutional Animal Care and Use Committee or the Ethics Committee on Animal Research of the University of Pécs. The number of animals and intensities of noxious stimuli used were kept to the minimum necessary to demonstrate consistent effects of the drug treatments.

2.2 Drug treatments and control vehicles

Two NTG preparations were used: Nitrolingual aerosol and Nitro Pohl solution for infusion (both obtained from Pohl-Boshkamp GmbH, Germany). Nitrolingual aerosol was freshly sprayed out into a foil-coated bottle on each experimental day. The solution contains 7.7 mg/ml NTG and was administered at a dosing volume of 1.3 ml/kg for a dose of 10 mg/kg intraperitoneally (i.p.). The vehicle control solution for Nitrolingual was clarified by data mining in patent literature (US 2011/0240508) and compounded at Gedeon Richter Plc. and comprised (in % w/w): Miglyol 812 (caprylic/capric triglyceride, Sasol Germany GmbH) 77.3%, Ethanol (96% Ph Eur.) 20%, Imwitor 988 (glyceryl caprylate, Sasol Germany GmbH) 2% and Peppermint oil (Sigma-Aldrich) 0.7%.

One ampoule Nitro Pohl solution for infusion was freshly opened on each experimental day. The solution contains 1 mg/ml NTG and was administered at a dosing volume of 10 ml/kg for a dose of 10 mg/kg i.p. In addition to NTG, the aqueous Nitro Pohl solution contains 49 mg/ml glucose monohydrate and its pH is set to be mildly acidic by dilute hydrochloric acid (pH 3-5.5 as declared by the manufacturer). Occasional pH testing of Nitro Pohl solution indicated pH of 3.8. As vehicle control for Nitro Pohl, we used Rindex 5 solution for infusion (Teva, Hungary), which has the following composition: glucose monohydrate 5500 mg (278 mmol/l), NaCl 397 mg (68 mmol/l), KCl 26 mg (3.5 mmol/l), $MgCl_2 \cdot 7H_2O$ 10.2 mg (0.5 mmol/l) and $CaCl_2$ 18 mg (1.25 mmol/l) in 100 ml aqueous solution (declared pH 3.5-6; measured pH 4.3). Various doses of sumatriptan succinate (synthesised at Gedeon Richter) were dissolved in physiological (0.9%) saline solution ("saline") and administered subcutaneously (s.c.) at a dosing volume of 10 ml/kg. Doses were calculated for free base. Topiramate (synthesised at Gedeon Richter) 80 mg/kg was dissolved in saline and administered i.p. at a dosing volume of 10 ml/kg. For anaesthesia, urethane was purchased from Spectrum 3D (Debrecen, Hungary) and dissolved in saline. It was used only in non-survival experiments.

2.3 Other materials

Materials required for immunohistochemistry were the following: paraformaldehyde (Molar Chemicals, Hungary), glycerol and ethylene glycol (Spectrum 3D, Debrecen, Hungary),

Diaminobenzidine (Sigma-Aldrich, Hungary; D5637), Triton X-100 and H_2O_2 (Sigma Chemical, Zwijndrecht, The Netherlands), normal goat serum (Sigma-Aldrich, Hungary), polyclonal antiserum raised against c-Fos (sc-52; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), biotinylated goat anti-rabbit IgG and avidine-biotin complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA), anti-nNOS antibody (Abcam, Cambridge, UK), EZ-DeWax Solution and Antigen Retrieval Citra Solution (Biogenex, Fremont, CA, USA), xylene, DPX and ethanol solutions (Merck, Leicester, UK).

2.4 Study logistics

Six types of studies were performed. In the first two studies, which included detection of light aversion and cranial blood flow, both NTG preparations were tried. However, based on the experience gained in these studies, the use of Nitrolingual was dismissed for the rest of the studies. Instead, in the immunohistochemistry studies and in the novel mouse orofacial pain test we carried out some pharmacological validation besides detecting the effects of NTG.

2.5 Light aversion assay

Light-aversive behaviour was examined in mice both in the early (0–30 min) and late phases (90–120 min) following administration of NTG. A modified light–dark box was custom made, similar to those used in recent publications (Recober, Kaiser, Kuburas, & Russo, 2010; Markovics et al., 2012). The mice (N=53) were individually tested in the light aversion chamber (60 cm length× 26 cm width× 29 cm height) with two equally-sized compartments: one brightly lit (1000 lux, thermal-neutral fiber optic source, Fiber-lite, Dolan-Jenner Inc., USA), painted white and lacking a top, the other not lit, painted black and fully enclosed. A small opening (7×7 cm) connected the two compartments. Two chambers were used in parallel to test a vehicle- and an NTG-treated mouse at the same time. After 1-hour conditioning period in the testing room, the animals were injected i.p. with NTG or vehicle and were immediately placed into the light-dark box. Two randomised experiments were performed with the two different formulations of NTG and their respective control vehicles (2x2 groups). In the experiment with Nitrolingual a third, physiological saline, group was also included. Initially 8 animals were dedicated to each group. However, in the Nitrolingual experiment the group sizes were extended to N=12-13 in a second run in order to draw reliable conclusions regarding presence or absence of difference from vehicle group with adequate statistical power. Both observation periods were equally 30 min long, which had been chosen on the basis of a series of previous experiments (Markovics et al., 2012). Following the early observation period, the mice were put back to their home cages and placed into the light–dark box again 90 min after treatment to see how they behave in the late period. The chamber was thoroughly cleaned and scrubbed with 70% ethanol between testing of each animal. All experiments were performed between 8.00 and 14.00 h. The experiments were recorded with a digital camera and evaluated later by an observer. The time spent in the light was measured with a stopwatch in each 5-min period and expressed in seconds. Finally, the percent time spent in the light compartment was calculated and plotted.

2.6 Cranial blood flow experiments

Mice were anaesthetised with urethane (1.5 g/kg i.p.) and placed onto a heating pad maintained at 38 °C. A cannula was inserted into the trachea and mice were breathing spontaneously throughout the experiment. The head was fixed and the skin and connective tissues covering the cranium were carefully removed. A small piece of a black cloth with an opening of the size of the exposed area was placed onto the head to reduce background noise. The cranial blood perfusion was investigated using a non-invasive laser Doppler scanner (PIM II System, Perimed AB, Sweden) through the closed cranium. The scanning device was positioned 18 cm above the head and the scanned region was extended to the top surface of both hemispheres. The scanner measures an averaged total microcirculatory blood perfusion including blood flows in capillaries, arterioles, venules and shunting vessels of meninges and superficial cortex. In this way an overall mean cranial blood flow could be assessed. The scanning procedure lasting for duration of approximately 1 min was automatically repeated continuously. Control baseline monitoring measurements were repeated until the mean perfusion value had stabilised. Then an NTG preparation (10 mg/kg Nitrolingual or Nitro Pohl) or corresponding vehicles were injected i.p. and the post-dose recording lasted for 4 hours, after which the animals were euthanized by overanaesthesia with i,p, injection of urethane. All four groups comprised six animals. Measurements from this instrument are expressed as arbitrary perfusion units. To enable comparison of results, perfusion changes were expressed as percentage of baseline measurement.

2.7 Immunohistochemistry study of c-Fos and nNOS in TNC and TRG

Four groups of six mice were injected i.p. either with NTG (3 groups with 10 mg/kg Nitro Pohl) or vehicle (10 ml/kg Rindex 5). Two groups treated with NTG received also treatment of sumatripan (2x5 mg/kg 30 min before and after NTG) or topiramate (80 mg/kg 30 min before NTG). Two hours following injection of NTG or vehicle, the mice were anaesthetised with 2 g/kg urethan (i.p.) and after induction of anaesthesia, within 5 minutes from urethane injection, they were perfused transcardially with 20 ml 0.1 M sodium phosphate-buffered saline (PBS; pH 7.6) followed by fixative (150 ml ice-cold 4% paraformaldehyde) solution. The whole brain with the rostral cervical spinal cord and TRGs was dissected and post-fixed for 24 h in 6% paraformaldehyde solution.

To study the caudal division of the spinal trigeminal nucleus, the medulla oblongata caudal to the obex with the rostral cervical spinal cord was embedded in 4% agar gel. Starting at the obex, 20 μm coronal sections were cut on a Lancer Vibratome (Ted Pella Inc., Redding, CA) and serially collected in 3 wells so that each well received sections at 60 μm intervals throughout the rostro-caudal extent of the brainstem and the spinal cord. Then, they were placed into anti-freeze solution consisting of 30% glycerol, 20% ethylene glycol and 0.1 M sodium phosphate buffer, and stored at -20 °C until further use. The first two series of sections were used for c-Fos and nNOS immunohistochemistry, respectively, while the third was kept as a reserve.

TRGs were dehydrated in 50 v/v%, 70 v/v%, 90 v/v%, and 100 v/v% ethanol solutions. Sectioning was carried out on paraffin-embedded accurately oriented and blocked samples.

Three series of five 4-μm-thick longitudinal sections each interspaced by 60 μm were collected and mounted on silan-pretreated slides and dried.

2.7.1 Free floating immunohistochemistry for c-Fos in the TNC

For c-Fos immunohistochemistry, sections were washed 6×10 min in PBS, followed by incubation in 0.5% Triton X-100 and in blocking buffer consisting of PBS and 2% normal goat serum, for 30 min respectively. Then sections were incubated overnight at room temperature in a polyclonal antiserum raised against c-Fos, diluted 1:500 in blocking buffer. After a wash in PBS, sections were treated with biotinylated goat anti-rabbit IgG diluted 1:200 in PBS containing 2% normal goat serum for 2 h at 20 °C (Gaszner et al., 2009). Sections were rinsed in cold PBS, incubated in avidin–biotin complex for 1 h at 20 °C, and rinsed with PBS for 3×10 min and with Tris buffer (pH 7.6) for 10 min. Finally, they were treated with 0.05% diaminobenzidine (DAB) in the Tris buffer with 0.00003% H_2O_2; the latter reaction was controlled under a stereomicroscope and stopped with Tris buffer. After 3×10 min washes in PBS, sections were mounted on gelatine-coated glass slides, air-dried, treated with xylene for 2×10 min and coverslipped with DPX mounting medium.

2.7.2 Free floating immunohistochemistry for nNOS in the TNC

For nNOS immunohistochemistry, sections were pretreated with 0.1 M PBS and 0.3% H_2O_2 in methanol for 30 min. The samples were washed twice in 0.1 M PBS containing 1% Triton X-100 (PBS-T) and incubated in blocking buffer consisting of PBS-T and 10% normal rabbit serum, for 1 h at room temperature. Then the sections were kept overnight at room temperature in polyclonal anti-nNOS antibody at a dilution of 1:300 in PBS-T solution containing 2 % normal rabbit serum. Between several rinses in cold PBS-T, the sections were treated with biotinylated rabbit anti-goat IgG at a dilution of 1:600 and the immunocytochemical reaction was visualized by Vectastain ABC Elite kit for 2 hours at room temperature, respectively. Samples were stained with nickel ammonium-sulphate-intensified DAB. The sections were dried on air overnight on glass slides, and coverslipped with DPX mounting medium.

2.7.3 Immunohistochemistry for c-Fos in the TRG

For c-Fos immunohistochemistry, rehydration was carried out by treating the slides in xylene for 2×10 min, followed by rinses in absolute ethanol and 96 v/v%, 70 v/v%, 50 v/v%, 30 v/v% ethanol solutions, respectively. Slides were then incubated in PBS for 3×10 min, followed by incubation in 0.5% Triton X-100 and in blocking buffer consisting of PBS and 2% normal goat serum, for 30 min, respectively. Subsequently, sections were incubated overnight at room temperature in a polyclonal antiserum raised against c-Fos, diluted 1:100 in blocking buffer (Gaszner et al., 2009). The second day of the c-Fos immunohistochemistry protocol was carried out as described above for free floating TNC sections, except mounting. TRG preparations were dehydrated by rinsing in 30 v/v%, 50 v/v%, 70 v/v%, 96 v/v% ethanol solutions, and in absolute alcohol respectively, treated with xylene for 2×10 min coverslipped with DPX mounting medium.

2.7.4 Immunohistochemistry for nNOS in the TRG

The paraffin embedded TRG sections on glass slides were deparaffinated by 2x5 min treatment with EZ-DeWax Solution. After rinsing in distilled water containing 50 mM Tris-buffer solution, the tissues were treated with Antigen Retrieval Citra Solution for 15 min according to the supplier's protocol. Sections were then encircled with a liquid blocker pap pen and stained as described above for TNC nNOS immunohistochemistry.

2.7.5 Digital imaging, cell counting

For each type of evaluation in TNC, approximately XX sections per animal interspaced by 60 µm and belonging to the first and second cervical segments of the spinal cord were selected according to Sidmann et al (1971), then 5 good quality sections per animal were designated for quantitative evaluation. Immunoreactive neurons in laminae I-III were evaluated. Also 5 longitudinal sections interspaced by 60 µm were selected from 6-8 sections covering the whole diameter of the ganglion for each type of evaluation in TRG. A random selection procedure was maintained throughout all experiments and all quantifications were performed by an experienced neuroanatomist blinded to group assignment of the samples. The contents of c-Fos in the TRG and TNC were determined by simple manual counting of all c-Fos-positive cell nuclei in non-edited digital images. Digital images were taken with a Nikon Microphot FXA microscope equipped with a Spot RT color digital camera (Nikon, Tokyo, Japan). nNOS immunoreactive neurons were counted in the TRG and TNC under a Zeiss Axio Imager M2 microscope. For the TNC, the absolute number of immunoreactive neurons per section was counted. For the TRG, the number of immunoreactive neurons was expressed in percentage of total number of cell nuclei. The observed absolute and relative counts were summed and averaged in each animal. Evaluation of 1, 3 and 2 animals from the TRG c-Fos, TRG nNOS and TNC nNOS studies, respectively, had to be omitted due to improper staining or injured sample. However, omissions did not exceed 1 (N=5-6 remaining) in any groups.

2.8 Thermal hyperalgesia of the paw measured with the hot plate test

The hot plate test was performed on two groups of ten mice by using an electronically controlled hot plate apparatus (Ugo Basile, Italy, Model Code: DS-37) heated to 46.5 °C (±0.3 °C). Animals were placed into a glass cylinder of 24 cm diameter on the heated surface, and the time between placing of the animal on the hot plate and the occurrence of licking of hind paws or jumping off the surface was recorded as response latency. The cut-off time was 60 s. The response latency was recorded 60 min before and 60, 120, 180 and 240 min following i.p. injection of NTG (10 mg/kg Nitro Pohl) or vehicle. Mice assigned to a vehicle- and an NTG-treated group (N=10 each) were treated and tested in parallel in a randomised arrangement without blinding.

2.9 Mechanical allodynia of the paw measured with von Frey filaments

We used a method essentially similar to that of Bates et al. (2010), except the strain of mice. Hind paw withdrawal thresholds were tested by von Frey filament stimulation of the plantar surface of hind paw using an up-and-down paradigm (Chaplan, Bach, Pogrel, Chung, & Yaksh, 1994). Mice were placed in plastic chambers with mesh metal floor and allowed a

habituation period of 30 min. A series of 10 von Frey hairs (Stoelting Co., USA) with incremental stiffness (ranging from 0.008 g to 2.0 g) was applied to the mid-plantar surface of the hind paw. The first testing filament had the stiffness of 0.16 g. A positive response was defined as licking, lifting or shaking of the paw upon stimulation. One trial comprised poking the paw five times with the same filament at few-second intervals. A trial was considered positive if the animal reacted at least twice. According to the up-and-down paradigm increasing or decreasing of the stiffness of the next filament by one step was dependent on negative or positive paw withdrawal response of the actual trial. The 50% paw withdrawal threshold (PWT expressed in grams) was determined from 5 critical test responses starting with the first positive response (Dixon, 1980). The experiments started with twenty mice. After a baseline measurement, animals with baseline threshold ≤0.6 g (N=4) were excluded from the study. The remaining animals (N=16) were assigned to a vehicle and an NTG treated group (N=8). NTG (10 mg/kg Nitro Pohl) or vehicle was injected and PWT was measured 60, 120, 180 and 240 min later. Mice assigned to these two groups were treated and tested in parallel in a randomised arrangement without blinding.

2.10 Orofacial pain sensitivity tested with von Frey filaments

The mice were placed into a 9-cm-long restraining glass cylinder so that only the head poked out and allowed a habituation period of 5 min. The restrainer allowed head and forepaw movements but prevented the animals from turning in it. A von Frey filament of 0.4 g force was used (Stoelting Co., USA). The filament was applied to the whisker pad on right side of the snout 12 times at approximately 90° angle until bent (Krzyzanowska et al., 2011). The responses were recorded and scored as follows: uni- or bilateral forepaw swipes across the face (1 point), aggression/biting of the probe following stimulus (0.25 points) or clear withdrawal of the head from the stimulus (0.25 points). The points (accumulated in the 12 trials) were summed for each animal separately for each testing time to give the overall "response score". The experiments started with sixty mice. After a baseline measurement animals with a score ≥2 (N=7) were excluded. The remaining animals (N=53) were assigned to five groups (N= 10-11) and NTG (10 mg/kg Nitro Pohl, in four groups) or vehicle (one group) was injected i.p. Thirty minutes later various doses of sumatriptan (0.3-3 mg/kg) or saline (control) solution was injected subcutaneously to the NTG-treated animals and saline also to the vehicle-treated animals. Then von Frey testing was repeated at 60, 90 and 120 min post-NTG. Mice assigned to different groups were treated and tested in parallel in a randomised arrangement. The experimenter was blind to the treatment during testing.

2.11 Data analysis and statistics

For different outcome measures data were expressed either as absolute values or percentages of baseline values. Results are plotted as the mean ± standard error of the mean (SEM). Where repeated measurements were performed statistical significance of differences was tested by two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni's post-test for time-matching samples. For immunohistochemistry data one-way ANOVA was performed after confirming normality of distribution by Kolgomorov-Smirnov test. In case of significance, all other groups were compared to NTG control by Dunnett's post-test. A p value less than 0.05 was considered significant in all tests.

3 Results

3.1 Light aversion assay

Mice injected with saline did not exhibit any overt tendency of change with regards to the average time spent in the light compartment ('light-time') either within the early or the late phase following the injection, although their time spent in light was less in the late phase (Fig. 1A-B). In contrast, the time spent in the light was significantly reduced by both Nitrolingual and its vehicle in the early phase observation period, suggesting rapid development of photophobia (Fig. 1A) elicited primarily by the solvent of Nitrolingual itself. Nevertheless, by the late phase there was no apparent difference in the light avoiding behaviour between the saline, Nitrolingual and vehicle groups (Fig. 1B). In the experiment with Nitro Pohl, apart from a moderate gradual decline of the light-time, the injection caused no overt effect either in the early or the late phase (Fig. 1C-D). Most importantly, we could not detect any significant differences between the NTG groups and their simultaneous vehicle controls in either experiment.

3.2 Cranial blood perfusion

Injection of Nitrolingual, as well as its vehicle initiated a progressive increase in the cranial blood perfusion as measured by laser Doppler scanning. The enhancement amounted approximately 20% by 120 min post dose and remained sustained till the end of the 4-hour observation period in both groups (Fig. 2A). Apart from the non-significant difference that the rise in cranial blood perfusion occurred somewhat faster in the Nitrolingual group, there was no overt difference between the changes elicited by Nitrolingual or its vehicle. In contrast, injection of Rindex, the vehicle for Nitro Pohl, caused no remarkable change in the cranial blood perfusion, apart from <5% decrease in the first hour, and there was no significant difference between Nitro Pohl and the corresponding vehicle group (Fig. 2B). Hence, again, we could not detect any significant effect of NTG compared to the relevant control groups. Therefore, having observed a remarkable effect with its solvent, we excluded Nitrolingual from our further studies.

3.3 Immunohistochemistry for c-Fos and nNOS

We combined verification of the effect of NTG with pharmacological validation by testing the sensitivity of this model to the abortive- and preventive- antimigraine drugs sumatriptan and topiramate, respectively. Quantification of c-Fos positive nuclei in the TNC indicated statistically significant two-fold increase after treatment with NTG (Nitro Pohl) compared with the vehicle control (Rindex) group (Fig. 3A). However, a statistically significant increase could not be detected in the TRG (Fig. 3B, ANOVA $p=0.31$). Compared to the NTG treated group, a high dose of sumatriptan (5 mg/kg, s.c.) administered before and after NTG injection did not reverse significantly the increase in c-Fos expression in TNC. Although an apparent trend towards normalisation might have been noticed, it was far from significance (Dunnett's adjusted $p=0.63$). In contrast, pretreatment with topiramate (80 mg/kg, i.p.) completely prevented the NTG-induced increase in c-Fos expression in TNC, which effect was significant (Fig.3A; $p=0.03$).

Immunostaining for nNOS did not reveal any significant alteration induced by NTG injection or any effect of the antimigraine drugs. The number of nNOS positive cells showed rather low variability in the TNC but much higher in the TRG (Fig. 3C-D).

3.4 Thermal hyperalgesia of the paw

Intraperitonal injecton of neither the vehicle nor Nitro Pohl caused any signs of pain related behaviour suggesting peritoneal irritation. Hot plate latencies of vehicle-treated mice were very stable over time with repeated testing four times up to 240 minutes post dose (Fig. 4A). NTG injection caused a clear-cut hyperalgesia, manifested in reduced defensive response latency, which effect was maximal and statistically significant at 60 min post dose. Nearly complete recovery was apparent by 180 min and there was no sign of a delayed secondary wave of hyperalgesia (Fig. 4A).

3.5 Mechanical allodynia of the paw

Changes in tactile hind paw withdrawal thresholds showed similar time course of the effect of NTG to that seen in the hot plate test (Fig. 4B). Although the mechanical threshold data of the vehicle control group displayed larger variability (coefficient of variation) and lower stability as compared to hot plate results, the apparent effect was also larger resulting in statistically significant changes at 60 min and 120 min post dose. The thresholds at 180-240 min after injection clearly indicated a tendency towards recovery without any sign of a late phase secondary sensitisation.

3.6 Orofacial pain sensitivity

Repeated recording of orofacial pain scores indicated no overt change in the pain-related behaviour of vehicle-treated animals (Fig. 5). However, mice treated with NTG exhibited a significant increase in nocifensive behaviour, which peaked at 60 min post-NTG and was declining later. The observed time-course was apparently similar to that seen in the plantar pain tests. Having established relatively short duration of action of NTG in the paw hyperalgesia/allodynia tests, we did not extend the study duration of the orofacial allodynia testing further than 2 hours after NTG. Instead, we included three further groups for pharmacological validation by a dose-response study of sumatriptan. Administration of sumatriptan 30 min before the first post-NTG testing remarkably suppressed the pain behaviour of mice. The dose-response relationship was apparent and flat at 60 min post NTG as the lowest dose of 0.3 mg/kg sumatriptan caused profound (more than 50%) inhibition. However, the effect of sumatriptan was not statistically significant at later time-points as the NTG-treated control group started to decline and the reversing effect of sumatriptan remained only partial (Fig. 5).

4 Discussion

Altogether we investigated 9 outcome measures in 6 studies and confirmed the utility of 4 of these for detecting the effects of NTG in mice. These outcome measures are (1) c-Fos expression in the TNC, (2) thermal hyperalgesia of the paw, (3) mechanical allodynia of the paw and (4) orofacial pain sensitivity. These might be useful to predict anti-migraine

effectiveness of drugs or target related genetic manipulations, provided that their sensitivity to anti-migraine drugs is proven in pharmacological validation experiments. Effectiveness of a single dose of NTG to influence the first 3 of these endpoints in mice has been proven previously (Bates et al., 2010; Goloncser & Sperlagh, 2014). However, some pharmacological validation by using sumatriptan has been provided only for the paw allodynia and hyperalgesia (Bates et al., 2010). Therefore, we included some groups to perform validation of those mouse tests that had no previous pharmacological validation data at all, i.e. in the immunohistochemistry and the orofacial pain tests. Nevertheless, it is worth to note that validation for predictive power of such migraine models would require the use of several different abortive and preventive medications with clinical proof of effectiveness, such as triptans, topiramate, propranolol and CGRP antagonists (Ashina et al., 2013) as well as investigation of some clinically failed negative controls. Extensive validation of these models was beyond the scope of the present investigations but might be the subject of further studies.

The protein product (c-Fos) of the proto-oncogene *c-fos* is a sensitive and widely applicable marker of neuronal activation in response to various stimuli and its enhanced expression in the TNC after NTG administration in rats has been reported by several groups (Tassorelli & Joseph, 1995; Pardutz et al., 2000; Knyihar-Csillik et al., 2008; Ramachandran et al., 2012). NTG-induced increase of nNOS protein expression in the TNC and TRG has also been demonstrated in rats (Pardutz et al., 2000; Srikiatkhachorn et al., 2002; Dieterle et al., 2011). Whereas we also proved the NTG-induced increase of c-Fos in the TNC, we could not confirm NTG-induced changes of nNOS expression in mice either in the TNC or in the TRG. Since nNOS inducing effects in rats were revealed by at least three different research groups, we may assume that the discrepancy can be a species specificity issue. Concerning NTG-induced c-Fos induction in TRG, we did not observe any significant effect in mice and we did not find any preceding literature data assessing this parameter in rats. However, c-Fos also proved not to be a suitable pain marker in sensory neurons of dorsal root ganglia of rats in contrast with the phosphorylated extracellular signal-regulated kinase which was induced both in dorsal horn and in sensory neurons by painful interventions (Gao & Ji, 2009). Having a significant NTG-induced increase only for c-Fos in the TNC, our attempt for pharmacological validation can be meaningfully interpreted only with regards to this outcome measure. The NTG-induced increase in c-Fos expression was sensitive to pretreatment with topiramate, which completely prevented the effect of NTG. Although preventive treatment by repeated dosing would have better mimicked the clinical setting, in this preliminary validation experiment administration of a single high dose was sufficient. On the contrary, our mouse NTG model with c-Fos expression readout was apparently insensitive or weakly sensitive to sumatriptan, administered 30 min before and after NTG, which did not produce a significant effect. In rats, Ramachandran et al. (2012) observed partial reversal of NTG-induced c-Fos increase in the TNC, but that model used much lower dose of NTG (80 μg/kg) by intravenous (i.v.) infusion. It remains an interesting question whether this difference in sensitivity to sumatriptan treatment is the consequence of species difference or the different provoking dose of NTG. In brief, c-Fos expression in TNC induced by NTG at the dose of 10 mg/kg i.p. in mice might be an utilisable outcome measure for detecting the effect of preventive treatments.

Our findings with regards to thermal hyperalgesia and tactile allodynia of the paw were in good concordance with those of Bates et al. (2010), showing maximal thermal hyperalgesia at 60 min and maximal or close to maximal tactile allodynia at 60-120 min post-NTG, and complete or partial recovery, respectively, by 4 hours post dose. Our results with the hot plate method showed greater stability but smaller percent reductions in latency than their results by the Hargraves method. On the other hand, the baseline thresholds in our tactile allodynia test in NMRI mice were more than two-fold higher than in their C57BL6 mice, which is in agreement with our unpublished experience on comparison of the two strains. However, starting from a higher control threshold, we observed also a greater NTG-induced drop in percentage terms, which provides a greater window for investigation of preventive effects. Here it is relevant to note that Pradhan et al. (2014) have shown the single dose (10 mg/kg i.p.) NTG-induced plantar allodynia of mice to be sensitive to both sumatriptan and topiramate, whereas the progressive sustained mechanical allodynia induced by repeated dosing was sensitive only to topiramate.

Measurement of orofacial pain sensitisation has apparently greater construct validity with regards to modelling migraine than measurements in other somatic areas. It reflects sensitisation in the innervations field of the trigeminal nerve, which is believed to play a central role in migraine generation (Messlinger et al., 2011). Spontaneous migraine is often accompanied by cephalic cutaneous allodynia, though extracephalic (somatic) allodynia is also prevalent (Bigal et al., 2008; Lipton et al., 2008; Bernstein & Burstein, 2012). Occurrence of orofacial pain hypersensitivity after NTG injection has been demonstrated in rats (Di et al., 2015; Greco et al., 2015), but not in mice. Our results show that pain-related behaviour scoring in mice can be used to detect orofacial pain sensitising effect of NTG. This orofacial hyperalgesia had similar time course to the plantar hyperalgesia with maximum at 60 minutes post NTG and a tendency of recovery afterwards. Furthermore, the NTG-induced orofacial hyperalgesia was significantly alleviated by sumatriptan administration. These data suggest, that measurement of orofacial pain can also be a useful outcome measure for modelling NTG-induced migraine-like headaches in mice; and this model is sensitive to triptans, like the plantar hyperalgesia/allodynia (Bates et al., 2010). It remains to be answered by extensive pharmacological validation experiments, whether the orofacial and the plantar (or other somatic) hyperalgesia/allodynia elicited by NTG exhibit differences in their pharmacological sensitivity, which might become an argument for favouring orofacial over plantar testing, or they prove to carry equal information in terms of modelling migraine.

We confirmed our previous finding (Markovics et al., 2012) that injection of Nitrolingual enhanced the cranial blood perfusion as measured by transcranial laser Doppler scanning over the hemispheres of mice. However, comparison of the results with the relevant vehicle indicated that the enhanced blood perfusion was not due to the effect of NTG but the vehicle itself caused such pronounced effect and there was no significant difference between Nitrolingual and its vehicle. Since the vehicle contains several pharmacologically active constituents, including ethanol and peppermint oil, its vasodilating effects might be attributed to these ingredients. Ethanol can cause vascular relaxation by activation of transient receptor potential (TRP) vanilloid 1 receptors and CGRP release (Nicoletti et al., 2008). Peppermint oil (which contains menthol and menthone) can cause vasodilation through CGRP release

caused by activation of the TRP ankyrin 1 and TRP menthol 8 receptors (Namer, Seifert, Handwerker, & Maihofner, 2005; Takaishi et al., 2012). More surprisingly, we could not demonstrate any remarkable effect of NTG on cranial blood perfusion when it was administered in an apparently inert vehicle (Nitro Pohl vs. Rindex 5), as neither the vehicle nor Nitro Pohl produced any overt change.

It is unclear if this lack of effect is due to species differences or the administration route or the inability of the technique to detect NTG-induced changes, as it averages blood flows in different depths and vessels or other experimental conditions, such as anaesthesia. In rats, typically meningeal blood vessel diameters have been assessed by imaging techniques and local cortical blood flow measurements were performed by laser Doppler flowmetry under anaesthesia. Cortical blood flow measurements after i.v. administration of small NTG doses (20-60 μg/kg) indicated increased flow (Read et al., 1999; Gozalov et al., 2008). Increased cortical laser Doppler flow was reported also after a large (10 mg/kg) i.v. dose of NTG (Srikiatkhachorn et al., 2002). Meningeal arterial diameters indicated strong dilations after a small i.v. NTG dose (Gozalov et al., 2008). Nevertheless, after an i.p. dose of 10 mg/kg in rats opposite changes, i.e. increased diameter of cortical and decreased diameter of meningeal arterioles were observed (Pryazhnikov et al., 2014). Hence, a possible explanation for lack of effect of NTG is that opposing vascular changes mutually neutralised in the net perfusion that was measured by the laser Doppler scanning technique. The impact of anaesthesia might also be considered. Gozalov et al., (2008) and Srikiatkhachorn et al., (2002) reported NTG-induced meningeal and cortical vasodilations in pentobarbital-anaesthetised rats, whereas Pryazhnikov et al. (2014) reported the opposing effects on cortical and meningeal arterioles in urethane- as well as ketamine-xylazine-anaesthetised rats. We also used urethane in our experiments, first, to reproduce the conditions of Markovics et al. (2012), a study comparing Nitrolingual to saline, second, to produce a stable and long-lasting anaesthesia with relatively preserved autonomic reflexes and cardiovascular tone (Janssen et al., 2004).

In mice, measurements of cranial vascular effects are more difficult than in rats due to their smaller size. A study applying intravital microscopy in pentobarbital-anaesthetised mice found that, in contrast with rats, meningeal vasodilating effect of CGRP could be detected only after preconstriction with endotheline-1 (Gupta et al., 2006). However, we could not find any reports of NTG-induced changes in cranial circulation of mice. The transcranial laser Doppler scanning seemed to be a promising and relatively non-invasive approach in mice. Nevertheless, although this method was apparently suitable to detect probably ethanol+menthol-induced enhancement of cranial blood flow in mice, it did not reveal 10 mg/kg i.p. NTG-induced changes in our experiments.

Photophobia is a common accompanying symptom of migraine, and light-avoiding behaviour is considered as a relevant outcome measure mimicking the migraineous state in mice. Light aversion could be induced by intracerebroventricular CGRP treatment in transgenic mice sensitized to CGRP by overexpression of the human receptor activity modifying protein 1 (hRAMP1) associated with functional CGRP receptors (Recober et al., 2009; Recober et al., 2010). In a previous study we showed that Nitrolingual induced significant light aversion when compared to a saline-treated group (Markovics et al., 2012). Significant differences were seen at the end of the early as well as the late phase of the study. In the present study, the

light aversive behaviour of saline control animals was less fluctuating and significant light aversion of Nitrolingual-treated mice was apparent only in the early phase. However, it turned out that the light aversive behaviour was attributable to the vehicle of Nitrolingual, as the genuine vehicle-treated animals exhibited a similar pattern to that with Nitrolingual. Furthermore, comparison of Nitro Pohl to its relevant vehicle control did not reveal any significant NTG-induced effect either in the early or in the late phase of the study. Hence, according to the present findings, it was not the NTG which induced light aversion in mice but the vehicle of Nitrolingual.

Doses and routes of administration of NTG varied between animal studies making the interpretation and comparison of the results difficult. We uniformly applied 10 mg/kg i.p. dose of NTG in all experiments. This dose selection was based on the report of Bates et al. (2010), who established in dose-response studies that this was the minimum dose essential for detecting overt hypersensitivity in both thermal hyperalgesia and mechanical allodynia tests in mice. The relevance of such large systemic NTG doses has been criticised as being 1000-fold higher than the doses provoking headache and migraine in volunteers and patients, respectively (Ramachandran et al., 2012; Jansen-Olesen, Tfelt-Hansen, & Olesen, 2013). In the human NTG model typically 2.4-10 μg/kg (0.12-0.5 μg/kg/min for 20 min) i.v. dose (Iversen & Olesen, 1996; Tvedskov, Tfelt-Hansen, Petersen, Jensen, & Olesen, 2010) or 0.5 mg (≈8 μg/kg) sublingual dose (Juhasz et al., 2005) is applied. Advocates of low-dose NTG proposed 80 μg/kg (4 μg/kg/min for 20 min) i.v. infusion in rats. After this dose, increased c-Fos expression in the TNC was evident (Ramachandran et al., 2012); but pain hypersensitivity has not been demonstrated so far. However, oral bioavailability of NTG in rats was low (F=1.6%) and associated with fast elimination ($T_{1/2}$=4 min) and strong first-pass effect (Yap & Fung, 1978). Several factors may contribute to that about 100-fold higher doses are needed in mice by i.p. administration to elicit pain hypersensitivity than the suggested i.v. dose in rats provoking c-Fos increase: (1) the first-pass effect may be also significant after i.p. administration (Lukas, Brindle, & Greengard, 1971), (2) allometric scaling between rats and mice might be an argument for higher doses in mice; and (3) the potential need for somewhat higher or longer exposures to elicit consistent pain hypersensitivity. In fact, almost all studies demonstrating NTG-induced pain hypersensitivity in healthy mice or rats used the dose of 10 mg/kg, i.p. (Tassorelli et al., 2003; Tassorelli et al., 2006; Bates et al., 2010; Pradhan et al., 2014; Di et al., 2015; Greco et al., 2015) or 15 mg/kg, i.p. (Goloncser & Sperlagh, 2014). The only exception applied 0.1 mg/kg i.p. dose and found short-lasting periorbital tactile threshold decrease in naïve rats (Oshinsky & Gomonchareonsiri, 2007). The time-course of headache ratings in healthy volunteers after 20-min-long infusion of NTG exhibits only an early peak, whereas that in migraine patients an early and a late peak. The second peak, the delayed headache, particularly if elicited by 0.5 μg/kg/min infusion rate, fulfils International Headache Society criteria of migraine (Tvedskov et al., 2004a; Tvedskov et al., 2004b). Our results in mice, in line with that of others (Bates et al., 2010; Goloncser & Sperlagh, 2014), displayed only an "early" peak at 60 min with a tendency towards recovery starting by 120 min. Assuming analogy with the human model this single peak in healthy mice may represent the early peak in healthy volunteers without a secondary headache generation. The time-course in

mice appears to be shorter than in rats, where the hyperalgesic effect is sustained beyond 4 hours post NTG (Tassorelli et al., 2003; Di et al., 2015).

There is a great interest in human models of migraine, which could be used as indicators of efficacy in early clinical development of anti-migraine drugs, particularly for prophylactics, for which proof-of-concept studies are very resource intensive (Tvedskov et al., 2004a; Tvedskov et al., 2004b). The human NTG model either in volunteers with detection of the early headache or in patients with the delayed migraineous headache could be a useful test. However, it is not unequivocally sensitive to all clinically proven preventive migraine drugs, probably depending on whether their targeted mechanisms are downstream or upstream from the NTG-induced effects in the migraine generation process. The NTG-induced early and late headache were both significantly reduced by valproate but were unaffected by pretreatment with clinically effective doses of propranolol (Tvedskov et al., 2004a; Tvedskov et al., 2004b). This latter finding might ruin the confidence in using the human NTG model in early clinical studies for dose finding or proof of effectiveness. If it were proven in further validation experiments that pharmacological sensitivity of the rodent NTG models with any of the feasible outcome measures predicts the sensitivity of the human model, then the rodent model can be used as a pre-screening test for drug candidates and mechanisms that are worth to investigate in the human NTG model with high confidence.

It is worth to note that the utilisable endpoints in rodents, either c-Fos in TNC or somatic and/or face allodynia/hyperalgesia, are different from the most important endpoint in volunteers or patients, which is reported (scored) headache. Although presence of allodynia has been detected in spontaneous migraine (Bigal et al., 2008), such measurements have not been reported from the human NTG experiments. Nevertheless, as the migraineous pain may be a manifestation of trigeminal or more universal pain hypersensitivity (Bernstein & Burstein, 2012), it is not unlikely that pharmacological sensitivity of the different endpoints used in rodents and human subject finally prove to be similar. Therefore, comparative validation by using distinguished positive and negative control drugs in the rodent and human NTG models might provide a good translational research tool supporting the development of new anti-migraine drugs with mechanisms liable to NTG-induced headache.

NTG is believed to function as a nitrogen monoxide (NO) donor via an enzymatic reaction catalysed by the mitochondrial aldehyde dehydrogenase enzyme (Chen, Zhang, & Stamler, 2002; Ignarro, 2002). In vascular smooth muscle cells, the released NO acts via the enzyme soluble guanylate cyclase (sGC) to stimulate the generation of cyclic guanosine-monophosphate (cGMP) and elicit vasodilation (Miller & Megson, 2007). However, sGC stimulation without NO mediation by low concentrations of NTG has also been proposed (Kleschyov et al., 2003). Multiple data suggest involvement of endogenous NO production and stimulation of the NO – sGC pathway during migraine attack (Olesen, 2008). Another mechanism which certainly plays a role in migraine generation is the release of CGRP in the trigeminovascular system. CGRP antagonists proved to effectively alleviate migraine (Edvinsson, 2008; Karsan & Goadsby, 2015). There is an intricate interplay between NTG or NO and CGRP. It has been suggested that NO causes release of CGRP (Strecker, Dux, & Messlinger, 2002) and *vice versa* CGRP elicits NO release (Li, Vause, & Durham, 2008), though the data from different sources are not unequivocal as reviewed by Olesen (2008).

Recently, it was shown that CGRP *per se* is not algogenic but becomes algogenic only after NTG treatment (Capuano, Greco, Navarra, & Tringali, 2014).

Release of CGRP and consequently increased regional or systemic plasma levels of CGRP were observed during headache phase of spontaneous migraine attacks (Goadsby, Edvinsson, & Ekman, 1990) and during NTG-induced migraineous attacks in patients (Juhasz et al., 2003). Nevertheless, the results on CGRP plasma levels during migraine attack are controversial, and a study using intrapatient comparison design failed to find any increase in jugular and cubital vein plasma levels (Tvedskov et al., 2005). No overt increase in systemic CGRP levels was observed during the early headache phase after NTG either in healthy volunteers or patients (Ashina, Bendtsen, Jensen, Schifter, & Olesen, 2001; Juhasz et al., 2003; Kruuse, Iversen, Jansen-Olesen, Edvinsson, & Olesen, 2010). Furthermore, while involvement of CGRP in NTG-induced hyperalgesia and c-Fos expression was proved in rats by the use of CGRP antagonists MK-8825 (Greco et al., 2013) and olcegepant (Ramachandran et al., 2014), olcegepant was ineffective in preventing the migraine attack in the patients induced by 20 min x 0.5 μg/kg/min infusion of NTG (Tvedskov, Tfelt-Hansen, Petersen, Jensen, & Olesen, 2010). These results could be interpreted in a way that the CGRP pathway is not involved in the human NTG model but is involved in the rat model either with the low-dose (4 μg/kg/min) infusion (Ramachandran et al., 2014) or the high-dose (10 mg/kg) i.p. injection (Greco et al., 2013) as well as in the natural human migraine. Therefore, the rat (or rodent) NTG models might be better in terms of predictive validity than the human NTG model. However, it should be noted that, in order to mimic an abortive paradigm, olcegepant was administered after the NTG infusion in the human study. Furthermore, olcegepant was effective in rats only by pre-treatment but not by post-treatment (Ramachandran et al., 2014). Therefore, investigating a CGRP antagonist (or antibody) in man also by pre-treatment would provide more reliable results with regards to presence or absence of involvement of CGRP in the early as well as the late headache.

In summary, we proved that c-Fos expression in the TNC, as well as somatic and facial pain sensitisation, are potentially useful endpoints in the mouse for detecting NTG-induced changes and modelling migraine. Further work is needed to confirm the predictive validity of these mouse models by extensive cross-validation using drugs and pharmacological tools by pre-treatment in the human and mouse NTG models and by comparisons with effectiveness in migraine patients. We could not confirm the utility of nNOS expression in the TNC or TRG, and also failed to show NTG-induced light avoidance in mice. For detection of NTG-induced vascular changes either different methods are needed instead of transcranial laser Doppler scanning or the mouse is not an appropriate species. Investigation of NTG effects using relatively inert vehicles and by comparison with adequate vehicle controls is highly recommended to avoid false conclusions in preclinical studies of NTG.

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Figure captions

Figure 1

Effects of two different formulations of nitroglycerin (NTG; 10 mg/kg, i.p.) compared to vehicle controls in the light aversion test. Nitrolingual (NL) was compared to both a saline group and a solvent mimicking „NL vehicle" group (A, B). Nitro Pohl was compared to its corresponding vehicle, Rindex (C, D). Data are presented as mean ± SEM (N=12-13/group in A-B and 8/group in C-D) of percent time spent in the light compartment of the light-dark box. Results were evaluated separately in the early 0-30 min (A, C) and late 90-120 min phases (B, D) following NTG injection. Abscissas show the time after NTG injection. Asterisks show statistically significant differences compared to the saline group (*$p<0.05$, ** $p<0.01$, ***$p<0.001$; two-way repeated measures ANOVA followed by Bonferroni test). There were no significant differences when NTG formulations were compared to their respective vehicle controls.

Figure 2

Effects of two different formulations of nitroglycerin (NTG 10 mg/kg, administered i.p. at 0 min) compared to their respective vehicle controls on cranial blood perfusion measured by Laser Doppler scanning. Data are presented as mean ± SEM (N=6/group) of percentage change relative to the baseline recording. There were no significant differences when the different NTG formulations (Nitrolingual: NL and Nitro Pohl) were compared to their respective vehicle controls (two-way repeated measures ANOVA followed by Bonferroni test).

Figure 3

Effects of nitroglycerin (NTG; 10 mg/kg i.p.) compared to vehicle (Veh) controls on quantitative immunohistochemistry for c-Fos and nNOS in the trigeminal nucleus caudalis (TNC) and trigeminal ganglia (TRG), and the effects of sumatriptan (Sum; 2x5 mg/kg, s.c.) and topiramate (Topi; 80 mg/kg, i.p.) in mice injected with NTG. Data are presented as mean ± SEM (N=5-6/group). Asterisks show statistically significant differences compared to the NTG group (*$p<0.05$; one-way ANOVA followed by Dunnett's test).

Figure 4

Effects of nitroglycerin (NTG; 10 mg/kg i.p.) compared to vehicle controls on thermal hyperalgesia (A) and mechanical allodynia (B) of the hind paw. Data are presented as mean ± SEM (N=10/group in A and 8/group in B). Abscissas represent the time after NTG injection. Asterisks show statistically significant differences (*$p<0.05$; **$p<0.01$; two-way repeated measures ANOVA followed by Bonferroni test).

Figure 5

Effects of nitroglycerin (NTG; 10 mg/kg, i.p.) compared to vehicle controls (Veh+Veh) on orofacial pain elicited by tactile stimuli, and alterations of the NTG-induced facial allodynia by different doses of sumatriptan (0.3-3 mg/kg, s.c.; NTG+Sum). Data are presented as mean ± SEM (N=10-11/group) Abscissa represents the time after NTG injection. Asterisks show statistically significant differences compared to the NTG+vehicle treated group (*$p<0.05$; **$p<0.01$; ***$p<0.001$; two-way repeated measures ANOVA followed by Bonferroni test). Arrows indicate the time of administration of drugs.