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The nitric oxide donor, isosorbide dinitrate, induces a cephalic cutaneous hypersensitivity, associated with sensitization of the medullary dorsal horn

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Abbreviations

BSA, bovine serum albumin

CGRP, Calcitonin gene related peptide

ERK, Extracellular signal-regulated kinases

ISDN, Isosorbide Dinitrate

MDH, medullary dorsal horn

NHS, normal horse serum

NTG, nitroglycerin

RM ANOVA, repeated measures analysis of variance

TBS, Tris-buffered saline

TX, Triton X

WDR, wide dynamic range

Abstract

Nitric oxide donors are known to produce headache in healthy as well as migraine subjects, and to induce extracephalic cutaneous hypersensitivity in rodents. However, little is known on the effect of nitric oxide donors on cephalic cutaneous sensitivity. Combining behavioral, immunohistochemical, and *in vivo* electrophysiological approaches, this study investigated the effect of systemic administration of the nitric oxide donor, isosorbide dinitrate (ISDN), on cephalic and extracephalic cutaneous sensitivity and on neuronal activation within the medullary dorsal horn (MDH) in the rat. Systemic administration of ISDN increased selectively the first phase and interphase of the facial formalin test, but had no effect on the hindpaw formalin one. Monitoring neuronal activity within the MDH with phospho-ERK1/2 immunoreactivity revealed that ISDN alone did not activate MDH neurons, but significantly increased the number of formalin-evoked phospho-ERK1/2-immunoreactive cells in the ipsilateral, but not contralateral, MDH. Using *in vivo* electrophysiological unit recordings, we show that ISDN administration never affected the spontaneous activity of trigeminal wide dynamic range neurons, but, facilitated C-fiber-evoked responses in half the neurons tested. This research demonstrates that a nitric oxide donor, isosorbide dinitrate, induces selectively cephalic hyperalgesia that arises as a consequence of central sensitization in pain pathways that subserve meningeal nociception. This model better mimics the clinical condition and offers another possibility of studying the role of nitric oxide donor in the pathophysiology of headache.

Keywords: Headache, pain, migraine, hyperalgesia, central sensitization, trigeminal

INTRODUCTION

Migraine is a common disorder affecting about 15% of the population (Stovner and Andree, 2010). Cutaneous hypersensitivity is a key feature of migraine (Bigal et al., 2008). It is a consequence of central sensitization in pathways that subserve meningeal pain (Bernstein and Burstein, 2012). Cutaneous hypersensitivity may occur within only the referred area of headache (that is the periorbital region) or spread throughout the face and scalp (Ashkenazi et al., 2007; Burstein et al., 2000; Cooke et al., 2007; Guy et al., 2010; Kitaj and Klink, 2005; Yoon et al., 2009) as well as the body and limbs (Burstein et al., 2000; Guy et al., 2010). About two-thirds of migraine patients exhibit cutaneous hypersensitivity over the course of their migraine attacks (Bigal et al., 2008). The prevalence and severity of cutaneous hypersensitivity are higher in chronic than episodic migraine patients (Bigal et al., 2008). Cutaneous allodynia has thus been suggested as a risk factor for disease progression (Bigal et al., 2008) or no remission (Manack et al., 2011). Therefore, assessing cutaneous sensitivity in animals in response to known migraine triggers has been proposed as a useful approach to modeling migraine (Boyer et al., 2014; Oshinsky and Gomonchareonsiri, 2007; Pradhan et al., 2014). For instance, in rats, dural stimulation with an inflammatory soup induces both cephalic and extracephalic allodynias (Boyer et al., 2014; Edelmayer et al., 2009; Oshinsky and Gomonchareonsiri, 2007), that is associated with trigeminospinal central sensitization (Boyer et al., 2014) and impaired descending inhibitory (Boyer et al., 2014) and/or facilitatory (Edelmayer et al., 2009) pain controls.

Systemic administration of nitroglycerin (NTG) is an extensively used model of migraine in both human and animals (Ashina et al., 2013). In healthy subjects, systemic NTG triggers a headache. In migraineurs, it induces, in addition, a delayed headache with migraine features (Ashina et al., 2013). Such NTG-induced migraine-like headache is associated with thermal

cephalic allodynia (de Tommaso et al., 2004). In rodents, systemic NTG produces extracephalic both thermal and mechanical allodynia (Bates et al., 2010; Brennan et al., 2013; Ferrari et al., 2016; Pradhan et al., 2014; Tassorelli et al., 2003), as well as hyperalgesia at the hindpaw formalin test (Greco et al., 2015; Tassorelli et al., 2003). However, this results are not in agreement with clinical studies reporting that migraine headache does not typically produce cutaneous hypersensitivity below the C8 dermatome (Ashkenazi et al., 2007). Surprisingly, though, only few studies have examined the effect of systemic administration of nitric oxide donors on cephalic cutaneous sensitivity. Moreover, they provided conflicting results. One study performed on only 3 restrained rats reported that systemic administration of NTG decreases the periorbital von Frey thresholds (Oshinsky and Gomomchareonsiri, 2007). Two other studies in mice found either a decrease (Farkas et al., 2016) or no change (Kaufmann et al., 2005) in the periorbital von Frey thresholds after systemic NTG. Similarly, either an increase in rats (Greco et al., 2015) or no change in mice (Grigoraay et al., 2013) in the duration of the face rubbing behavior was found after formalin injection into the upper lip in rats that had been pre-treated with NTG. Finally, two studies found that NTG administration raises the duration of the face rubbing behavior induced by the subcutaneous injection of Calcitonin gene related peptide (CGRP) into the upper lip (Capuano et al., 2014; Yao and Sessle, 2008). Therefore, combining behavioral, immunohistochemical, and *in vivo* electrophysiological approaches, this study investigated the effect of systemic administration of the nitric oxide donor, isosorbide dinitrate (ISDN), on cephalic and extracephalic cutaneous sensitivities and on the medullary dorsal horn (MDH) neuronal activation. ISDN was selected because: i) it reliably produces headache in migraineurs but less often in healthy subjects (Bellantonio et al., 1997; Castelleno et al., 1998), ii) it is less hypertensive than NTG (Manabe et al., 2001), iii) it has an injectable form, so the same solution can be injected in humans as well as animals, allowing comparison between preclinical and clinical results, and iv) it does not need to be

dissolved in alcohol and propylene glycol, thereby reducing non-specific effects (Olesen and Jansen-Olesen, 2012).

EXPERIMENTAL PROCEDURES

Animals

Animal experiments were performed according to the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983), the Directive 2010/63/UE of the European Parliament and the Council on the protection of animals used for scientific purpose. Protocols for animal care and use applied in this work were approved by the appropriate local committee at the University of Clermont-Ferrand-Auvergne (no. CE 28-12).

Adult male Sprague-Dawley rats (250–275 g; Charles River, L'Arbresle, France) were raised at 23 ± 1 °C in plastic cages (3-4 rats per cage; held in Iffa- Credo units) with soft bedding and water and food *ad libitum* in a 12-h/12-h dark/light cycle for at least one week before experiment. All efforts were made to minimize the number of animals used. Numbers of animals were selected according to previous experience (Boyer et al., 2014; Lapirot et al., 2011; Miraoucourt et al., 2009; Raboisson and Dallel, 2004), that is a balance between usual sample sizes in the field and the need to reduce the use of animals in pain experiments. Experiments were performed on 74 animals (6-8 rats/group). All experimenters were blind to treatment conditions. Rats were randomized into treatment groups before any assessment.

Behavioral testing

Habituation

Animal were tested in light conditions, between 11:00 and 19:00 h, in a quiet room, at 23-24°C. Test boxes (30 x 30 x 30 cm) had 3 mirrored sides. Animals were habituated for 2 days in the test environment. During each day, animals were first placed in this test box for 30-min to minimize stress. They were then gently restrained and the experimenter simulated an

injection into the vibrissae area or the hindpaw, using a similar syringe as that in the real test. During behavioral tests, rats had access to neither water nor food. Each rat was used only once. At the end of the experiment, the rat was killed by intraperitoneal (i.p.) injection of a lethal dose of urethane (>1.5 g/kg) and death was confirmed by permanent cessation of cardio-respiratory functions.

Face formalin test

Animals were randomly included into 4 groups (6-8 rats/group). Rats were injected with formalin (50 µl, 1% into the right vibrissa pad; Raboisson and Dallel, 2004) and then placed into the test box for a 45-min period. This observation period was divided into 15 3-min blocks. We computed a nociceptive score for each block: the time (in seconds) the animal spent rubbing the vibrissa pad with only the forepaw. The rubbing behavior was recorded using a videocamera. Formalin was injected either 2 h after i.p. administration of saline or 1, 2 or 4 h after i.p. administration of isosorbide dinitrate (ISDN, 10 mg/kg, Sanofi-aventis, France).

Hindpaw formalin test

Rats were randomly included into 2 groups (8 rats/group) and received formalin (50 µl, 1%) into the right plantar hindpaw (Abbott et al., 1995). After injection, animals were placed into the test box for a 45-min period. This observation period was divided into 15 3-min blocks. We computed a nociceptive score for each block: the time (in seconds), the animal spent licking the right hindpaw. The licking behavior was recorded using a videocamera. Formalin was injected 2 h after intraperitoneal administration of either saline or ISDN (10 mg/kg).

Immunocytochemistry

MDH activation was studied using phospho-extracellular signal-regulated kinase (ERK) expression. Rats were randomly included into 2 groups (8 rats/group) and received formalin (50 μ l, 1%) into the right vibrissa pad 2 h after i.p. administration of either saline or ISDN (10 mg/kg). Five min after formalin injection rats were anesthetized with urethane (1.5 g/kg, i.p.) and perfused transcardially with heparinized saline (25 IU heparin/mL, at 37°C) and then phosphate-buffered solution (0.1 M, pH 7.6 at 10°C) containing 4% paraformaldehyde + 0.03% picric acid for 15 min. The brain was then removed and proceeded as previously described (Alvarez et al., 2009; Miraucourt et al., 2009; Peirs et al., 2016). To characterize the population of pERK1/2 immunopositive cells, double immunofluorescence labeling for pERK1/2 and the neuronal marker NeuN was performed in one set of MDH slices.

To analyze bright-field images of representative labeling, we used a charge-coupled device color video camera (Sony DXC-950P) connected to a Nikon Optiphot-2 microscope. Images were then transformed into Adobe PhotoShop (CS2) files to adjust brightness and contrast before setting the image scale.

In each rat, we computed the number of phospho-ERK⁺ neurons within the different MDH laminae at 7 different rostrocaudal planes, between levels 0 and 2400 caudal to the obex. Given the 400- μ m inter-plane interval between planes, cells could not be counted twice. Our own myelo-architectural together with the Paxinos and Watson atlas (2007) were used to delineate MDH. Data are the number of labeled cells/section that were analyzed in each rat.

Electrophysiology

Animal preparation

Animal surgery for in vivo electrophysiological recordings within the MDH were performed under 2% halothane in a NO₂/O₂ mixture as previously described (Boyer et al., 2014; Lapirot

et al., 2011). For the remainder of the experiment, anesthesia was reduced to 0.6-0.7% halothane.

Unitary extracellular recordings

Glass microelectrodes (7–10 Mohm) filled with 5% NaCl containing pontamine sky blue were used to record single-unit activities within the right MDH. The brainstem was explored 0.4 - 2.1 mm laterally to the midline and between the frontal planes AP - 0.8 and AP - 2.4 mm from the interaural line (Paxinos and Watson, 2007). The electrophysiological setup was essentially as described previously (Boyer et al., 2014; Lapirot et al., 2011). Mechanical noxious (pinch with forceps) and innocuous (paint brush) stimulations of cutaneous receptive fields were performed to characterize wide-dynamic range (WDR) neurons (Boyer et al., 2014; Lapirot et al., 2011). Every neuron responding to stimuli from innocuous to noxious intensity with progressively increasing firing rates was classified as WDR neuron. Electrical stimuli (2-ms duration) were delivered to the cutaneous receptive field through two subcutaneous stainless steel needle electrodes and the thresholds for A- and C-fiber-evoked responses determined. A- and C-fiber-evoked responses within poststimulus histograms were dissociated based on their latencies: responses with a latency > 30 ms were considered as C-fiber-evoked (Boyer et al., 2014; Lapirot et al., 2011).

Only C-fiber-evoked responses were analyzed. One neuron was recorded per animal, and only neurons with stable spike amplitude or waveform over the whole experimental procedure were included in the present report.

C-fiber-evoked responses to peripheral electrical stimuli

Sequences of 30 electrical stimuli (at 0.66 Hz) were applied to the receptive field at 1.5 time the threshold for C-fiber activation at 15-min intervals (Boyer et al., 2014; Lapirot et al., 2011). This interval was the shortest that allowed full recovery of the responses to baseline levels.

ISDN was slowly injected (10 µg/min) into the jugular vein over 120 min after two stable (variation in C-fiber response < 10%) control sequences. C-fiber-evoked responses were normalized to baseline – the two control sequences before ISDN administration – and were considered as potentiated when they were $\geq 120\%$ of baseline. The criterion that was selected for potentiation of the neuronal responses corresponds to more than 4 times the mean standard deviation of the two successive stable – that is, a variation in C-fiber response <10% – control sequences.

Statistical analysis

Data on formalin induced rubbing behavior were analyzed using one-way analysis of variance (ANOVA) followed by Fisher LSD post-hoc test (normally distributed data) or Kruskal-Wallis ANOVA on ranks followed by Dunn's post-hoc test (non-normally distributed data). Data on p-ERK-immunoreactive cells were analyzed using either paired Student t test, one-way repeated measures (RM) ANOVA followed by Fisher LSD post-hoc test (normally distributed data) or two-way ANOVA followed by Duncan's post-hoc test, with treatment and lamina or level as factors (non-normally distributed data). The effect of ISDN administration on C-fiber-evoked responses was assessed by comparing values at different delays (15, 30, 45, 60, 75, 90, 105 and 120 min) after administration with those before or baseline using one-way RM ANOVA followed by Dunnett's post-hoc test. The level of significance was set at $P < 0.05$. Normally distributed data are expressed as mean \pm standard error of the mean (SEM) while non-normally distributed data are presented in the figures as median, first and third quartiles (boxes) and minimum–maximum (whiskers).

Histological analysis

At the end of experiments, a pontamine sky blue solution was injected to mark the position of the recording site. The rat was then killed by intravenous lethal dose of chloral hydrate (10%), the brain was removed and prepared for histological examination (see Chebbi et al., 2014). fixed in a 10% formalin solution for seven days. The tissue was frozen, cut into serial 100- μ m-thick sections, and stained with neutral red. The position of the recording sites were determined according to the Paxinos and Watson atlas (2007).

RESULTS

Effect of systemic administration of ISDN on facial and hindpaw formalin test

In saline treated rats ($n = 8$), subcutaneous injection of formalin into the right vibrissa pad causes 2-phases nociceptive rubbing response: an early, short-lasting phase 1 (0-3 min) and a late, long-lasting phase 2 (15-30 min), separated by an interphase with no rubbing (Fig. 1A). The durations of phase 1 and 2 rubbing were 35 ± 10 sec and 132 ± 13 sec, respectively.

When the facial formalin test was performed 1 h after ISDN injection ($n = 6$), no change was observed in either phase (Fig. 1). The durations of phase 1 and 2 rubbing were 38 ± 11 sec and 112 ± 10 sec, respectively (Fig. 1). On the other hand, animals tested 2 h ($n = 8$, one-way ANOVA followed by Fisher LSD post-hoc test, $P < 0.01$) or 4 h ($n = 8$, one-way ANOVA followed by Fisher LSD post-hoc test, $P < 0.05$) after ISDN injection showed a significant enhancement in the duration of the rubbing behavior during phase 1. Two hours after ISDN injection, the durations of phase 1 and 2 rubbing were 76 ± 9 sec and 131 ± 26 sec, respectively. Four hours after ISDN injection, the durations of phase 1 and 2 rubbing were 68 ± 12 sec and 129 ± 27 sec, respectively. In addition, there was very little interphase when the test was performed 2 h after ISDN injection, the rubbing behavior being significantly increased during

the second block (Kruskal-Wallis ANOVA on ranks followed by Dunn's post-hoc test, $P < 0.01$, Fig. 1A).

In the saline group ($n = 8$), subcutaneous injection of formalin into the hindpaw also causes a 2-phases nociceptive licking response: an early, short-lasting phase 1 (0-3 min) and a late, long-lasting phase 2 (12-30 min). The duration of phase 1 and 2 liking are presented in Fig. 2. Rats tested 2 h after ISDN administration showed no change in either phase of the hindpaw formalin test.

Effect of systemic administration of ISDN on MDH neuronal activity

ERK1/2 activation was used as a marker of MDH neuronal activation. To characterize the population of pERK1/2 immunopositive cells, double immunofluorescence labeling for pERK1/2 and the neuronal marker, NeuN, was performed. As shown in Fig. 3, virtually all pERK1/2 immunopositive cells were also immunoreactive for NeuN, indicating that pERK1/2 was induced in MDH neurons.

In saline treated rats, formalin injection into the vibrissa pad resulted in a strong ERK1/2 phosphorylation in the ipsilateral (Fig. 4A, C), but not contralateral, MDH. Immunostaining was localized within dendrites, soma cytoplasm and nuclei. The number of phospho-ERK1/2-immunoreactive cells/section on the ipsilateral side (6.5 ± 1.4) was much larger (paired Student's t test, $P < 0.01$) than that on the contralateral (1.1 ± 0.6) side. Within ipsilateral MDH, ERK1/2 phosphorylation predominated in lamina I and outer lamina II (II_o) (one-way RM ANOVA followed by Fisher LSD post-hoc test, $F_{7,14} = 24.441$, $P < 0.001$). None or very few phospho-ERK1/2 cells were observed in the inner lamina II (II_i) and in laminae III-V.

ISDN injection enhanced significantly the number phospho-ERK1/2-immunoreactive cells/section evoked by formalin injection only on the ipsilateral MDH. The number of phospho-ERK1/2 cells/section on ipsilateral MDH (11.3 ± 1.8) was much larger (paired

Student's *t* test, $P < 0.001$) than that on contralateral MDH in the same rats (0.8 ± 0.3). Within ipsilateral MDH, phospho-ERK1/2-immunoreactive cells tended to be concentrated within laminae I-II_o (one-way RM ANOVA followed by Fisher LSD post-hoc test, $F_{7,14} = 39.112$, $P < 0.001$, Fig. 4B, C). Finally, the number of phospho-ERK1/2-immunoreactive cells/section in the lamina I-II_o was significantly [two-way ANOVA followed by Duncan's post-hoc test, with treatment ($F_{1,42} = 6.169$, $P < 0.05$) and lamina ($F_{2,42} = 43.810$, $P < 0.001$) as factors] larger in ISDN treated rats than in saline treated rats.

Effect of systemic administration of ISDN on the C-fiber-evoked responses of the trigeminal WDR neurons

A total of 12 WDR neurons were recorded within the MDH. None of them exhibited spontaneous activity. All had an ipsilateral receptive field that included the maxillary and/or the ophthalmic regions. They respond to both innocuous and noxious mechanical stimuli and increased their firing rate as stimulus intensity increased into the noxious range. When percutaneous electrical stimuli were applied to the center of the cutaneous receptive field of these WDR neurons, responses due to peripheral activation of A- and C-fibers could be observed for all recorded neurons. Mean thresholds and latencies of C-fiber-evoked responses were 7.8 ± 1.0 mA and 74.2 ± 5.1 ms, respectively. Computed conduction velocities (~ 0.5 m.sec⁻¹) were in the range of those previously reported for C-fibers (Boyer et al., 2014; Lapirot et al., 2011).

ISDN administration did not change the level of spontaneous activity of trigeminal WDR neurons ($n = 12$). But, it facilitated C-fiber-evoked responses (RM ANOVA followed by Dunnett's post-hoc test, $n = 6$, $P < 0.01$). Fig. 5A illustrates the typical facilitation of C-fiber-evoked response. Such facilitation of C-fiber-evoked responses was observed in 6 of 12 WDR neurons. Data from the facilitated trigeminal WDR neurons are summarized in Fig. 5B. The

facilitation was significant at 60 min (RM ANOVA followed by Dunnett's post-hoc test, $n = 6$, $P < 0.05$) and reached a peak within 105 min (45.9 ± 10.9 % of baseline, RM ANOVA followed by Dunnett's post-hoc test, $n = 6$, $P < 0.01$), and lasted for at least 120 min.

DISCUSSION

The present study investigated whether the nitric oxide donor, isosorbide dinitrate (ISDN), leads to significant: i) alterations in the cephalic and extracephalic cutaneous sensitivities, and ii) changes in MDH neuronal activity. Using the facial and hindpaw formalin tests, we found that systemic administration of ISDN increases selectively the first phase and interphase of the facial formalin test, but does not change either phase of the hindpaw formalin one. Monitoring neuronal activity within MDH with phospho-ERK1/2 immunoreactivity shows that ISDN alone does not activate, but significantly increases the number of formalin-induced phospho-ERK1/2 cells on the ipsilateral, but not the contralateral, MDH. Accordingly, extracellular unit recordings show that ISDN does not activate, but potentiates the facial C-fiber-evoked responses of trigeminal WDR neurons.

ISDN induced selectively cephalic but not extracephalic hyperalgesia

Systemic NTG was previously shown to induce extracephalic heat and mechanical allodynia in mice (Bates et al., 2010; Brennan et al., 2013; Farkas et al., 2016; Pradhan, et al., 2014) and rats (Ferrari et al., 2016; Harrington et al., 2011; Tassorelli et al., 1995). The effect of systemic NTG on the hindpaw formalin test has already been investigated by only one other laboratory (Greco et al., 2015; Tassorelli et al., 1995, 2005) who reported that NTG increases selectively the second phase of the hindpaw formalin test. Using ISDN, another nitric oxide donor, we found here no change in either phase of the hindpaw formalin test. Nevertheless, our results are consistent with clinical findings reporting no extracephalic cutaneous hypersensitivity in healthy subjects (Thomsen et al., 1996) and migraineurs (de Tommaso et al., 2004) after NTG administration.

The development of extracephalic cutaneous hypersensitivity has been attributed to sensitization of trigeminovascular thalamic nociceptive neurons (Bernstein and Burstein, 2012), activation of descending pain-facilitating processes from the rostral ventromedial medulla (Edelmayer et al., 2009), impairment of descending pain inhibitory controls (Boyer et al., 2014) and/or sensitization of the spinal dorsal horn (Boyer et al., 2014). Of note, in the two latter studies, dural nociception with inflammatory soup could produce an extracephalic cutaneous hypersensitivity only if it had a high-intensity (Edelmayer et al., 2009) or was repeated (Boyer et al., 2014). Thus, the nitric oxide-donor concentration, we used here, might not be sufficient to impair the descending pain modulatory controls and/or sensitize the spinal dorsal horn. In line with this hypothesis, low doses of NTG (0.5 or 1 mg/kg) fail to induce extracephalic cutaneous hypersensitivity (Bates et al., 2010, Brennan et al., 2013; but see Ferrari et al., 2016).

The discrepancies between our and other results (Greco et al., 2015; Tassorelli et al., 1995; 2005) in the hindpaw formalin test, could also be attributed to methodological differences. On the one hand, we used the duration of licking behavior as pain index, while the other laboratory (Greco et al., 2015; Tassorelli et al., 1995) used the flinching behavior. Previous findings suggest that the generation of licking and flinching behaviors result from the activation of different neural pathways. Indeed, mid-thoracic spinal transection has no effect on formalin-induced flinching (Coderre et al., 1994), but completely inhibits formalin-induced licking (Wheeler-Aceto and Cowan, 1991). Different effects on flinching and licking have also been observed with various pharmacological drugs, e.g. systemic naloxone (Wheeler-Aceto and Cowan, 1993), amitriptyline (Sawynok and Reid, 2001), phenobarbital (Abbott et al., 1995) (increase and decrease, respectively), as well as spinal neurotoxins 5,6-dihydroxytryptamine and 6-hydroxydopamine (Tjølsen et al., 1991) (no effect and decrease, respectively). Similarly, rats exposed during early life to liposaccharide displayed enhanced formalin-induced flinching but not licking at the adult age (Zouikr et al., 2014). On the other hand, the observed effects

with NTG might be non-specific, caused by substances used to dissolve NTG. Indeed, NTG usually must be dissolved in a mixture of alcohol and propylene glycol. Because very large amounts of NTG are necessary, the amount of injected alcohol and propylene glycol are considerable (Olesen and Jansen-Olesen, 2012).

Our results show that the administration of ISDN is nevertheless able to induce cephalic hyperalgesia. We found that systemic administration of ISDN selectively increases the first phase and interphase, but not the second phase, of the facial formalin test. This effect was significant 2 h after ISDN injection and lasted for at least 4 h. The development of ISDN-induced cephalic hyperalgesia resembles that of delayed migraine headache (Ashina et al., 2007). The present findings are in agreement with previous studies showing that NTG facilitates CGRP-induced face rubbing behavior (Capuano et al., 2014; Yao and Sessle, 2008). However, they are not consistent with previous findings that noted either no change on both phases (Grigoraay et al., 2013) or an increase in the duration of the rubbing behavior during only the second phase of the facial formalin test (Greco et al., 2015) after systemic NTG (10 mg/kg). These conflicting data may in part be explained by differences in species [mice (Grigoraay et al., 2013) vs. rat (Greco et al., 2015)], nitric oxide donors [ISDN (present study) vs. NTG (Greco et al., 2015)] or measured pain behavior: we measured the duration of the face rubbing responses with the forepaw and not with the hindpaw, while Greco et al. (2015) measured the duration of both the rubbing and scratching behavior evoked by the forepaw and hindpaw, respectively.

The first phase of the formalin response is generally attributed to chemical activation of nociceptors, whereas the second phase is due to the release of local endogenous mediators responsible for the sensitization of nociceptors and central nociceptive neurons (Raboisson and Dallel, 2004). The preferential effect of ISDN on the first phase of the facial formalin test suggests that the most important mechanism underlying ISDN-induced cephalic hyperalgesia

is increasing available nitric oxide at peripheral sites and/or central terminals of meningeal nociceptors within MDH.

Our observation that systemic administration of nitric oxide donor induces hyperalgesia in the maxillary division is consistent with clinical studies indicating that patients develop cephalic hypersensitivity, not only in the ophthalmic area, but also in maxillary regions during migraine attack (Ashkenazi et al., 2007; Cooke et al., 2007; Guy et al., 2010; Kitaj and Klink, 2005; Yoon et al., 2009). Similarly, preclinical studies have described both dura-sensitive neurons with mandibular and maxillary receptive fields and dura-sensitive neurons that can expand their cutaneous receptive fields to these areas after chemical stimulation of the meninges (Boyer et al., 2014; Burstein et al., 2000; Yamamura et al., 1999). The presence of such neurons may explain the hypersensitivity of the in maxillary region during meningeal stimulation in awake patients (Wirth and Van Buren, 1971; Yamamura et al., 1999) as well as during headache attacks.

ISDN does not activate, but sensitize MDH neurons

ERK1/2 activation was used here as an anatomical marker of MDH neuronal activation (Gao and Ji, 2009). ERK1/2 activation also contributes to pain hypersensitivity (Gao and Ji, 2009). In the medullary and spinal dorsal horns, it is activated by mechanical, thermal and chemical noxious, but not innocuous, stimuli (Ji et al., 1999; Miraucourt et al., 2009). We found no or very few phospho-ERK1/2-immunoreactive cells in the MDH of saline- and ISDN-pretreated rats, contralateral to the formalin injection side. Similarly, results from our electrophysiological study revealed that no trigeminal WDR neuron started to fire spontaneously after ISDN administration. In addition, neither NTG (Capuano et al., 2014; Yao and Sessle, 2008) nor ISDN (present study), alone, caused any significant change in the face rubbing behavior. This suggests that ISDN *per se* does not activate MDH neurons. Of note, however,

immunohistochemical and electrophysiological studies have obtained conflicting results on NTG-induced activation of MDH neurons. While some authors (Jones et al., 2001; Martin and Martin, 2001; Offenhauser et al., 2005; Tassorelli et al., 2005) failed to detect any NTG-induced Fos expression, others observed Fos expression in rats (Knyihar-Csillik et al., 2008; Pardutz et al., 2000; Tassorelli et al., 1995) as well as mice (Bates et al., 2010; Brennan et al., 2013) after a single NTG injection. Interestingly, studies in which Fos expression was detected, used rather high doses (10 mg/kg) of NTG (Bates et al., 2010; Brennan et al., 2013; Knyihar-Csillik et al., 2008; Pardutz et al., 2000; Tassorelli et al., 1995, but see Ramachandran et al., 2012) whereas those where Fos expression was not detected used low doses (< 5 mg/kg) of NTG (Jones et al., 2001; Martin and Martin, 2001; Offenhauser et al., 2005; Tassorelli et al., 2005). Similarly, electrophysiological studies reported either no change (Jones et al., 2001) or an increase in the ongoing activity of trigeminal WDR neurons (Feistel et al., 2013; Koulchitsky et al., 2004; Lambert et al., 2000) following systemic administration of NTG. Therefore, as already suggested above, it is possible that we used here a low dose of ISDN, that is, sufficient to sensitize, but not to activate, MDH nociceptive neurons. The different chemical structures of the two drugs might also contribute to this difference between NTG- and ISDN-induced neuronal activation. Indeed, ISDN has a longer onset and duration of action than NTG (Willis et al., 1976). Moreover, the hypotensive effect of ISDN is 2000 times smaller than that of NTG (Manabe et al., 2001). Finally, the present and previous studies used different anatomical markers of neuronal activation. While almost all previous studies used Fos protein as an anatomical marker of neuronal activation (see above), we used phospho-ERK1/2. Although, both Fos protein and ERK1/2 phosphorylation are good markers for nociception-induced neuronal activation within the spinal dorsal horn and MDH, they differ in many ways (Gao and Ji, 2009): compared with Fos protein, ERK1/2 phosphorylation is more dynamic with a quicker induction, different cellular and subcellular localizations and a closer correlation to pain

behavior. Thus, phospho-ERK1/2 appears to be a better marker than Fos protein for central sensitization (Gao and Ji, 2009).

The other main finding of the present study is that ERK1/2 activation in MDH, ipsilateral to the formalin injection site was clearly potentiated by ISDN. Phospho-ERK1/2-immunoreactive cells were confined almost exclusively within the most superficial laminae (I and outer lamina II) of the MDH that contain predominantly nociceptive specific neurons (Sessle, 2000). Similarly, Fos response to infraorbital injection of capsaicin can be potentiated by NTG (Jones et al., 2001). Accordingly, our *in vivo* electrophysiological results show that ISDN facilitates C-fiber-evoked responses in 50% of trigeminal WDR neurons. Infusion of NTG (Jones et al., 2001) or sodium nitroprusside (Koulchitsky et al., 2004) also potentiates the responses to electrical stimulation of the face, but the effects of nitric oxide donors varies between neurons suggesting that only a fraction of MDH neurons is readily under the control of nitric oxide (Feistel et al., 2013). The present results are also consistent with a previous report showing NTG infusion results in a delayed increase in the mechano-sensitivity, with no significant change in the ongoing discharges, of primary afferent meningeal nociceptors (Zhang et al., 2013). Consistently, upon NTG, the decrease in periorbital von Frey thresholds following repeated dural inflammation appears to be potentiated (Oshinsky and Gomomchareonsiri, 2007) and that in spontaneous allodynia rats to be extended, from 1.5 to 5 h (Oshinsky et al., 2012). Altogether, these data suggest that nitric oxide does not activate MDH neurons directly but rather facilitates their response to peripheral nociceptive inputs. Thus our results support the statement that nitric oxide-related increased activity of the MDH is mediated by central facilitation of trigeminal neuronal response to afferent traffic that originate from peripheral nociceptors (Zhang et al., 2013) or to increased presynaptic activity on central terminal of meningeal nociceptors (Oshinsky and Gomomchareonsiri, 2007). Thus, NO, alone, does not appear to be sufficient to cause migraine pain. Nevertheless, migraine patients may have an additional level of susceptibility such that a basal neuronal response can lead to headache.

In summary, the present study characterizes for the first time the behavioral and neuronal effects of the administration of the nitric oxide donor, ISDN. We found that ISDN induces cutaneous hypersensitivity at cephalic but not extra-cephalic level that is associated with sensitization but not activation of MDH neurons. This model better mimics the clinical

condition and offers another possibility to study the role of nitric oxide donor in the physiopathology of headache.

Conflict of interest

None of the authors have any conflict of interest to declare

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Highlights

- Isosorbide dinitrate induces a cephalic but not extracephalic hyperalgesia.
- Isosorbide dinitrate does not activate, but sensitize medullary dorsal horn neurons.
- This animal model better mimics the clinical features of migraine.

Author's contribution

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: PL and RD. Acquisition of data: JMFR, LD, AD, PL, JLM. Analysis and interpretation of data: JMFR, LD, AD, PL, JLM and RD. Drafting of the manuscript: RD. All authors have approved the final version of the manuscript.

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Figure legends

Fig. 1. Effect of the systemic administration of isosorbide dinitrate (ISDN) on the facial formalin test. **A.** Time courses of the facial rubbing response to subcutaneous injection of formalin (50 μ l, 1%) into the vibrissa pad in rats previously intraperitoneally injected with saline or ISDN. ISDN (10 mg/kg) was injected 1 (n = 6), 2 (n = 8), and 4 h (n = 8), and saline 2 h (n = 8) before formalin. The recording time was divided into 15 blocks of 3 min, and a nociceptive score was determined for each block by measuring the number of seconds that the animals spent rubbing the injected area with the ipsilateral forepaw. The first phase of rubbing activity was measured during the first block (*i.e.*, 0 - 3 min) and the second phase was identified between blocks 4 and 10 (*i.e.*, 15 - 30 min) after the formalin injection. **B.** Bar histograms of the duration of the phases 1 and 2 of the rubbing behavior in the four groups of rats. Statistical analysis was performed using one-way ANOVA followed by Fisher post-hoc LSD test (normally distributed data, $F_{3,26} = 3.917$, $P < 0.05$). * $P < 0.05$; ** $P < 0.01$.

Fig. 2. Effect of the systemic administration of isosorbide dinitrate (ISDN) on the hindpaw formalin test. **A.** Time courses of the hindpaw licking responses to subcutaneous injection of formalin (50 μ l, 1%) into the plantar hindpaw in rats previously intraperitoneally injected with saline (n = 8) or ISDN (n = 8, 10 mg/kg) 2 h before formalin. **B.** Bar histograms of the duration of licking behavior during the phases 1 and 2 of the hindpaw formalin test.

Fig. 3. Fluorescence images of pERK-1/2 immunopositive cells (red) and NeuN positive neurons (green) in the MDH. The merged image on the right shows that virtually all pERK1/2 cells were immunoreactive for NeuN, indicating that pERK1/2 expression was restricted to neurons. Bar is 50 μ m.

Fig. 4. Distribution of phospho-ERK-1/2-immunoreactive cells within the medullary dorsal horn (MDH). **A.** Examples of phospho-ERK1/2 immunolabeling in the MDH of control (saline) and ISDN injected rats. ISDN (10 mg/kg) or saline were intraperitoneally injected 2 h before subcutaneous injection of formalin (50 μ l, 1%) into the right vibrissa pad. Subcutaneous formalin triggers ERK1/2 phosphorylation within almost exclusively laminae I-IIo of the ipsilateral MDH. No phospho-ERK1/2-immunoreactive cell is observed in the contralateral MDH. **B.** Examples showing the enhanced phospho-ERK1/2 immunolabeling in laminae I-IIo of the ipsilateral MDH following subcutaneous injection of formalin (50 μ l, 1%) into the right vibrissa pad after an intraperitoneal injection of ISDN (10 mg/kg). Again no phospho-ERK1/2-immunoreactive cell is observed within the contralateral MDH. **C.** Boxplots showing the number of phospho-ERK1/2-immunoreactive cells/section in the different laminae of the ipsilateral and contralateral MDH following subcutaneous injection of formalin (50 μ l, 1%) into the right vibrissa pad, 2 h after an intraperitoneal injection of ISDN (10 mg/kg) or saline (n = 8/group). I-IIo: laminae I and outer II; Ii: inner laminae II; III-V: laminae III-V. The data are presented as median (thick line), first and third quartiles (boxes) and minimum–maximum (whiskers). Comparisons between the two groups were performed using two-way ANOVA followed by Duncan's post-hoc test, with treatment and lamina as factors. Bar is 100 μ m*** P < 0.001.

Fig. 5. Effect of the systemic administration of isosorbide dinitrate (ISDN) on C-evoked responses of trigeminal WDR neurons. **A.** Histograms of C-fiber-evoked responses in one trigeminal WDR neuron to the 30 successive percutaneous electrical stimulations (1.5 x C-fiber threshold intensity, 0.66 Hz) recorded before (left) and 105 min (right) after intravenous injection of ISDN (10 mg/kg). **B.** Summary bar histograms (mean \pm standard error of the mean) of C-fiber-evoked responses before and 15, 30, 45, 60, 75, 90, 105 and 120 min following

infusion of ISDN. C-fiber-evoked responses are normalized to those before ISDN administration. Note that ISDN administration potentiates C-fiber-evoked responses. Comparisons were performed using RM ANOVA followed by Dunnett's post-hoc test ($F_{5,35}=3.453, P < 0.01$). * $P < 0.05$, ** $P < 0.01$. C. Microphotograph showing the histologically confirmed (arrow) recording locus of one trigeminal WDR neuron within the MDH.









