Orofacial Antinociceptive Effect of Nifedipine in Rodents Is Mediated by TRPM3, TRPA1, and NMDA Processes

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Aims: To test for the possible antinociceptive effect of nifedipine in rodent models of acute and chronic neuropathic orofacial pain and the possible involvement of TRP- and NMDA-related processes in this effect. **Methods:** Acute nociceptive behavior was induced by administering formalin, cinnamaldehyde, glutamate, capsaicin, or acidified saline to the upper lip or hypertonic saline to the cornea of Swiss mice. Acute nociceptive behavior was also induced by formalin injected into the TMJ or mustard oil injected into the masseter muscle of Wistar rats. The chronic pain model involved infraorbital nerve transection (IONX) in Wistar rats to induce mechanical hypersensitivity, which was assessed with von Frey hair stimulation of the upper lip. The effects of pretreatment with nifedipine or vehicle (control) were tested on the nociceptive behaviors. Docking experiments were also performed. Statistical analysis included one-way ANOVA followed by Tukey post hoc test and two-way ANOVA followed by Bonferroni post hoc test (statistical significance $P < .05$). **Results:** Nifedipine produced significant antinociceptive effects in all of the acute nociceptive behaviors except that induced by capsaicin. The antinociceptive effects were attenuated by NMDA, TRPA1, or TRPM3 receptor antagonists. The IONX animals developed facial mechanical hypersensitivity, which was significantly reduced by nifedipine. The docking experiments suggested that nifedipine may interact with TRPM3 and NMDA receptors. **Conclusion:** The present study has provided novel findings in a variety of acute and chronic orofacial pain models showing that nifedipine, a selective inhibitor of L-type Ca^{2+} channels, can suppress orofacial nociceptive behavior through NMDA, TRPA1, and TRPM3 receptor systems. *J Oral Facial Pain Headache 2020;34:174–186. doi: 10.11607/ofph.2491*

Keywords: *acute, neuropathic, orofacial pain, rodents*

Orofacial pain states, especially when chronic and neuropathic, are often difficult to manage effectively, partly because the underlying nociceptive mechanisms are not well understood.[1] Since the transmission of nociceptive signals depends on several types of ion channels, a recent research focus has been on peripheral nociceptive mechanisms. These include processes involving transient receptor potential (TRP) family channels, voltage-dependent calcium ion (Ca^{2+}) channels, and N-methyl-D-aspartate (NMDA) receptors. These processes have become key targets for the development of new analgesic approaches.[2]

The presence of long-lasting (L)–type Ca^{2+} channel subunits has been reported in trigeminal ganglion neurons, and it has been suggested that this presence may be associated with orofacial nociceptive transmission.[3] Ca^{2+} channels are upregulated in orofacial pain states, and the blocking of these channels may provide therapeutic benefits for orofacial pain management.[4] Nifedipine is a selective inhibitor of 1,4-dihydropyridine-derived L-type voltage-dependent Ca^{2+} channels (VDCCs), and previous studies have shown that nifedipine has an antinociceptive action.[5–8] This effect appears to be related to actions involving the hypothalamic pituitary adrenal axis,[9] spinal mechanisms,[10] and blockade of transmembrane-inward movements of Ca^{2+},[11] but not related to nifedipine's hypotensive effect.[10] Furthermore, the antinociceptive action of nifedipine has been reported to be related also to a

blockade of electrically evoked Ca^{2+} transients in peripheral sensory nerves[12] and to a blockade of neurotransmitter release in the central nervous system (CNS).[13]

However, the effect of nifedipine on nociceptive processes underlying acute and chronic orofacial pain has not yet been reported. Therefore, this study aimed to test for the possible antinociceptive effect of nifedipine in rodent models of acute and chronic (neuropathic) orofacial pain and the possible involvement of TRP- and NMDA-related processes in this effect.

Materials and Methods

Animals and Study Overview

Swiss albino male mice (20 to 25 g, 42 days old) and Wistar male rats (250 to 300 g, 42 days old) from the experimental animal facility at the University of Fortaleza were kept at 22°C (12-hour light/dark cycle) in a specific pathogen-free (SPF) facility in Techniplast individually ventilated cages (five animals per cage) with free access to autoclaved water and a standard pellet diet (Purina). The experimental protocols followed the ethical guidelines of the Brazilian Council for the Control of Animal Experimentation (CONCEA) and were approved by the university Animal Research Ethics Committee under entry no. 005/2016.

All behavioral tests (Fig 1) were performed blinded and during the morning. Mice were used for experiments involving previously described procedures for testing orofacial nociception in mice via the administration of formalin,[14] cinnamaldehyde,[14] capsaicin,[14] glutamate,[14] acidified saline,[14] or hypertonic saline[15] in different groups. Rats were used for experiments involving orofacial nociceptive tests previously described in rats: the temporomandibular joint (TMJ) formalin test,[16] mustard oil–induced orofacial muscular nociception,[17] and facial mechanical hypersensitivity following transection of the infraorbital nerve (IONX).[18] Sample sizes of experimental groups were based on those used in the authors' previous studies assessing drug effects in orofacial pain models and documenting statistically significant group differences.[14,19] The behaviors were analyzed by an independent observer blind to the drug and type of treatment. All animals were euthanized at the end of the experiments.

This study was designed to test the effects of nifedipine in acute and chronic orofacial pain models. In the acute models, nociceptive behavior was assessed following administration of an algesic chemical to the upper lip (formalin, cinnamaldehyde, capsaicin, glutamate, or acidified saline), eye (hypertonic saline), TMJ (formalin), or masseter muscle (mustard oil) of animals pretreated with nifedipine or vehicle. In some of these acute experiments, nifedipine administration was preceded by administration of the TRPA1 antagonist camphor, the NMDA antagonist ketamine, or the TRPM3 antagonist mefenamic acid (Fig 1). In the chronic neuropathic pain model produced by IONX, nifedipine or vehicle was administered several days postoperation to test the effects on nociceptive behavior, expressed as facial mechanical hypersensitivity (Fig 1). Details for each model are provided below.

Drugs and Doses

Nifedipine (Neo Fedipina) was dissolved in 0.1% dimethyl sulfoxide (DMSO; Dinâmica) and tested at 2.5, 5, and 10 mg/kg. These doses were chosen based on previous findings of the antinociceptive effectiveness of different doses of nifedipine.[10] The doses of other drugs were also based on previous studies, noted below, that have shown the efficacy of these doses in eliciting or modifying nociceptive behavior. Nociception was elicited with formaldehyde (1%[14]), acidified saline (2% acetic acid, pH 1.981[14]), or hypertonic saline (5 M sodium chloride [NaCl][15]), which were purchased from Dinâmica. Cinnamaldehyde (13.2 µg/lip[14]), capsaicin (2.5 µg/lip, dissolved in ethanol, DMSO, and distilled water, 1:1:8[14]), glutamate (25 mM[14]), and mustard oil (20% mineral oil[17]), all of which were purchased from Sigma-Aldrich, were also used.

To test whether NMDA or TRP receptor processes were involved in nifedipine effects, NMDA and TRP receptor antagonists were also used. These antagonists may have antinociceptive effects when administered at sufficient doses, but they were administered in the present study at much lower doses than those previously shown to elicit these antinociceptive effects.[14,20,21] These antagonists were the NMDA receptor antagonist ketamine (0.1 mg/kg[20]; Cetamin, Syntec), the TRPA1 receptor antagonist camphor (7.6 mg/kg[14]; Sigma-Aldrich), and the TRPM3 receptor antagonist mefenamic acid (0.1 mg/kg[18]; Sigma-Aldrich). Other drugs were used for general anesthesia during formalin injection into the TMJ or during infraorbital nerve transection (IONX) surgery; these drugs were ketamine (100 mg/kg)[14] and xylazine (10 mg/kg[14]; Sedomin, König do Brasil). In addition, diazepam (DZP; 3 mg/kg[22]; Valium, Roche) was used in the assessment of motor performance (see below).

Drug solutions were freshly prepared before each experiment. All drugs except for nifedipine, capsaicin, and mustard oil were dissolved in isotonic saline.

Formalin-Induced Orofacial Nociception

Mice received 1% formalin (20 µL, subcutaneous [sc]) injected with a 27-gauge needle into the right upper lip (perinasal area). Nociception was quantified as time the mouse spent rubbing the site of injection with the

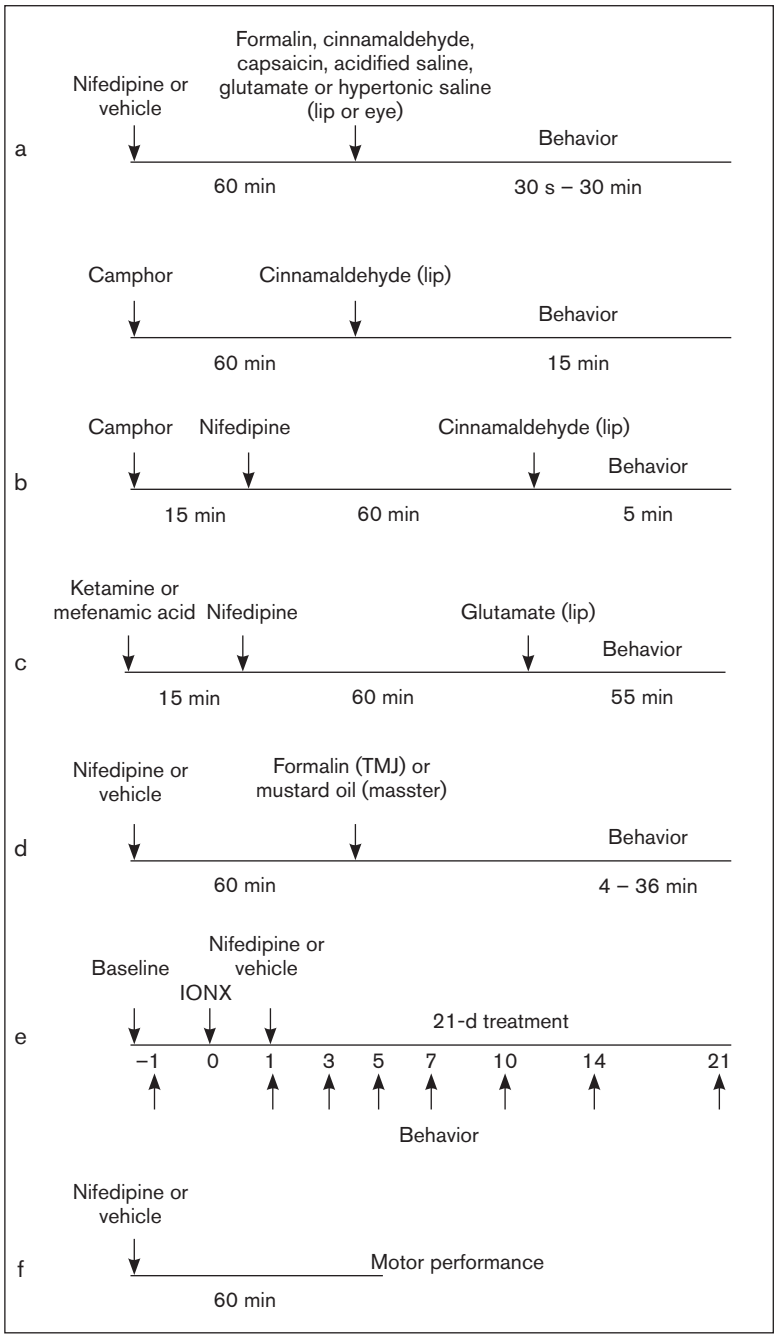


Fig 1 Flowchart of animal experiments. The time ranges for some of the behavioral assessments reflect the different behavioral testing periods used in the different models. **(a to d)** Acute orofacial pain models. **(e)** Chronic pain model following infraorbital nerve transection (IONX). **(f)** Assessment of motor performance.

fore or hind paw. Recording of nociceptive behavior began immediately (0 to 5 minutes) following formalin injection (phase 1) and was continued for up to 30 minutes (duration of 15 to 30 minutes; phase 2).[14] To assess the effect of the test drug, mice (n = 6/group) were pretreated with vehicle control (0.1% DMSO in 0.9% NaCl; 10 mL/kg, per os) or nifedipine (2.5, 5, or 10 mg/kg) 60 minutes before the formalin injection (Fig 1a). A naïve group (n = 6) was also included.

Cinnamaldehyde-Induced Orofacial Nociception

Mice received an injection via a 27-gauge needle of 20-µL cinnamaldehyde (13.2 µg/lip) into the right upper lip (perinasal area). Nociception was analyzed in terms of time the mouse spent rubbing the injected area with the ipsilateral fore or hind paw during the first 5 minutes after cinnamaldehyde injection.[14] The mice were pretreated with vehicle control (10 mL/kg), nifedipine (2.5 mg/kg, per os) (Fig 1a), or camphor (7.6 mg/kg, sc) (Fig 1b) at 60 minutes before the injection of cinnamaldehyde (n = 6/group). A fourth group (n = 6) received camphor (7.6 mg/kg, sc) 15 minutes before the nifedipine and subsequent administration of cinnamaldehyde (60 minutes after nifedipine) (Fig 1c). Camphor is a well-documented TRPA1 antagonist[14] and was used to test for possible involvement of TRPA1 receptors in the antinociceptive effects of nifedipine in this test.

Capsaicin-Induced Orofacial Nociception

Mice received capsaicin (20 µL, 2.5 µg) injected with a 27-gauge needle into the right upper lip (perinasal area). Nociception was quantified as the time the mouse spent rubbing the site of injection with the fore or hind paw for 10 to 20 minutes after the capsaicin injection[14] (Fig 1a). Nifedipine and vehicle control were administered in a similar manner and at the same doses as described previously for the formalin test (n = 6/group). A naïve group (n = 6) was also included.

Acidified Saline–Induced Orofacial Nociception

Mice received acidified saline (20 µL, 2% acetic acid, pH 1.98) injected with a 27-gauge needle into the right upper lip (perinasal area). Nociception was quantified as the time the mouse spent rubbing the site of injection with the fore or hind paw over the 20-minute period following the acidified saline injection[14] (Fig 1a). Nifedipine and vehicle control were administered (n = 6/group) in a

similar manner as noted above (nifedipine 2.5, 5, and 10 mg/kg 60 minutes before injection). A naïve group (n = 6) was also included.

Glutamate-Induced Orofacial Nociception

Mice received glutamate (20 µL, 25 mM) via a 27-gauge needle into the right upper lip (perinasal area). Nociception was quantified as the time the mouse spent rubbing the site of injection with the fore or hind paw during the 15-minute period after the glutamate injection[14] (Fig 1a). Nifedipine and vehicle control were administered (n = 6/group) in a similar manner as described above. A naïve group (n = 6) was also included.

Since nifedipine showed a marked antinociceptive effect in the glutamate test and because nifedipine is a TRPM3 agonist and modulates glutamatergic transmission,[23,24] this test was also chosen to assess the possible involvement of NMDA and/or TRPM3 receptors. Thus, two additional groups of mice (n = 6/group) were pretreated with antagonists of NMDA and TRPM3 receptors. These were, respectively, ketamine (0.1 mg/kg sc) and mefenamic acid (0.1 mg/kg, sc), injected 15 minutes before the administration of nifedipine (2.5 mg/kg, per os) (Fig 1d).

Eye-Wiping Test

Corneal nociception was induced in mice by instillation of one drop (20 µL) of hypertonic saline (5 M NaCl) into the left corneal surface. The number of eye wipes performed with the ipsilateral fore paw during the first 30 seconds was recorded[15] (Fig 1a). The mice (n = 6/group) were pretreated with the vehicle control (10 mL/kg, per os) or nifedipine (2.5, 5, or 10 mg/kg) 60 minutes before instillation of hypertonic saline. A naïve group (n = 6) was also included.

TMJ Formalin Test

Rats were acclimated individually in a glass test chamber (30 × 30 × 30 cm) for 30 minutes to minimize stress. The animals (n = 6/group) were pretreated (10 mL/kg, per os) with nifedipine (2.5 mg/kg) or vehicle control and anesthetized 60 minutes later with ketamine (100 mg/kg, intraperitoneal [ip]) and xylazine (10 mg/kg, ip). The left TMJ was injected with 50 µL of 1.5% formalin via a Hamilton syringe and a 30-gauge needle. A sham group receiving 0.9% NaCl (50 µL) and a naïve group were also included (n = 6/group).

After their recovery from anesthesia (20 minutes), the animals were returned individually to the test chamber, and nociception was quantified as asymmetrical rubbing of the orofacial region with the ipsilateral fore or hind paw and as head flinching (intermittent and reflexive shaking of the head).[16] The time that the rat spent rubbing the orofacial region was registered 12 times at 3-minute intervals (Fig 1e), and head flinching was registered by its absence or presence.

Mustard Oil–Induced Orofacial Muscular Nociception

Rats were acclimated individually in a glass test chamber (30 × 30 × 30 cm) for 30 minutes to minimize stress. The rats (n = 6/group) were pretreated (10 mL/kg, per os) with vehicle control or nifedipine (2.5 mg/kg) 60 minutes before injection of mustard oil (20%; 20 µL) into the left masseter muscle, delivered via a Hamilton syringe and a 30-gauge needle (Fig 1e). A sham group that received pretreatment saline and a naïve group (n = 6/group) were also included. The ipsilateral hind-paw shaking was quantified by counting the number of paw shakes in 30-second intervals during the first 4 minutes following the mustard oil injection.[17]

Assessment of Mechanical Sensitivity Following IONX

Rats were anesthetized with ketamine (100 mg/kg, ip) and xylazine (10 mg/kg, ip) to expose the left infraorbital nerve (ION) at its entry into the infraorbital foramen by way of an intraoral incision (2 mm) in the oral mucosa of the left fronto-lateral maxillary vestibulum, as previously described.[18] The ION was lifted from the maxillary bone and cut (IONX) without damaging adjacent nerves and vessels. Subsequently, the animals were returned to their cages and fed with mash and chow. The animals were monitored daily in the postoperative period. Rats were divided into two groups (n = 6/group): nifedipine (2.5 mg/kg, per os) and vehicle control (10 mL/kg, per os). Naïve and sham-operated animals (n = 6/group) were used as controls.

The rats were acclimated, trained, and tested for facial mechanical sensitivity (head withdrawal threshold) 1 day prior to nerve transection (baseline) and on postoperative days 1, 3, 5, 7, 10, 14, and 21, as previously described.[18] A single dose of nifedipine or vehicle control (n = 6/group) was administered by gavage at each postoperative day (Fig 1f). Mechanical sensitivity of the left whisker pad skin was assessed using von Frey hairs before and 60 minutes after the nifedipine or vehicle treatment. The head-withdrawal threshold to mechanical stimulation of the whisker pad skin was defined as the minimum force needed to evoke an escape more than three times as a result of five stimuli.

Assessment of Motor Performance

To assess whether pretreatment with nifedipine could produce any motor deficit or deficient coordination, groups of mice (n = 6/group) were pretreated (10 mL/kg, per os) with nifedipine (2.5 or 10 mg/kg)

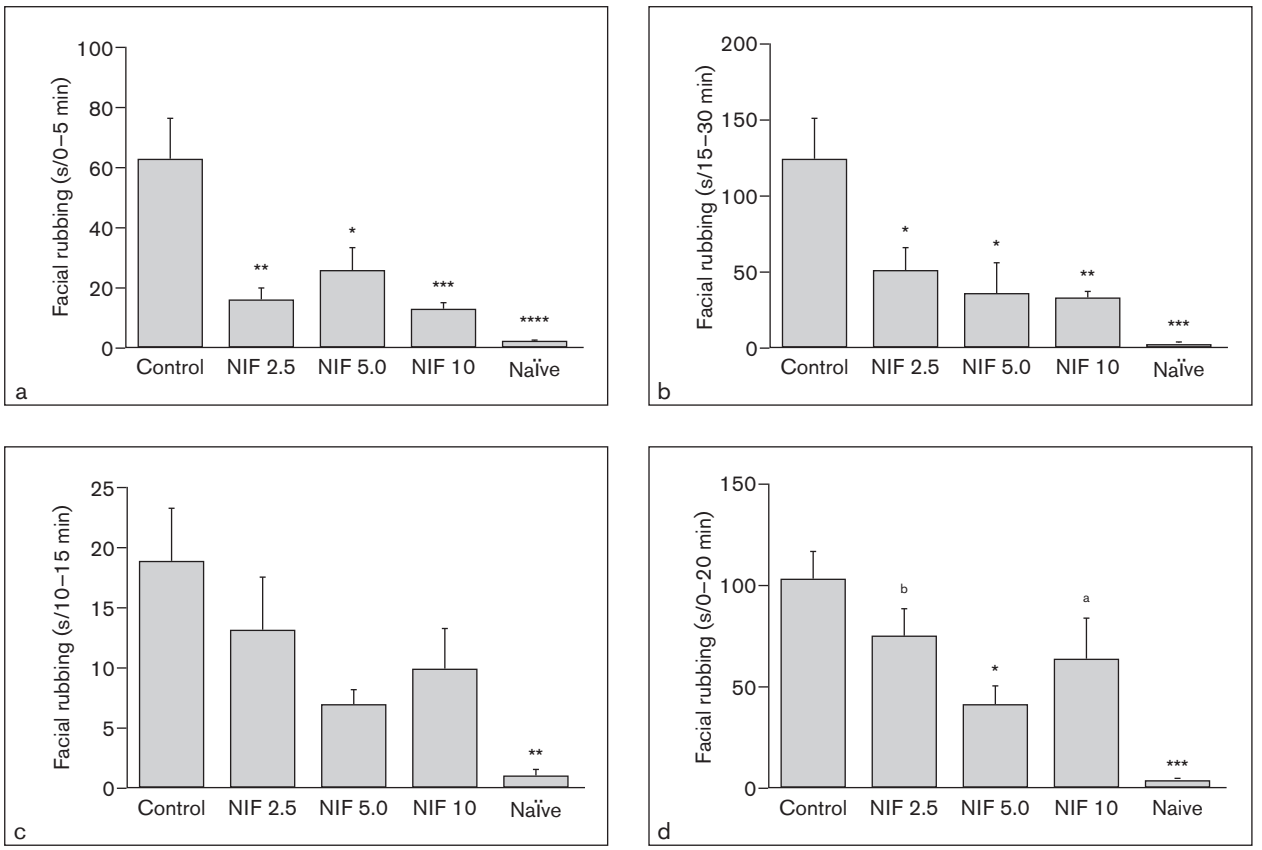


Fig 2 Effect of preadministration of nifedipine (NIF; 2.5 mg/kg; 5 mg/kg; 10 mg/kg) on formalin-, capsaicin-, and acidified saline–induced orofacial nociception in mice. Results are expressed as mean value ± standard error of the mean. **(a)** Phase 1 and **(b)** phase 2 of formalin test. **(c)** Capsaicin test. **(d)** Acidifed saline test. ANOVA followed by Tukey test was used for statistical analyses. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001 vs control. [a]*P* < .05, [b]*P* < .01 vs naïve.

or vehicle (control), and their motor performance was evaluated after a 60-minute period (Fig 1g) through the grip-force assay and the rotarod apparatus, as described previously.[25] DZP (3 mg/kg, ip; n = 6) was used as a positive control.

Molecular Docking Study

Molecular docking is a valuable, inexpensive, and speedy technique in computational chemistry that analyzes the interaction between a small molecule and a protein at the atomic level. It has become an increasingly important tool for drug discovery.[26,27] The interactions between nifedipine and NMDA, TRPV1, TRPA1, and TRPM3 receptors were analyzed using molecular docking, which consists of the use of a computational software with an algorithm for coupling two molecules seeking to form a stable complex. The three-dimensional structures of nifedipine, NMDA, TRPV1, TRPA1, and TRPM3 are available from PubChem (4485), Protein Data Bank (NDMA: 5FXG; TRPV1: 3J5P; TRPA1: 3J9P), and UniProt (TRPM3: Q9HCF6). The docking was performed using two approaches: *(1)* Hex 8.0.0 software (http://hex.loria.fr/), which performs the fittings automatically by seeking all the possible binding sites of nifedipine around the receptor surface based on the loss of intrinsic energy after interaction between the two molecules in certain positions; and *(2)* PyMOL v. 1.4.7 software (http://www.pymol.org/), which allows a detailed investigation of the complexes formed by chemical binding, the amino acid residues involved, and the conformational nuances. The parameters used inside the software interface for the fitting process were: correlation type = shape only; calculation device = CPU; FFT mode = 3D fast life; grid dimension = 0.6; receptor range = 180; ligand range = 180; twist range = 360; and distance range = 40.

Statistical Analyses

The results are presented as mean ± standard error of the mean (SEM) of each group of six animals. Normality of the distribution was confirmed (Kolmogorov-Smirnov), and data were submitted to one-way analysis of variance (ANOVA) followed by Tukey post hoc test or submitted to two-way ANOVA followed by Bonferroni post hoc test. For analyses of nifedipine (and vehicle) effects in IONX animals, the baseline was normalized, and all values postadministration of

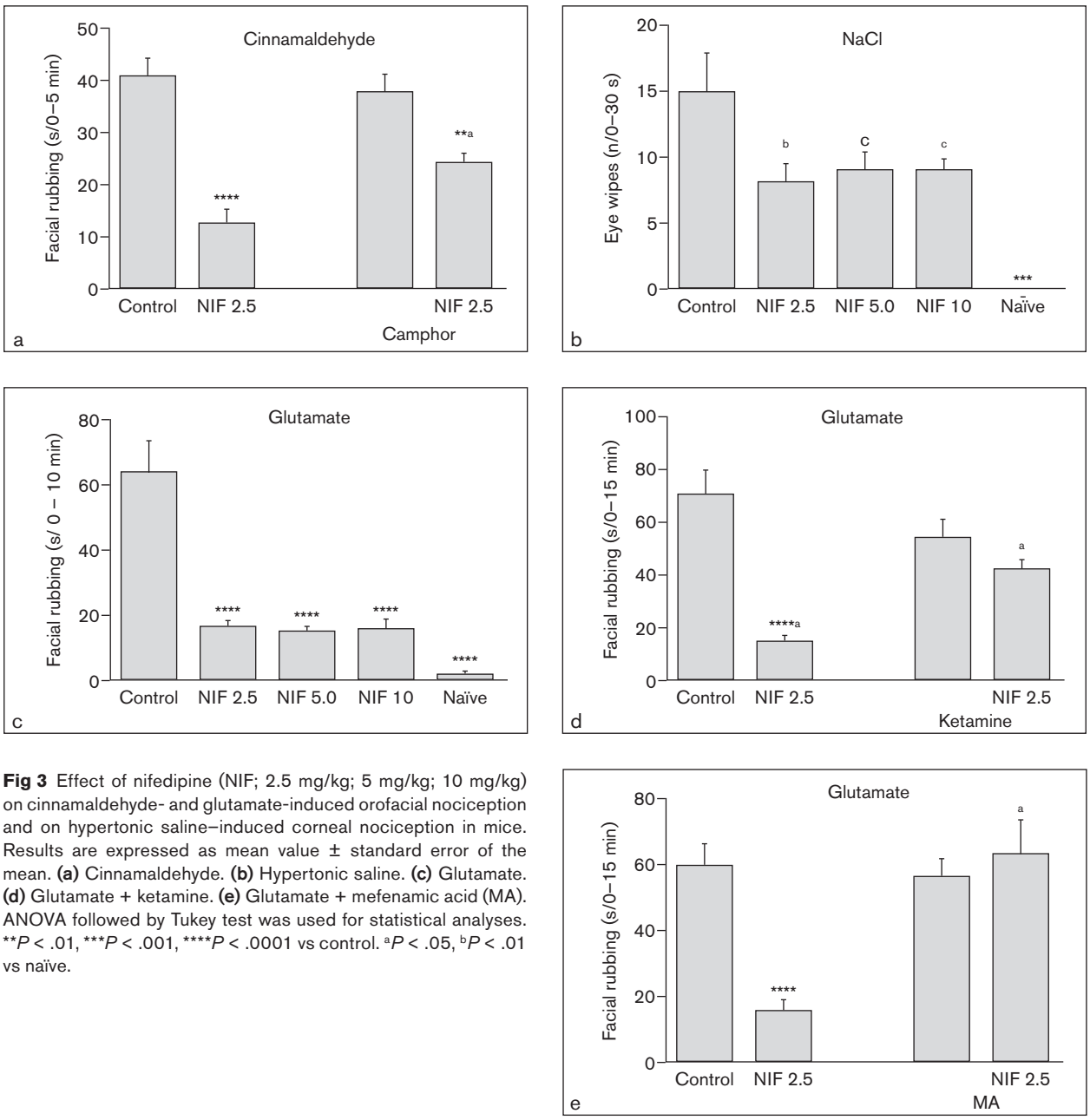


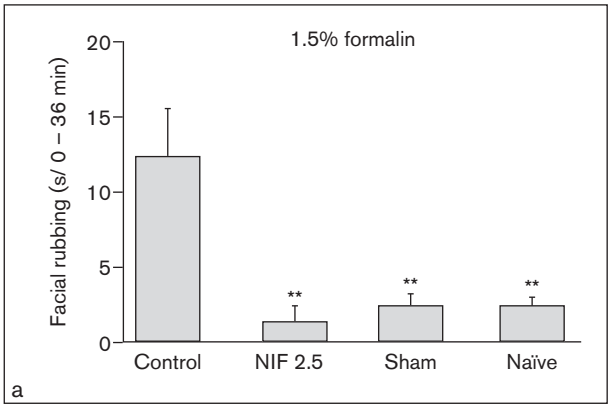
Fig 3 Effect of nifedipine (NIF; 2.5 mg/kg; 5 mg/kg; 10 mg/kg) on cinnamaldehyde- and glutamate-induced orofacial nociception and on hypertonic saline–induced corneal nociception in mice. Results are expressed as mean value ± standard error of the mean. **(a)** Cinnamaldehyde. **(b)** Hypertonic saline. **(c)** Glutamate. **(d)** Glutamate + ketamine. **(e)** Glutamate + mefenamic acid (MA). ANOVA followed by Tukey test was used for statistical analyses. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$ vs control. $^{a}P < .05$, $^{b}P < .01$ vs naïve.

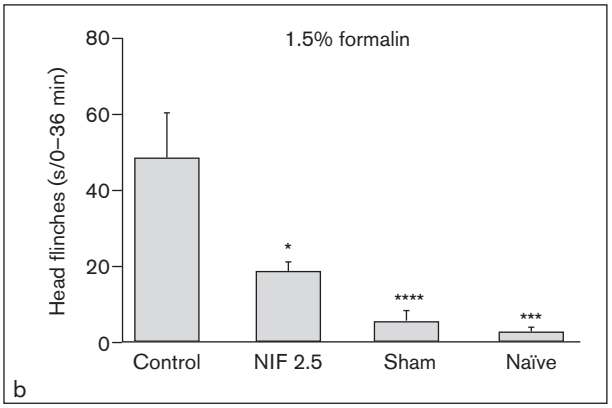
nifedipine or vehicle were expressed as percentage change from baseline. The level of statistical significance was set at 5% ($P < .05$).

Results

Formalin, cinnamaldehyde, capsaicin, acidified saline, or glutamate injection into the upper lip of mice induced face rubbing, but naïve animals showed no evidence of this nociceptive behavior (Figs 2 and 3). Pretreatment with nifedipine was associated with a reduction in face-rubbing behavior induced by the application of formalin, glutamate, cinnamaldehyde, or acidified saline when compared to the respective vehicle controls, with no dose-response effect (Figs 2 and 3). Nifedipine had no significant effect on the nociceptive behavior evoked by the application of capsaicin (Fig 2c) to the upper lip or on the number of eye wipes induced by local application of hypertonic saline to the corneal surface (Fig 3b). Camphor (TRPA 1 antagonist) had no antinociceptive effect itself, but pretreatment with camphor before nifedipine significantly attenuated the antinociceptive effect of nifedipine (Fig 3a).

Since the orofacial antinociceptive effect of nifedipine was especially evident in the glutamate test, the possible participation of NMDA and TRPM3





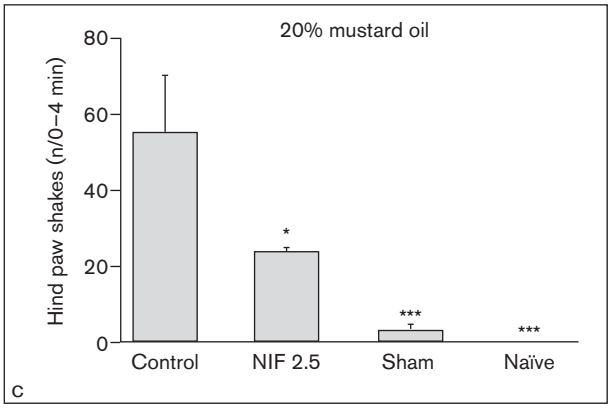


Fig 4 Effect of nifedipine (2.5 mg/kg) on **(a and b)** formalin-induced nociception in the TMJ and **(c)** mustard oil–induced craniofacial nociception in rats. Results are expressed as mean value ± standard error of the mean. ANOVA followed by Tukey test was used for statistical analyses. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001 vs control.

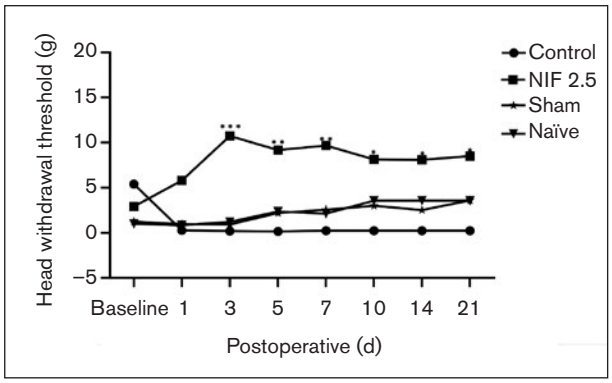


Fig 5 Antihyperalgesic effect of repeated nifedipine (2.5 mg/kg) treatment after infraorbital nerve transection. ANOVA followed by Bonferroni test was used for statistical analysis. **P* < .05, ***P* < .01, ****P* < .001 vs control.

receptors in this effect was analyzed using this test. Ketamine (noncompetitive antagonist of NMDA receptor) had no effect itself on the glutamate-induced nociceptive behavior, but its application before nifedipine administration partially reduced the antinociceptive effect of nifedipine (Fig 3d). Mefenamic acid (TRPM3 antagonist) also had no effect itself on the nociceptive behavior, but its application before nifedipine administration prevented the antinociceptive effect of nifedipine (Fig 3e). Ketamine and mefenamic acid produced no significant antinociceptive effects themselves (Fig 2d).

Naïve and sham animals showed no evidence of nociceptive behavior in the formalin and glutamate tests. The injection of formalin into the TMJ induced the nociceptive behaviors of head flinching and face rubbing. As shown in Figs 4a and 4b, pretreatment with nifedipine at 2.5 mg/kg—but not with vehicle control—significantly reduced the formalin-induced behaviors. Intramuscular injection of mustard oil into the masseter also produced an immediate and intense nociceptive behavior manifested as hind-paw shaking. Nifedipine (but not vehicle control) administered prior to mustard oil injection resulted in a significant attenuation of the shaking behavior (Fig 4c).

Left IONX produced sustained hypersensitivity to facial mechanical stimulation that was reflected in a reduced mechanical withdrawal threshold for 21 days. The thresholds in sham-operated and naïve rats did not significantly change. To investigate the effects of nifedipine on the mechanical withdrawal threshold in IONX rats, nifedipine (2.5 mg/kg) or vehicle control was administered at postoperative days 1, 3, 5, 7, 10, 14, and 21. Nifedipine significantly reversed the reduced mechanical threshold on postoperative days 3 to 21 when compared to control (Fig 5).

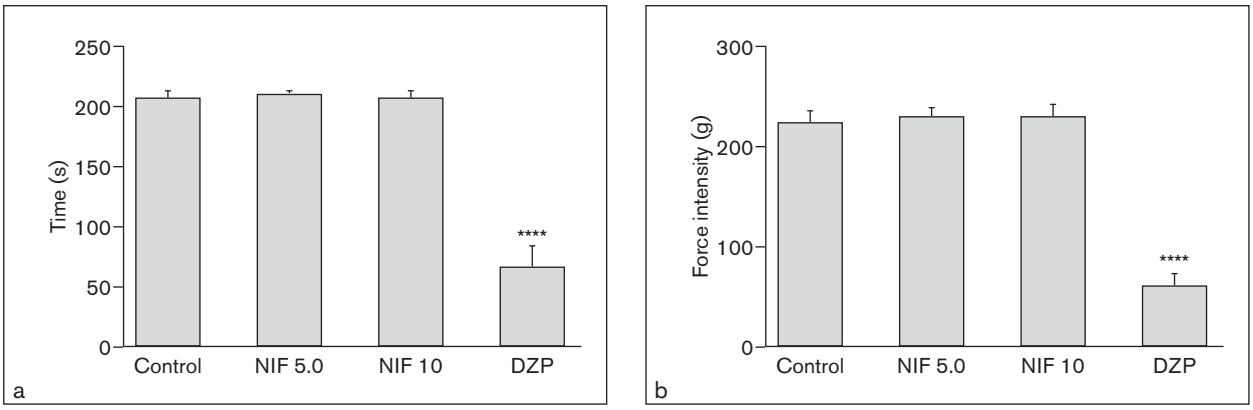


Fig 6 Effect of nifedipine (NIF; 5.0 mg/kg; 10 mg/kg) on the **(a)** rotarod test and **(b)** grip force assay. Results are expressed as mean value ± standard error of the mean. ANOVA followed by Tukey test was used for statistical analysis. DZP = diazepam (mg/kg). ****$P < .0001$ vs control, NIF 5.0, and NIF 10.

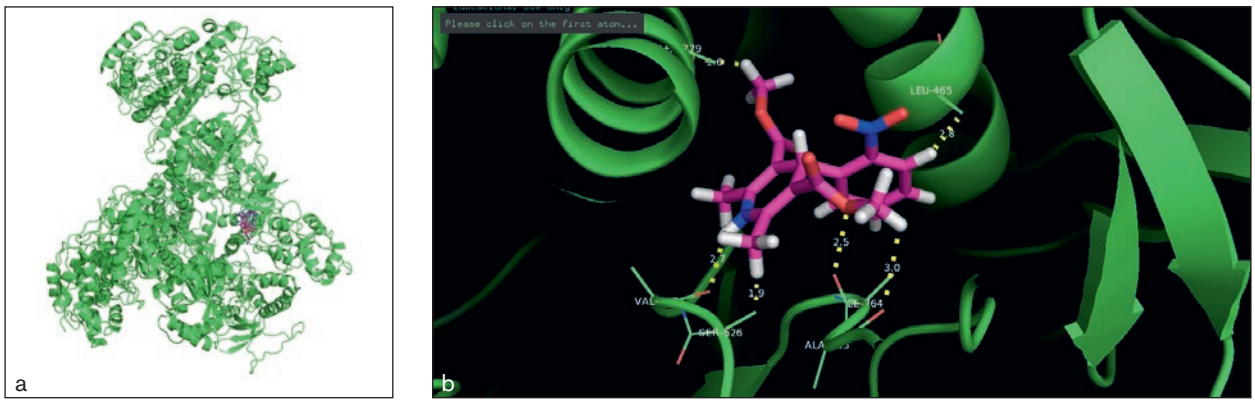


Fig 7 Overlapping of the seven most energetic formed clusters. **(a)** The fit between nifedipine and the NMDA receptor has high reproducibility and specificity. **(b)** Binding site between NMDA and nifedipine showing the amino acids involved in ligand recognition.

Unlike DZP, which disrupted motor performance, nifedipine did not cause any alteration in motor coordination or grip-strength force when assessed in the rotarod and grip-strength tests (Fig 6).

In the docking experiments, the interaction between nifedipine and the NMDA receptor showed high reproducibility. The software searched for 50,000 possible interactions and analyzed the 10 most stable complexes formed with higher stabilizing energy. When complexing with the binder, the intrinsic vibration strength of the receiver is stabilized, generating a negative balance and a loss of free energy strength caused by the bond. Seven of 10 complexes showed the ligand interacting at the same site on the receptor (Fig 7a), and it was possible to note the involvement of amino acids Leu465, Ser526, Val527, Ile764, Ala765, and Ala779 by their residues, establishing six chemical bonds capable of stabilizing nifedipine at the interaction site (Fig 7b). For comparison, molecular docking was also performed to test for any possible interaction between nifedipine and the TRPV1 receptor, which in vivo (Fig 2c) did not show evidence of a strong interaction. In this simulation, a lower number of amino acids was involved (Ala680, Ile679, and Asn687) at the interaction site (Fig 8a), resulting in a low number of chemical bonds and low energy affinity. There was also low compatibility between nifedipine and the TRPA1 receptor (Fig 8b). A molecular docking assessment between nifedipine and the TRPM3 receptor was also performed (Fig 8c). A high reproducibility of fit was found, and it was possible to note the involvement of eight amino acids by their residues: Tyr940, Asn942, Arg983, Asp986, Gln1130, Gln1133, Leu1134, and Thr1137. In contrast, the interaction between nifedipine and the TRPM3 channel was strong, indicating high chemical stabilization of the formed complex. All interaction strengths between nifedipine and NMDA, TRPV1, or TRPM3 receptors were found to be in line with the data of the in vivo studies.

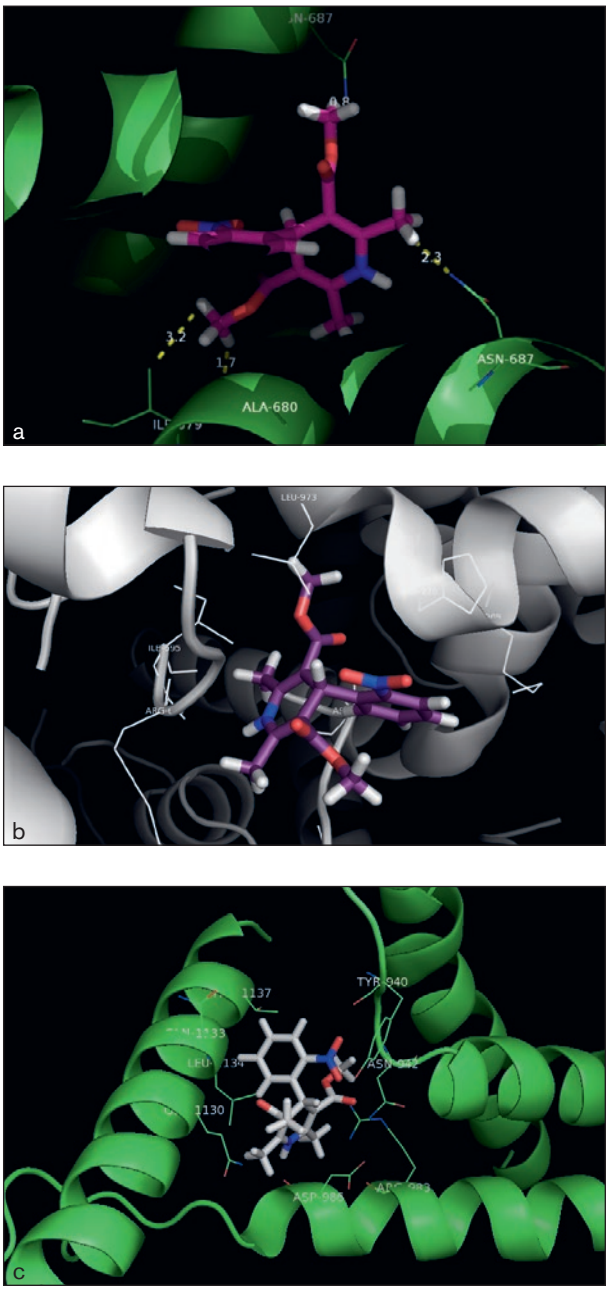


Fig 8 (a) Site of interaction between nifedipine and TRPV1 receptor showing the low number of amino acids involved and the ligand binding. **(b)** Amino acid residues involved in interaction between nifedipine and TRPA1 receptor, with side chains of TRPA1 structure. **(c)** Amino acid residues involved in interaction between TRPM3 (green) and nifedipine (white): Tyr940, Asn942, Arg983, Asp986, Gln1130, Gln1133, Leu1134, and Thr1137.

Discussion

A single experimental pain model is not able to replicate the complex nature of pain or predict the clinical efficacy of analgesics.[28] A major challenge for a model to be predictive and clinically useful is the heterogeneity of patient populations in their symptoms and pharmacology, reflecting in part differences in pathophysiology.[29] However, several nociceptive models used together may point to preclinical findings that provide valuable insight into the mechanism of action of a drug.[30] Thus, although only a limited number of behaviors were assessed in the present study, one of its strengths was its use of a variety of acute and chronic orofacial pain models to test for the possible antinociceptive effects of nifedipine.

This study has indeed provided novel findings with these different pain models and documented that oral administration of nifedipine reduces orofacial acute nociceptive behavior induced by formalin, mustard oil, glutamate, cinnamaldehyde, and acidified saline (but not by capsaicin), and that it is also effective in an orofacial neuropathic pain model induced by IONX. This study also assessed the possible effect of nifedipine on motor performance in the rotarod and grip-strength tests, since it is well established that drugs that produce CNS depression and nonspecific muscle relaxation effects may reduce motor coordination, and such effects could invalidate data obtained from behavioral tests assessing the potential analgesic effects of drugs.[30] However, unlike the disruptive motor effects of DZP, no significant alteration in motor performance after pretreatment with nifedipine was found. Therefore, the analgesic profile of nifedipine does not seem to be related to effects on motor coordination and/or grip strength. Nifedipine is a blocker of L-type Ca^{2+} channels and its antinociceptive effect has been previously demonstrated in other nociceptive models,[8,10] but to the present authors' knowledge, this is the first time that its antinociceptive effect in orofacial pain models has been documented.

The TRPA1 receptor is involved in many forms of acute and chronic pain in the orofacial region, and its activation is associated with the development of inflammation.[31] McNamara et al[32] demonstrated that formalin activates primary afferent sensory neurons through a direct action on TRPA1 receptors. In the present study, nifedipine significantly reduced facial rubbing in both phases of the formalin test when the TRPA1 antagonist formalin was injected into the upper lip of mice and also significantly reduced nociceptive behaviors induced by formalin injection into the rat TMJ. In addition, it significantly attenuated the hind-paw shaking behavior induced by another TRPA1 receptor agonist, mustard oil, when it was injected into the masseter muscle. These results suggest that nifedipine may act at least in part as a TRPA1 receptor antagonist, although this view contrasts with Fajardo et al,[33] who reported that nifedipine is a TRPA1 receptor agonist. It is also noteworthy that while TRPA1 has been described as the preferred target of formalin, Shields et al[34] have demonstrated that the ablation of most nociceptive sensory

neurons (including those expressing TRPA1) results in little change in the biphasic formalin response. More recently, Fischer et al[35] reported that formalin can excite nociceptors via a TRPA1-independent pathway. Taken together, this information suggests that additional pathways other than just those involving TRPA1 participate in the antinociceptive effect of nifedipine, and several other processes are indeed suggested from the findings of the present study, as discussed below.

The TRPV1 channel is another TRP receptor involved in orofacial nociceptive mechanisms. This receptor can be activated by the application of capsaicin to peripheral tissues, and the capsaicin-induced orofacial nociceptive test is a valid and reliable method that has often been used for the study of trigeminal pain mechanisms and for testing analgesic drugs.[36] Nifedipine, at several doses that were effective in the other nociceptive tests used in this study, did not significantly inhibit nociceptive behavior induced by capsaicin applied to the upper lip, indicating that its antinociceptive effect may not be dependent on the TRPV1 receptor. However, Castro-Júnior et al[37] reported that the peripheral administration of nifedipine decreases the nociceptive behavior induced by intraplantar capsaicin administration to the hind paw. This divergence in findings may be related to the facts that in the present study, the dose of nifedipine was lower than in the earlier hind-paw study, and that nifedipine was administered systemically and not peripherally to orofacial tissues.

Orofacial nociception and pain can be produced by algesic chemical application to several unique target tissues, such as the meninges, cornea, tooth pulp, oral/nasal mucosa, masticatory muscles, and TMJ.[38] One such algesic chemical is hypertonic saline, which is a very effective noxious stimulus in humans and laboratory animals. For example, hypertonic saline injected into orofacial tissues, including the cornea, evokes pain in humans and activates nociceptive primary afferents and nociceptive neurons in the trigeminal subnucleus caudalis of rats.[39] There is evidence that this may occur due to activation of the TRPV1 receptor by hyperosmotic stress.[40] In the present study, nociceptive behavior occurred when 5 M NaCl was applied to the cornea, but pretreatment with nifedipine at several different doses failed to inhibit this nociceptive behavior, suggesting that the antinociceptive effect of nifedipine may not involve TRPV1 receptor mechanisms. This possibility is supported by the present molecular docking study, which demonstrated the low affinity of nifedipine for the TRPV1 receptor. It is noteworthy that only three amino acids (Ala680, Ile679, and Asn687) were involved in the interaction between nifedipine and TRPV1, and, according to Carnevale and Rohacs,[41] these are not included in the most important side chains for recognition of the ligand by TRPV1.

There is evidence that acid-sensitive ion channels (ASICs) play an important role in the regulation of nociceptive transmission from orofacial tissues.[42] In the present study, nifedipine at an intermediate dose significantly decreased orofacial nociceptive behavior induced by acidified saline applied to the upper lip, suggesting that blockade of ASICs may be one of the processes involved in the antinociceptive effect of nifedipine. Recently, Gan et al[43] demonstrated that group I metabotropic glutamate receptors (mGluRs) sensitize ASIC receptors in dorsal root ganglion neurons and contribute to acidosis-evoked pain. In addition, blockers of glutamatergic ionotropic receptors (eg, NMDA) may modulate ASIC receptors.[44]

The possible role of glutamate receptors was investigated in the present study by testing whether nifedipine had an antinociceptive action on glutamate-evoked nociceptive behavior. Glutamate is an excitatory neurotransmitter that plays an important role in the transduction of nociceptive information, including from the orofacial region, and glutamatergic NMDA receptors are integrally involved in several physiologic functions, including pain.[45] It was found that the nociceptive behavior induced by the injection of glutamate into the upper lip was strongly reduced by preadministration of nifedipine. These results point to a possible antagonistic action of nifedipine on glutamatergic receptors. Although nifedipine presents low and irregular bioavailability after oral administration,[46] it can easily cross the brain-blood barrier.[47] Since nifedipine was administered systemically in the present study, its site of action could have been within the CNS or even in peripheral orofacial tissues, since glutamate-sensitive nociceptive afferents occur in these tissues.[48] Therefore, further studies are needed to investigate nifedipine's site(s) of action in relation to orofacial nociceptive mechanisms.

Furthermore, with the present objective of investigating the possible modulation of NMDA receptors in the effect of nifedipine, mice were pretreated with the NMDA receptor antagonist ketamine in the glutamate test group. This drug presented no antinociceptive effect itself (at the doses used), but significantly reduced the antinociceptive effect of nifedipine when administered beforehand. The involvement of NMDA receptors was corroborated by the molecular docking study, which indicated the existence of a strong interaction between nifedipine and NMDA receptors. The findings from the analysis of the interaction site, the strength of the six stabilizing chemical bonds, the amount of amino acids involved, and the high binding strength are all features that provide strong support for a link between nifedipine and NMDA. Therefore, these findings suggest that the antinociceptive effect

of nifedipine on orofacial nociceptive processes may be closely associated with NMDA receptor modulation. Moreover, since nifedipine is also a selective inhibitor of L-type Ca^{2+} channels, this modulatory effect may involve inhibition of L-type Ca^{2+} channels. This possibility is supported by Fossat et al,[48] who showed that an interaction between NMDA receptors and L-type Ca^{2+} channels may contribute to sensitization mechanisms underlying nociceptive processes in the spinal cord.

The transient receptor potential melastatin 3 (TRPM3) is a Ca^{2+}-permeable nonselective cation channel expressed in neuronal and nonneuronal cells, and although less extensively studied than other thermosensitive TRP receptors, it has been recently identified as a nociceptive heat receptor.[49] It is also noteworthy that Zamudio-Bulcock et al[24] demonstrated that TRPM3 channels are modulators of glutamatergic transmission in the developing brain. Furthermore, Wu et al[50] reported that the TRPM3 receptor has a functional role in the release of glutamate, but its specific function in controlling the synaptic release of glutamate has not yet been determined. Nifedipine has been identified as a TRPM3 agonist,[51] and the present study revealed that mefenamic acid (a TRPM3 antagonist) could itself produce no antinociceptive effect (at the dose used), yet inhibited the orofacial antinociceptive effect of nifedipine on the orofacial nociceptive behavior induced by glutamate. The molecular docking study also showed a strong interaction between nifedipine and the TRPM3 channel, further suggesting that the effect of nifedipine is at least partly dependent on the TRPM3 channel. Other findings also pointing to nifedipine as a TRPM3 agonist have come from cell culture studies.[24]

Following IONX, it was found that rats exhibited prolonged mechanical hyperalgesia in the vibrissal pad, which is consistent with previous studies.[18,52] Using this rodent model of facial neuropathic pain, it was found that treatment with nifedipine could reduce facial mechanically induced hypersensitivity. This effect may be due to an antagonistic action of nifedipine on $Ca_v1.2$ L-type voltage-dependent Ca^{2+} channels, since nifedipine has been shown to be a selective $Ca_v1.2$–channel blocker.[53] These channels are multimeric protein elements comprising a pore-forming alpha-1 subunit, and by accessory alpha-2/delta, beta, and sometimes gamma subunits.[54] Since injury to the trigeminal nerve leads to upregulation of $Ca_v\alpha2\delta1$ and to the development of a neuropathic pain state,[4] this action of nifedipine has clinical relevance, since neuropathic pain in the orofacial region is often the clinical manifestation of trigeminal nerve injury following oral therapeutic procedures.[52]

Further studies are warranted to test the effects of nifedipine in other acute and chronic pain models in order to provide additional preclinical data bearing on its potential utility for managing acute or chronic pain conditions. Given the enormous costs of the development of novel analgesics, drug repurposing has become a reasonable alternative approach to new drug development.[55] The present results indicate that nifedipine, a successful antihypertensive drug, might prove to be a clinically effective analgesic drug in acute and chronic pain states and that this effect may be due to the modulation of several different receptors and processes, including TRPA1, TRPM3, and NMDA.

Conclusions

The present study used a variety of acute and chronic orofacial pain models to document for the first time that nifedipine, a selective inhibitor of L-type Ca^{2+} channels, can suppress orofacial nociception through NMDA, TRPA1, and TRPM3 receptor systems. While the findings suggest the possible involvement of L-type Ca^{2+} channels through direct or indirect actions in some of these receptor mechanisms, further studies are needed to investigate the possible NMDA and TRP receptor pathways and direct or indirect actions involved, as well as the possible interactions of nifedipine with L-type Ca^{2+} channels in these receptor mechanisms.

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References

1. Sessle BJ. Are cannabinoids effective for orofacial pain states? J Oral Facial Pain Headache 2015;29:5–6.
2. Bannister K, Kucharczyk M, Dickenson AH. Hopes for the future of pain control. Pain Ther 2017;6:117–128.
3. Goto T, Oh SB, Takeda M, et al. Recent advances in basic research on the trigeminal ganglion. J Physiol Sci 2016;66: 381–386.
4. Li KW, Yu YP, Zhou C, et al. Calcium channel $\alpha2\delta1$ proteins mediate trigeminal neuropathic pain states associated with aberrant excitatory synaptogenesis. J Biol Chem 2014;289: 7025–7037.
5. Martín MI, del Val VL, Colado MI, Goicoechea C, Alfaro MJ. Behavioral and analgesic effects induced by administration of nifedipine and nimodipine. Pharmacol Biochem Behav 1996;55:93–98.
6. Zbuzek VK, Cohen B, Wu W. Antinociceptive effect of nifedipine and verapamil tested on rats chronically exposed to nicotine and after its withdrawal. Life Sci 1997;60:1651–1658.

7. Hunt TE, Wu WH, Zbuzek VK. The effects of serotonin biosynthesis inhibition on nicotine and nifedipine-induced analgesia in rats. Anesth Analg 1998;87:1109–1112.
8. Chang E, Chen X, Kim M, Gong N, Bhatia S, Luo ZD. Differential effects of voltage-gated calcium channel blockers on calcium channel alpha-2-delta-1 subunit protein-mediated nociception. Eur J Pain 2015;19:639–648.
9. Esmaeili-Mahani S, Vahedi S, Motamedi F, Pourshanazari A, Khaksari M, Ahmadiani A. Involvement of hypothalamic pituitary adrenal axis on the analgesic cross-tolerance between morphine and nifedipine. Pharmacol Biochem Behav 2007; 86:806–812.
10. Wong CH, Wu WH, Yarmush J, Zbuzek VK. An antinociceptive effect of the intraperitoneal injection of nifedipine in rats, measured by tail-flick test. Life Sci 1993;53:PL249–PL253.
11. Bustamante D, Miranda HF, Pelissier T, Paeile C. Analgesic action of clonixin, nifedipine and morphine using the formalin test. Gen Pharmacol 1989;20:319–322.
12. Kawashiri T, Egashira N, Kurobe K, et al. L type Ca^{2}+ channel blockers prevent oxaliplatin-induced cold hyperalgesia and TRPM8 overexpression in rats. Mol Pain 2012;8:7.
13. Kania BF, Kowalczyk M, Brytan M, Tomaszewska D, Przekop F. The inhibition of experimentally induced visceral hyperalgesia by nifedipine—A a voltage-gated Ca(2+) channels blocker (VGCCs) in sheep. Res Vet Sci 2009;86:285–292.
14. Magalhães FEA, Batista FLA, Serpa OF, et al. Orofacial antinociceptive effect of Mimosa tenuiflora (Willd.) Poiret. Biomed Pharmacother 2018;97:1575–1585.
15. Farazifard R, Safapour F, Sheibani V, Javan M. Eye-wiping test: A sensitive animal model for acute trigeminal pain studies. Brain Res Brain Res Protoc 2005;16:44–49.
16. Roveroni RC, Parada CA, Cecília M, Veiga FA, Tambeli CH. Development of a behavioral model of TMJ pain in rats: The TMJ formalin test. Pain 2001;94:185–191.
17. Ro JY, Capra N, Masri R. Development of a behavioral assessment of craniofacial muscle pain in lightly anesthetized rats. Pain 2003;104:179–185.
18. Kumar N, Cherkas PS, Varathan V, et al. Systemic pregabalin attenuates facial hypersensitivity and noxious stimulus-evoked release of glutamate in medullary dorsal horn in a rodent model of trigeminal neuropathic pain. Neurochem Int 2013; 62:831–835.
19. Cairns BE, Sessle BJ, Hu JW. Evidence that excitatory amino acid receptors within the temporomandibular joint region are involved in the reflex activation of the jaw muscles. J Neurosci 1998;18:8056–8064.
20. Araújo JRC, Júnior JMAM, Damasceno MBMV, et al. Neuropharmacological characterization of frutalin in mice: Evidence of an antidepressant-like effect mediated by the NMDA receptor/NO/cGMP pathway. Int J Biol Macromol 2018;112: 548–554.
21. Klose C, Straub I, Riehle M, et al. Fenamates as TRP channel blockers: Mefenamic acid selectively blocks TRPM3. Br J Pharmacol 2011;162:1757–1769.
22. Quintans Júnior LJ, Santana MT, Melo MS, et al. Antinociceptive and anti-inflammatory effects of Costus spicatus in experimental animals. Pharm Biol 2010;48:1097–1102.
23. Malafoglia V, Traversetti L, Del Grosso F, et al. Transient Receptor Potential Melastatin-3 (TRPM3) mediates nociceptive-like responses in hydra vulgaris. PLoS One 2016;11:e0151386.
24. Zamudio-Bulcock PA, Everett J, Harteneck C, Valenzuela CF. Activation of steroid-sensitive TRPM3 channels potentiates glutamatergic transmission at cerebellar Purkinje neurons from developing rats. J Neurochem 2011;119:474–485.
25. Nascimento SS, Camargo EA, DeSantana JM, et al. Linalool and linalool complexed in β-cyclodextrin produce anti-hyperalgesic activity and increase Fos protein expression in animal model for fibromyalgia. Naunyn Schmiedebergs Arch Pharmacol 2014;387:935–942.
26. Meng XY, Zhang HX, Mezei M, Cui M. Molecular docking: A powerful approach for structure-based drug discovery. Curr Comput Aided Drug Des 2011;7:146–157.
27. Vilar S, Sobarzo-Sanchez E, Santana L, Uriarte E. Molecular docking and drug discovery in β-adrenergic receptors. Curr Med Chem 2017;24:4340–4359.
28. Okkerse P, van Amerongen G, de Kam ML, et al. The use of a battery of pain models to detect analgesic properties of compounds: A two-part, four-way, randomised, placebo-controlled, crossover study. Clin Ther 2015;37:e14.
29. Berge OG. Predictive validity of behavioural animal models for chronic pain. Br J Pharmacol 2011;164:1195–1206.
30. Le Bars D, Gozariu D, Cadden SW. Animal models of nociception. Pharmacol Rev 2001;53:597–652.
31. Raboisson P, Dallel R. The orofacial formalin test. Neurosci Biobehav Rev 2004;28:219–226.
32. McNamara CR, Mandel-Brehm J, Bautista DM, et al. TRPA1 mediates formalin-induced pain. Proc Natl Acad Sci U S A 2007;104:13525–13530.
33. Fajardo O, Meseguer, V, Belmonte, C, Viana F. TRPA1 channels: Novel targets of 1,4-dihydropyridines. Channels (Austin) 2008;2:429–438.
34. Shields SD, Cavanaugh DJ, Lee H, Anderson DJ, Basbaum AI. Pain behavior in the formalin test persists after ablation of the great majority of C-fiber nociceptors. Pain 2010;151:422–429.
35. Fischer MJ, Soller KJ, Sauer SK, Kalucka J, Veglia G, Reeh PW. Formalin evokes calcium transients from the endoplasmatic reticulum. PLoS One 2015;10:e0123762.
36. Pelissier T, Pajot J, Dallel R. The orofacial capsaicin test in rats: Effects of different capsaicin concentrations and morphine. Pain 2002;96:81–87.
37. Castro-Junior CJ, Milano J, Souza AH, et al. Phα1β toxin prevents capsaicin-induced nociceptive behavior and mechanical hypersensitivity without acting on TRPV1 channels. Neuropharmacology 2013;71:237–246.
38. Hargreaves KM. Orofacial pain. Pain 2011;152(3 suppl): s25–s32.
39. Carstens E, Kuenzler N, Handwerker HO. Activation of neurons in rat trigeminal subnucleus caudalis by different irritant chemicals applied to oral or ocular mucosa. J Neurophysiol 1998;80:465–492.
40. Ciura S, Bourque CW. Transient receptor potential vanilloid 1 is required for intrinsic osmoreception in organum vasculosum lamina terminalis neurons and for normal thirst responses to systemic hyperosmolality. J Neurosci 2006;26:9069–9075.
41. Carnevale V, Rohacs T. TRPV1: A target for rational drug design. Pharmaceuticals (Basel) 2016;9. pii: E52.
42. Gu Q, Lee LY. Acid-sensing ion channels and pain. Pharmaceuticals (Basel) 2010;3:1411–1425.
43. Gan X, Wu J, Ren C, Qiu CY, Li YK, Hu WP. Potentiation of acid-sensing ion channel activity by peripheral group I metabotropic glutamate receptor signaling. Pharmacol Res 2016;107:19–26.
44. Tikhonova TB, Nagaeva EI, Barygin OI, Potapieva NN, Bolshakov KV, Tikhonov DB. Monoamine NMDA receptor channel blockers inhibit and potentiate native and recombinant proton-gated ion channels. Neuropharmacol 2015;89:1–10.
45. Honda K, Noma N, Shinoda M, et al. Involvement of peripheral ionotropic glutamate receptors in orofacial thermal hyperalgesia in rats. Mol Pain 2011;7:75.

46. Emara LH, Badr RM, Elbary AA. Improving the dissolution and bioavailability of nifedipine using solid dispersions and solubilizers. Drug Dev Ind Pharm 2002;28:795–807.
47. Janicki PK, Siembab D, Paulo EA, Krzaścik P. Single-dose kinetics of nifedipine in rat plasma and brain. Pharmacology 1988;36:183–187.
48. Fossat P, Sibon I, Le Masson G, Landry M, Nagy F. L-type calcium channels and NMDA receptors: A determinant duo for short-term nociceptive plasticity. Eur J Neurosci 2007;25:127–135.
49. Thiel G, Rubil S, Lesch A, Guethlein LA, Rössler OG. Transient receptor potential TRPM3 channels: Pharmacology, signaling, and biological functions. Pharmacol Res 2017;124:92–99.
50. Wu SW, Fenwick AJ, Peters JH. Channeling satiation: A primer on the role of TRP channels in the control of glutamate release from vagal afferent neurons. Physiol Behav 2014;136:179–184.
51. Wagner TF, Loch S, Lambert S, et al. Transient receptor potential M3 channels are ionotropic steroid receptors in pancreatic beta cells. Nat Cell Biol 2008;10:1421–1430.
52. Iwata K, Imamura Y, Honda K, Shinoda M. Physiological mechanisms of neuropathic pain: The orofacial region. Int Rev Neurobiol 2011;97:227–250.
53. Liu Y, Harding M, Dore J, Chen X. $Ca_v1.2$, but not $Ca_v1.3$, L-type calcium channel subtype mediates nicotine-induced conditioned place preference in mice. Prog Neuropsychopharmacol Biol Psychiatry 2017;75:176–182.
54. Catterall WA. Structure and regulation of voltage-gated Ca2+ channels. Annu Rev Cell Dev Biol 2000;16:521–555.
55. Sisignano M, Parnham MJ, Geisslinger G. Drug repurposing for the development of novel analgesics. Trends Pharmacol Sci 2016;37:172–183.