



## Involvement of voltage-gated sodium channels blockade in the analgesic effects of orphenadrine

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### ARTICLE INFO

#### Article history:

Received 7 July 2008

Received in revised form 18 December 2008

Accepted 9 January 2009

#### Keywords:

Orphenadrine

Sodium channel subtypes

Local anesthetic

Analgesia

DRG sensory neurons

Toxic effects

### ABSTRACT

Orphenadrine is a drug acting on multiple targets, including muscarinic, histaminic, and NMDA receptors. It is used in the treatment of Parkinson's disease and in musculoskeletal disorders. It is also used as an analgesic, although its mechanism of action is still unknown. Both physiological and pharmacological results have demonstrated a critical role for voltage-gated sodium channels in many types of chronic pain syndromes. We tested the hypothesis that orphenadrine may block voltage-gated sodium channels. By using patch-clamp experiments, we evaluated the effects of the drug on whole-cell sodium currents in HEK293 cells expressing the skeletal muscle (Nav1.4), cardiac (Nav1.5) and neuronal (Nav1.1 and Nav1.7) subtypes of human sodium channels, as well as on whole-cell tetrodotoxin (TTX)-resistant sodium currents likely conducted by Nav1.8 and Nav1.9 channel subtypes in primary culture of rat DRG sensory neurons. The results indicate that orphenadrine inhibits sodium channels in a concentration-, voltage- and frequency-dependent manner. By using site-directed mutagenesis, we further show that orphenadrine binds to the same receptor as the local anesthetics. Orphenadrine affinities for resting and inactivated sodium channels were higher compared to those of known sodium channels blockers, such as mexiletine and flecainide. Low, clinically relevant orphenadrine concentration produces a significant block of Nav1.7, Nav1.8, and Nav1.9 channels, which are critical for experiencing pain sensations, indicating a role for sodium channel blockade in the clinical efficacy of orphenadrine as analgesic compound. On the other hand, block of Nav1.1 and Nav1.5 may contribute to the proconvulsive and proarrhythmic adverse reactions, especially observed during overdose.

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### 1. Introduction

Orphenadrine is an anticholinergic agent used mainly in the treatment of Parkinson's disease to alleviate some of the troublesome symptoms of the disease, especially the involuntary resting tremor [44,45]. In addition to this use, the drug has a long history in the clinics as a muscle relaxant [19]. The mechanism of action for such effect remains unclear, but may be related in part to sedative effects; orphenadrine exerts unspecific antagonist activity at the phencyclidine binding site of N-methyl-D-aspartate (NMDA) receptors, one of the subtypes of glutamate receptors [24]. A study reported that orphenadrine is able to protect cultured cerebellar neurons from excitotoxicity following direct exposure of neurons [17].

Orphenadrine is used also as an analgesic both alone and in association with non-steroidal anti-inflammatory drugs [21]. In a human model of capsaicin-dependent inflammatory pain obtained with laser somatosensory-evoked potentials, orphenadrine citrate was able to exert an analgesic/anti-hyperalgesic effect in a low dose (30 mg/day), which was predominantly due to central/spinal mechanisms [42]. A central action of orphenadrine was thus proposed, but the detailed mechanisms are unknown.

Orphenadrine is a monomethylated derivative of diphenhydramine, an antihistaminic drug. Since histamine plays an important role in pain processes, it is possible that the analgesic action of orphenadrine may be related to histamine antagonism. However, diphenhydramine was also shown to block voltage-gated sodium channels [22], suggesting that other pharmacological properties may contribute to antinociceptive effects of orphenadrine. Both physiological and pharmacological evidence have demonstrated a critical role for voltage-gated sodium channels in many types of chronic pain syndromes, because these channels play a fundamental role in the excitability of neurons in the central and

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peripheral nervous systems [10]. The recent findings strengthen this view, since a gain of function of the Nav1.7 channel, expressed at high levels in nociceptive dorsal root ganglion (DRG) neurons, was shown to cause primary erythralgia and paroxysmal extreme pain disorder, which are two inherited pain syndromes linked to SCN9A mutations and responsive to lidocaine, mexiletine and carbamazepine treatment [15,30,49]. Conversely, loss of function of Nav1.7 channels results in an inherited channelopathy characterized by total insensitivity to pain of any type [9]. Moreover, expression levels of the Nav1.3 channel isoform increase in DRG following a neuronal injury or an inflammatory insult [3]. Finally, knocking-out the Nav1.8 or Nav1.9 channel isoforms in mice has been shown to cause deficits in thermal and mechanical pain perception [1,7,35].

In the current study, we tested the hypothesis that orphenadrine may block voltage-gated sodium channels. We evaluated the effects of the drug on muscle, cardiac and neuronal human subtypes of sodium channels that are heterologously expressed in HEK293 cells, and elucidated the molecular mechanism of block by orphenadrine by using specific voltage-clamp protocols and sodium channel site-directed mutagenesis. We also tested orphenadrine on tetrodotoxin (TTX)-resistant sodium currents in sensory neurons dissociated from rat dorsal root ganglions (DRGs). A major result of this study indicates that inhibition of voltage-gated TTX-sensitive or resistant sodium channels in DRG neurons likely contributes to analgesic/anti-hyperalgesia effects of orphenadrine.

## 2. Methods

### 2.1. Mutagenesis and expression of recombinant sodium channels

Full-length cDNA encoding wild-type (WT) hNav1.4 (skeletal muscle isoform), hNav1.5 (cardiac isoform), and hNav1.1 (central and peripheral neuron isoform) channels were subcloned in the mammalian expression vector pRc-CMV or pCMV-Script [5,31,48], and hNav1.7 (peripheral neuron isoform) was subcloned in a modified pcDNA3/pBR222 expression vector [23].

The F1586C mutation of hNav1.4 was engineered by standard two-step PCR-based site-directed mutagenesis. All PCRs were performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) for high-fidelity amplification. The complete coding region of channel mutant cDNA was sequenced to exclude any polymerase errors.

Transient expression of WT hNav1.5 and hNav1.7 in HEK293 cells was achieved by 10  $\mu$ g plasmid transfection using the calcium phosphate coprecipitation method [12]. These channels were co-transfected with the gene reporter CD8 in a 10:1 plasmid mass ratio. Cells that were identified with microbeads coated with anti-CD8 antibody (Dyna, Norway) were used for patch-clamp experiments 36–96 h after transfection. Permanent expression of WT hNav1.1, WT hNav1.4 as well as F1586C mutant was achieved in HEK293 cells by the same transfection method followed by clone selection with geneticin (GIBCO-Invitrogen, Italy).

### 2.2. Primary cultures of sensory neurons

Dissociation of DRG neurons from 5- to 6-week-old male Wistar rats was performed as previously described [7]. Briefly, the rats were deeply anesthetized with halothane and sacrificed by cutting the carotid arteries in accordance with the Guide for the Care and Use of Laboratory Animals and Institutional Guidelines. Excised thoraco-lumbar DRGs were incubated for 45 min. in Hank's balanced salt solution (HBSS) supplemented with

2 mg/ml of collagenase Type IA (Sigma) at 37 °C. After incubation, the DRGs were rinsed several times with HBSS and gently triturated through the smoothed tip of Pasteur pipettes. Neurons were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml penicillin-streptomycin, 2 mM L-glutamine, 25 mM glucose, 25 ng/ml nerve growth factor (Invitrogen) and 2 ng/ml glial-derived neurotrophic factor (Invitrogen). Neurons were incubated in a humidified atmosphere (5% CO<sub>2</sub>, 37 °C) for 14–20 h before recording.

### 2.3. Voltage-clamp studies

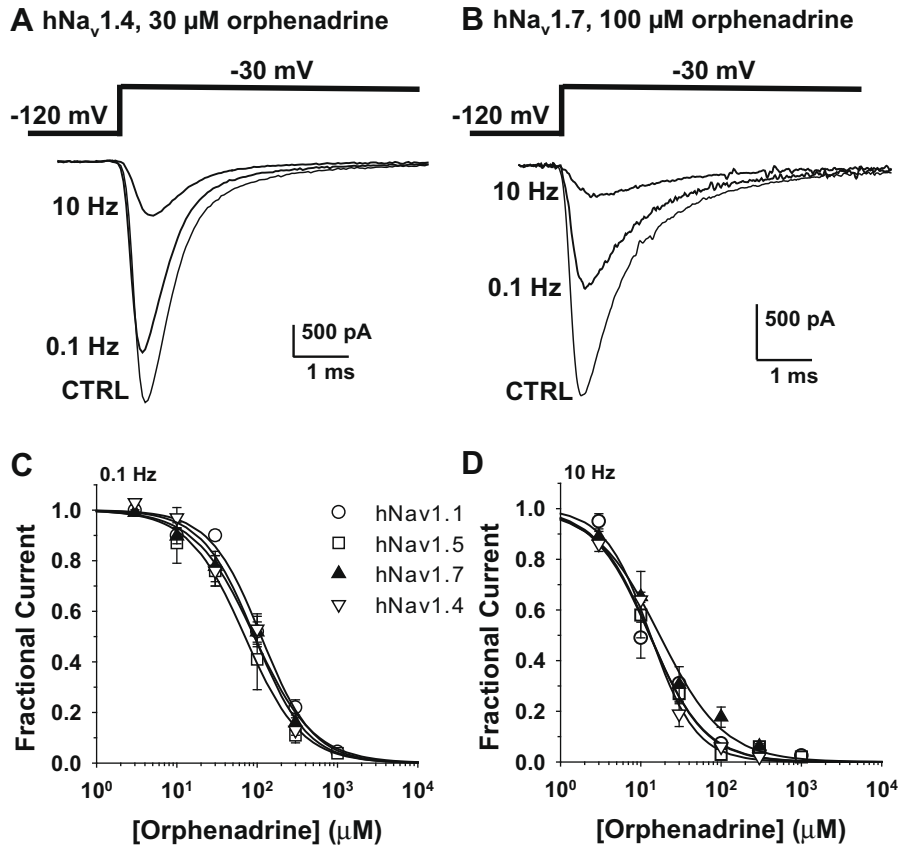
Whole-cell sodium currents ( $I_{Na}$ ) were recorded at room temperature (20–22 °C) using Axopatch 1D or 200B amplifiers (Axon Instruments, Union City, CA, USA). Voltage-clamp protocols and data acquisition were performed with pCLAMP software (version 6.0, 9.2, or 10.0, Axon Instruments) through a 12-bit A-D/D-A interface (Digidata 1200 or 1440A, Axon Instruments). Patch pipettes had resistance ranging from 1 to 3 M $\Omega$ . Currents were low-pass filtered at 2 kHz (–3 dB) by the four-pole Bessel filter of the amplifier and were digitized at 10–20 kHz.

For recordings in HEK293 cells, after rupturing the patch membrane, a 25-ms long test pulse at –30 mV was applied from the holding potential (HP) of –120 mV at a low frequency until stabilization of  $I_{Na}$  amplitude and kinetics was achieved (typically 5 min) [12]. Data were considered for analysis only from cells exhibiting a series resistance errors <5 mV. Little (<5%) or no run-down was observed within the experiments. Specific voltage protocols and analysis procedures are described in Section 3.

In DRG neurons, TTX-resistant sodium currents were recorded using specific voltages, allowing separation of high-voltage activated (HVA) currents (known as SNS or PN3) and low-voltage activated (LVA) currents (known as NaN or SNS2). With TTX and La<sup>3+</sup> in the bath solution, the HVA current is mainly supported by Nav1.8 sodium channels, whereas the LVA current results mainly from activation of Nav1.9 sodium channels [7,8,33]. The HVA current was elicited from a holding potential of –55 mV to a test pulse of –10 mV. At this HP, the Nav1.9 channels are mostly inactivated. At 0.1 and 2 Hz stimulation frequencies, the leak and capacitive currents were subtracted online using the P/4 protocol of pClamp software. At 10 Hz stimulation frequency, subtraction was performed offline using an adequately scaled current response to hyperpolarizing stimulation. The LVA current was elicited from an HP of –100 to –60 mV at 0.1 Hz stimulation frequency. At –60 mV, the Nav1.8 channels are closed, allowing activation of solely Nav1.9 channels. The use of fluoride in the pipette solution increases amplitude and negatively shifts the voltage dependence of LVA currents [33]. A P/6 protocol was used for leak and capacitive current subtraction.

### 2.4. Drugs and solutions

All reagents as well as hydrochloride salts of mexiletine and orphenadrine were purchased from Sigma-Aldrich (Milan, Italy), except differently indicated. For patch-clamp recordings of heterologously expressed sodium channels, the pipette solution contained in mM 120 CsF, 10 CsCl, 10 NaCl, 5 EGTA and 5 Hepes, and the pH was set to 7.2 with CsOH, while the bath solution contained in mM 150 NaCl, 4 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 Hepes and 5 glucose. The pH was set to 7.4 with NaOH. For patch-clamp recordings in DRG sensory neurons, the pipette solution contained in mM 100 CsCl, 30 CsF, 8 NaCl, 2.4 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 EGTA, 10 Hepes, 4 ATP, and 0.4 GTP, and the pH was set to 7.3 with NaOH. The bath solution contained in mM 131 NaCl, 3 KCl, 2.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 Hepes,



**Fig. 1.** Dose-dependent and use-dependent block of four sodium channel subtypes by orphenadrine. The block of sodium currents by orphenadrine was assessed 3 min after drug application by measuring the reduction of  $I_{Na}$  elicited with a 25-ms long test pulse from  $-120$  to  $-30$  mV at stimulation frequencies of 0.1 and 10 Hz. (A) Effects of 30  $\mu$ M orphenadrine on hNav1.4 currents. (B) Effects of 100  $\mu$ M orphenadrine on hNav1.7 currents. The concentration–response curves for orphenadrine block were constructed at 0.1 (C) and 10 Hz (D) and fitted with Eq. (1). Each data point is the mean  $\pm$  SEM of at least three cells. The calculated  $IC_{50}$  and nH values  $\pm$  SE of the fit are reported in Table 1.

10 glucose, 0.5 tetrodotoxin (TTX), and 5  $\mu$ M  $La^{3+}$ . The pH was set to 7.35 with NaOH.

Orphenadrine was diluted in bath solution at a desired concentration, and the pH was adjusted to 7.4. The patched HEK293 cell was continuously exposed to a stream of control or drug-supplemented bath solution flowing out from a plastic capillary. During recordings, the DRG neurons were perfused with bath solution at a flow rate of 5 ml/min. The bath solution was recycled to limit sparing of TTX. Both perfusion systems allowed application of drug in less than 1 min.

**2.5. Statistical analysis**

Average data are presented as means  $\pm$  SEM, and statistical difference between the means was evaluated using Student’s unpaired or paired *t*-test, with  $P < 0.05$  considered as significant.

**3. Results**

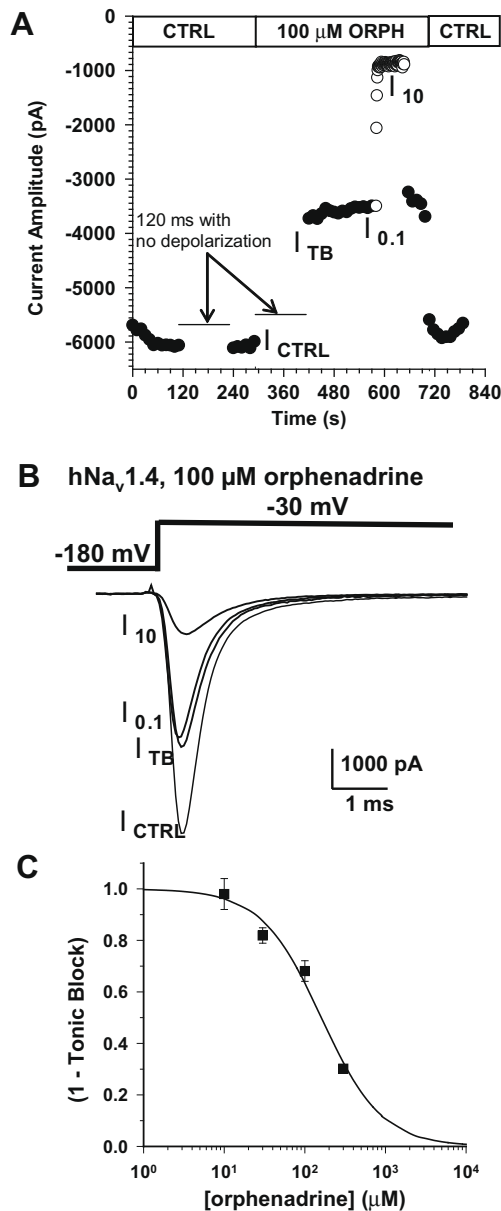
**3.1. Dose- and frequency-dependent block of four sodium channel subtypes by orphenadrine**

We tested orphenadrine on four sodium channel subtypes encoded by different genes. While the hNav1.4 channel (SCN4A gene) is expressed exclusively in skeletal muscle, the hNav1.5 channel (SCN5A gene) is the main cardiac isoform and is expressed also in some areas of the central nervous system as well as in immature or denervated skeletal muscle, the hNav1.1 channel (SCN1A gene) is expressed in central and peripheral neurons and in cardiac myocytes, and the hNav1.7 channel (SCN9A) is predominantly expressed in peripheral neurons, including the DRG where it is concentrated in small C fiber nociceptors [4]. Wild-type hNav1.1, hNav1.4, hNav1.5, and hNav1.7 channels

**Table 1**  
 $IC_{50}$  values for orphenadrine block of four human voltage-gated sodium channel isoforms and the F1586C hNav1.4 mutant.

	$IC_{50}$ at 0.1 Hz ( $\mu$ M)	nH at 0.1 Hz	$IC_{50}$ at 10 Hz ( $\mu$ M)	nH at 10 Hz	$V_{1/2}$ (mV)
hNav1.4	92.9 $\pm$ 12.0	1.3 $\pm$ 0.2	13.2 $\pm$ 1.1	1.5 $\pm$ 0.2	-72.3 $\pm$ 0.2 (17)
hNav1.5	70.4 $\pm$ 6.1	1.2 $\pm$ 0.1	13.0 $\pm$ 1.2	1.3 $\pm$ 0.2	-79.7 $\pm$ 0.7 (17)
hNav1.1	110.3 $\pm$ 10.2	1.3 $\pm$ 0.1	12.9 $\pm$ 2.1	1.2 $\pm$ 0.2	-71.0 $\pm$ 0.2 (18)
hNav1.7	92.1 $\pm$ 9.1	1.2 $\pm$ 0.1	17.2 $\pm$ 1.9	1.1 $\pm$ 0.1	-62.1 $\pm$ 0.7 (20)
F1586C	206 $\pm$ 10	1.4 $\pm$ 0.1	127 $\pm$ 14	1.2 $\pm$ 0.2	-64.4 $\pm$ 0.2 (24)

The half-maximum inhibitory concentration ( $IC_{50}$ ) and the slope factor (nH) values were calculated using Eq. (1) at the HP of  $-120$  mV from the concentration/response relationships shown in Figs. 1 and 4. The half-maximum inactivation potential ( $V_{1/2}$ ) was calculated from the fit with the Boltzmann equation of steady-state inactivation relationships obtained as in Fig. 3B using the cells (number indicated within brackets) used for determination of  $IC_{50}$  value.



**Fig. 2.** Affinity of orphenadrine for resting, closed hNav1.4 channels. (A) Time course of peak  $I_{Na}$  in a HEK293 cell expressing hNav1.4 channels. The cell was held at the HP of  $-180$  mV and depolarized for 25 ms to  $-30$  mV at 0.1 Hz (plain circles) and 10 Hz (open circles), except in correspondence to arrows where the HP was maintained with no depolarization. Tonic block was measured in the presence of  $100 \mu\text{M}$  orphenadrine at the first pulse depolarization. Little or no block was observed at 0.1 Hz, whereas a huge use-dependent block was observed at 10 Hz. (B) Traces of hNav1.4  $I_{Na}$  measured at the times reported in (A):  $I_{CTRL}$  was measured just before application of the drug,  $I_{TB}$  corresponds to tonic block,  $I_{0.1}$  corresponds to the steady-state block reached at 0.1 Hz, and  $I_{10}$  corresponds to the steady-state block at 10 Hz. (C) Concentration–response curve was constructed for tonic block ( $I_{TB}/I_{CTRL}$ ) of hNav1.4  $I_{Na}$  currents measured as in (A). Each data point is the mean  $\pm$  SEM of at least three cells. The relationship was fitted with Eq. (1), where the calculated  $IC_{50}$  corresponds to affinity to resting, closed channels  $K_R$ . The  $K_R$  value  $\pm$  SE of the fit was  $161 \pm 23 \mu\text{M}$ . The calculated slope factor  $nH \pm$  SE of the fit was  $1.2 \pm 0.2$ .

were transiently or permanently expressed in HEK293 cells, and the resulting  $I_{Na}$  were recorded with patch-clamp technique in the whole-cell configuration [12]. Externally applied orphenadrine produced both tonic- and use-dependent block of  $I_{Na}$  elicited by depolarizing pulses to  $-30$  mV from a holding potential (HP) of  $-120$  mV. Tonic block was assayed 3 min after drug application by measuring the reduction of  $I_{Na}$  elicited at 0.1 Hz,

whereas use-dependent block was further obtained by increasing stimulation frequency to 10 Hz. By applying this protocol in the absence of drug, no significant change in the  $I_{Na}$  amplitude was observed (not shown). Fig. 1A shows representative examples of hNav1.4 current traces recorded before (control) and after application of  $30 \mu\text{M}$  orphenadrine. The drug reduced the amplitude of peak  $I_{Na}$  by  $24 \pm 6\%$  at 0.1 Hz and  $81 \pm 5\%$  at 10 Hz ( $n = 4$ ). Fig. 1B shows representative examples of hNav1.7 current traces recorded in the presence of  $100 \mu\text{M}$  orphenadrine. The drug reduced the amplitude of peak  $I_{Na}$  by  $48 \pm 4\%$  at 0.1 Hz and  $82 \pm 4\%$  at 10 Hz ( $n = 4$ ). The inhibitory effect of orphenadrine was dose-dependent. The concentration–response curves were fitted with the first-order binding function,

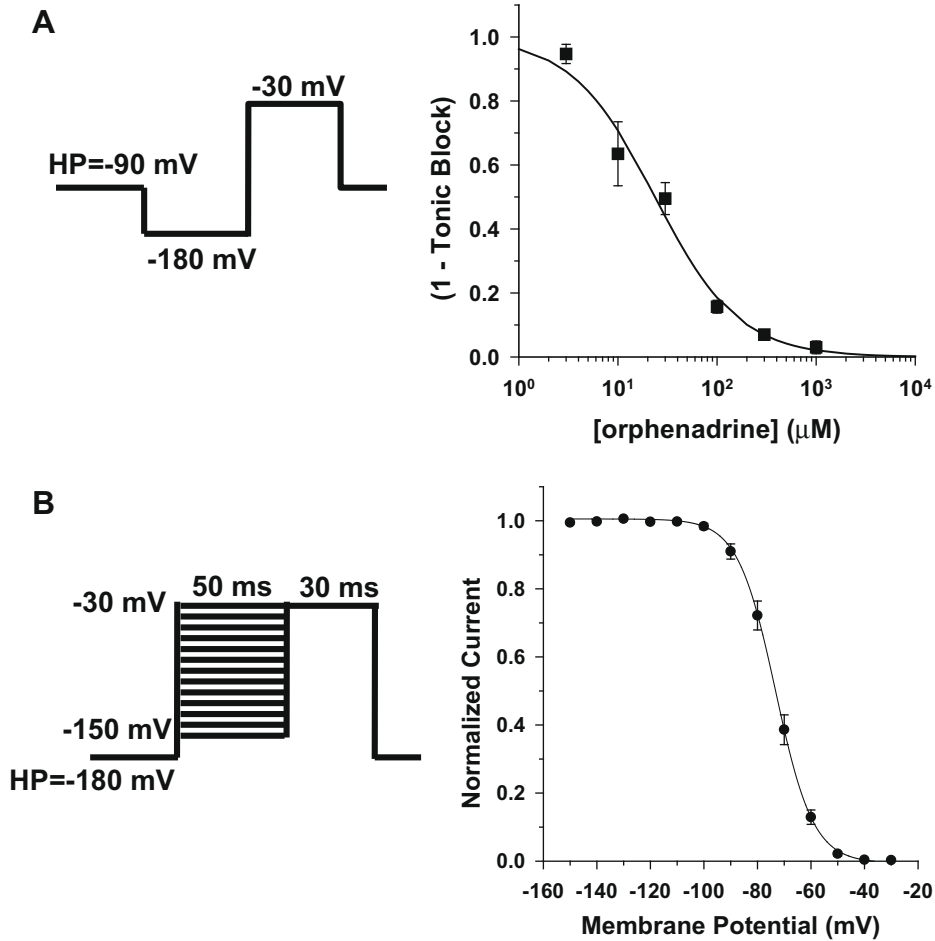
$$I_{DRUG}/I_{CONTROL} = 1 / \{1 + ([drug]/IC_{50})^{nH}\} \quad (1)$$

where  $IC_{50}$  ( $\mu\text{M}$ ) is the half-maximum inhibitory concentration, and  $nH$  is the slope factor (Fig. 1C and D). The values of  $IC_{50}$  and  $nH$  are reported in Table 1. Little or no difference was found between the four sodium channel isoforms. Compared to the effects of the well-known sodium channel blockers, mexiletine and flecainide, on hNav1.4 in the same experimental conditions, orphenadrine was equipotent to flecainide and more potent than mexiletine at 0.1 Hz, while its  $IC_{50}$  at 10 Hz was about 3-fold smaller than that of the two other drugs indicating a high frequency-dependent profile [12,13]. The block of orphenadrine of sodium channel was completely reversible (see below).

### 3.2. State-dependent binding affinities of orphenadrine to hNav1.4 channels

According to the modulated receptor hypothesis, use-dependent block can be explained from different binding affinities to the closed and open/inactivated channels [20]. For a similar reason, the block of  $I_{Na}$  at the HP of  $-120$  mV probably reflects the combination of binding to both the resting and inactivated sodium channels at this HP [12,50]. We calculated the drug-binding affinities to resting ( $K_R$ ) and inactivated ( $K_I$ ) sodium channels using hNav1.4 to allow direct comparison with data that were previously obtained with mexiletine and flecainide. To evaluate  $K_R$ , we first measured tonic block of the channels while maintaining the cell hyperpolarized at  $-180$  mV for 120 s (prepulse) and, only after that, the cell was depolarized at 0.1 Hz frequency (Fig. 2A and B). At the HP of  $-180$  mV, the entire population of the channels is in the closed state, ready to open in response to the first pulse depolarization. No change in  $I_{Na}$  occurred in the absence of drug. In the presence of drug, a reduction of  $I_{Na}$  amplitude, labeled tonic block (TB), was observed on the first pulse after the 120-s long prepulse, which reflects binding to closed channels. Little or no additional block was observed at 0.1 Hz. At 10 Hz, a huge use-dependent block developed, which was reversed on turning back to 0.1 Hz stimulation. Finally, TB can be fully reversed by washing out the drug. We calculated the  $K_R$  as the  $IC_{50}$  value of concentration–response curves for TB occurring during the prepulse (Fig. 2C). The calculated  $K_R$  for orphenadrine was  $161 \pm 23 \mu\text{M}$ . For comparison, the  $K_R$  values for mexiletine and flecainide on hNav1.4 channels were 800 and  $480 \mu\text{M}$ , respectively [12,13]. Thus, orphenadrine binds to the resting sodium channels with a relatively high affinity.

Because inactivated channels are non-conducting, calculation of affinity constant for inactivated channels can be only indirect. We calculated the TB at HP =  $-90$  mV using the same protocol as in Fig. 2A, except a 35-ms long hyperpolarized pulse at  $-180$  mV was introduced before the test pulse at  $-30$  mV to allow channels inactivated at  $-90$  mV to recover from inactivation, thereby assuring that the reduction of  $I_{Na}$  was attributable only to closure of



**Fig. 3.** Affinity of orphenadrine for inactivated hNav1.4 channels. (A) Concentration–response curve was constructed for  $I_{TB}/I_{CTRL}$  measured as in Fig. 2A, except the HP was  $-90$  mV and a 35-ms long hyperpolarized pulse at  $-180$  mV was introduced before the test pulse at  $-30$  mV to allow channels inactivated at  $-90$  mV to recover from inactivation, thereby assuring that the reduction of  $I_{Na}$  was attributable only to closure of drug-bound channels. Each data point is the mean  $\pm$  SEM of at least three cells. The relationship was fitted with Eq. (1), where the calculated  $IC_{50}$  value corresponds to apparent affinity constant ( $K_{APP}$ ). The  $K_{APP}$  value  $\pm$  SE of the fit was  $23.6 \pm 3.4 \mu M$ . The calculated slope factor  $nH \pm$  SE of the fit was  $1.0 \pm 0.1$ . (B) Steady-state inactivation curve of  $I_{Na}$  in HEK293 cells expressing hNav1.4.  $I_{Na}$  was evoked by a 20-ms long test pulse to  $-30$  mV after a 50-ms long conditioning pulse to potentials ranging from  $-150$  to  $-30$  mV in 10 mV increments; the HP was  $-180$  mV. The peak  $I_{Na}$  recorded during the test pulse was plotted against the conditioning pulse potential. The relationship was fitted with the Boltzmann equation,  $I_{Na}/I_{Na,max} = 1/(1 + \exp[(V - V_{1/2})/S])$ , where  $V_{1/2}$  (mV) is the half-maximum inactivation potential, and  $S$  (mV) is the slope factor. Each data point is the mean  $\pm$  SEM of the 18 cells used for determination of  $K_{APP}$ . The  $V_{1/2}$  value  $\pm$  SE of the fit was  $-73.2 \pm 0.1$  mV. The calculated slope factor  $S \pm$  SE of the fit was  $-7.0 \pm 0.1$  mV. From this relationship, the proportions of closed ( $h$ ) and inactivated ( $1 - h$ ) channels were determined at  $-90$  mV and inserted together with  $K_R$  and  $K_{APP}$  values into Eq. (2) to calculate the affinity for inactivated channel ( $K_I$ ), which was  $2.2 \mu M$ .

drug-bound channels (Fig. 3A). In these conditions, the  $IC_{50}$  value for TB was  $23.6 \pm 3.4 \mu M$  ( $K_{APP}$  in Eq. (2)). Using Bean’s equation derived from the modulated receptor hypothesis,

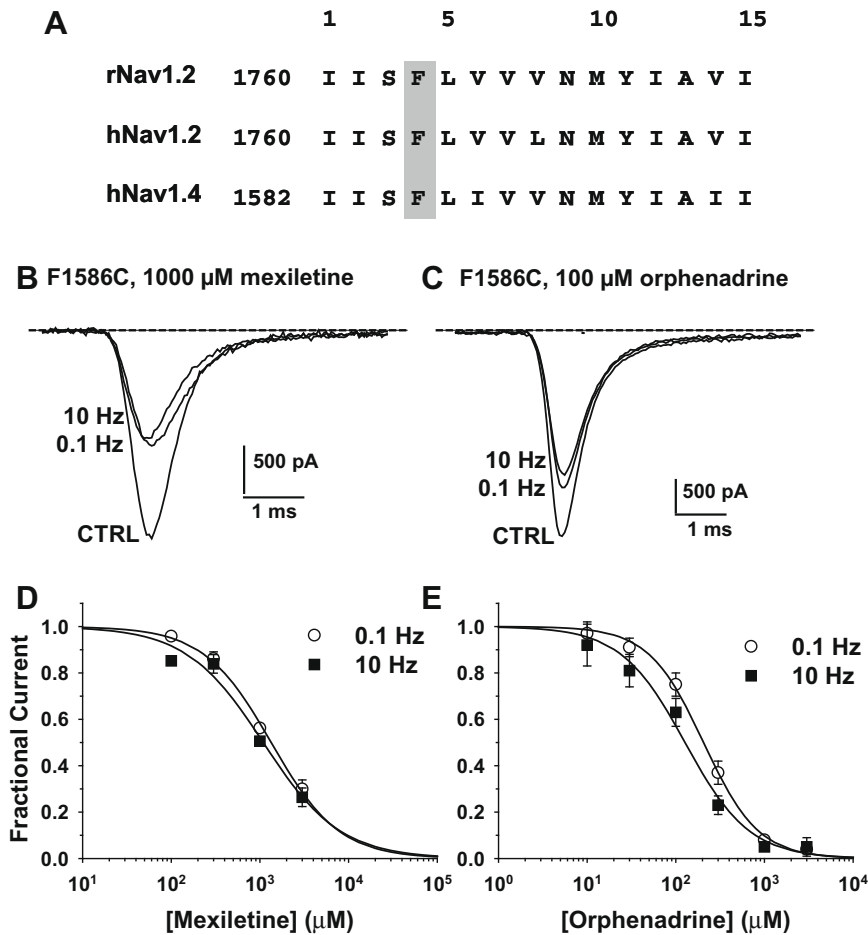
$$K_I = (1 - h) \cdot (1/K_{APP} - h/K_R)^{-1} \tag{2}$$

where  $K_{APP}$  is the apparent affinity constant at the potential considered, and the terms  $h$  and  $(1 - h)$  are the proportions of closed and inactivated channels at this potential as determined from steady-state inactivation curves (Fig. 3B) [2]. With  $h = 0.9197$  at  $-90$  mV, the calculated  $K_I$  value was  $2.2 \mu M$ . Compared to mexiletine ( $K_I = 6 \mu M$ ) and flecainide ( $K_I = 18 \mu M$ ) on hNav1.4 channels, orphenadrine appeared as a potent blocker of sodium channels inactivated from the closed state [13].

### 3.3. Orphenadrine binds to the local anesthetic receptor

Sodium channel block characteristics suggest that orphenadrine may bind to the local anesthetic receptor within the channel pore. In particular, the aromatic moiety and the charged amine of LAs were proposed to interact with two aromatic residues of the seg-

ment 6 of domain IV (Phe1764 and Tyr1771 in the rat Nav1.2 channel) through hydrophobic and  $\pi$ -cation interactions [39]. The phenylalanine residue appeared as the more important amino acid for inactivated channel block. This amino acid is conserved in the various mammalian voltage-dependent sodium channel subtypes (Fig. 4A). We engineered the corresponding F1586C mutation into the hNav1.4 template. The mutant expresses well in HEK cells and clones with permanent expression were obtained. Similarly to F1764A in Nav1.2, the F1586C mutation in hNav1.4 positively shifted the voltage dependence of channel availability by 7.9 mV (Table 1). The local anesthetic mexiletine was first tested on the F1586C mutant to verify whether this amino acid is critical for LA binding in hNav1.4 channels (Fig. 4B and D). The  $IC_{50}$  values for block of F1586C channels by mexiletine were  $1340 \mu M$  at 0.1 Hz and were  $1089 \mu M$  at 10 Hz, compared to the  $IC_{50}$  values of  $235 \mu M$  at 0.1 Hz and  $37 \mu M$  at 10 Hz for blocking wild-type (WT) hNav1.4 channels in similar conditions [12]. Thus, mexiletine was 6-fold (0.1 Hz) and 29-fold (10 Hz) less potent in blocking F1586C mutants than WT channels. Consequently, the  $IC_{50}$  value for F1586C blockade at 0.1 Hz was greater than the  $K_R$  of mexile-



**Fig. 4.** Binding of orphenadrine and mexiletine to the local anesthetic receptor. (A) Alignment of amino acidic sequence within the 6th segment of domain IV of the  $\alpha$  sodium channel subunit of rat neuronal (rNav1.2), human neuronal (hNav1.2), and human skeletal muscle (hNav1.4) channels. The phenylalanine residue, originally found in rNav1.2 to be critical for binding of local anesthetics, is conserved among sodium channel subtypes and among species. The corresponding F1586C mutation was introduced into the hNav1.4 template. (B and C) Representative traces of  $I_{Na}$  recorded in HEK293 cells expressing the F1586C hNav1.4 channel. Mexiletine and orphenadrine block was assessed 3 min after drug application by measuring the reduction of  $I_{Na}$  elicited with a 25-ms long test pulse from  $-120$  to  $-30$  mV at stimulation frequencies of 0.1 and 10 Hz. (D and E) Concentration–response curves for mexiletine and orphenadrine block were constructed at 0.1 and 10 Hz using the protocol described above, and fitted with Eq. (1). Each data point is the mean  $\pm$  SEM of at least three cells. For mexiletine, the calculated  $IC_{50}$  values  $\pm$  SE of the fit were  $1340 \pm 72$   $\mu$ M at 0.1 Hz and  $1089 \pm 181$   $\mu$ M at 10 Hz. The calculated slope factors  $nH \pm$  SE of the fit were  $1.1 \pm 0.1$  at 0.1 Hz and  $1.0 \pm 0.2$  at 10 Hz. For orphenadrine, the calculated  $IC_{50}$  and  $nH$  values are reported in Table 1.

tine for WT channels ( $\sim 800$   $\mu$ M, see ref. [12]), and the use dependence was almost zeroed by the mutation, confirming that the phenylalanine residue in position 1586 is critical for binding of LAs to inactivated channels. Importantly, the effects of orphenadrine on F1586C recapitulated those of mexiletine (Fig. 4C and E). At 0.1 Hz, 100  $\mu$ M orphenadrine was needed on F1586C currents to obtain an effect similar to 30  $\mu$ M on WT channels. The concentration–effect relationships of orphenadrine on F1586C sodium currents indicated a  $IC_{50}$  value of  $206 \pm 10$   $\mu$ M at 0.1 Hz and of  $127 \pm 14$   $\mu$ M at 10 Hz, which are about 2-fold and 10-fold greater than those measured for WT channels. As for mexiletine, the  $IC_{50}$  value for F1586C blockade was greater than the  $K_R$  for WT channels, and very little use-dependent block was observed on F1586C currents.

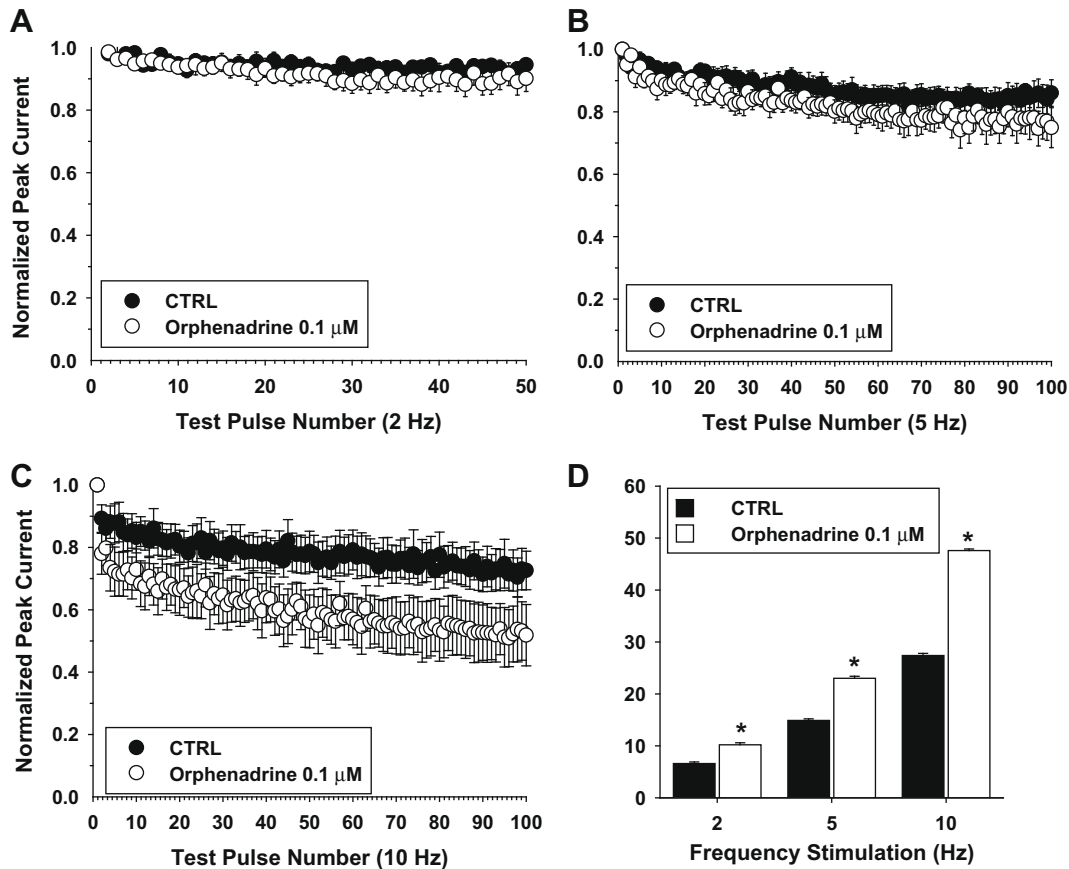
#### 3.4. Use-dependent block of hNav1.7 channels by clinical dose of orphenadrine

The serum concentrations of orphenadrine used clinically for analgesia are in the range of 0.1–0.4  $\mu$ g/ml, corresponding to 0.03–0.13  $\mu$ M [26]. Thus, we tested the effects of 0.1  $\mu$ M orphenadrine on hNav1.7 channels using various stimulation frequencies

and a holding potential of  $-90$  mV (Fig. 5). At this HP, significant use-dependent reduction of sodium currents was observed in the absence of drug at 5 and 10 Hz frequencies. However, the reduction of sodium currents was significantly accentuated in the presence of the low drug concentration. Steady-state reduction of peak sodium current was significantly greater with the drug compared to control condition at all the three stimulation frequencies tested (Fig. 5D).

#### 3.5. Effects of orphenadrine on TTX-resistant sodium currents in DRG neurons

Other than the tetrodotoxin-sensitive Nav1.7 channel isoform, the tetrodotoxin-resistant Nav1.8 and Nav1.9 subtypes are expressed in DRG neurons, and are involved in pain sensation [1,27,33,35,40]. Both these channels have proved very difficult to express in heterologous systems, thus we studied the effects of orphenadrine on TTX-resistant sodium currents in primary cultures of DRG neurons [36]. In sensory neurons, the Nav1.8 channels conduct a HVA sodium current that is mainly responsible for action potential rising phase. In contrast, the Nav1.9 channel is the molecular correlate of a persistent, LVA sodium current that is potenti-



**Fig. 5.** Use-dependent block of hNav1.7 channels by the clinically relevant 0.1 μM concentration of orphenadrine. (A–C) Sodium currents were elicited with a 12-ms long test pulse at –30 mV from the holding potential of –90 mV at 2, 5, or 10 Hz in control conditions (CTRL), then in the presence of 0.1 μM orphenadrine. The peak current amplitude was normalized with respect to the first test pulse peak current amplitude and plotted against the test pulse number. Each point is the mean ± SEM from five cells. (D) The last 10 amplitude values (pulse number 41–50 at 2 Hz and 91–100 at 5 and 10 Hz) were averaged to calculate the percentage of use-dependent inhibition at steady state. Paired Student’s *t*-test indicated significant differences (at least *P* < 0.01) between CTRL and drug at the three stimulation frequencies tested.

ated by inflammatory mediators, thereby increasing sensory neuron excitability during peripheral inflammation. Because of their peculiar voltage dependence, the two channels can be distinguished by using specific protocols.

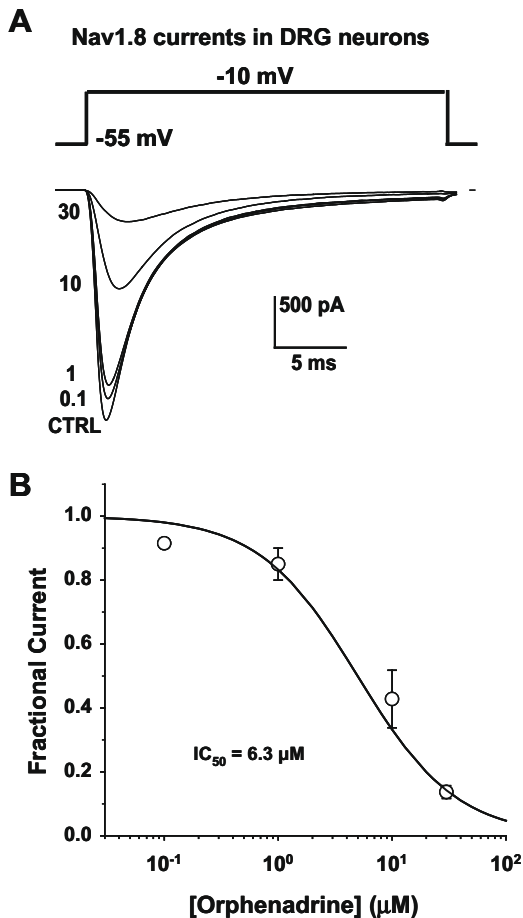
For recording of Nav1.8 currents, we used a depolarized holding potential (–55 mV) that pushes Nav1.9 channel into a slow-inactivated state. A depolarized HP also allows to record HVA currents of limited amplitude (1–5 nA) to improve voltage clamp. Representative examples of HVA currents are shown in Fig. 6. Please note that, as expected for Nav1.8 channels, HVA current activation and inactivation were slower with respect to sodium currents recorded in HEK cells. At 0.1 Hz stimulation, 0.1 μM orphenadrine had a small effect on current amplitude, whereas 1, 10 and 30 μM orphenadrine produced a significant reduction (Fig. 6A). The IC<sub>50</sub> value for inhibition of Nav1.8 channels in these conditions was 6.3 μM (Fig. 6B). We also studied the use-dependent inhibition of Nav1.8 currents by orphenadrine (Fig. 7). In the absence of drug, current amplitude is stable at 0.1 Hz stimulation, but is greatly reduced by increasing the stimulation frequency to 2 and 10 Hz. As previously observed in Fig. 6, using 0.1 Hz stimulation, orphenadrine produced little tonic block at 0.1 μM but a huge current inhibition at 10 μM. In addition, use-dependent reduction of sodium currents at 2 and 10 Hz was significantly greater in the presence of 0.1 and 10 μM of orphenadrine.

To record LVA currents, we used a holding potential of –100 mV and a test pulse at –60 mV. In the presence of

TTX to block TTX-sensitive sodium channels and La<sup>3+</sup> to block T-type calcium channels, the LVA currents are mainly supported by Nav1.9 channels [7]. The Nav1.9 channels are rather atypical with respect to other sodium channel subtypes, generating little-inactivating, persistent sodium currents at hyperpolarized potentials (Fig. 8). Using fluoride as the main intracellular anion, amplitude of the LVA current increased dramatically within 3–5 min after patch membrane rupture [8,34]. However, current amplitude ran down soon after, reaching a more stable low amplitude level, as shown in Fig. 8A. When applied on the maximally activated Nav1.9 current, orphenadrine (10 μM) reduced current amplitude by 29.4% (Fig. 8B). The drug effect was partly reversed upon washout, while currents were entering the run-down process. When applied on the residual current after run-down, orphenadrine reversibly decreased Nav1.9 currents by 28.7% (Fig. 8C). In average, the inhibition of Nav1.9 currents by 10 μM orphenadrine was 28.5 ± 0.9% (*n* = 5).

#### 4. Discussion

Orphenadrine is a drug acting on multiple targets, including histaminic, muscarinic, and NMDA receptors, as well as the noradrenaline reuptake system, although it shows lower affinity with respect to known specific ligands [24,37,41,46]. It was introduced into the market as a medication for Parkinson’s disease, providing control of symptoms when used as monotherapy, but its psycho-



**Fig. 6.** Tonic block of Nav1.8 channels by orphenadrine in rat DRG sensory neurons. (A) High-voltage activated (HVA) currents were recorded in primary culture of rat DRG neurons with whole-cell patch-clamp technique by depolarizing the cell to  $-10$  mV for 25 ms from a holding potential of  $-55$  mV every 10 s (frequency stimulation of 0.1 Hz). With tetrodotoxin in the bath solution, the HVA currents are supported mainly by the Nav1.8 sodium channel subtype. Current traces were recorded in a representative neuron before (CTRL) and after successive application of 0.1, 1, 10, and 30  $\mu$ M orphenadrine. (B) Concentration–response curve for tonic block of Nav1.8 channels by orphenadrine was constructed at 0.1 Hz using the protocol described in A and fitted with Eq. (1). Each data point is the mean  $\pm$  SEM from at least three DRG neurons. The calculated  $IC_{50}$  values  $\pm$  SE of the fit were  $6.3 \pm 1.4$   $\mu$ M, and the slope factor  $nH$  was  $0.97 \pm 0.19$ .

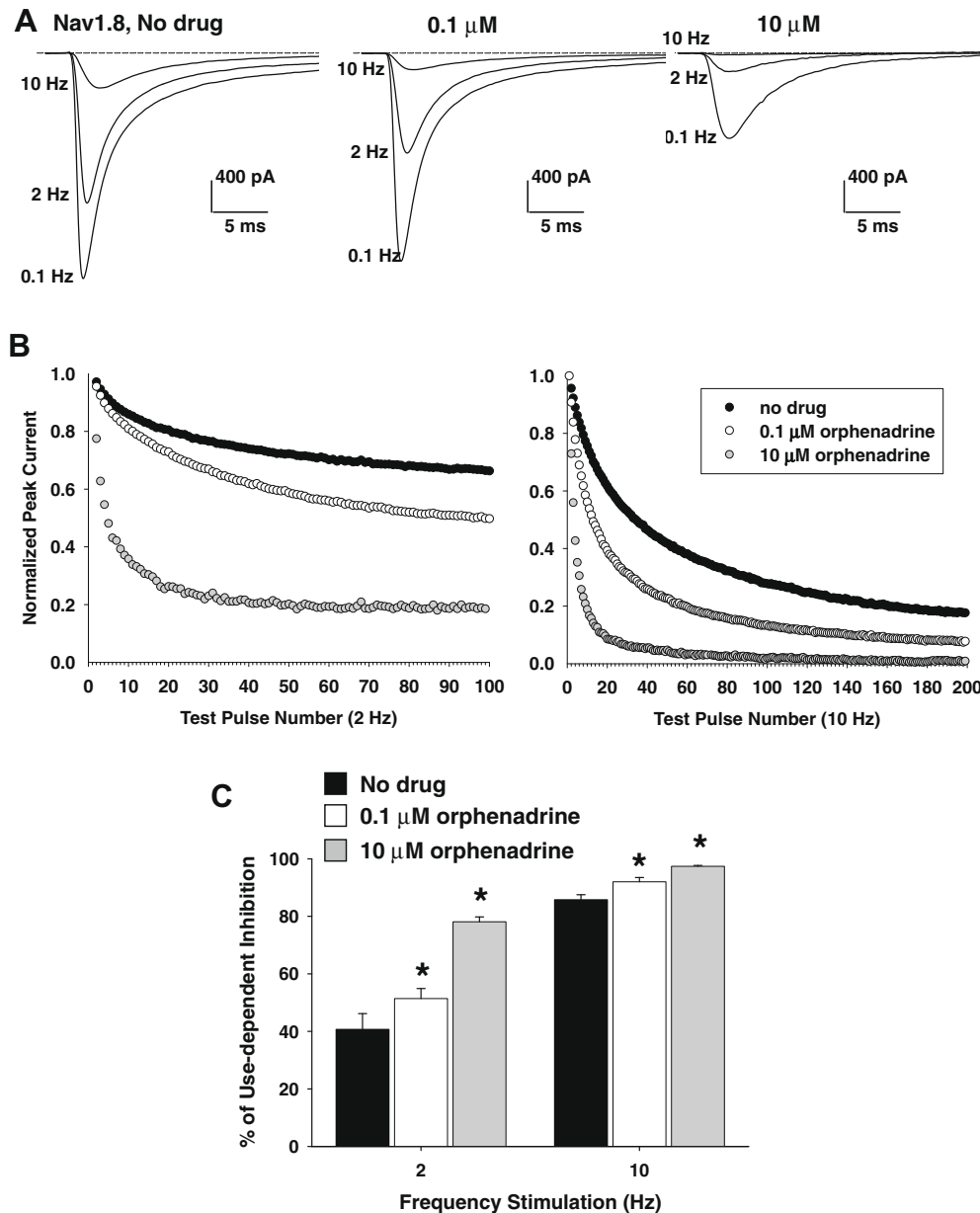
toxic, cognitive and autonomic adverse events may limit its use in some patients [29]. Randomized trials have also demonstrated muscle relaxing properties of orphenadrine without impairment of normal muscle tone or voluntary movements [6]. Orphenadrine is also used as analgesic either alone or in combination with paracetamol/acetaminophen [21]. The antinociceptive effect of orphenadrine was investigated in mice, suggesting that the drug may reduce different types of nociceptive transmissions. In humans, orphenadrine proved benefits with respect to placebo against shoulder, neck, and low back pain, as well as acute and chronic painful musculoskeletal conditions [21]. More recently, orphenadrine was able to exert an analgesic/anti-hyperalgesic effect in a human model of capsaicin-dependent inflammatory pain [42]. The exact molecular mechanism by which orphenadrine induces analgesia is still unknown. It is possible that antihistaminergic properties and NMDA-receptor inhibition may play a role for the observed analgesic effects. Our results strongly support the inhibition of voltage-gated sodium channels as a contributor to analgesic action of orphenadrine.

We demonstrate that orphenadrine blocks voltage-gated sodium channels with a mechanism analogous to a local anesthetic: effect is concentration, voltage, and frequency-dependent. Moreover, the F1586C mutation, located at a position putatively involved in LA binding, greatly reduces  $I_{Na}$  inhibition by orphenadrine and zeroes use-dependence, as it does for mexiletine. This result indicates that the phenylalanine is important for the binding of orphenadrine to inactivated sodium channels. The molecule of orphenadrine has a chemical structure similar to sodium channel blockers, like the local anesthetic lidocaine, the antiarrhythmic mexiletine, and the anticonvulsant phenytoin, which consists of an aromatic hydrophobic tail linked to a hydrophilic tertiary amine group by ester chain. Its  $pK_a$  ( $9.05 \pm 0.01$ ) and  $\log P$  ( $3.78 \pm 0.01$ ) values indicate that neutral form is highly lipophilic, while the charged form predominates at physiological pH (percentage of ionization is 97.8% at pH 7.4, as calculated with Handerson–Hasselbach equation). Voltage- and use-dependent block are related to different affinities for closed and inactivated channels. The  $K_R$  for orphenadrine is about 5-fold and 3-fold lower compared to those of mexiletine and flecainide, while the  $K_I$  is 3-fold and 9-fold reduced, respectively [12,13]. These results indicate that orphenadrine is a potent blocker of closed and inactivated sodium channels. On one hand, the superior lipophilia of orphenadrine may favor access of the drug to closed channels; on the other hand, the diphenyl structure would strengthen drug-channel interactions at the binding site, especially to the inactivated channel. Indeed, it has been found that other diphenyl compounds have a binding affinity to the inactivated sodium channels  $\sim 100$ -fold higher than to the resting channels [25].

The use-dependence block of sodium channels may contribute to the clinical efficacy of orphenadrine as analgesic compound. Indeed, it has been hypothesized for many years that voltage-gated sodium channels might play specialized roles in nociception and pain mechanisms [10]. It is clear from animal studies that Nav1.7, Nav1.8 and Nav1.9 all play important roles in inflammatory and neuropathic pain. Voltage-gated sodium channels in sensory neurons have been implicated in several chronic painful neuropathies that arise from peripheral nerve injury [27,40]. Human studies have shown that Nav1.7 is crucial for experiencing physiological pain sensations since gain-of-function mutations in the *SCN9A* gene encoding this channel subtype can result in severe chronic pain sensations [15]. Many types of pain syndromes appear to reflect neuronal hyperexcitability, so the use-dependent block of sodium channel is thought to be effective in the treatment of chronic pain [14]. We observed a significant use-dependent block of hNav1.7 channels at the hp of  $-90$  mV with the clinically relevant 0.1  $\mu$ M concentration of orphenadrine, suggesting that clinical doses may produce a very significant block of high-frequency action potential firing in depolarized, physiologic or pathologic conditions. We also demonstrated that low doses of orphenadrine block tetrodotoxin-resistant sodium channels in DRG sensory neurons, which are conducted by Nav1.8 and Nav1.9 channel subtypes. Because orphenadrine blocks both tetrodotoxin-sensitive (Nav1.1, Nav1.4, and Nav1.7) and tetrodotoxin-resistant (Nav1.5; Nav1.8, Nav1.9) channel subtypes, it is quite probable that the block of the entire cohort of sodium channels expressed in sensory neurons may contribute to its analgesic action.

On the other hand, blood concentrations of orphenadrine greater than 0.5  $\mu$ g/ml ( $\sim 0.2$   $\mu$ M) may cause toxic reactions [28]. Such toxic effects are frequently observed because orphenadrine is widely available, and the drug can be deliberately abused for its analgesic, stimulating, and euphoriant effects, as well as for suicide purpose [18,38]. Besides anticholinergic side effects, orphenadrine can produce both central and peripheral





**Fig. 7.** Use-dependent block of Nav1.8 channels by orphenadrine in rat DRG sensory neurons. (A) HVA sodium currents were recorded in a representative rat DRG neuron as in Fig. 6 using 0.1, 2, and 10 Hz stimulation frequency before and after successive applications of 0.1 and 10 μM orphenadrine. (B) Time course of use-dependent reduction of Nav1.8 currents in the same neuron as in A at 2 (left) and 10 (right) Hz stimulation frequency before and after successive applications of 0.1 and 10 μM orphenadrine. (C) Mean ± SEM percentage of use-dependent Nav1.8 current reduction measured in four DRG neurons before and after successive applications of 0.1 and 10 μM orphenadrine. The symbol \* indicates significant difference (at least  $P < 0.05$ ) with respect to control condition (no drug) calculated with paired Student's *t*-test.

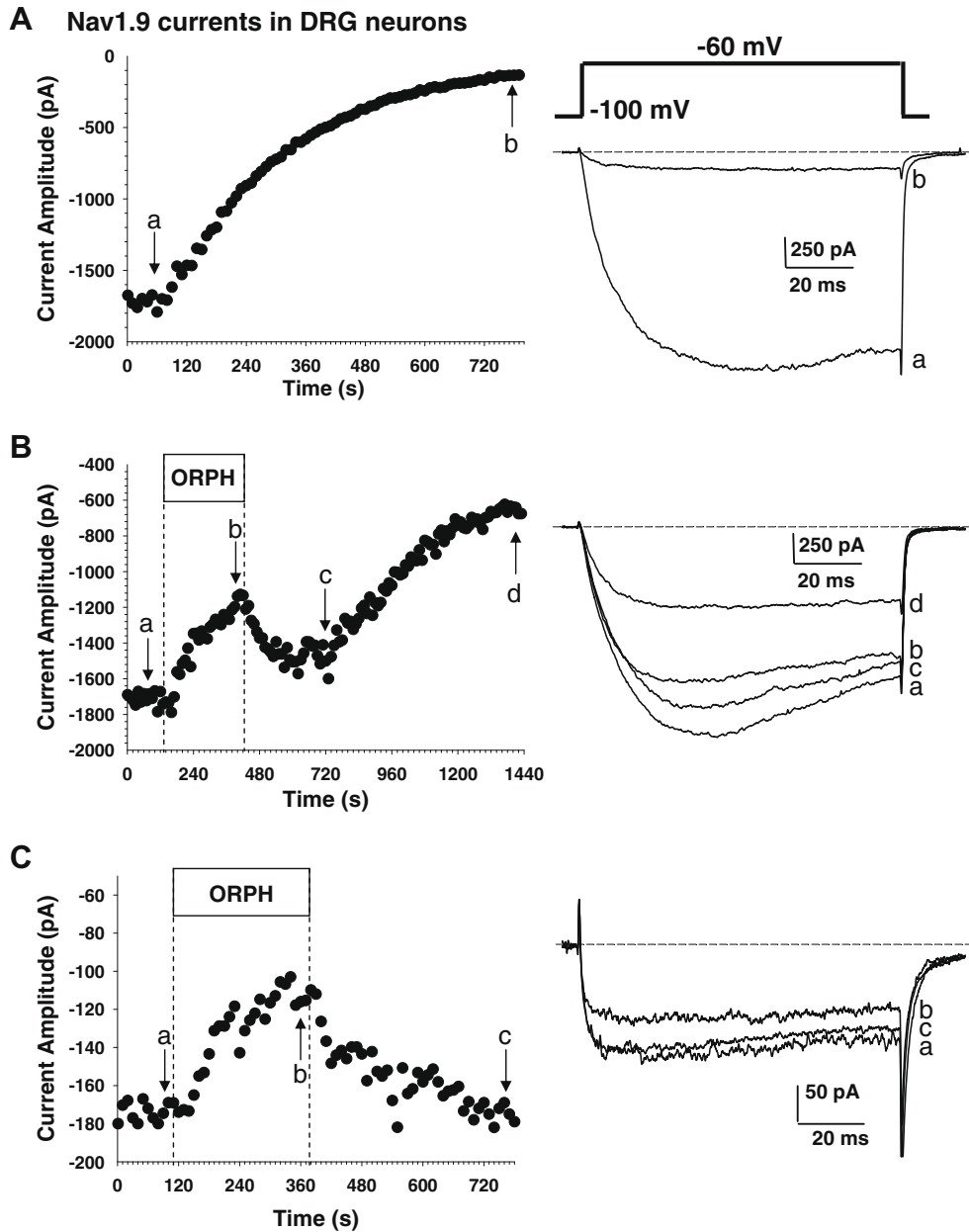
adverse reactions, including generalized tonic-clonic seizures and life-threatening arrhythmias [11,16]. Low dose of orphenadrine was also shown to precipitate long QT and Torsades-de-Pointes tachycardia in a patient with congenital long QT syndrome [32]. The recent studies suggest that cardiac and neuronal toxicities may be linked to the action of orphenadrine on HERG channels, which contribute to the action potential repolarization phase in the heart and to spike-frequency accommodation in the nervous system [43,47]. Our results show that orphenadrine inhibits the sodium channel subtypes expressed in heart and central neurons. Although sodium channel blockers may be used as antiarrhythmics and anticonvulsants for their use-dependent mechanism of action, exaggerated inhibition of sodium currents by high orphenadrine doses would induce

proarrhythmic and proconvulsive effects, especially because the drug displays a relatively high affinity to resting channels.

In conclusion, our study shows that orphenadrine blocks different subtypes of voltage-gated sodium channels at clinically relevant doses, including the Nav1.7, Nav1.8 and Nav1.9 channel subtypes that are primarily involved in nociception. These results indicate a new mechanism likely contributing to its analgesic effect. On the other hand, the inhibition of sodium channels in various tissues may be a source for toxic reactions.

**Conflict of interest**

The authors declare no conflict of interest.



**Fig. 8.** Effects of orphenadrine on Nav1.9 channels in rat DRG sensory neurons. (A) Low-voltage activated (LVA) currents were recorded in primary culture of rat DRG neurons with whole-cell patch-clamp technique by depolarizing the cell to  $-60$  mV for 100 ms from a holding potential of  $-100$  mV every 10 s (frequency stimulation of 0.1 Hz). With tetrodotoxin and  $\text{La}^{3+}$  in the bath solution, the LVA currents are conducted mainly by the Nav1.9 sodium channel subtype. (A) Representative time course of the characteristic run-down of Nav1.9 currents recorded with intracellular fluoride in a DRG neuron. Right panel shows current traces extracted at time a (maximum activity) and b (after run-down). (B) Time course of current amplitude when  $10 \mu\text{M}$  orphenadrine was applied on the maximally activated Nav1.9 currents in a representative DRG neuron. Right panel shows current traces extracted at time a (maximum Nav1.9 current activation), b (during application of orphenadrine), c (under drug washout), and d (after run-down). (C) Time course of current amplitude when  $10 \mu\text{M}$  orphenadrine was applied on Nav1.9 currents after run-down in a representative DRG neuron. Right panel shows current traces extracted at time a (after run-down), b (during application of  $10 \mu\text{M}$  orphenadrine), and c (under drug washout).

## Acknowledgements

We are grateful to Prof. Enzo Wanke (Milan) for the gift of hNav1.7 plasmid. We thank Dr. Claudio Bruno (Bari) for measurement of orphenadrine logP and  $\text{pK}_a$ , and Jizhe Hao (Marseille) for help with primary culture of DRG neurons. This study was supported by Telethon-Italy (Grant GGP04140 to D.C.C.).

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