

Block of Neuronal Na⁺ Channels by Antidepressant Duloxetine in a State-dependent Manner

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ABSTRACT

Background: Duloxetine is a mixed serotonin–norepinephrine reuptake inhibitor used for major depressive disorder. Duloxetine is also beneficial for patients with diabetic peripheral neuropathy and with fibromyalgia, but how it works remains unclear.

Methods: We used the whole cell, patch clamp technique to test whether duloxetine interacts with the neuronal Nav1.7 Na⁺ channel as a potential target. Resting and inactivated Nav1.7 Na⁺ channel block by duloxetine were measured by conventional pulse protocols in transfected human embryonic kidney cells. The open-channel block was determined directly using inactivation-deficient mutant Nav1.7 Na⁺ channels.

Results: The 50% inhibitory concentration (IC₅₀) of duloxetine for the resting and inactivated wild-type hNav1.7 Na⁺ channel were 22.1 ± 0.4 and 1.79 ± 0.10 μM, respectively (mean ± SE, n = 5). The IC₅₀ for the open Na⁺ channel was 0.25 ± 0.02 μM (n = 5), as determined by the block of persistent late Nav1.7 Na⁺ currents. Similar open-channel block by duloxetine was found in the muscle Nav1.4 isoform (IC₅₀ = 0.51 ± 0.05 μM; n = 5). Block by duloxetine appeared *via* the conserved local anesthetic receptor as determined by site-directed mutagenesis. Finally, duloxetine elicited strong use-dependent block of neuronal transient Nav1.7 Na⁺ currents during repetitive stimulations.

Conclusions: Duloxetine blocks persistent late Nav1.7 Na⁺ currents preferentially, which may in part account for its analgesic action.

What We Already Know about This Topic

- ❖ Duloxetine, a mixed serotonin–norepinephrine reuptake inhibitor, is indicated to treat chronic pain, but its analgesic mechanisms are unclear.

What This Article Tells Us That Is New

- ❖ Duloxetine blocks persistent late Na⁺ currents preferentially, and this may participate in its analgesic effects.

CONVENTIONAL antidepressants exert diverse pharmacological actions in four broad categories: (1) norepinephrine-reuptake inhibitors, (2) selective serotonin-reuptake inhibitors, (3) atypical antidepressants, and (4) monoamine oxidase inhibitors.¹ Duloxetine (Cymbalta®; Eli Lilly and Company, Indianapolis, IN) belongs to a new type of antidepressant classified as mixed serotonin/norepinephrine-reuptake inhibitors. This relatively novel antidepressant was approved by the U.S. Food and Drug Administration in 2004 to treat major depressive disorder and generalized anxiety disorder. Duloxetine has also been used successfully to treat patients with neuropathic pain syndromes such as painful diabetic peripheral neuropathy and fibromyalgia.^{2,3} Although the exact mechanisms in humans are unknown, both antidepressant activities and pain inhibitory properties of duloxetine are believed to be related to its potentiation of serotonergic and noradrenergic activity in the central nervous system.⁴

Other traditional antidepressants, including amitriptyline, have been prescribed for decades to patients with pain syndromes.⁵ One potential target for the analgesic action of amitriptyline is the voltage-gated Na⁺ channel responsible for the generation of action potentials.^{6–9} Whether the antidepressant duloxetine also interacts with the Na⁺ channel remains uncertain because the chemical structure of duloxetine does not resemble that of the tricyclic antidepressant (TCA) amitriptyline or the local anesthetic (LA) lidocaine (fig. 1). If duloxetine indeed also targets the Na⁺ channel, such a result will definitely help us understand how the antidepressant duloxetine works as a nonselective analgesic for

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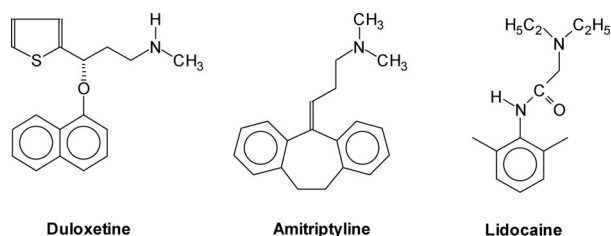


Fig. 1. Chemical structures of duloxetine, amitriptyline, and lidocaine. Antidepressant duloxetine contains a secondary amine, a thiophene ring, and a naphthylene moiety. Antidepressant amitriptyline is a tricyclic antidepressant drug with a tertiary amine. Local anesthetic lidocaine is an amide-containing drug with a tertiary amine and a phenyl ring. The hydrophobicity expressed as logP of the octanol/water partition coefficient of the molecule is 1.81 (lidocaine), 5.1 (amitriptyline), and 4.72 (duloxetine).

chronic pain. This in turn will provide crucial information for the future design of novel pain therapeutics with multiple molecular targets. A literature search on PubMed found no peer-reviewed studies regarding the action of duloxetine on Na^+ channels.

There are a total of nine α -subunit Na^+ channel isoforms (Nav1.1–1.9) found among mammalian excitable tissues, including the central nervous system, peripheral nervous system, muscle, and heart.^{10,11} The Na^+ channel protein contains four homologous domains (D1–D4), each with six transmembrane segments (S1–S6). Upon depolarization, Na^+ channels go through rapid transitions from the resting to the open state and then to the inactivated state.¹² The open state of the Na^+ channel in general has a very brief open time of ~ 0.5 ms. Interactions between local anesthetics and Na^+ channels are highly state-dependent, as indicated by a modulated receptor hypothesis.¹³ Seven of nine Na^+ channel isoforms are considered neuronal types and are expressed mainly in the nervous system. With respect to nociception, Nav1.7 is of special interest among Na^+ channel isoforms. For example, it is well known that a loss of function of neuronal Nav1.7 Na^+ channels in humans could render people unable to experience pain.¹⁴ It is noteworthy that missense mutations of the human (h) Nav1.7 isoform could cause inherited painful channelopathies, such as primary erythralgia and paroxysmal extreme pain disorder.^{15,16} This is particularly true when the hNav1.7 mutations instigate small but devastating persistent late Na^+ currents as a result of minor defects in fast Na^+ channel inactivation (*i.e.*, gain of function).¹⁷ A recent article likewise suggests that fibromyalgia may be due to Nav1.7 channelopathies in dorsal root ganglion neurons, because this channel is poised as a molecular gatekeeper of pain detection at peripheral nociceptors.¹⁸ We therefore set out to test whether the Nav1.7 Na^+ channel is sensitive to duloxetine block. To a lesser extent, we also included muscle Nav1.4 Na^+ channel isoform in this study for comparison because its receptor site for local anesthetics has been well established.¹⁹ We first sought to determine whether duloxetine blocks neuronal resting, open, and inactivated Na^+ channels in a state-dependent manner. In addition,

we tried to find out whether duloxetine targets a common LA receptor within the inner cavity of Na^+ channels. Lastly, we sought to determine whether duloxetine, like traditional LAs, can elicit strong use-dependent block during repetitive pulses.

Materials and Methods

Cultures of HEK293 Cells Stably Expressing Na^+ Channels. Human embryonic kidney (HEK) 293 cells were maintained at 37°C in a 5% CO_2 incubator in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (HyClone, Logan, UT), 1% penicillin and streptomycin solution (Sigma, St. Louis, MO), 3 mM taurine, and 25 mM HEPES (Invitrogen). HEK293 cell lines heterologously expressing rat rNav1.4 wild-type Na^+ channels and rat (r) inactivation-deficient Nav1.4 Na^+ channels (rNav1.4-L435W/L437C/A438W, abbreviated as WCW mutant; *i.e.*, mutation at position Leu₄₃₅Leu₄₃₇Ala₄₃₈ \rightarrow Trp₄₃₅Cys₄₃₇Trp₄₃₈) were reestablished from frozen vials as described previously.²⁰ We define the term “inactivation-deficient Na^+ channels” to mean mutant Na^+ channels with a substantial defect in fast inactivation. Other inactivation-deficient Na^+ channel mutants (*e.g.*, Ile/Phe/Met \rightarrow Gln/Gln/Gln at the domain D3–D4 linker) have been used to study open-channel blockers in *Xenopus laevis* oocytes,²¹ but such mutant channels do not express well in mammalian HEK293 cells.²²

Transient Transfection of HEK293 Cells with Wild-type Na^+ Channels and Inactivation-deficient Na^+ Channels with WCW Mutations. Human hNav1.7 clone²³ was used to create an inactivation-deficient hNav1.7-WCW homologous mutant by triple mutations at L396W/L398C/A399W positions. Previous investigations indicated that WCW mutations do not significantly affect the local anesthetic binding affinity toward the open Na^+ channel.²⁴ For example, the open-state affinity of mexiletine in the WCW persistent late Na^+ currents is comparable with those generated by sea anemone α -toxin or by chemical modifications. Both of these treatments will cause wild-type Na^+ channels to generate persistent late Na^+ currents, thereby indicating that mexiletine block of the open Na^+ channels is not dependent on the specific treatments used. These results suggest that such a WCW mutant is suitable for the detailed study of drugs that target the LA receptor within the open Na^+ channel. An additional S6 mutant channel (rNav1.4-WCW-F1579K) in the rNav1.4-WCW clone was generated by site-directed mutagenesis as described previously.²⁰ This specific residue in rNav1.4 channels (Phe1579) is located in the middle of S6 segments and is known to be critical for binding with LAs.^{25,26} Our attempts to create a homologous rNav1.4-F1579K mutant in the hNav1.7-WCW backbone were so far unsuccessful. Various gain-of-function mutations that caused Nav1.7 channelopathies were not attempted because these mutants carry persistent late Na^+ currents too small to be studied in detail.

HEK293t cells were grown to ~50% confluence and transfected by calcium phosphate precipitation.²⁷ Transient transfection of wild-type and mutant Na⁺ channel clones (5–10 μ g) along with β 1-plasmid (10–20 μ g) and reporter CD8-plasmid (1 μ g) was adequate for current recording. The r β 1 or h β 1 subunit was included in channel expression as described previously.²⁸ Preliminary experiments indicated that coexpression of the β 1 subunit did not affect binding affinities of duloxetine. Cells were replated 15 h after transfection in 35-mm dishes, maintained at 37°C in a 5% CO₂ incubator, and used after 1–4 days. Transfection-positive cells were identified with CD8-immunobeads bound on the cell surface under an inverted microscope (diameter, 4–5 μ m; Dynabeads; Invitrogen).

Solutions and Chemicals. Cells were perfused with an extracellular solution containing 65 mM NaCl, 85 mM choline chloride, 2 mM CaCl₂, and 10 mM HEPES (titrated to pH 7.4 with tetramethylammonium-OH). The pipette (intracellular) solution consisted of 100 mM NaF, 30 mM NaCl, 10 mM EGTA, and 10 mM HEPES (titrated to pH 7.2 with cesium-OH). In some experiments, we reduced the Na⁺ concentration to 13 mM in the pipette solution (substituting NaF and NaCl with CsF) to measure directly the block of inward Na⁺ currents by duloxetine. Duloxetine-HCl was purchased from AK Scientific, Inc. (Mountain View, CA). The drug was dissolved in dimethyl sulfoxide at 100 mM and stored at 4°C. Final duloxetine concentrations up to 30 μ M were made by serial dilution. Dimethyl sulfoxide (up to 1%) in the bath solution had little effect on Na⁺ currents.

Electrophysiology, Data Acquisition, and Statistics. The whole cell configuration of a patch clamp technique²⁹ was used to record Na⁺ currents in HEK293 cells at room temperature (22 \pm 2°C). Electrode resistance ranged between 0.5 and 1.0 M Ω . Command voltages were elicited with pCLAMP9 software and delivered by Axopatch 200B (Molecular Devices, Inc., Sunnyvale, CA). Cells were held at –140 mV and dialyzed for 10 to 15 min before current recording. This was necessary because we found that drugs such as LAs could enhance and bind preferentially with the inactivated state at holding potentials of approximately –120 mV.³⁰ The capacitance and leak currents were cancelled with the patch clamp device and by P/–4 subtraction. Peak currents at +30 mV were 2–20 nA for the majority of cells. Access resistance was 1–2 M Ω under the whole cell configuration; series resistance compensation of more than 85% usually resulted in voltage errors of up to 3 mV at +30 mV. Concentration-response studies were typically performed at +30 or +50 mV for the outward Na⁺ currents. Such recordings allowed us to avoid the complication of series resistance artifacts and to circumvent inward Na⁺ ion loading.³¹ Curve fitting was performed by Origin software (OriginLab Corp, Northampton, MA) with a Hill equation for concentration-response studies or with a single exponential function for the use-dependent studies. An unpaired Student *t* test was used to evaluate estimated parameters

(mean \pm SEM or fitted value \pm SE of the fit); *P* values of less than 0.05 were considered statistically significant.

Results

Resting versus Inactivated Block of Na⁺ Channels by Duloxetine in HEK293T Cells Expressing hNav1.7 and rNav1.4 Isoforms. We used the same pulse protocols for Nav1.7 and Nav1.4 isoforms because the activation and steady-state inactivation parameters are comparable in these two isoforms; *i.e.*, activation threshold is near –40 to –50 mV and the 50% steady-state inactivation occurs near –60 to –70 mV.^{23,28} Figure 2A shows that duloxetine at 3 μ M blocked approximately 5% of peak hNav1.7 Na⁺ currents at +30 mV when the cell was held at –140 mV (see figure 2, inset, for pulse protocol). This block of peak Na⁺ currents was defined as the resting block of Na⁺ channels at –140 mV holding potential before Na⁺ channel activation at the test pulse.³² However, if the cell was first depolarized to –70 mV for 10 s at a conditioning pulse (fig. 2, inset), the level of block was significantly enhanced; ~70% of peak Na⁺ currents were inhibited as measured by a brief test pulse (fig. 2B). Such a pulse protocol was used to measure the level of drug binding with inactivated Na⁺ channels directly.³⁰ This type of block by drug was defined as inactivated block. An interpulse (–140 mV for 50 ms) was inserted between the conditioning pulse and the test pulse (inset). More block occurs with this pulse protocol because most drug-bound inactivated Na⁺ channels do not recover during the interpulse, whereas the drug-free inactivated channels recover readily during this period (control trace). Block of rNav1.4 Na⁺ currents by duloxetine was comparable with hNav1.7 counterparts, as shown in figure 2C, for the resting block when the cell was held at –140 mV or for the inactivated block when the cell was applied with a conditioning voltage of –70 mV for 10 s (fig. 2D).

For concentration-response curves, we measured the block of peak Na⁺ currents at various duloxetine concentrations ranging from 0.1 to 30 μ M, normalized with the control peak currents, plotted against concentrations, and fitted with a Hill equation. Figure 3 shows such concentration-response curves for hNav1.7 Na⁺ channels. The 50% inhibitory concentrations (IC₅₀) and the Hill coefficients for hNav1.7 Na⁺ channels were obtained from curve fitting and are listed in table 1. The IC₅₀ for the resting block of hNav1.7 Na⁺ channels was measured 22.1 \pm 0.4 μ M, whereas the IC₅₀ for the inactivated block was 1.79 \pm 0.10 μ M (*n* = 5). Our data clearly show that the inactivated state of hNav1.7 Na⁺ channels has a lower IC₅₀ value than the resting counterpart (*P* < 0.001), with a ratio of 12.3.

Open-channel Block of Persistent Late hNav1.7 Na⁺ Currents by Duloxetine. Once activated, voltage-gated Na⁺ channels have a very brief open time (~0.5 ms) because of fast inactivation (*e.g.*, fig. 2).¹² A number of diseased Nav1.7 channels generated persistent late Na⁺ currents, but these currents are generally small for the investigation of the

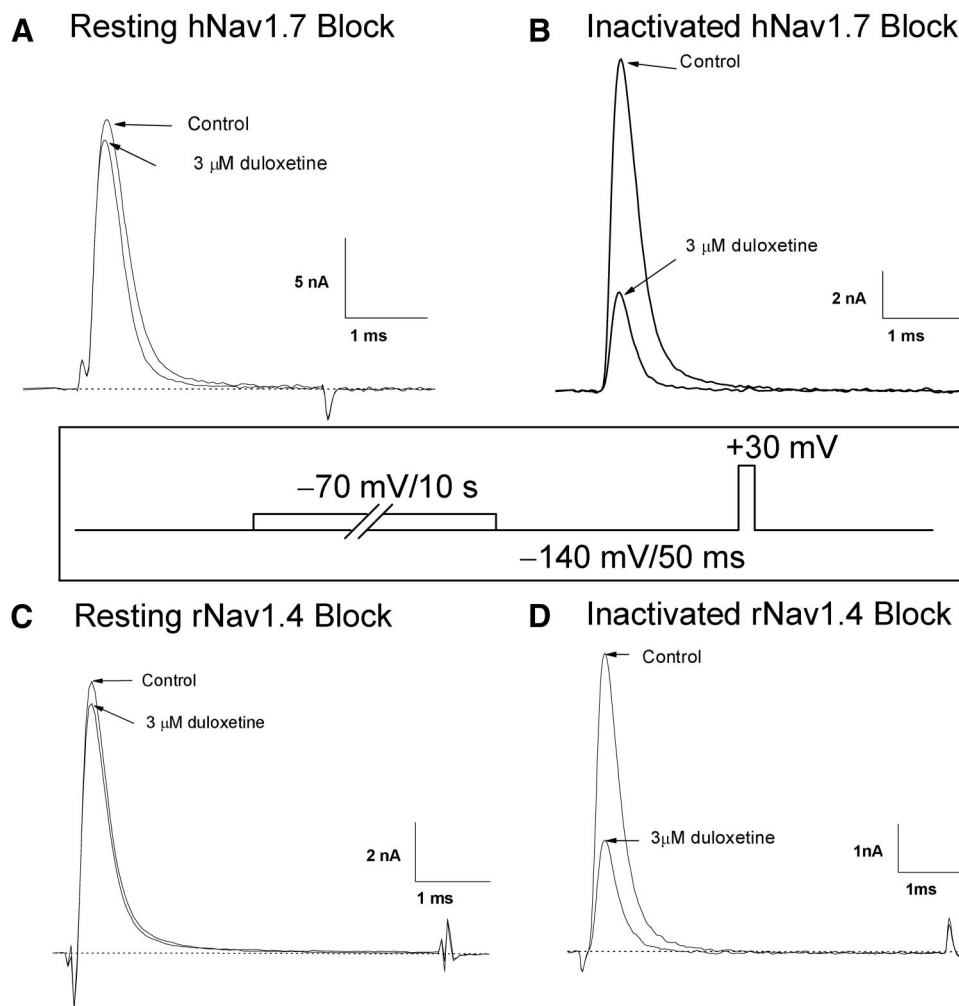


Fig. 2. Resting and inactivated block of Na^+ channels by duloxetine in human embryonic kidney cells expressing neuronal Nav1.7 and Nav1.4 isoforms. (A) Traces of wild-type hNav1.7 currents shown illustrate the resting block by $3 \mu\text{M}$ duloxetine at the holding potential of -140 mV , whereas traces shown (B) show the inactivated block by $3 \mu\text{M}$ duloxetine after a conditioning pulse of -70 mV for 10 s . Pulse protocols are shown in the inset. Currents were evoked every 30 s by a 5-ms test pulse at $+30 \text{ mV}$ before and after duloxetine wash-in. Block of peak current by duloxetine reached its steady state generally within $3\text{--}5 \text{ min}$, and this block was readily reversible after drug wash-out, usually within 5 min . (C) Traces of wild-type rNav1.4 currents shown illustrate resting block by $3 \mu\text{M}$ duloxetine, whereas traces shown on D show inactivated block by $3 \mu\text{M}$ duloxetine. Current traces in four panels were recorded from separate cells. h = human.

drug effects.^{16,33} To study the open Na^+ channel block by duloxetine directly, we therefore created fast inactivation-deficient hNav1.7 mutant Na^+ channels homologous to those used to study the open-channel block by various clinic drugs earlier.^{20,34}

Figure 4A shows the open-channel blocking phenotype of inactivation-deficient hNav1.7-WCW Na^+ currents at various concentrations of duloxetine ranging from 0.1 to $30 \mu\text{M}$. Without drugs (fig. 4A, trace 0), the current traces show that a significant portion of Na^+ currents are maintained at the end of 100-ms pulse. With the drug treatment, the higher the drug concentrations, the greater the block of the persistent late Na^+ currents. Because a further increase in pulse duration will induce slow inactivation of inactivation-deficient Na^+ channels, the reduction in late Na^+ currents should be considered as approximations for steady-state block of open

Na^+ channels. Concentration-response curves were constructed for the block of the peak (closed circle) and persistent late currents (open circle) by duloxetine as shown in figure 4B. Curve fitting was performed by using the Hill equation; the corresponding IC_{50} values and Hill coefficients are listed in table 1. Evidently, duloxetine has the highest potency toward the open Na^+ channels, with an IC_{50} value of $0.25 \pm 0.02 \mu\text{M}$ ($n = 5$) and a Hill coefficient of 1.24 ± 0.10 . Further kinetic analyses of the time-dependent block at various duloxetine concentrations (*e.g.*, fig. 4A) revealed that the decaying time courses were concentration-dependent and were best fitted by a single exponential function. The plot of $1/\tau$ versus concentration shown in figure 4C yields a linear correlation with an intercept (corresponding to off-rate, k_{-1}), $y = 49.5 \text{ s}^{-1}$, and a slope (corresponding to on-rate, k_1) of $129.1 \text{ s}^{-1} \mu\text{M}^{-1}$. The calculated equilibrium dis-

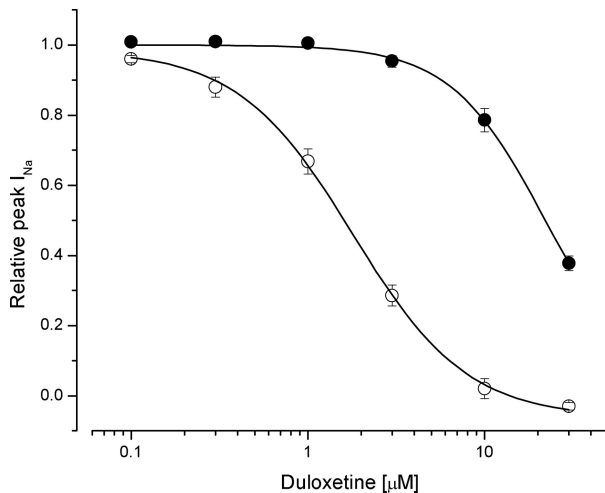


Fig. 3. Concentration-response curve of resting and inactivated block of wild-type Nav1.7 Na⁺ channels by duloxetine. Relative potency of duloxetine of the resting and inactivated Na⁺ channels in neuronal hNav1.7 isoform (● and ○, respectively) was compared. Data were taken from experiments at various drug concentrations with pulse protocols described in Fig. 2. Data were then fitted with a Hill equation, $y = 1/[1 + (x/IC_{50})^{n_H}]$; values of the IC₅₀ (50% inhibitory concentration) and the Hill coefficient (n_H) are listed in table 1. h = human.

sociation constant K_D (i.e., k_{-1}/k_1) gives a value of 0.38 μM, which is close to the IC₅₀ value obtained from the concentration-response curve.

For peak current block, the IC₅₀ value was estimated to be 7.5 ± 1.4 μM ($n = 5$) with a Hill coefficient of 0.77 ± 0.12 (fig. 4B; table 1). It is not clear why IC₅₀ values for the resting block of duloxetine in wild-type hNav1.7 Na⁺ channels are greater than those in inactivation-deficient hNav1.7 Na⁺ channels (22.1 vs. 7.5 μM, respectively). One explanation is

that we may underestimate the peak amplitude in inactivation-deficient rNav1.4 Na⁺ channels. This could take place if significant block of Na⁺ channels occurs during channel opening when fast inactivation is hampered.²⁰ However, we could not rule out the possibility that the resting block is greater in inactivation-deficient hNav1.7Na⁺ channels because of nonspecific mutational effects.

To investigate whether inward Na⁺ currents were blocked differently from outward Na⁺ currents, we reduced the Na⁺ concentration from 130 to 13 mM in the pipette solution. Inward currents were generated by a −20-mV pulse for 100 ms and recorded. Under these conditions, the concentration-response curve for duloxetine yielded an IC₅₀ of 0.33 ± 0.05 μM ($n = 5$), which was comparable with that measured at +50 mV for the outward currents (fig. 4B; $P > 0.05$). This result also suggested that the open-channel block by duloxetine is not voltage-dependent. The lack of significant difference in IC₅₀ values between the outward and inward Na⁺ currents was further confirmed by a reversed ramp protocol (from +100 mV to −140 mV in 200 ms). Both outward and inward currents were blocked equally between −50 to +100 mV in the presence of 1 μM duloxetine (data not shown).

A Common Duloxetine Binding Site within Na⁺ Channel Isoforms? For comparison, we also examined duloxetine block of inactivation-deficient rNav1.4-WCW mutant channels. An IC₅₀ value of 0.51 ± 0.05 μM ($n = 5$) for the open block of rNav1.4-WCW mutant channels was obtained from the concentration-response curve (fig. 5A, squares; table 1). This value is approximately twice that of hNav1.7-WCW mutant channels (0.25 ± 0.02 μM, $n = 5$; $P < 0.05$), indicating that duloxetine may be isoform-selective, albeit modestly.

Table 1. Estimated Values of IC₅₀ and Hill Coefficient in Resting, Open, and Inactivated Na⁺ Channels Heterologously Expressed in HEK293T Cells

Na ⁺ Channel Isoforms	IC ₅₀ of Duloxetine			
	Resting Block (Peak Current)	Open Block (Late Na ⁺ Current)	Inactivated Block	Ratio
hNav1.7 Wild-type	22.1 ± 0.4 [1.62 ± 0.05]	N.A.	1.79 ± 0.10 [1.34 ± 0.10]	12.3
hNav1.7-WCW Mutant	7.5 ± 1.4 [0.77 ± 0.12]	0.25 ± 0.02 [1.24 ± 0.10]	N.A.	30.0
rNav1.4 Wild-type	20.6 ± 0.5 [1.96 ± 0.08]	N.A.	2.24 ± 0.09 [1.90 ± 0.13]	9.2
rNav1.4-WCW Mutant	17.5 ± 0.9 [1.31 ± 0.09]	0.51 ± 0.05 [1.34 ± 0.12]	N.A.	34.3
rNav1.4-WCW/F1579K Mutant	25.1 ± 1.0 [1.61 ± 0.11]	6.19 ± 0.15 [1.86 ± 0.07]	N.A.	4.1

Values are presented as mean ± SE (micromolar and [Hill coefficient]). In cells expressing wild-type Na⁺ channels, currents were evoked by a 5-ms test pulse at +30 mV. In cells expressing Nav1.7-WCW, Nav1.4-WCW, and Nav1.4-WCW/F1579K mutant channels, Na⁺ currents were evoked by a 100-ms test pulse at +30 mV. Values were derived as described in figures 3, 4B, and 5A, with $n = 5$ (for hNav1.7 isoform) or 6 (rNav1.4 isoform). The ratios of IC₅₀ (50% inhibitory concentration) values for the resting over the open or the inactivated state of corresponding Na⁺ channels are listed. Note that block of transient peak currents may be overestimated because it may include the open-channel block during channel activation, particularly at high concentrations. As a result, Hill coefficient may also deviate from unity predicted for a single drug receptor. WCW represents mutations at L396W/L398C/A399W positions. For both Na⁺ channel isoforms, IC₅₀ values for resting block are significantly larger than those for inactivated block, which in turn are significantly larger than those for the open block ($P < 0.05$).

h = human; HEK = human embryonic kidney; N.A. = not applicable; r = rat.

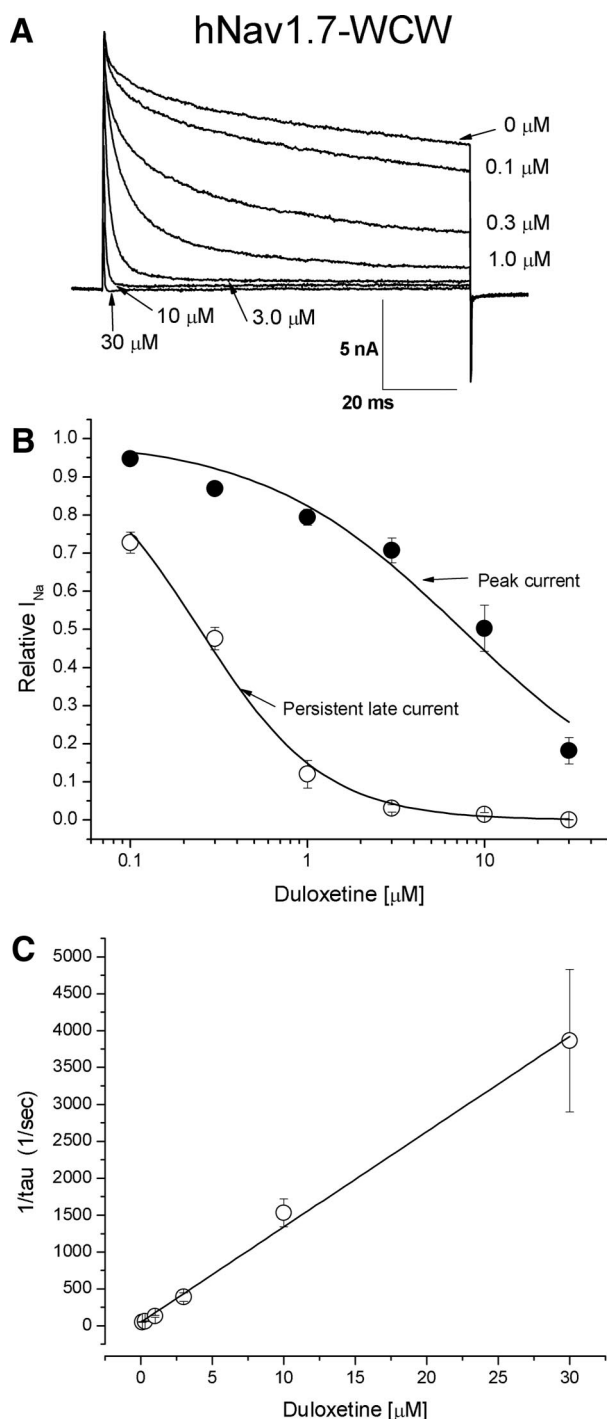


Fig. 4. Open-channel block of persistent late hNav1.7-WCW mutant Na^+ currents by duloxetine. (A) Currents of inactivation-deficient mutant channels were evoked by a 100-ms test pulse at +30 mV. Current traces of hNav1.7-WCW channels were recorded before and after the application of duloxetine at various concentrations. Traces are labeled with corresponding duloxetine concentrations. (B) Concentration-response curves of peak and persistent late currents in the presence of various drug concentrations were constructed. Peak and late currents (near the end of 100-ms pulse) were measured, normalized to the control trace without drug, plotted against the duloxetine concentration, and fitted with a Hill equation. The resting-channel IC_{50} (peak current block) and the Hill coefficient (in parentheses) was 7.5 ± 1.4 (0.77 ± 0.12) and the

The block of voltage-gated Nav1.4 and Nav1.7 Na^+ channels by duloxetine is clearly state-dependent (table 1) and is comparable with those found previously by LAs³² or TCAs.³⁴ Both LAs and TCAs are known to target a specific receptor within the Na^+ channel α -subunit. The LA/TCA receptor in the Na^+ channel includes a critical phenylalanine residue, Phe1579, in the rNav1.4 isoform.²⁶ We therefore sought to determine whether Phe1579 is involved in binding of duloxetine. Figure 5B shows the block of inactivation-deficient rNav1.4-F1579K Na^+ currents at various concentrations of duloxetine. The F1579K mutation significantly reduced the potency of duloxetine as an open-channel blocker. Concentration-response curves were constructed, and the IC_{50} values and Hill coefficients were obtained by curve fitting (fig. 5A; circles) and are listed in table 1. These data illustrate that the duloxetine potency toward the open rNav1.4-WCW/F1579K Na^+ channels is reduced, with an IC_{50} value of $6.19 \mu M$, compared with the value of $0.51 \mu M$ without this Phe1579 mutation. Introduction of a positive charge at position Phe1579 by lysine substitution therefore diminishes the duloxetine binding by a factor of 12.1, suggesting that Phe1579 forms a part of the common receptor for duloxetine, LAs, and TCAs.^{26,35} In addition, the reduction in the resting block as measured with the peak current amplitude is comparably less with a factor of 1.43 (25.1 vs. $17.5 \mu M$, table 1). This phenomenon of lesser reduction on the resting block is similar to that of F1579K mutation found for LAs and TCAs.

Use-Dependent Block of Neuronal hNav1.7 Na^+ Channels by Duloxetine. Most LAs and TCAs are capable of eliciting use-dependent block of Na^+ currents. We therefore determined whether duloxetine displays a similar use-dependent phenotype in hNav1.7 channels during repetitive pulses. We found that duloxetine indeed elicited strong use-dependent block of neuronal hNav1.7 Na^+ currents. Figure 6A shows that duloxetine at $10 \mu M$ produced rapid use-dependent block of peak hNav1.7 Na^+ currents when stimulated at 5 Hz. A total of 20 pulses were applied; each pulse depolarized the cell to +50 mV for 20 ms. Figure 6B shows the time course of the use-dependent block of the peak Na^+ currents by duloxetine that reached a steady-state block of $89.7 \pm 0.3\%$ ($n = 6$).

We then investigated the role of the pulse duration on the use-dependent block by varying the duration from 0.5 to 20 ms. Figure 7A shows that repetitive pulses, even with a du-

open-channel block IC_{50} (late current block) was 0.25 ± 0.02 (1.24 ± 0.10) ($n = 5$). (C) The decaying phases of the Na^+ currents in the presence of various duloxetine HCl concentrations, as shown in A, were each normalized to the control ($0 \mu M$) and the resulting trace was fitted by a single exponential function. The normalization was applied to remove the slow -decaying component found in the control. The inverse of the time constant, τ , was finally plotted against the corresponding duloxetine concentration (y intercept: $49.5 s^{-1}$; slope: $129 s^{-1} \mu M^{-1}$; linear correlation coefficient, $r = 0.996$). h = human; IC_{50} = 50% inhibitory concentration.

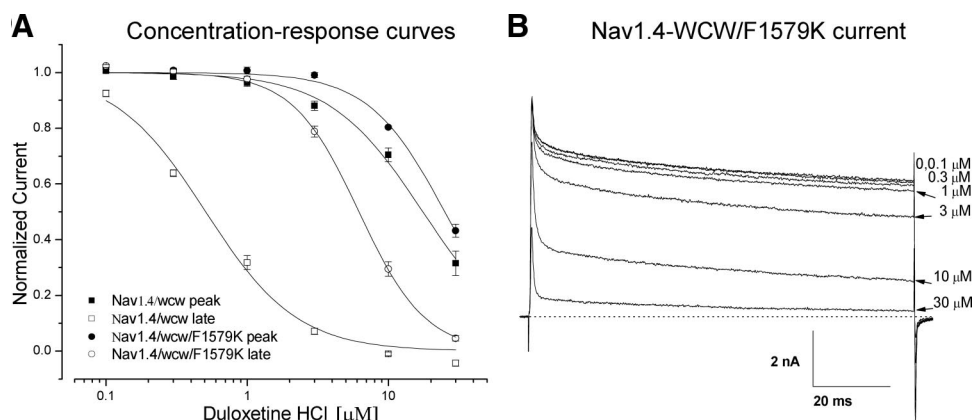


Fig. 5. Block of rNav1.4-WCW and rNav1.4-WCW/F1579K mutant Na⁺ channels in the presence of duloxetine. (A) Concentration-response curves of rNav1.4-WCW channels were determined in a manner as described in Fig. 4B. The peak currents (■) and maintained rNav1.4-WCW Na⁺ currents near the end of the 100-ms pulse (□) were measured, normalized with respect to the control current, and plotted against the corresponding duloxetine concentration ($n = 5$). The fitted IC_{50} (50% inhibitory concentration) values and Hill coefficients are listed in table 1. (B) Superimposed representative rNav1.4-WCW/F1579K Na⁺ current traces were recorded before and after application with various concentrations of duloxetine. Currents were evoked by a 100-ms test pulse at +30 mV. The concentration-response curve shows that the persistent late rNav1.4-WCW/F1579K Na⁺ currents were much less sensitive to drug block compared with their rNav1.4-WCW counterparts shown in Fig. 5A (○ vs. □) ($n = 5$). The fitted IC_{50} values and the Hill coefficient are listed in table 1. $r = \text{rat}$.

ration as short as 1 ms, elicit a nearly maximal use-dependent block of Na⁺ currents by 10 μM duloxetine compared with the duration of 4 ms shown in figure 7B. The level of the use-dependent block of Na⁺ currents was measured after repetitive pulses with different pulse durations, and plotted against the pulse number. In the absence of duloxetine, repetitive pulses with a duration as long as 20 ms produced no reduction of the peak currents (fig. 7C, ►). In the presence of 10 μM duloxetine, significant use-dependent block was detected with a pulse duration of 0.5 ms. Maximal use-dependent block was achieved for a pulse duration as short as ~ 1 ms and remained relatively constant for durations up to 20 ms (fig. 7C). For a 1-ms repetitive pulse protocol, the total depolarization time is 20 ms, whereas for a 20-ms protocol, the total depolariza-

tion time is 400 ms. This result implies that duloxetine blocked open hNav1.7 Na⁺ channels preferentially during repetitive pulses. The inactivated state of hNav1.7 Na⁺ channels did not seem to play a significant role in this use-dependent phenomenon; otherwise, longer pulse duration should enhance duloxetine binding and generate a larger use-dependent block. It is noteworthy that at a reduced concentration of 0.3 μM , we observed no significant use-dependent block, suggesting that normal open Na⁺ channels with a brief open time are rather resistant to duloxetine block near its therapeutic plasma concentration.

Recovery from Use-dependent Block of hNav1.7 Na⁺ Channels versus Recovery from Open-channel Block of hNav1.7 Na⁺ Channels. Recovery from the use-dependent block of hNav1.7 Na⁺ channels was measured at various

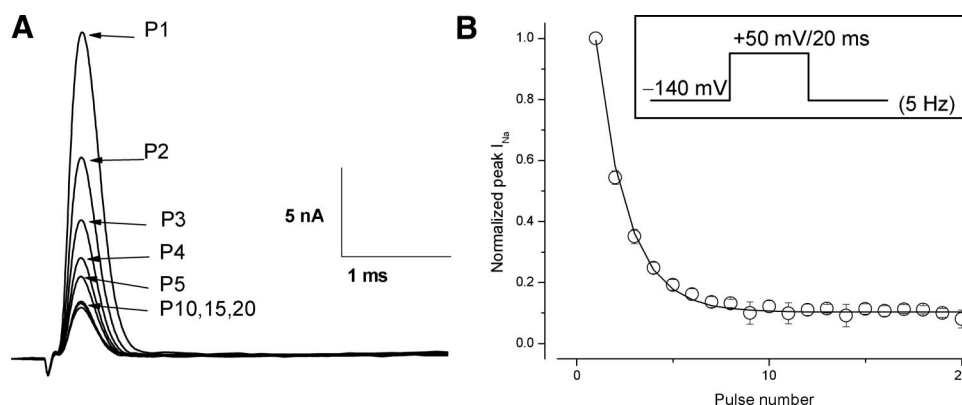


Fig. 6. Use-dependent block of wild-type hNav1.7 Na⁺ currents by 10 μM duloxetine in human embryonic kidney cells. (A) Superimposed current traces correspond to pulse 1, 2, 3, 4, 5, 10, 15, and 20. Pulses (+50 mV for 20 ms) were applied repetitively at 5 Hz in the presence of 10 μM duloxetine. Peak currents as shown in A were measured, normalized with respect to the peak amplitude at pulse 1, and plotted against the corresponding pulse number (B). The pulse protocol is shown in the inset. The solid line is the best fit of a single exponential function with a time constant (τ) of 1.61 ± 0.07 pulse for duloxetine ($n = 6$). h = human.

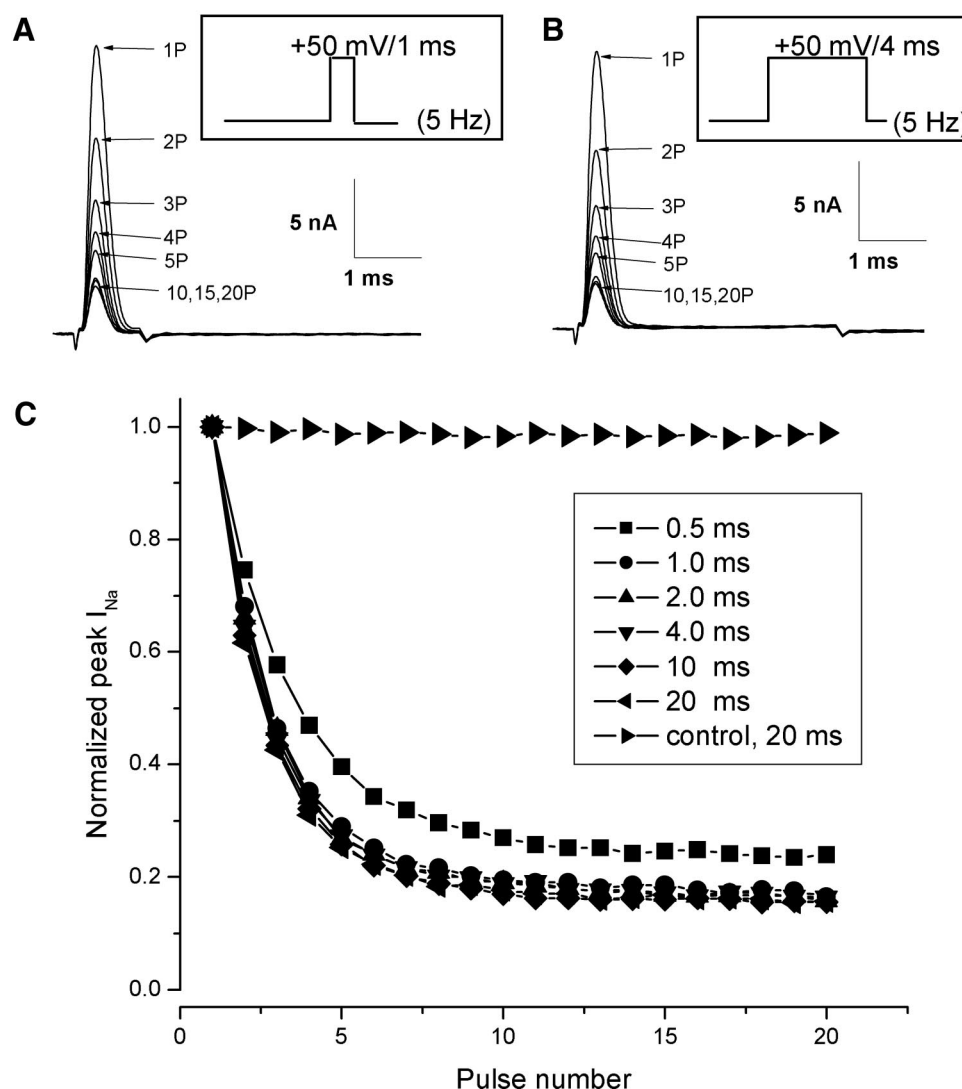


Fig. 7. Use-dependent block *versus* pulse duration: Repetitive pulses were applied in the presence of 10 μM duloxetine as described in Fig. 6, except that the pulse duration was shortened to 1 ms (A, inset) or 4 ms (B, inset). Superimposed current traces correspond to pulses 1, 2, 3, 4, 5, 10, 15, and 20. Peak currents under various pulse durations were measured during repetitive pulses, normalized with respect to the peak amplitude at pulse 1, and plotted against the corresponding number (C). Durations as short as 0.5 ms elicited significant use-dependent block; nearly maximal block was achieved with a duration of 1 ms. Notice that without the drug, no use-dependent block was evident with a pulse duration up to 20 ms (\blacktriangleright).

time intervals after repetitive pulses. Figure 8A shows the time course of recovery from the use-dependent block after 20 repetitive pulses (+50 mV for 20 ms at 5 Hz). Peak Na^+ currents recovered with a time constant of 5.7 ± 0.1 s ($n = 8$) in the presence of 10 μM duloxetine. Without the drug, there was no use-dependent block of peak currents during repetitive pulses (fig. 7C, \blacktriangleright).

Recovery time course from the open-channel block of inactivation-deficient hNav1.7-WCW currents in the presence of 3 μM duloxetine was measured by a two-pulse protocol. A conditioning pulse of +50 mV with a duration of 100 ms was applied first, followed by an interpulse of -140 mV with variable time intervals. A second brief test pulse of +30 mV was then employed to generate the Na^+ currents. Figure 8B shows the time courses of recovery at various intervals

after the conditioning pulse. In the absence of duloxetine, most peak Na^+ currents (more than 85%) recovered rapidly within 100 ms (open circles). In the presence of 3 μM duloxetine, peak currents recovered slowly with a time constant of 5.3 ± 0.3 s ($n = 5$). This value is comparable with the recovery time constant of the use-dependent block induced by duloxetine (fig. 8A), suggesting that the recovery of use-dependent block follows the recovery time course of open-channel block.

Discussion

We have examined the state-dependent block of neuronal Nav1.7 and muscle Nav1.4 Na^+ channels induced by antidepressant duloxetine. Our results show, for the first time, that this mixed serotonin/norepinephrine reuptake inhibitor blocks the open state of Na^+ channels preferentially over the

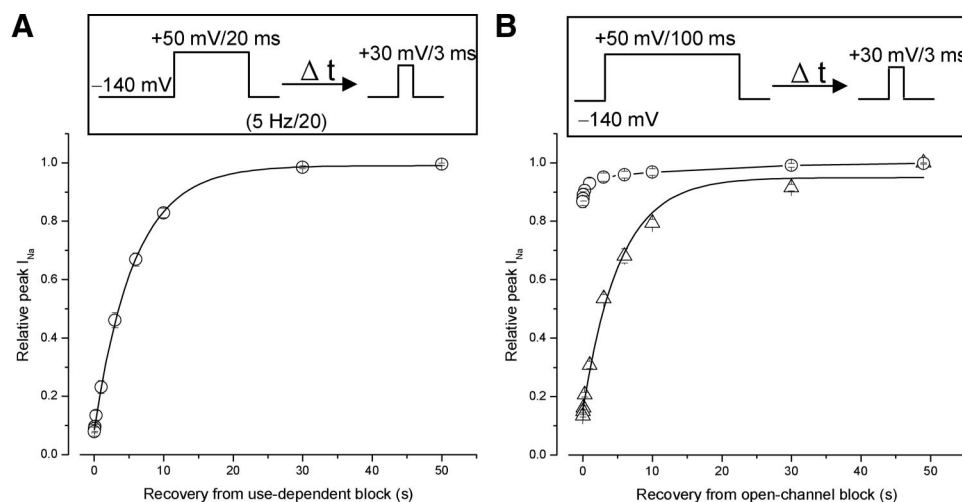


Fig. 8. Recovery from use-dependent block of wild-type hNav1.7 Na⁺ currents versus from open-channel block of hNav1.7-WCW persistent late Na⁺ currents. (A) Recovery time courses for the use-dependent block of hNav1.7 wild type were measured by a brief test pulse (+30 mV for 3 ms) at various time intervals after 20 repetitive pulses (+50 mV for 20 ms at 5 Hz; inset) with 10 μ M duloxetine. Peak currents were determined, normalized with respect to the peak amplitude measured without repetitive pulses, and plotted against the time interval. Data ($n = 8$) were best fitted by a single exponential function with a time constant of 5.7 ± 0.1 s. (B) Recovery time courses from the open-channel block induced by 3 μ M duloxetine were measured by a brief test pulse (+30 mV for 3 ms; inset) at various time intervals after a conditioning pulse of +50 mV for 100 ms (Δ). The data were fitted by a single exponential function with a time constant of 5.3 ± 0.3 s ($n = 5$). Without duloxetine, most peak Na⁺ currents (more than 85%) recovered rapidly within 100 ms (\circ). h = human.

resting and inactivated counterparts. In a recent meeting, Rolland *et al.*³⁶ described their biophysical characterization of duloxetine activity on voltage-gated Na⁺ channels involved in pain transmission. The authors found that the IC₅₀ value for Nav1.7 is 16 μ M at a holding potential of -100 mV. They also found a preference of duloxetine for the inactivated state of Na⁺ channels. These findings are consistent with data shown in table 1. However, the potent Nav1.7 open Na⁺ channel block by duloxetine was not addressed in their studies. Like traditional local anesthetics, duloxetine can also elicit strong use-dependent block of wild-type Nav1.7 Na⁺ currents during repetitive pulses. The duloxetine binding site within the Na⁺ channel seems to overlap the known LA/TCA receptor. The significance and implications of these findings are discussed next.

Duloxetine Blocks hNav1.7 Na⁺ Channels in a Highly State-dependent Manner. Block of hNav1.7 Na⁺ channels by duloxetine is highly state-dependent. The IC₅₀ values for hNav1.7 Na⁺ channels follow the order of open (0.25 μ M; 1 \times) < inactivated (1.79 μ M; $\sim 7\times$) < resting states (22.1 μ M; $\sim 88\times$) (table 1). Duloxetine shows comparably weak resting and modest inactivated affinities toward the muscle rNav1.4 channel (~ 20 and ~ 2 μ M, respectively; table 1). However, the IC₅₀ values for the open-channel block of duloxetine were at the submicromolar level for both neuronal hNav1.7 and muscle rNav1.4 Na⁺ channels, demonstrating that the open state of these isoforms is especially sensitive to duloxetine block. It is noteworthy that duloxetine seems more potent toward hNav1.7 open channels (IC₅₀ = 0.25 μ M) than toward rNav1.4 counterparts (0.51 μ M; $P < 0.05$),

suggesting a modest isoform-selective action of duloxetine for the open configuration.

Despite its high potency toward persistent late hNav1.7 Na⁺ currents, we observed no significant block of wild-type transient peak Na⁺ currents at 0.3 μ M duloxetine (fig. 3, closed circle), signifying that normal open Na⁺ channels with intact fast inactivation are rather resistant to duloxetine block near its therapeutic plasma concentration range (0.09–0.3 μ M). The fast inactivation of Na⁺ channels seems to play a protective role in duloxetine block. This could happen if the fast inactivation keeps the open state of Na⁺ channels for less than 1 ms and thereby prevents the brief open channel from duloxetine block. In this report, we used the fast inactivation-deficient hNav1.7-WCW and rNav1.4-WCW mutant Na⁺ channels to measure directly the open-channel block by duloxetine, an approach used previously to study the TCA block of open Na⁺ channels.³⁴ The pharmacological actions of duloxetine on Na⁺ channels are indeed very similar to those of TCAs, which elicit strong use-dependent block of wild-type Na⁺ currents and have the highest affinities toward the open state of Na⁺ channels. However, the IC₅₀ ratio of inactivated versus open states for amitriptyline is only approximately two-fold (0.51 vs. 0.26 μ M), much less than the seven-fold difference found for duloxetine (1.79 vs. 0.25 μ M). In this respect, duloxetine acts more like an open Na⁺ channel blocker such as flecainide or ranolazine, described previously in transfected HEK293 cells.^{27,37}

Duloxetine Targets the LA/TCA Receptor in Na⁺ Channels.

Because duloxetine, TCAs, and LAs act so similarly, we tested the idea that all these structurally distinct drugs (fig. 1) perhaps target the same receptor in Na⁺ channels. This no-

tion was strongly supported by the result from receptor mapping. We found that rNav1.4-WCW/F1579K persistent late Na^+ currents were quite insensitive to duloxetine block, contrary to that found with rNav1.4-WCW counterparts (fig. 5). The increase in IC_{50} measured approximately 12-fold (table 1, $P < 0.001$). The magnitude of such increase agrees with results reported earlier for LAs, in which the block was generally reduced by a factor of 10 or more when the residue F (phenylalanine) at the LA receptor was mutated to alanine or lysine.^{26,35} With respect to the lysine substitution at Phe1579, the reduction in drug binding is probably due to the charge-charge repulsion introduced by the protonated lysine side chain and cationic duloxetine (*i.e.*, protonated form of duloxetine). Accordingly, the binding site for duloxetine may be near the cation binding site within the inner cavity found in K^+ and Na^+ channels.^{19,38} This cation binding site is critical for Na^+ permeation within the inner cavity, and the occupation of this site by duloxetine would explain the block of Na^+ permeation. In any event, our finding is thus consistent with the notion that the LA receptor site accommodates all three distinct types of drugs (duloxetine, amitriptyline, and lidocaine) during Na^+ channel opening, probably through electrostatic interaction between the cationic drug and π electrons at Phe1579.²⁶ Additional receptor mapping studies for duloxetine will be required to confirm and to delineate other contact points of the duloxetine receptor. Finally, it is noteworthy that this common receptor has been considered vulnerable to drug overdose,³⁵ including drugs such as LAs, TCAs, anticonvulsants, class 1 antiarrhythmics, and antiepileptics. Therefore, adverse side effects in terms of the use-dependent and inactivated Na^+ channel block during duloxetine overdose should not be overlooked.

Clinical Significance of Open Na^+ Channel Block by Duloxetine? The use of duloxetine as an analgesic agent has proven beneficial for patients with painful diabetic peripheral neuropathy and fibromyalgia.³⁹ How duloxetine works as an analgesic agent for neuropathic pain, however, remains largely unclear. To date, studies attempting to define the plasma levels of duloxetine that are associated with clinical improvement of depression and neuropathic pain have not been firmly established. Nonetheless, average minimum and maximum steady-state plasma concentrations for a daily oral dose of 60 mg duloxetine are measured at 27.0 and 89.5 ng/ml (*i.e.*, 0.09 and 0.30 μM), respectively.[§] The listed IC_{50} value measured for duloxetine block of the open hNav1.7 Na^+ channels (0.25 μM ; table 1) is within this estimated plasma concentration range. Our results thus support a working hypothesis that, in addition to the potentiation of serotonergic and noradrenergic activity in the central nervous system, duloxetine may target persistent late neuronal Na^+ currents for its efficacy in neuropathic pain. This hypothesis is consistent with the fact that persistent late neuro-

nal Nav1.7 Na^+ currents, even those small in size, can elicit abnormal impulses and cause painful channelopathies.^{16,17} Furthermore, a recent report also suggested that fibromyalgia pain in patients may be due to Nav1.7 gain-of-function channelopathies in dorsal root ganglion neurons.¹⁸ Such a suggestion seems to be in agreement with the clinical efficacy of duloxetine for fibromyalgia and the block of persistent late Nav1.7 Na^+ currents by duloxetine described here. This blocking action of antidepressant duloxetine on persistent late Na^+ currents could work in concert with its other pharmacological actions on central and peripheral pain pathways. Duloxetine may therefore act as an analgesic agent through a number of molecular targets, and this “polypharmacology” is probably therapeutically essential.⁴⁰ Although not yet tested, it may be sensible to determine in the near future whether duloxetine will be beneficial for patients with Nav1.7 channelopathies such as primary erythromalgia and paroxysmal extreme pain disorder.

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