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Fluoxetine Blocks $Na_v1.5$ Channels Via a Mechanism Similar to That of Class 1 Antiarrhythmics

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Abbreviations: CNS, central nervous system; DAT, dopamine transporter; HP, holding potential; IC50, dose producing 50% maximum current inhibition; OCD, obsessive compulsive disorders; MAT, monoamine transporter; NET, norepinephrine transporter; SCD, sudden cardiac death; SERT, serotonin transporter; SSRI, selective serotonin re-uptake inhibitor

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Abstract

The voltage-gated $Na_v1.5$ channel is essential for the propagation of action potentials in the heart. Malfunctions of this channel are known to cause hereditary diseases. It is a prime target for class 1 antiarrhythmic drugs and a number of antidepressants. The purpose of the present study was to investigate the $Na_v1.5$ blocking properties of fluoxetine, a selective serotonin re-uptake inhibitor. $Na_v1.5$ channels were expressed in HEK-293 cells, and Na^+ currents were recorded using the patch-clamp technique. Dose-response curves of racemic fluoxetine (IC_{50} = 39 μM) and its optical isomers had similar IC_{50} (40 and 47 μM for the (+) and (–) isomer, respectively). Norfluoxetine, a fluoxetine metabolite, had a higher affinity than fluoxetine, with an IC_{50} of 29 μM. Fluoxetine inhibited currents in a frequency-dependent manner, shifted steady-state inactivation to more hyperpolarized potentials, and slowed the recovery of $Na_v1.5$ from inactivation. Mutating a phenylalanine (F1760) and a tyrosine (Y1767) in DIVS6, significantly reduced the affinity of fluoxetine and its frequency-dependent inhibition. We used a non-inactivating $Na_v1.5$ mutant to show that fluoxetine displays open-channel block behavior. The molecular model of fluoxetine in $Na_v1.5$ was in agreement with mutational experiments, in which F1760 and Y1767 were found to be the key residues in binding fluoxetine. We concluded that fluoxetine blocks $Na_v1.5$ by binding to the class 1 antiarrhythmic site. The blocking of cardiac Na^+ channels should be taken into consideration when prescribing fluoxetine alone, or in association with other drugs that may be cardiotoxic or for patients with conduction disorders.

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Introduction

Fluoxetine (Prozac) is a selective serotonin re-uptake inhibitor (SSRI) (Wong *et al.*, 1995) that is widely prescribed for the treatment of Central Nervous System (CNS)-linked cognitive, emotional, and behavioral disorders. Since its discovery in 1974 (Wong *et al.*, 1974), the beneficial psychotropic effects of fluoxetine have led to its being used to treat disorders other than depression, including obsessive compulsive disorders (OCD) and bulimia nervosa (Wong *et al.*, 1995). The multiple side effects of fluoxetine (Sghendo and Mifsud, 2012) have raised questions about its supposed selective 5-HT-mediated effect. While fluoxetine inhibits serotonin transporter (SERT) in the low nanomolar range (Torres *et al.*, 2003), its therapeutic effect appears only at a much higher plasma and brain concentrations (Muscettola *et al.*, 1978; Bolo *et al.*, 2000). At low micromolar concentrations, fluoxetine also targets other proteins and inhibits several types of ion channels and receptors, including the nicotinic acetylcholine receptor (Hennings *et al.*, 1999; Eisensamer *et al.*, 2003), voltage-gated Ca^{2+} channels (Deák *et al.*, 2000; Pacher *et al.*, 2000), volume-regulated anion channels (Maertens *et al.*, 2002), neuronal Na^{+} channels (Lenkey *et al.*, 2006), and hERG, a cardiac K^{+} channel (Thomas *et al.*, 2002). The inhibition of the hERG K^{+} channel by fluoxetine occurs via two different mechanisms: (i) direct channel blockade and (ii) disruption of channel protein trafficking (Rajamani *et al.*, 2006). This may explain some of the cardiovascular side effects observed during chronic fluoxetine treatments, including bradycardia and QT prolongation (Pacher and Kecskemeti, 2008; Timour *et al.*, 2012). Dysfunctions of $Na_v1.5$, which are responsible for the rapid upstroke of the action potential caused by the rapid entry of Na^{+} ions into cardiomyocytes, also lead to arrhythmia complications. The

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prolongation of QT intervals may be due to the improper inactivation of the $Na_v1.5$ as in Romano-Ward syndrome (LQT3), while the reduction of Na^+ currents through $Na_v1.5$ may lead to arrhythmias such as Brugada syndrome (Herbert and Chahine, 2006). The major cause of the higher mortality rate in psychiatric patients *versus* the general population is sudden cardiac death (SCD), which mainly results from arrhythmias that occur during treatments with psychotropic drugs. It has been reported that fluoxetine decreases the maximum rate of rise of the depolarization phase (V_{max}) of ventricular cell preparations (Pacher *et al.*, 2000; Magyar *et al.*, 2003), but little is known about the direct effect of fluoxetine on the biophysical properties of $Na_v1.5$.

In the present study, we investigated the electrophysical properties of fluoxetine (racemic and enantiomers) and its metabolite norfluoxetine, as well as other psychotropic drugs, on $Na_v1.5$ stably expressed in HEK-293 cells. We showed that racemic fluoxetine, its metabolite norfluoxetine, and its enantiomers act as potential antagonists of human $Na_v1.5$ unlike the other classes of antidepressants tested.

We also studied the effect of the F1760C and Y1767C mutations of the class I antiarrhythmic binding site on the use-dependent blockade of cardiac Na^+ channels by fluoxetine and showed that fluoxetine behaves like a class I antiarrhythmic drug.

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Materials and Methods

Cell cultures

Human embryonic kidney (HEK-293) cells stably expressing human $Na_v1.5$ were used as previously described (Huang *et al.*, 2011). In brief, the cells were grown under standard tissue culture conditions (5% CO_2, 37°C) in high-glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin, and 10 mg/ml of streptomycin (Gibco-BRL Life Technologies, Burlington, ON). For experiments with the F1760C, Y1767C, and L409C/A410W mutants, the HEK-293 cells were transiently transfected with the pcDNA3.1 vector containing mutant $Na_v1.5$ cDNA (5 μg) or with the empty vector pIRES/CD8 (5 μg) in 10 cm petri dishes using the calcium phosphate method as previously described (Huang *et al.*, 2011). Transfected cells were briefly preincubated with CD8 antibody-coated beads (Dynabeads M450 CD8-a, (Life Technologies Inc., Burlington, ON, Canada) before recording currents. HEK-293 cells expressing the pIRES/CD8 vector were decorated with CD8 beads, which were used to identify cells for recording Na^+ currents.

Whole-cell patch-clamp recordings

Macroscopic Na^+ currents from HEK-293 cells were recorded using the whole-cell patch-clamp technique. Patch-clamp recordings were obtained using low-resistance, fire-polished electrodes (<1 MΩ) made from 8161 Corning borosilicate glass coated with Sylgard (Dow-Corning, Midland, MI) to minimize electrode capacitance. Currents were recorded using an Axopatch 200 amplifier with the pClamp software (Molecular Devices Sunnyvale, CA). The series resistance was 70-80% compensated. Whole-cell currents

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were filtered at 5 kHz, digitized at 10 kHz, and stored on a microcomputer equipped with an analog-to-digital converter (Digidata 1300, Molecular Devices). The cells were allowed to stabilize for 5 min after the whole-cell configuration was established before recording the currents. The experiments were performed at room temperature (22°C). The pipettes were filled with an intracellular solution composed of 35 mM NaCl, 105 mM CsF, 10 mM EGTA, and 10 mM Cs-HEPES. The pH was adjusted to 7.4 with CsOH. The external solution was composed of 150 mM NaCl, 2 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with NaOH.

The drugs were applied using a constantly running ValveLink8.2® gravity-driven perfusion system (Automate Scientific, Berkeley, CA) equipped with a glass syringe with a 250 µM tip. Different concentrations of the same drug were applied on the same cell. We used silicone-free tubing since we had observed changes in fluoxetine concentrations when silicon tubing was used, most likely because fluoxetine adheres to silicone, which can change the applied concentrations considerably.

The peak current amplitudes at different drug concentrations were subtracted from the value obtained with the control solution and were normalized to the control value in order to obtain the dose-response curves and IC_{50} values. Each point on the dose-response curves represents the mean of inhibition calculated from all recorded cells at a specific drug concentration. The values were fit to a Hill equation of the following form:

$$(I_{control} - I_{fluoxetine})/I_{control} = ax^b/(c^b + x^b),$$

where I is the peak current, a is the maximum inhibition, b is the Hill coefficient, c is the IC_{50}, and x is the concentration of agonist. To obtain activation curves, Na^+ conductance (G_{Na}) was calculated from the peak current (I_{Na}) using the following equation: $G_{Na} =$

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$I_{Na}/(V - E_{Na})$, where V is the test potential and E_{Na} is the reversal potential. Normalized G_{Na} values were plotted against the test potentials. To obtain the inactivation curves, the peak current was normalized to the maximal value and was plotted against the conditioning pulse potential. Steady-state activation and inactivation curves were fit to a Boltzmann equation of the following form:

$$G/G_{max}\ (or\ I/I_{max}) = 1/[1 + \exp\ (V_{1/2} - V/k_v],$$

where G is the conductance, I is the current, $V_{1/2}$ is the voltage at which the channels are half-maximally activated or inactivated, and k_v is the slope factor. To determine the recovery from inactivation, the test pulse peak current (I_{test}) was normalized to the corresponding prepulse current (I_{cont}). I_{test}/I_{cont} was plotted against the pulse interval and was fitted to a double or triple exponential function of the following form:

$$I/I_{max} = A_1(1 - \exp\ (-t/\tau_1)) + A_2(1 - \exp\ (-t/\tau_2))$$

or

$$I/I_{max} = A_1(1 - \exp\ (-t/\tau_1)) + A_2(1 - \exp\ (-t/\tau_2)) + A_3(1 - \exp\ (-t/\tau_3)),$$

where τ_1, τ_2 and τ_3 are the time constants, t is the time and A_1, A_2 and A_3 are the amplitudes of the time constants.

The results were analyzed using a combination of pCLAMP software v10.2 (Molecular Devices), Microsoft Excel, and SigmaPlot v11.0 (SSPSS, Chicago, IL).

Statistical analysis

Results are expressed as means ± SEM. Statistical significance was calculated using Student's unpaired *t* test, and the level of statistical significance was set at $P < 0.05$. The

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statistical significances for the IC_{50} were calculated using R software and the *drc* package (The R Foundation for Statistical Computing, Vienna, Austria).

Drugs

Racemic fluoxetine, S (+) fluoxetine, S (–) fluoxetine, norfluoxetine, and (+) fenfluramine were from Sigma-Aldrich (St. Louis, MO). Nisoxetine and methylphenidate were from Tocris Bioscience (Bristol, UK). Stock solutions (5 mM) were prepared in water and were diluted in the external solution prior to use.

Homology modeling of fluoxetine binding site in the $Na_v1.5$

The human cardiac $Na_v1.5$ was modeled in the closed and open states based on the closed Na_vAb (3RVY.pdb) and open Na_vMs (3ZJZ.pdb) X-ray structures (Payandeh *et al.*, 2011; Bagnéris *et al.*, 2013). To describe the symmetric positions of residues in four homologous domains in the channel, we used a universal residue-labeling scheme (Zhorov and Tikhonov, 2004). A residue is labeled by its domain number (1-4), segment (i, inner helix S6; o, outer helix S5; p, P-loop), and the relative number from the N end of a transmembrane helix or from the DEKA-locus positions 1p50, 2p50, etc. For example, $F^{4i15(1760)}$ designates phenylalanine in the domain IV inner helix, 15 positions downstream from the start of the segment. In some cases, the sequence-based residue number is included in the label in parentheses.

The alignment of bacterial Na_vAb and Na_vMs with eukaryotic sodium channels was taken as previously proposed (Payandeh *et al.*, 2011; McCusker *et al.*, 2012; Tikhonov and Zhorov, 2012). An insertion downstream from the DEKA locus was proposed (Tikhonov and Zhorov, 2012), however, in our models, this insertion was not introduced

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as the ligand was docked in the pore and residues above the DEKA locus would not affect ligand binding. The models contained the pore region (S5, P, and S6) of the human $Na_v1.5$. The closed model also contained the L4-5 linker (the linker between domain 4 and 5) because it is available in the X-ray structure. The extracellular linkers between P-loops and transmembrane helices were truncated to match the length of the X-ray structure templates, which does not affect ligand docking in the inner pore as they are distant. Ionizable residues were modeled as neutral, but the ionizable residues of DEKA locus were modeled as charged. S-fluoxetine was modeled as protonated, since its ammonium group has a pKa of ~10.

All calculations were performed using the ZMM program (www.zmmsoft.com, ZMM Software Inc., Flamborough, Ontario, Canada). The nonbonded energy was calculated using the AMBER force field (Weiner *et al.*, 1984, 1986) with a cutoff distance of 8 Å. Atomic charges at fluoxetine were calculated with the MOPAC software using the semi-empirical method AM1 (Dewar *et al.*, 1985). The hydration energy was calculated by using the implicit-solvent method (Lazaridis and Karplus, 1999). Electrostatic energy was calculated using the environment- and distance-dependent dielectric function without desolvation energy (Garden and Zhorov, 2010). The DEKA locus was loaded with an explicit water molecule, which was initially constrained to the Asp and Lys side chains, subsequently once constraints were removed, the water did not move away from the DEKA locus. The Monte Carlo minimization (MCM) method (Li and Scheraga, 1987) was used to optimize the models. All torsional angles of the protein and ligand were allowed to vary during energy calculations, while bond angles were rigid in the protein and flexible in the ligand. To prevent large deviations of the channel

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models from the X-ray structure templates during energy minimizations, the α-carbons of the model were constrained to the template using a flat-bottom energy function that allows atoms to deviate penalty-free up to 1 Å, but imposes a penalty of 10 kcal mol^{-1} $Å^{-1}$ for larger deviations. All molecular images were created using MVM. No specific energy terms were used for cation-π interactions, which were accounted for with partial negative charges at the aromatic carbons (Bruhova *et al.,* 2008).

The homology models were first MC-minimized without ligand until the 3000 consecutive energy minimizations did not improve the apparent global minimum found. The optimal binding modes of S-fluoxetine were searched by a two-stage random-docking approach. In the first stage, 60 000 different binding modes of the ligand were randomly generated within a cube with 14 Å edges. This sampling volume covered the entire inner pore including the domain interfaces. Each binding mode was MC-minimized for only 5 steps to remove steric overlaps with the protein. Energetically favorable conformations within 200 kcal/mol from the apparent global minimum were accumulated and then clustered based on ligand generalized coordinates. In the second stage, the 500 energetically best conformations found in the first stage were further MC-minimized for 1000 MC-minimization steps. The energetically most favorable ligand-receptor complexes within 4 kcal/mol were collected and analyzed.

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Results

Fluoxetine and its optical isomers block the $Na_v1.5$ channel

We studied the effect of fluoxetine on $Na_v1.5$ stably expressed in HEK-293 cells. Figure 1A shows an example of whole-cell current traces before (control) and after superfusion of 25 and 100 μM racemic fluoxetine. Fluoxetine inhibited Na^+ currents, with a maximum blockade occurring at 100 μM. The inhibition was partially reversible. The superfusion of increasing concentrations of fluoxetine (1, 10, 25, 50, 100, and 200 μM) showed that the blockade by fluoxetine was dose dependent. The dose-response curves (Fig. 1B) showed that the sensitivities of the optical isomers were similar, with an IC_{50} of 39.4 μM for racemic fluoxetine, and 40.0 μM and 46.7 μM for the (+) and (–) isomers, respectively. When the cells were maintained at a holding potential of -90 mV instead of -140 mV, where a proportion of the channels are inactivated, the affinity of racemic fluoxetine for $Na_v1.5$ significantly increased with an IC_{50} of 4.7 μM. Surprisingly, norfluoxetine, a fluoxetine metabolite, displayed a significantly higher affinity than fluoxetine, with an IC50 of 29.5 μM at holding potential of -140 mV. Surprisingly, norfluoxetine, a fluoxetine metabolite, displayed a significantly higher affinity than fluoxetine, with an IC50 of 29.5 μM at holding potential of -140 mV.

The effects of three other monoamine transporters (MAT) -targeting drugs were also tested using HEK-293 cells stably expressing $Na_v1.5$. The norepinephrin reuptake inhibitor, nisoxetine, the dopamine reuptake inhibitor, methylphenidate, and fenfluramine, which like fluoxetine, targets SERT, were all less effective in blocking the channels than fluoxetine, with IC_{50} of 104.5, 618.7 and 203.5 μM, respectively at a holding potential of -140 mV (Fig. 1C). The inhibition potency of these three compounds

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was also increased at a holding potential of -90 mV, with IC_{50} of 20.2, 239.5 and 65.5 μM for nisoxetine, methylphenidate and fenfluramine, respectively (Fig. 1C).

Effect of fluoxetine on the steady-state gating properties of $Na_v1.5$ channels

The availability of Na^+ channels following depolarization depends on a number of parameters, including the membrane potential. Fewer channels become available as the membrane potential progressively becomes more depolarized. This is due to the buildup of channels in the inactivated non-conducting state. We studied this phenomenon using a double-pulse protocol: a 500 ms conditioning pulse to voltages ranging from –140 mV to 0 mV, and a test pulse to –30 mV. The current measured following the test pulse is an indicator of the fraction of available channels. The normalized currents following the test pulse were plotted against the conditioning voltage (Fig. 2A). Fluoxetine (30 μM) significantly shifted the $V_{1/2}$ of inactivation of $Na_v1.5$ by 6.7 mV toward more hyperpolarized voltages and resulted in a less steep slope factor (Table 1 and Fig. 2A).

We also investigated the effect of fluoxetine on the steady-state activation of $Na_v1.5$. The activation curves were derived from I/V curves (see Materials and Methods). The activation curves of $Na_v1.5$ in the absence and presence of 30 μM fluoxetine were plotted against voltage (Fig. 2A). Fluoxetine did not significantly shift the midpoint of steady-state activation but had a little effect on the slope factor by reducing its steepness.

Fluoxetine slows the recovery from inactivation of $Na_v1.5$ channels

A prominent characteristic of many class 1 antiarrhythmics is their ability to slow the recovery from inactivation of drug-modified Na^+ channels. We used a two-pulse protocol

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to investigate the effect of fluoxetine on the recovery from inactivation. We used a 40 ms, –30 mV conditioning pulse and a 20 ms, –30 mV test pulse, with an interval ranging from 0.1 to 4000 ms to induce recovery from inactivation. The amplitudes of the Na^+ currents measured following the test pulse were then normalized to the control currents and were plotted against the duration of the recovery interval. Channels that recovered from inactivation displayed a progressive increase in currents following the increase in the recovery interval (Fig. 2B). The recovery from inactivation of $Na_v1.5$ after fluoxetine treatment was strongly slowed with the appearance of a third time constant. In comparison, the control curve had a τ_1 and τ_2 of 1.50 and 9.13 ms, respectively, while the fluoxetine had a τ_1, τ_2 and τ_3 of 1.63, 14.90 and 1598.23 ms, respectively (Table 1).

Fluoxetine blocks $Na_v1.5$ channels in a use-dependent manner

During depolarization, Na^+ channels cycle from the resting to the activated and inactivated states. However, when they are subjected to a train of depolarizing pulses, the number of channels available to open is reduced because they are gradually trapped in the inactivated state. This phenomenon is referred to as use-dependence or "frequency-dependent" current reduction. In the presence of a drug, further decreases in currents are likely due to the accumulation of drug-modified channels. For example, lidocaine, a class 1 antiarrhythmic drug, is known to cause the use-dependent inhibition of Na^+ channels. We tested the effect of rapid pulsing on $Na_v1.5$ by applying a series of 50 short 10 ms depolarizing –30 mV pulses. We first characterized the effect of fluoxetine on $Na_v1.5$/WT, and then on the $Na_v1.5$/F1760C and $Na_v1.5$/Y1767C mutant channels. We previously reported that these highly conserved residues of the D4S6 directly contribute

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to local anesthetic binding to cardiac channels (O'Leary and Chahine, 2002). As shown in Figure 3A, in the absence of fluoxetine, there was no significant change in the availability of $Na_v1.5$/WT channels when they were pulsed up to 10 Hz. However, in the presence of 30 μM fluoxetine, the availability of $Na_v1.5$/WT channels was dramatically reduced by 44% (P50/P1) when they were pulsed at 2 Hz (Fig. 3B-C) in comparison with the control without drug. When 5 and 10 Hz pulses were used, 30 μM fluoxetine reduced the currents of the $Na_v1.5$/WT by 58% and 67%, respectively compared to the control without drug. To further investigate the role of class 1 antiarrhythmic binding in the current block caused by fluoxetine, we inserted the F1760C or Y1767C mutation into $Na_v1.5$. As shown in Figure 3B-C, 30 μM fluoxetine reduced the current by 8%, 15%, and 20% when $Na_v1.5$/F1760C were pulsed at 2, 5, and 10 Hz, respectively, in comparison with the control without drug. The Y1760C mutation almost completely prevented the use-dependent inhibition of fluoxetine, with a maximal current inhibition of 5% when pulsed at 10 Hz. These results indicated that fluoxetine blocks $Na_v1.5$/WT currents in a use-dependent manner, and that the F1760C and Y1767C mutations dramatically reduce the use-dependent inhibition.

Fluoxetine had a lower affinity for $Na_v1.5$/F1760C mutant channels

We studied the effect of the F1760C and Y1767C mutations on the concentration-dependent block of $Na_v1.5$ currents by fluoxetine. Figure 4A shows examples of current traces recorded from $Na_v1.5$/WT and the mutant channels before and after a treatment with 50 μM fluoxetine. As shown in Figure 4B, while the IC_{50} value of fluoxetine for

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$Na_v1.5$/Y1767C (50.1 μM) was slightly higher to that of $Na_v1.5$/WT (39.4 μM), the IC_{50} value for $Na_v1.5$/F1760C (82.8 μM) was more than twice that of the WT channel.

Fluoxetine act as an open-channel blocker

To investigate the role of inactivation in the blockade of $Na_v1.5$ by fluoxetine in greater detail, we used $Na_v1.5$/L409C/A410W mutant stably expressed in HEK-293 cells. These channels exhibit a significant reduction in fast inactivation in HEK-293 (Wang *et al.*, 2013). A large persistent current was detected in the absence of fluoxetine (Fig. 5A). We applied different concentrations of fluoxetine and determined the IC_{50} at the peak current and at the end of the test pulse (90-100 ms). The block at the end of the pulse represents the affinity of the fluoxetine for open channels. As shown in Figure 5B, the IC_{50} (3.5 μM) at the end of the pulse was slightly lower than the IC_{50} at the peak current (9.6 μM), suggesting that fluoxetine is an open-channel blocker.

Molecular modeling of fluoxetine in the $Na_v1.5$

To discover the molecular details of the fluoxetine binding site, we have homology modeled the pore domain of the cardiac $Na_v1.5$ in the closed and open states based on the X-ray structures of bacterial Na channels, Na_vAb (Arcobacter butzleri sodium channel) (Payandeh *et al.*, 2011) and Na_vMs (Magnetococcus sp sodium channel) (Bagnéris *et al.*, 2013), respectively (see PDB file in Data Supplement). A random sampling approach was used to search for the energetically most favorable binding modes of fluoxetine in the $Na_v1.5$. 60 000 random orientations of fluoxetine were seeded inside the channel within a volume to cover the entire pore cavity and inner helix interfaces (Fig. 6A, B). After two

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rounds of Monte-Carlo energy minimizations, the energetically best fluoxetine complexes bound inside the inner pore. Fluoxetine adopts two distinct binding modes, a horizontal and a vertical binding mode (Fig. 6C-F). These two binding modes were energetically favorable in both the closed and the open channel pore.

Fluoxetine resembles a three-pointed star with a chiral center in the middle linking three arms comprising an ammonium group, a benzene ring, and a trifluoromethyl benzene ring. In both of the two binding modes of fluoxetine, its ammonium group localizes to the channel's central axis under the DEKA locus, near the focus of the P-helices (Figure 1C-F). Just one position upstream of the DEKA locus, a ring of QGFS residues in position p49 (see description of relative number scheme in Methods) favorably interact with fluoxetine since their side chains face downward into the pore. Particularly, $Q^{1p49(372)}$ and $S^{4p49(1712)}$ form favorable electrostatic contacts with fluoxetine's nitrogen, each contributing 4-9% to the ligand-receptor energy. The ammonium group of fluoxetine was also attracted by the two negatively charged residues of the DEKA locus, which outweighed the repulsion from the Lys of the DEKA locus. Further, the backbone carbonyl groups of residues two to three positions upstream of the DEKA locus (positions p47 and p48) also stabilize fluoxetine.

The horizontal and vertical binding modes are distinguished by the two benzene arms of fluoxetine. In the vertical binding mode (Fig. 6 C, E), one benzene arm is parallel and the other arm is perpendicular to the pore axis. In this mode, one benzene ring π-stacks with $Y^{4i22(1767)}$ and the other interacts with $F^{4i15(1760)}$. Y^{4i22} and F^{4i15} were found to be the two most significant residues in binding fluoxetine; each contributes 16-33% to the ligand-receptor energy. In the horizontal binding mode (Fig. 6 D, F), the two benzene

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arms point away ~45-90^{o} from the pore axis. The ligand leans against the P-loop and protrudes between the III-IV domain inner helices. Here, one of the benzene rings π-stacks with F^{4i15}, while the other arm extends towards $F^{3p49(1236)}$ and IIIS6. In the horizontal binding mode, F^{4i15} has the strongest interaction with fluoxetine contributing 23-33% to the ligand-receptor energy, while other residues contributed <10%.

The closed Na_vAb-based and open Na_vMs-based models exhibit similar channel geometry, except for the intracellular half of the inner helices of the open state that bend to widen the pore. In both the closed and open channel models, residues in position i15 (which includes F^{4i15}) and position i22 (which includes Y^{4i22}) are pore-facing. Thus, a vertical and a horizontal binding mode of fluoxetine were found in both the closed and open pore. However, in terms of energy, the horizontal binding mode was favored in the closed state, because the fluoxetine experienced ligand strain in the narrower closed pore. On the other hand, the fluoxetine preferred to adopt the vertical binding mode in the open state as it formed better ligand-receptor contacts with F^{4i15} and Y^{4i22}. In summary, the molecular model of fluoxetine in $Na_v1.5$ was in agreement with mutational experiments, suggesting that F1760 and Y1767 are the two key residues for its binding.

Discussion

In the present study, we characterized the effects of fluoxetine, a widely used antidepressant drug, on $Na_v1.5$, the cardiac voltage-gated Na^+ channel.

Our results showed that racemic fluoxetine and its optical isomers are equally effective blockers of $Na_v1.5$ when current were recorded at a holding potential of -140 mV. Similar results have been reported for cardiac voltage-gated Ca^{2+} channels in canine

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ventricular cardiomyocytes, where both fluoxetine enantiomers have similar IC_{50} (Magyar *et al.*, 2003). We also conducted dose-response curves experiments for racemic fluoxetine in HEK-293 at a holding potential of -90 mV, which is near the resting potential of cardiomyocytes. These experiments showed that the IC_{50} of fluoxetine is eight time lower at a holding potential of -90 mV compared to -140 mV, going from 39.4 μM to 4.7 μM. In a manner that is hard to explain, these data are in contradiction with those published by Rajamani et al. who reported that fluoxetine does not inhibit Na^+ currents in HEK-293 cells expressing $Na_v1.5$ (Rajamani *et al.*, 2006). However, our IC_{50} of 4.7 μM is very similar with that published by Harmer et al., who reported an IC_{50} of 4.9 μM using IonWorks™ assays from $hNa_v1.5$-expressing CHO cells maintained at a holding potential of -90 mV (Harmer *et al.*, 2011). These results suggest that the holding potential of the cell is very important to the affinity of fluoxetine for the channel, as it has been also shown in rat hippocampi neurons (Lenkey *et al.*, 2006), suggesting that the fluoxetine binds with higher affinity to inactivated than to resting channels.

In the nervous system, fluoxetine primary targets SERT, which, together with dopamine transporter (DAT) and norepinephrine transporter (NET), make up the three major MAT classes. In order to investigate the effect of other MAT-targeting drugs, we investigated the effect of nisoxetine (NET-targeting drug) (Tejani-Butt, 1992), methylphenidate (DAT-targeting drug) (Han and Gu, 2006), and fenfluramine (SERT-targeting drug) (Cosgrove *et al.*, 2010) on $Na_v1.5$ currents. Our results showed that the affinity of these drugs for $Na_v1.5$ is dependent of the holding potential. The IC_{50} of nisoxetine, methylphenidate and fenfluramine are respectively 5, 2.5 and 3 times lower at a holding potential of -90 mV compared to -140 mV. Like the fluoxetine, the decrease of

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IC_{50} at more depolarized potential suggests a higher affinity of these three compounds to inactivated than to resting channels. Furthermore, these compounds also exhibited a use-dependent inhibition, especially nisoxetine with a significant current reduction of 26%, 36% and 38% when pulsing at 2, 5 and 10 Hz, respectively (data not shown). However, these three compounds are still less potent than fluoxetine to inhibit $Na_v1.5$.

The present study was designed to investigate the biophysical mechanism of the $Na_v1.5$ block by fluoxetine as well as the possible pro-arrhythmic properties of this drug. A major finding of our work was that fluoxetine shifts the steady-state inactivation curve by 6.7 mV toward more hyperpolarized values, indicating that it binds to the inactivated state of $Na_v1.5$, as is the case with neuronal Na^+ channels (Lenkey *et al.*, 2006). In addition to a tonic block, fluoxetine decreased $Na_v1.5$ currents in a use-dependent manner when pulsing at 2, 5 and 10 Hz, The affinity of fluoxetine for $Na_v1.5$ thus appears to be modulated by the state of the channel, which rapidly switches between the open and inactivated configurations, leading to the progressive accumulation of inactivated $Na_v1.5$. Use-dependence occurs because drug-modified channels slowly recover only at hyper-polarized voltages. Class 1 antiarrhythmic drugs and local anesthetics have a similar effect (Chahine *et al.*, 1992). We thus determined whether fluoxetine could inhibit Na^+ currents by mutating residues in the class 1 antiarrhythmic drug binding site. Amino acids situated near the cytoplasmic ends of the membrane-spanning S6 α-helixes of all four homologous domains (D1S6-D4S6) form the cytoplasmic entrance of the pore and contribute to the binding sites of both the native inactivation gate and class 1 antiarrhythmic drugs. We previously reported that two highly conserved residues of the D4S6 segment (F1760, Y1767) contribute directly to the local anesthetic binding site of

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cardiac Na^+ channels (O'Leary and Chahine, 2002). We showed that both mutations (F1760C and Y1767C) markedly reduced the frequency-dependent effect, with the Y1767C mutation having the greatest effect. However, in tonic block, the F1760C increased the IC_{50} of fluoxetine more significantly than the Y1767C. These results showed that these residues of D4S6 are an integral part of the binding site of fluoxetine, as is the case with many class 1 antiarrhythmic drugs. Our data also suggest that F1760 appears to be more involved in binding fluoxetine when the channel is in the resting state, while Y1767 appears to be key for fluoxetine binding when the channel is in the open/inactivated sate.

Molecular modeling fluoxetine in $Na_v1.5$ was in agreement with mutational experiments, in which $F^{4i15(1760)}$ and $Y^{4i22(1767)}$ were found to be the key residues in binding fluoxetine. However, the models predicted that the ligand is able to assume two energetically favorable binding modes. The vertical binding mode was favored in the open state model, while the horizontal mode in the closed state model. This could suggest that open channel block involves both $F^{4i15(1760)}$ and $Y^{4i22(1767)}$ as visualized in the vertical binding mode. With the same assumption, the horizontal binding mode could represent resting channel block with $F^{4i15(1760)}$ as the essential residue. Fluoxetine share similarities to local anesthetics. Both are drugs sensitive to mutations at $F^{4i15(1760)}$ and $Y^{4i22(1767)}$. Structurally, fluoxetine resembles most classical local anesthetics in approximate size and by possessing an ammonium group and a benzene ring. Fluoxetine adopts similar binding modes in the closed channel homology model as QX-314, cocaine, and tetracaine (Bruhova *et al.*, 2008; Tikhonov and Zhorov, 2012). Since fluoxetine can protrude between the III-IV inner helix interface while in the horizontal binding mode, it could

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suggest that fluoxetine may enter or exit through the III-IV domain interface pathway from the extracellular side of the membrane as it has been demonstrated with local anesthetics (Qu *et al.*, 1995; Sunami *et al.*, 2001). Experiments with fluoxetine with a quaternarized ammonium could reveal whether the ligand can block from the extracellular side.

The blockade of the $Na_v1.5$ by fluoxetine should be taken into consideration when prescribing this drug. Blocking the cardiac Na^+ channel may cause an intracardiac conduction delay, which may in turn cause a prolongation of the QRS complex on the electrocardiogram (Delk *et al.*, 2007). Given the association between QRS prolongation and mortality, and the potential for drug-induced arrhythmia, caution is required when prescribing fluoxetine (Thanacoody and Thomas, 2005; Delk *et al.*, 2007), especially given that inhibiting the $Na_v1.5$ by as little as 10% may cause a prolongation of the QRS complex in humans (Cordes *et al.*, 2009). However, a question remains as to how to transpose the significance of the IC_{50} value of fluoxetine to a pathophysiological setting. The IC_{50}/fC_{max} ratio, where fC_{max} represents the unbound (free) plasma concentration in a clinical setting, of a drug that evokes a QRS or a change in QT has been proposed as a tool for determining whether a drug can be safely prescribed (Redfern *et al.*, 2003; Harmer *et al.*, 2011). An IC_{50}/fC_{max} ratio above 30 to 100 has been shown to ensure a suitable degree of safety in terms of drug-induced QRS complex prolongation. The fC_{max} for fluoxetine is 93 nM (Harmer *et al.*, 2011). Thereby, when we mimic the membrane potential of cardiomyocytes in patch-clamp by imposing a holding potential of -90 mV to HEK-293 cells, the IC_{50}/fC_{max} ratio is 50. This is within the 30 to 100 margin and it should act as a safety flag for a possible cardiotoxicity.

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Furthermore, in the case of fluoxetine, fC_{max} may not be a good indicator of actual plasma concentrations of total $Na_v1.5$ blockers in vivo since norfluoxetine, an active metabolite of fluoxetine, has a higher affinity for $Na_v1.5$ than fluoxetine itself. Given that norfluoxetine has a half-life of more than a week compared to 70 h for a single dose of fluoxetine (Schepens, 1996), there is a possibility of a long-lasting additive effect on cardiac Na^+ channels. In fact, in the calculation of the IC_{50}/fC_{max} ratio, we should take into consideration the unbound (free) plasma concentration of norfluoxetine. Despite the lack of information about the fC_{max} after a single dose, it is known that the plasma concentration of total (unbound and bound) fluoxetine and norfluoxetine at steady state are very similar after chronicle treatment (91 to 302 ng/ml and 72 to 258 ng/ml, respectively) (U.S. Food and Drug Administration). These suggest that the IC_{50}/fC_{max} ratio following fluoxetine treatment is probably underestimated.

In conclusion, caution should be taken when prescribing fluoxetine at same time as other Na^+ channel inhibitors such as class 1 anti-arrhythmic drugs, especially class 1A and 1C drugs. In addition, fluoxetine should be prescribed with extreme care for patients suffering from ventricular conduction disorders or liver disease. Indeed, as the liver is the primary site of fluoxetine metabolism, its impairment, such cirrhosis, affects the elimination half-life of fluoxetine and norfluoxetine (Schenker *et al.*, 1988).

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Authorship Contributions:

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Footnotes

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Figure legends:

Figure 1

Tonic block of $Na_v1.5$/WT currents. **(**A) Superimposed I_{Na} recordings obtained before and after perfusion with two concentrations of racemic fluoxetine from a holding potential of -140 mV. The dashed line represents zero current. (B) Dose-response curves of the inhibitory effect of norfluoxetine and different optical isomers of fluoxetine on $Na_v1.5$/WT currents. HEK-293 cells stably expressing $Na_v1.5$/WT were perfused with different concentrations of norfluoxetine, racemic fluoxetine, S(+) fluoxetine or R(–) fluoxetine. There was no significant difference between the IC_{50} of racemic fluoxetine (IC_{50} = 39.4 ± 2.0 μM, n = 3-7) and its two optical isomers (IC_{50} = 40.0 ± 2.6 μM, n = 6-14 and 46.7 ± 3.1 μM, n = 3-10). However, norfluoxetine had a significant lower IC_{50} (29.5 ± 1.0 μM, n = 8-15). The IC_{50} of fluoxetine was significantly reduced to 4.7 ± 0.5 μM (n = 7-10) when recorded at a holding potential of -90 mV (*B*, open triangle). (C) Dose-response curves of the inhibitory effect of nisoxetine (n = 3-11), methylphenidate (n = 4-9), and fenfluramine (n = 4-6) on $Na_v1.5$/WT currents recorded at a holding potential of -140 or -90 mV. The IC_{50} of the three drugs at a holding potential of -90 mV were significantly lower than those recorded at -140 mV. Insets in *B* and *C* show the IC_{50} for each compound. The values were fitted to a Hill equation. Currents were elicited from a holding potential of –140 mV or -90 mV, and a–30 mV test pulse lasting 50 ms was delivered every 5 s. ***$P < 0.001$

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Figure 2

Gating properties of $Na_v1.5$/WT treated with fluoxetine. (A) Voltage-dependence of steady-state activation and inactivation of $Na_v1.5$. Cells were perfused with Ringer's solution as a control (activation, n = 14; inactivation, n = 19) or with 30 µM racemic fluoxetine (activation, n = 18; inactivation, n = 17). Activation curves were elicited with 50 ms depolarizing steps from –100 to 80 mV in 10 mV increments. Cells were held at a holding potential of –140 mV. Fluoxetine caused no significant shift in the activation curve. Steady-state inactivation was determined using 4 ms test pulses to –30 mV after a 500 ms prepulse to potentials ranging from –140 mV to 0 mV (see the *inset* under the inactivation curves for the protocol). The application of 30 µM fluoxetine induced a significant –6.7 mV shift of the inactivation curve (***$P < 0.001$) (Table 1). The activation and inactivation curves were fitted to a single Boltzman function (see Materials and Methods). (B) Recovery from inactivation of $Na_v1.5$ in the absence (n = 10) or presence (n = 9) of 30 µM fluoxetine. The cells were depolarized to –30 mV for 40 ms from a holding potential of –140 mV to inactivate all the Na^+ channels. Test pulses were then applied to –30 mV for 20 ms to measure current amplitudes, with an interval ranging from 0.1 to 4000 ms. The resulting curves were fitted to a double (control) or a triple (+ fluoxetine) exponential equation, which yielded two or three time constants (τ_1, τ_2, τ_3). The application of 30 µM fluoxetine strongly slowed the recovery from inactivation with the appearance of a third time constant (Table 1).

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Figure 3

Frequency-dependent inhibition. (A) Representative whole-cell traces recorded from $Na_v1.5$/WT (+/- 30 μM fluoxetine), $Na_v1.5$/F1760C (+ 30 μM fluoxetine), and $Na_v1.5$/Y1767C (+ 30 μM fluoxetine) when pulsing at 10 Hz. The dashed line represents zero current. (B) Use-dependent blocks of $Na_v1.5$/WT, $Na_v1.5$/F1760C, and $Na_v1.5$/Y1767C currents in the presence of 30 μM fluoxetine. A 50 pulse train was applied at –30 mV for 10 ms from a holding potential of –140 mV when pulsing at 2 Hz, 5 Hz, and 10 Hz. Peak currents were measured, normalized to the peak amplitude at P_1, and plotted against the corresponding pules. (C) Relative currents amplitudes (P_{50}/P_1) of the 50th sweep recorded from $Na_v1.5$/WT, $Na_v1.5$/F1760C and $Na_v1.5$/Y1767C. After the fluoxetine treatment, $Na_v1.5$/WT (n = 8) currents were significantly reduced by 44%, 58%, and 67% compared to the control when pulsing at 2, 5, and 10 Hz, respectively (***$P < 0.001$). Fluoxetine significantly reduced $Na_v1.5$/F1760C (n = 11) currents by 15% and 20% when pulsing at 5 and 10 Hz (###$P < 0.001$), respectively, and $Na_v1.5$/Y1767C (n = 6) currents by 5% when pulsing at 10 Hz (φ φ $P < 0.01$) compared with control. There was no significant use-dependent inhibition of $Na_v1.5$/WT (n = 7), $Na_v1.5$/F1760C (n = 5), or $Na_v1.5$/Y1767C (n = 6) currents before the fluoxetine treatment. The controls curves of $Na_v1.5$/WT, $Na_v1.5$/F1760C and $Na_v1.5$/Y1767C without fluoxetine treatment were removed from the graphic *B* and *C* for clarity.

Figure 4

Tonic block of $Na_v1.5$/F1760C and $Na_v1.5$/Y1767C by fluoxetine. (A) Representative whole-cell traces recorded from $Na_v1.5$/WT (left), $Na_v1.5$/F1760C (middle), and

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$Na_v1.5$/Y1767C (right) channels before and after the application of 50 μM fluoxetine. The dashed line represents zero current. (B) Dose-response curves of the inhibitory effect of racemic fluoxetine on $Na_v1.5$/WT, $Na_v1.5$/F1760C, and $Na_v1.5$/Y1767C. The IC_{50} values of $Na_v1.5$/F1760C (83 μM) and $Na_v1.5$/Y1767C (50 μM) were significantly higher than $Na_v1.5$/WT value (39 μM), $^{**}P < 0.01$ and $^{***}P < 0.001$. The different concentrations of drugs were applied using a perfusion system. Currents were elicited from a holding potential of –140 mV with a 50 ms test pulse at –30 mV delivered every 5 s. Insets in *B* shows the IC_{50} for each compound. Normalized current (I_{Na}) values were fitted to a Hill equation.

Figure 5

Open-channel block of $Na_v1.5$ by fluoxetine. (A) Superimposed I_{Na} recordings obtained following the application of different concentrations of fluoxetine on $Na_v1.5$/L409C/A410W expressing cells. The dashed line represents zero current. (B) Dose-response curves of the inhibitory effect of fluoxetine on $Na_v1.5$/L409C/A410W at the peak current (blue circle) and 90-100 ms after the beginning of the pulse (green square). The IC_{50} value at the end of the pulse (3.5 μM) was significantly lower than the IC_{50} value at the peak current (9.6 μM) ($^{***}P < 0.001$). Currents were elicited from a holding potential of –140 mV with a 50 ms test pulse at 0 mV delivered every 5 s. Normalized current (I_{Na}) values were fitted to a Hill equation. Dotted grey boxes represent the peak current (left boxe) and the 90-100 ms (right boxe) areas used to construct the dose-response curves.

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Figure 6

Searching for the binding site of fluoxetine in the closed and open $Na_v1.5$. The P-loops and S6 helices of domains I, II, III, and IV are colored blue, orange, green, and violet, respectively. The outer helices and the L45 linker are shown as gray strands. The side chains of residues in the DEKA locus, $Q^{1p49(372)}$, $S^{4p49(1712)}$, $F^{4i15(1760)}$ and $Y^{4i22(1767)}$ are shown as sticks with yellow carbons. The water molecule at the DEKA locus is rod-shaped. (A and B) The side and extracellular views of the randomly generated starting points of fluoxetine in the closed $Na_v1.5$. Fluoxetine is presented in wire-frame with gray carbons. For clarity, only 6000 of the 60,000 starting points are shown. (C-F) The side views of the lowest energy vertical (C and E) and horizontal (D and F) binding modes of fluoxetine in the closed (C and D) and open (E and F) channel. Fluoxetine is shown in thick sticks with gray carbons. The side chain of $F^{3p49(1236)}$ is shown in D and F. For clarity the outer helices are not shown in C–F (see PDB file in Data Supplement).

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Table 1. ***Biophysical properties of Nav1.5 channels***

	$Na_v1.5$/WT Control		$Na_v1.5$/WT Fluoxetine		
	Mean ± sem	***n***	**Mean ± sem**		***n***
Steady-state activation					
$V_{1/2}$, mV	- 43.49 ± 1.46	14	- 41.52 ± 1.17		18
kv	- 6.13 ± 0.32	14	7.28 ± 0.17	**	18
Steady-state inactivation					
$V_{1/2}$, mV	-87.34 ± 0.94	19	- 94.04 ± 1.64	***	17
kv	6.37 ± 0.19	19	7.67 ± 0.29	***	17
Recovery from inactivation					
τ_1	1.50 ± 0.1	10	1.63 ± 0.1		6
A_1	76.3 ± 2.7	10	35.7 ± 2.1		6
τ_2	9.13 ± 1.0	10	14.90 ± 2.6		6
A_2	23.7 ± 0.8	10	22.0 ± 2.1		6
τ_3	–	–	1598.23 ± 41.6		6
A_3	–	–	42.3 ± 1.7		6

$V_{1/2}$, midpoint for activation or inactivation; *kv*, slope factor for activation or inactivation; τ, time constant; *A*, fraction of the τ components (%); *n*, number of cells. Values are means ± sem, $^{**}P < 0.01$, $^{***}P < 0.001$

Figure 1

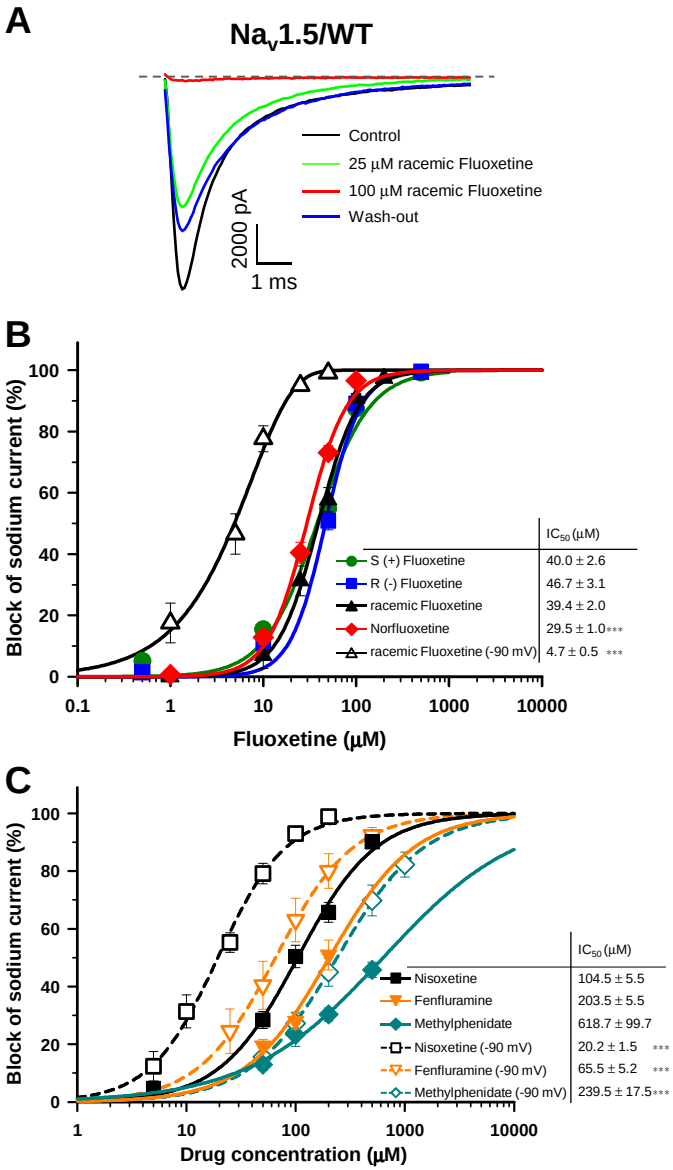

Figure 2

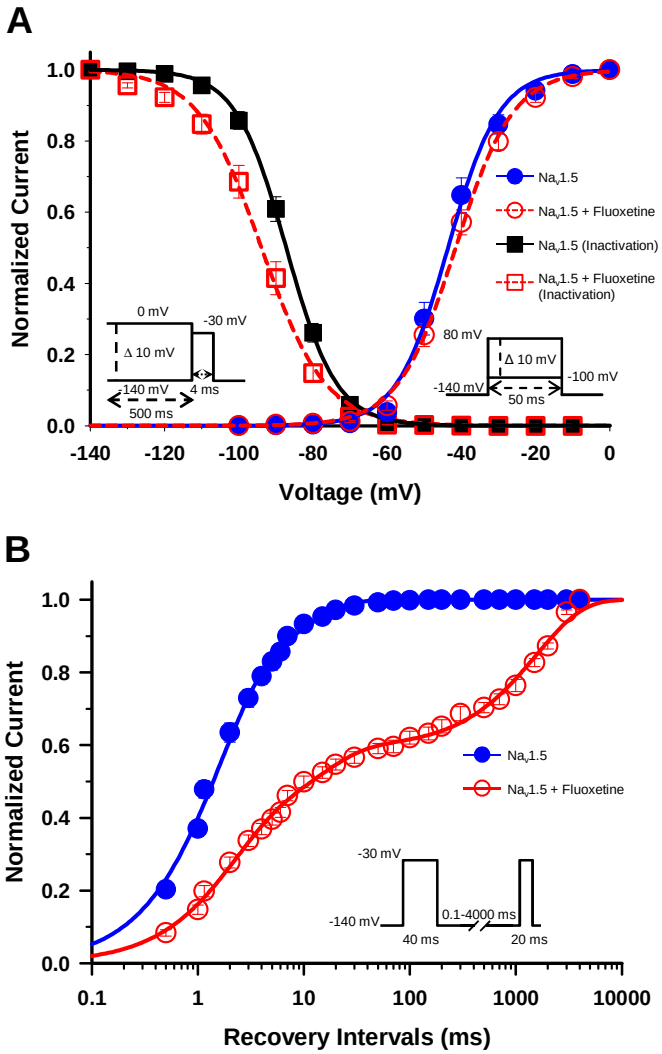

Figure 3

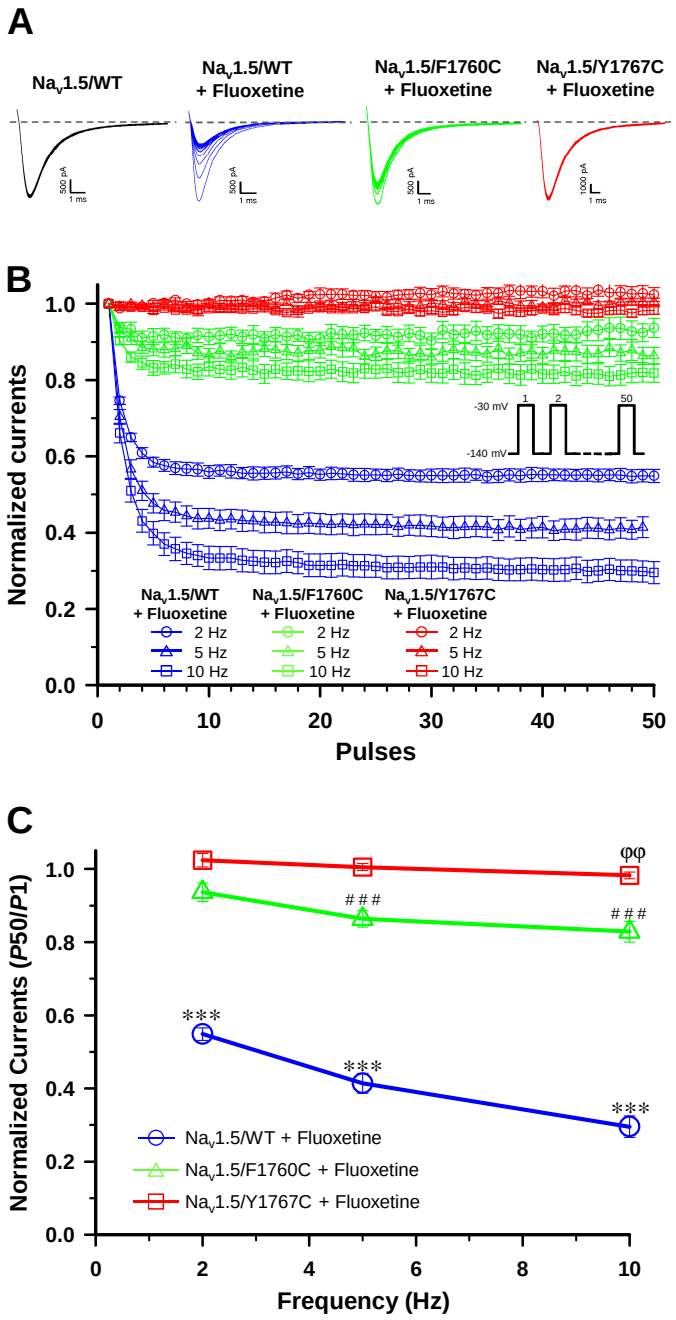

Figure 4

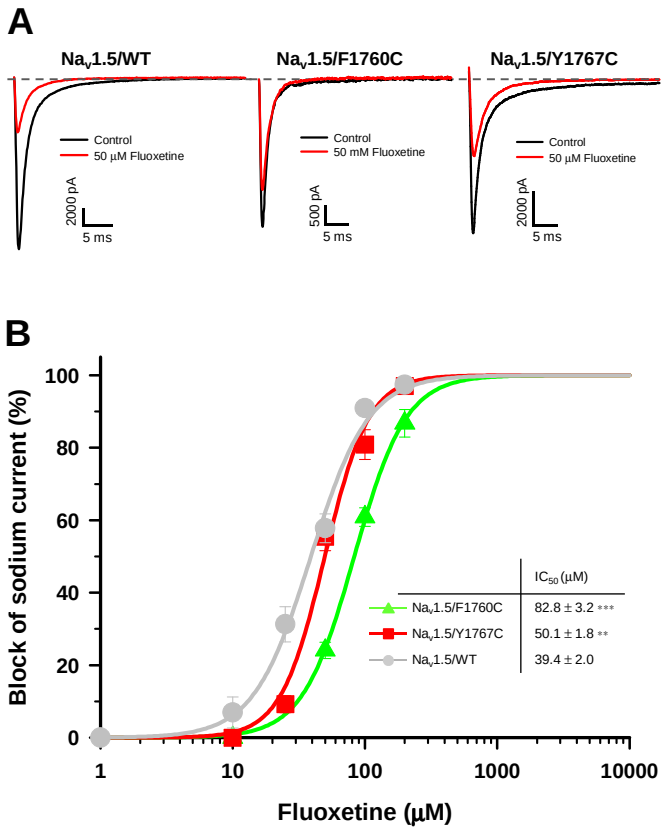

Figure 5

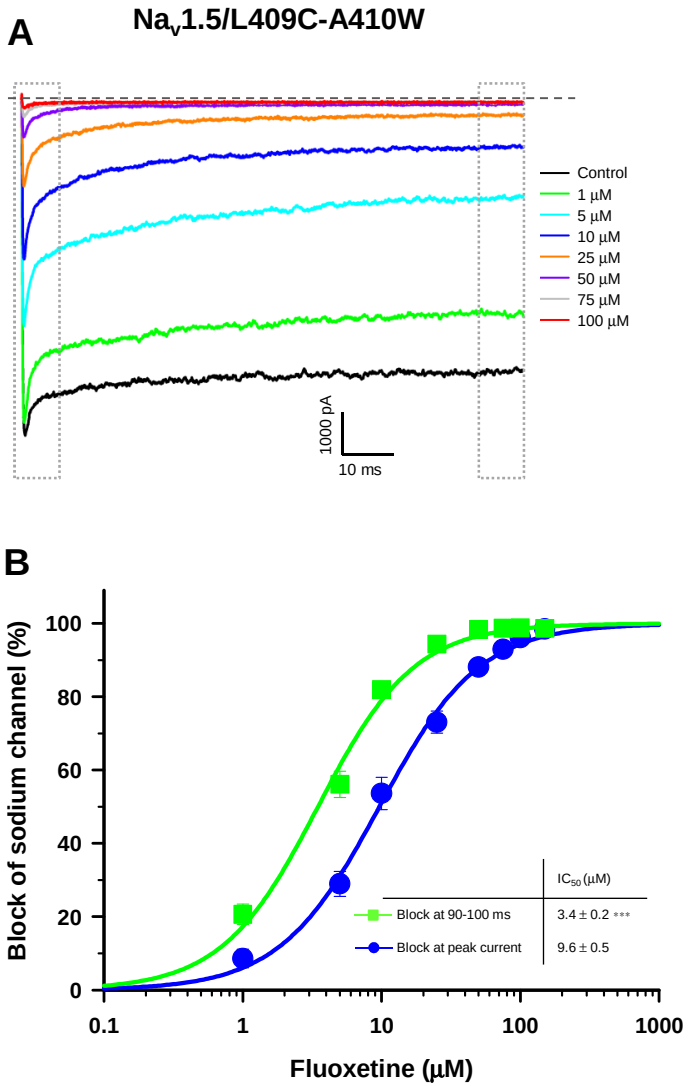

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Figure 6