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Mutational and In Silico Analyses for Antidepressant Block of Astroglial Inward-Rectifier Kir4.1 Channel[S]

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ABSTRACT

Drug interaction with target proteins including ion channels is essential for pharmacological control of various cellular functions, but the majority of its molecular mechanisms is still elusive. We recently found that a series of antidepressants preferentially block astroglial K^+-buffering inwardly rectifying potassium channel (Kir) 4.1 channels over Kir1.1 channels. Here, using electrophysiological analyses of drug action on mutated Kir4.1 channel as well as computational analyses of three-dimensional (3D) arrangements of the ligands (i.e., bidirectional analyses), we examined the underlying mechanism for the antidepressant-Kir4.1 channel interaction. First, the effects of the selective serotonin reuptake inhibitor fluoxetine and the tricyclic antidepressant nortriptyline on chimeric and site-directed mutants of Kir4.1 expressed in *Xenopus laevis* oocytes were examined using the two-electrode voltage-clamp technique. Two amino acids, Thr128 and Glu158, on pore and transmembrane domain 2 helices were critical for the drug inhibition of the current. The closed and open conformation models of the Kir4.1 pore suggested that both residues faced the central cavity, and they were positioned within a geometrical range capable of interacting with the drugs. Second, to represent molecular properties of active ligands in geometric terms, a 3D quantitative structure-activity relationship model of antidepressants was generated, which suggested that they share common features bearing a hydrogen bond acceptor and a positively charged moiety. 3D structures and physicochemical features of receptor and ligand were fitted together. Our results strongly suggest that antidepressants interact with Kir4.1 channel pore residues by hydrogen bond and ionic interactions, which account for their preferential inhibitory action on Kir4.1 current. This study may represent a possible general approach for the understanding of the mechanism of ligand-protein interactions.

The inwardly rectifying potassium (Kir) channels family contributes to a wide variety of physiological function, such as the maintenance of the resting membrane potential, regulation of the action potential duration, receptor-dependent inhibition of cellular excitability, and epithelial K^+ transport system (Isomoto et al., 1997; Kubo et al., 2005). Despite the intense molecular and physiological studies on the Kir channels, pharmacological studies are limited (Spassova and Lu, 1998; Yamada et al., 1998; Kobayashi et al., 2004; Kikuta et al., 2006; Liu et al., 2007; Ohno et al., 2007; Su et al., 2007; Rodríguez-Menchaca et al., 2008). In particular, little is known about the interaction of small organic chemicals with Kir channels (Rodríguez-Menchaca et al., 2008). Like previous informative studies in other ion channels (Yellen et al., 1991; Ragsdale et al., 1994, 1996; Hockerman et al., 1995; Peterson et al., 1997; Lees-Miller et al., 2000; Mitcheson et al., 2000; Hille, 2001; Lenaeus et al., 2005; Hosaka et al., 2007), the studies on the drug-Kir channel interactions will facilitate our current understandings of the structure-function relationship and regulation of Kir channels.

The cytoplasmic pore of Kir2.1 has been reported to be a drug-binding site for chloroquine (Rodríguez-Menchaca et al., 2008). It still remains to be determined whether residues within the central cavity as deep as the internal entrance to the selectivity filer of Kir channels contribute pharmacological action of small organic chemicals. The drug-binding site has not been identified within the central cavity of Kir channels. These regions are of special concern in studies of ion

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ABBREVIATIONS: Kir, inwardly rectifying potassium; 3D, three-dimensional; E_K, K^+ equilibrium potential; QSAR, quantitative structure-activity relationship; TM, transmembrane; WT, wild type.

permeation, channel gating, and block by blocking cation (Isomoto et al., 1997; Nichols and Lopatin, 1997; Thompson et al., 2000; Yi et al., 2001; Bichet et al., 2003, 2004; Lu, 2004; Chatelain et al., 2005; Kurata et al., 2006; Rapedius et al., 2007; Robertson et al., 2008). In other K^+ channels, many small organic compounds are known to interact with the residues, leading the block or facilitation of the channel activity (Yellen et al., 1991; Mitcheson et al., 2000; Lenaeus et al., 2005; Hosaka et al., 2007).

We have previously shown that several clinically useful antidepressants, including tricyclic antidepressants such as nortriptyline and desipramine and selective serotonin reuptake inhibitors such as fluoxetine and sertraline, block Kir4.1 channels rather than other Kir channels (Kir1.1, Kir2.1, and Kir3.1) (Ohno et al., 2007; Su et al., 2007). Given the relative simplicity and similarity of Kir channels (Isomoto et al., 1997; Kubo et al., 2005), the specificity of block by such structurally diverse compounds is surprising.

In this study, we first performed the electrophysiological studies using mutant Kir4.1 channels and identified two main amino acid residues, Thr128 and Glu158, in the channel pore cavity to be critical for the drug-channel interaction. The closed and open conformation models of the Kir4.1 pore suggested that both amino acids faced the central cavity. Next, to understand its functional groups of ligands and their assumed arrangement, we generated a 3D quantitative structure-activity relationship (QSAR) model of active antidepressants. This model represented molecular properties of a series of active antidepressants in geometric terms, which are involved in the interaction. Resultant 3D structures of receptor and ligand fit together in total agreement between experimental data with channel mutagenesis, pharmacophore of ligands, and physicochemical feature of drug interaction sites. Using the bidirectional approach from both receptor and ligands, we characterized the interaction between receptor and ligands and show that antidepressant interacts with Kir4.1 channel pore residues by ionic and hydrogen bond interactions. This study may further represent a possibly general approach for the understanding of the mechanism of ligand interactions with various target proteins.

Materials and Methods

Molecular Biology. Rat Kir4.1 and rat Kir1.1 cDNA subcloned into pGEM-HEfx and pBlueScript SK(−) vector, respectively, were used. CH414, where the transmembrane 1 (TM1) to transmembrane 2 (TM2) region of Kir4.1 was replaced with the homologous region from Kir1.1, was constructed by ligating a polymerase chain reaction fragment Val83–Ala177 from Kir1.1 to Met1–Leu69 and Phe165–Val379 of Kir4.1. Conversely, CH141 that contains the TM1–TM2 region of Kir4.1 in the Kir1.1 sequence was constructed by ligating a Leu70–Thr164 polymerase chain reaction fragment from Kir4.1 to Met1–Thr82 and Ile178–Met391 of Kir4.1 (Fig. 1B). CH414 and CH141 were subcloned into pGEM-HEfx vector and subjected to cRNA preparation. Site-directed mutagenesis was carried out using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instruction. All of the wild-type, chimeric, and point-mutated constructs were confirmed by direct DNA sequencing. cRNAs for injection into oocytes were prepared with T7 or T3 RNA polymerase (Invitrogen, Carlsbad, CA) in the presence of $m^7G(5')ppp(5')G$ RNA-capping analog (Invitrogen) after linearization of the expression constructs.

Isolation of Oocytes and Injection of cRNA. *Xenopus laevis* oocytes were maintained and treated in accordance with the guidelines for the use of laboratory animals of Osaka University Graduate School of Medicine. Isolation of *X. laevis* oocytes and injection with cRNA were performed as described previously (Inanobe et al., 2001; Hosaka et al., 2007). The cells were injected with 5 to 150 ng of cRNA of wild-type (WT) Kir4.1, WT Kir1.1, CH414, CH141, or each mutant, and incubated at 18°C in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6, with NaOH) supplemented with 50 μg/ml gentamicin.

Electrophysiology. Membrane currents were recorded from oocytes with a conventional two-microelectrode voltage-clamp technique (Inanobe et al., 2001; Hosaka et al., 2007) 2 to 4 days after cRNA injection. The glass microelectrodes had a resistance of 0.6 to 1.0 MΩ when filled with 3 M KCl. Oocytes were perfused with a solution containing 45 mM KCl, 45 mM NaCl, 3 mM $MgCl_2$, and 5 mM HEPES, pH 7.4. Niflumic acid (150 μM) was also included to inhibit endogenous Cl^- channel currents. Oocytes were voltage-clamped at −20 mV ($\sim E_K$ in 45 mM $[K^+]_o$) and stepped to −80 mV for 1 or 5 s to elicit inward K^+ currents, upon which the effects of fluoxetine or nortriptyline was examined. A ramp voltage-clamp from −100 to +40 mV (1 s in duration) was applied to estimate changes in reversal potential before and after drug application. The clamp voltage and associated macroscopic currents were recorded with a Geneclamp 500 amplifier, fed through a digitizer (Digidata 1322A), and stored in a computer with the data acquisition system Clampex 9.2 (all from Molecular Devices, Sunnyvale, CA). The signals were also monitored on a dual-beam oscilloscope and recorded on a thermal array recorder (RTA-1100; Nihon Koden, Tokyo, Japan). All experiments were performed at room temperature (22–25°C).

Molecular Modeling, 3D Pharmacophore Modeling, and Drug-Docking Simulations. Homology models of a Kir4.1 pore region (amino residues 62–172) were generated with version 6.0 of the program MODELER in Discovery Studio 1.7 suite (Accelrys, Inc., San Diego, CA) using tetramers of Kv1.2 (Protein Data Bank number 2A79) and KirBac1.1 and KirBac3.1 (1P7B and 1XL4, respectively) crystal structures as templates for open and closed conformations, respectively. Amino acid sequences of KcsA, Kv1.2, KvAP, KirBac1.1, KirBac1.3, and mammalian Kir channels including Kir4.1 were aligned by the program T-coffee, and the alignment was used as the initial condition to increase the accuracy of homology modeling. Using the CHARMm-based force field implemented in the suite, the models were refined by steepest descent and conjugate gradient energy minimizations. During model-building and refinement, a constraint was applied to the carbonyl backbone of the selectivity filter to keep the model structures comparable with the original structures.

An activity-based pharmacophore hypothesis accounting for drug interaction with Kir4.1 was produced with Catalyst implemented in the suite. The drugs and their half concentrations of inhibition (IC_{50}) of Kir4.1 expressed in human embryonic kidney 293 cells were used as follows: sertraline at 7.2 μM, fluoxetine at 15.2 μM, nortriptyline at 16.0 μM, desipramine at 55.0 μM, amitriptyline at 62.1 μM, imipramine at 98.4 μM, and fluvoxamine at 196.0 μM (Su et al., 2007; Ohno et al., 2007; K. Furutani, personal communication). The tetracyclic antidepressant mianserin and the 5-hydroxytryptamine$_{1A}$ agonist buspirone that do not interact with Kir4.1 channels were used as negative controls. Diverse conformational models for each drug were generated and analyzed for features such as hydrogen bond acceptor and donor, hydrophobe, and positively and negatively charged features with HypoGen/HipHop operation in the Catalyst environment. Best-fitted conformations of these drugs were then selected by a Catalyst compare/fit operation.

The compounds were built using the Catalyst 2D/3D visualizer and were minimized to the closest local minimum by the CHARMm-based force field. Diverse conformational models for fluoxetine and nortriptyline were produced and then subjected to docking analysis using CDOCKER within the suite. Because amino acids responsible for the interaction with drugs were located in the transmembrane

domains, the central cavity was selected for the search site for docking of the drugs within the open conformation model of Kir4.1.

Drugs. Fluoxetine hydrochloride and nortriptyline hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions (10 mM) were prepared with distilled water, stored at 4°C until the day of the experiment, and diluted in the bathing solution before the experiments. All other reagents were obtained from commercial sources.

Analysis. For analysis, the recorded signals were reproduced offline and analyzed with Clampfit 9.2 (Molecular Devices). In each cell, an excessive concentration (1~3 mM) of Ba^{2+}, which totally blocked Kir channels, was applied at the end of each experiment. The Kir channel currents were measured by subtracting the Ba^{2+}-resistant currents from the total current. The response to the test drug was expressed as the current ratio, $I_{Drug}/I_{Control}$, which was obtained by dividing the current recorded in the presence of the drug at the end of each voltage step with the equivalent current recorded in the absence of the drug. The IC_{50} value of each drug was determined by fitting the concentration-response data to Hill's equation as follows: $f(D) = 1/\{1 + (D/IC_{50})^{nH}\}$, where $f(D)$ is the current ratio in the presence of the drug at a given concentration (D), and n_H is the Hill coefficient.

Results

Drugs Interact with Kir4.1 Channels in the Pore Domain. Kir4.1 is inhibited by fluoxetine and nortriptyline (see structures in Fig. 1A), whereas Kir1.1 is unaffected by either drug (Ohno et al., 2007; Su et al., 2007). We first examined whether the interaction sites for fluoxetine or nortriptyline were located in the pore-forming region, including its two TM regions (TM1 and TM2) or in the intracellular domains (i.e., N or C terminus) of Kir4.1 channels. We constructed two chimeras of Kir4.1-Kir1.1 in which the TM1 and TM2 regions were exchanged (the chimera CH414 contains the TM1–TM2 region of Kir1.1 with Kir4.1 termini, whereas the chimera CH141 contains the TM1–TM2 region of Kir4.1 with Kir1.1 termini) (Fig. 1B).

In oocytes bathed in control solution, Kir4.1, Kir1.1, CH414, and CH141 channels exhibited inward K^+ currents during the −80 mV voltage-clamp steps (Fig. 1C). As seen previously when these channels were expressed in human embryonic kidney 293T cells (Ohno et al., 2007; Su et al.,

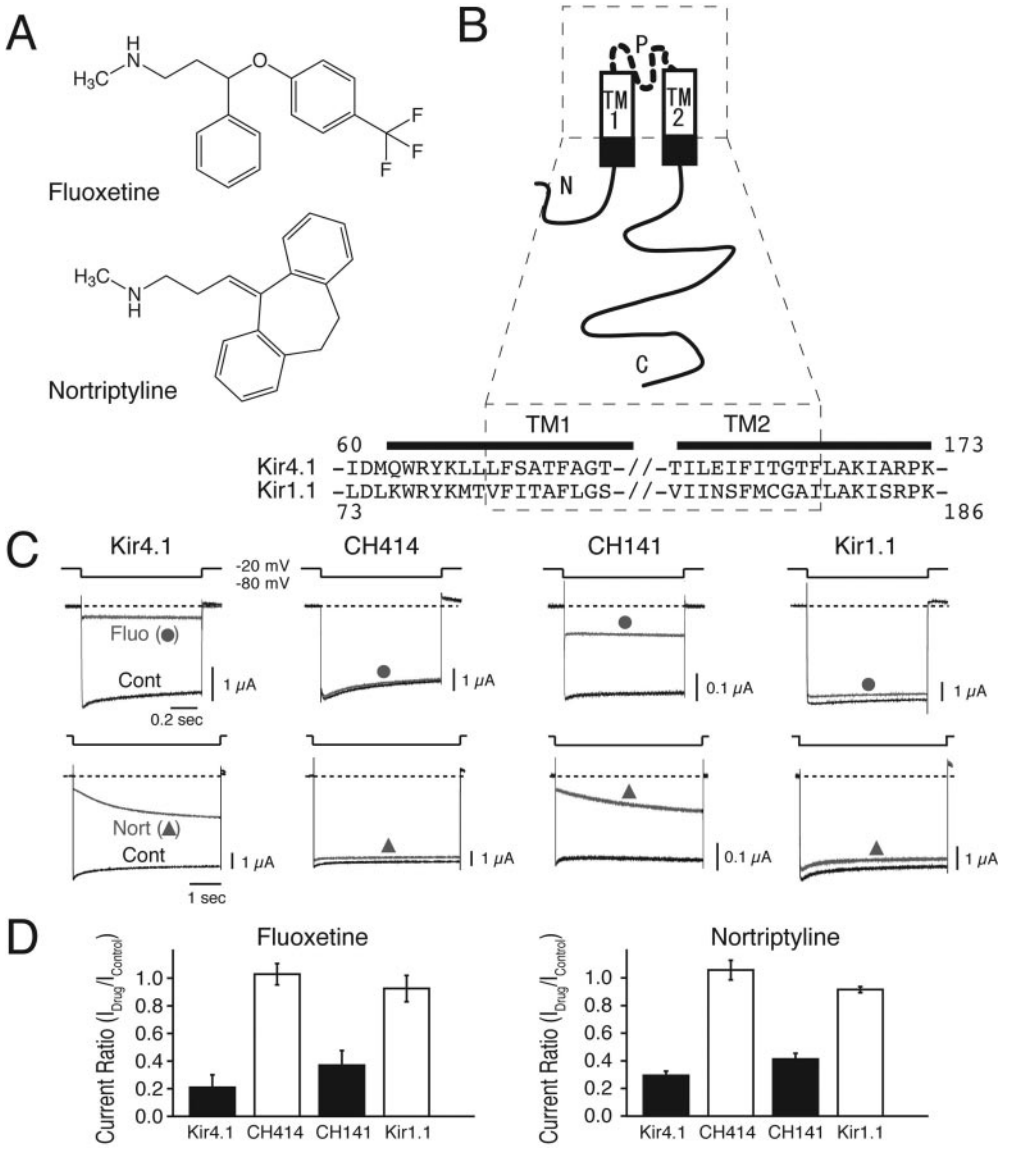

Fig. 1. Effects of fluoxetine and nortriptyline on Kir4.1, Kir1.1 and related chimera (CH414 and CH141) channels expressed in *X. laevis* oocytes. A, chemical structures of fluoxetine and nortriptyline. B, representation of the polypeptide chain topology of the Kir4.1–Kir1.1 chimeric channel and partial sequences of the parent channels around the pore-forming region. To construct the chimeric channels, the TM1, pore helix, TM2 region of Kir4.1, or Kir1.1 was exchanged to form CH414 or CH141. C, representative responses of Kir4.1, CH414, CH141, and Kir1.1 channels to 300 μM fluoxetine (Fluo) or 100 μM nortriptyline (Nort). The oocytes were voltage-clamped at −20 mV (~E_K) in 45 mM $[K^+]_o$ and stepped to −80 mV for 1 or 5 s. Superimposed traces represent currents recorded before (Cont) and after (steady state) the drug application. D, effects of fluoxetine (300 μM) and nortriptyline (100 μM) on Kir4.1, CH414, CH141, and Kir1.1 channels. The drug response was expressed as the current ratio (at −80 mV) $I_{Drug}/I_{Control}$, which was obtained by dividing the current recorded in the presence of the drug with the equivalent current recorded in the absence of the drug. Symbols represent the mean ± S.E.M. of three to nine separate experiments.

2007), fluoxetine (300 μM) and nortriptyline (100 μM) inhibited the wild-type Kir4.1 currents by approximately 70 to 80%, but they had a negligible effect upon Kir1.1 currents (Fig. 1, C and D). Inhibition of Kir4.1 channels was concentration-dependent and reversible with IC_{50} values of 76.4 μM (Hill coefficient = 1.81 ± 0.32, n = 5–11) for fluoxetine and 92.7 μM (Hill coefficient = 1.73 ± 0.34, n = 3–5) for nortriptyline (Supplementary Fig. S1). The effect of fluoxetine was voltage-independent (Fig. 1C). On the other hand, the action of nortriptyline was apparently voltage-dependent, where inhibition of Kir4.1 currents at the beginning of the voltage step was approximately 80% and then gradually reduced during the 5-s step to −80 mV (Fig. 1C).

Similar to wild-type Kir1.1, the CH414 currents were hardly affected by fluoxetine (300 μM) or nortriptyline (100 μM) (Fig. 1, C and D). In contrast, CH141 currents were effectively inhibited by fluoxetine and nortriptyline (Fig. 1, C and D). These results suggest that the interaction sites for both fluoxetine and nortriptyline are located within the TM1–TM2 region of Kir4.1.

Alanine-Scanning Mutagenesis Search for Drug Interaction Sites. To identify the amino acid residues in Kir4.1 that may be responsible for its interaction with fluoxetine and nortriptyline, we systematically replaced with alanine each of the residues of the channel pore cavity, from the pore helix to the selectively filter (Thr127–Thr128) and also in the TM2 helix (Ile148–Thr164) and examined the effects of the drugs on the mutants. Most of these Kir4.1 mutants retained electrophysiological properties comparable with the wild-type channel. The exception was E158A, which exhibited reduced inward rectification (Supplementary Fig. S2).

Figure 2 illustrates the results of this alanine scan on drug-induced block. In wild-type Kir4.1 channels, fluoxetine (300 μM) and nortriptyline (100 μM) reduced the inward K^+ current at −80 mV by ~80 and ~70%, respectively (Fig. 2). Substitutions at Thr128 located between the pore helix and selectivity filter and Glu158 located close to the center of the cavity markedly reduced the effects of fluoxetine and nortriptyline (Fig. 2). A lesser reduction of inhibition was observed with I159A. Besides Thr128 and Glu158, the substitution of most other amino acid residues in Kir4.1 to alanine did not significantly affect the drug action (Fig. 2).

Because Q150A and L151A usually exhibited only small currents, we could not examine the drug effect on these mutants. Therefore, we further substituted Gln150 and Leu151 to several amino acids residues other than alanine (Fig. 3). When Leu151 was converted to isoleucine, valine, or asparagine, these mutants elicited enough currents, and we found that L151N, but not L151I or L151V, showed reduced sensitivities to both fluoxetine and nortriptyline (Fig. 3, A and B). On the other hand, point mutants at Gln150, including Q150N, lacked functional expression (Fig. 3A).

Although the F160A substitution had an interesting effect upon block by nortriptyline, whereas instantaneous block at the onset of the voltage step remained unchanged, steady-state block was dramatically decreased (Fig. 2A and open columns in Fig. 2B, right). Phe160 may be involved in the time-dependent interaction between nortriptyline and the Kir4.1 channel.

Interactions of Kir4.1-Blocking Drugs with the Kir4.1 Channel Pore. To further explore how these drugs might interact with the Kir channel, we first generated a pharmacophore of the antidepressants that inhibit the Kir4.1 channel currents (Ohno et al., 2007; Su et al., 2007). We then tried to identify a conformational image of the interaction between the blockers and Kir4.1 channels.

The IC_{50} values of seven different Kir4.1 blockers (fluoxetine, fluvoxamine, nortriptyline, amitriptyline, desipramine, imipramine, and sertraline) were used as the index of biological activity (Ohno et al., 2007; Su et al., 2007), and we constructed a ligand-based pharmacophore model for Kir4.1 channel blockers by 3D QSAR method (Guner, 2000). The active Kir4.1 blockers apparently possess common molecular features such as positively ionizable and hydrophobic features, as well as a hydrogen bond acceptor. Based on this pharmacophore hypothesis, we generated diverse conformations of all compounds and then analyzed the three-dimensional spatial arrangements of these chemical features with the Catalyst program. The resulting hypothetical pharmacophore consisted of three hydrophobic (hydrophobe 1, 2, and 3) and one positively ionizable feature (Fig. 4A), which was able to predict acceptable IC_{50} values (R^2 = 0.766; n = 7) compared with experimental data (see Supplementary Fig. S3).

A conformer fit to this hypothetical pharmacophore was then developed for fluoxetine and nortriptyline. Both of them could be reasonably superimposed onto this assembly of four-point features (Fig. 4A). A trifluoromethyl group of fluoxetine and one of the benzene rings of nortriptyline fit onto hydrophobe 1. Fluorine has the greatest electronegativity of all the elements and may act as a hydrogen bond acceptor. An aromatic ring is partially charged and also functions as a hydrogen bond acceptor. Because Catalyst does not support these structural descriptors, we speculate that these molecular features of the drugs function as either a hydrophobic feature or a hydrogen bond acceptor.

Models of the closed and open conformations of the Kir4.1 pore region were developed with KirBac1.1, KirBac3.1, and Kv1.2 as templates (Fig. 3, B and C). Both models suggest that the side chains of Thr128 and Glu158 are exposed to the central cavity, where they could directly interact with fluoxetine and nortriptyline. One the other hand, the side chain of Gln150 is located at the intrasubunit interface between TM1, TM2, and the pore helices, interacting with the hydroxyl groups of Ser122 and Ser125 of pore helix in the models (Fig. 3C). Because the mutants at Gln150 lacked functional expression (Fig. 3A), Gln150 may be critical for the normal Kir4.1 function. The side chain of Leu151 partially exposes to the central cavity (Fig. 3C), providing the possibilities of its function in the drug-channel interaction, the direct association or the support for the drug-binding site in its correct configuration.

Further insights into the interaction between fluoxetine and nortriptyline and the pore region of Kir4.1 were obtained with docking simulations (Fig. 4B). The geometrical arrangement of both blockers indicated that they could be located within the central cavity sandwiched between a hydroxyl group of Thr128 and a carboxyl group of Glu158 on two different channel subunits diagonally opposed across the channel pore. At this position, it is unlikely that the drugs can reach close to the side chain of Leu151.

The Structural Basis for Drug Block Being Confined to Kir4.1. Docking simulations (Fig. 4B) and alanine-substi-

tution experiments (Fig. 2B) have identified Thr128 and Glu158 as main sites for interaction with fluoxetine and nortriptyline within the TM2 region of Kir4.1 channels. The alignment of the amino acid sequences of different Kir channels shows that threonine is conserved among the Kir subunits at this position. On the other hand, the amino acids corresponding to Glu158 in Kir4.1 are not conserved with Asn171 in Kir1.1, Asp172 in Kir2.1, and Asp173 in Kir3.1, respectively (Fig. 5A).

The susceptibilities of Kir channels to the drugs may correlate with the presence of a negatively charge in the position corresponding to Glu158 of Kir4.1 in the middle of TM2. To test that Glu158 of Kir4.1 is important for block by antidepressants, Asn171 of Kir1.1 was mutated to aspartic acid, glutamine, and glutamic acid, and we examined the effect of fluoxetine and nortriptyline on the mutants (Fig. 5, B and C). Although the glutamine substitution (N171Q) had little effect, the substitution of Asn171 with aspartic acid or glutamic acid allowed both drugs to block Kir1.1 mutants with a tendency for the potency of glutamic acid > aspartic acid (Fig. 5, B and C). The drugs also inhibited the currents of N171E and N171D Kir1.1 mutants in the same way as their block of Kir4.1 wild-type channels, with voltage-independent block by fluoxetine and voltage-dependent block by nortriptyline. These results indicate that these drugs require a negatively charged carboxyl group for high-affinity interaction, whereas the length of the side chain is secondary in the interaction.

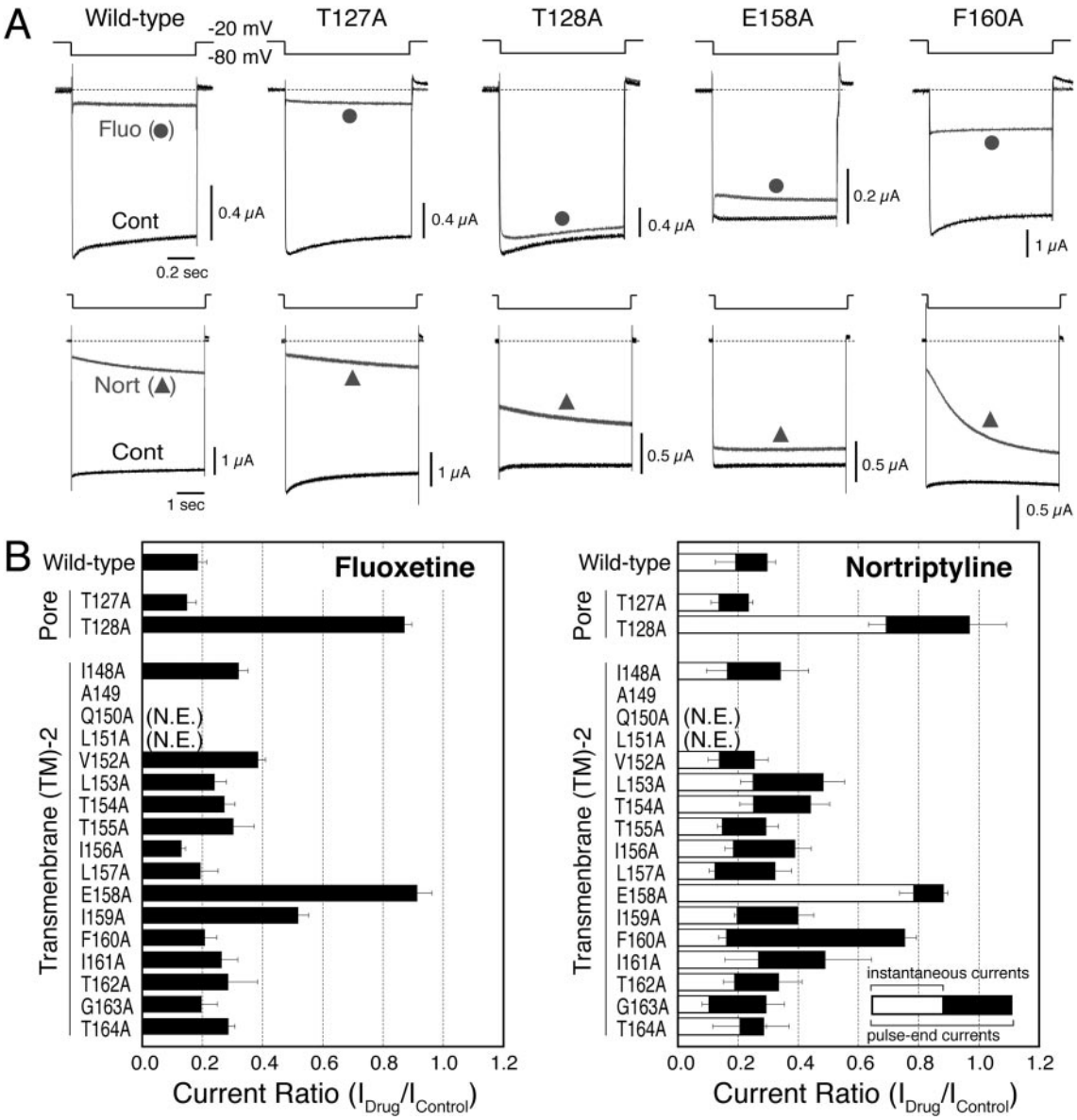

Fig. 2. Effects of alanine-scanning mutagenesis of Kir4.1 on inhibition induced by fluoxetine and nortriptyline. A, representative responses of wild-type and Kir4.1 mutants (i.e., T127A, T128A, E158A, and F160A) to 300 μM fluoxetine (Fluo, top row) or 100 μM nortriptyline (Nort, bottom row). The oocytes expressing the channels were voltage-clamped at −20 mV (~E_K) in 45 mM $[K^+]_o$ and stepped to −80 mV for 1 or 5 s. Superimposed traces were obtained before and after (steady state) the drug application. B, effects of fluoxetine (300 μM) and nortriptyline (100 μM) on mutated Kir4.1 channels in each of which an individual residue that contributes to the central cavity (Thr127 and Thr128 situated between the pore-helix and the selectivity filter and residues Ile148 to Thr164 in the TM2 helix) was replaced with alanine. The drug response was expressed as the current ratio (at −80 mV) $I_{Drug}/I_{Control}$ (Fig. 1D). N.E., mutant channel that lacked functional expression. Symbols represent the mean ± S.E.M. of three to six separate experiments.

Discussion

Drug-Channel Interactions underlying Kir4.1 Channel Block by Antidepressants. In this report, we examined the underlying mechanism for the interactions between antidepressant and Kir4.1 channel. The drug-binding site for antidepressant is the central cavity of channel pore. A position is very different from the previously identified chloroquine-binding site in Kir2.1 channel, in which the drug binds to the cytoplasmic end of the conduction pathway of the channel (Rodríguez-Menchaca et al., 2008). The alanine-scanning mutagenesis analysis shows that Thr128 and Glu158 of the Kir4.1 channel pore are crucial for antidepressants block (Fig. 2). Thr128 and Glu158 of Kir4.1 are equivalent to the positions that contribute strongly to the electrostatic profile along the pore (e.g., Thr142 and Asp172 of Kir2.1, respectively) (Robertson et al., 2008) and probably provide a favorable environment for cation.

Alanine-scan method has the power to identify significant residues, but it cannot identify the contribution of them to the drug's actions. To this end, we constructed a number of mutant Kir4.1 channels, for example T128L, T128I, E158D, E158N, and E158Q. However, these mutants exhibited currents that were too small (data not shown). Therefore, we turned to ligand-based 3D QSAR analyses and docking simulations using homology models to obtain further insights into the interaction. This bidirectional approach was crucial for the understanding the mechanism of drug-channel interaction. Molecular features of the putative interaction sites at Thr128 and Glu158 complemented those of the hypothetical pharmacophore developed for the Kir4.1-blocking antidepressants with multiple hydrophobes and one positively charged feature (Fig. 4A). Therefore, Glu158 can interact with the amine moiety of fluoxetine or nortriptyline with an ionic bond. Although Thr128 can interact with the *p*-trifluoromethyl group of fluoxetine and the benzene ring of the dibenzocycloheptane structure of nortriptyline with a hydrogen bond, both of these moieties are known to function as hydrogen-bond acceptors.

Docking simulation techniques suggest that antidepressants are positioned at the top of the Kir4.1 pore cavity where Thr128 and Glu158 are located (Fig. 4B). The distance between oxygen atoms of the hydroxyl group of Thr128 and the carboxyl group of Glu158 on channel subunits diagonally opposed in the 4-fold axis of the Kir4.1 tetramer was ~9.6 Å. This is close to the distance between the centers of the hydrogen bond acceptor and the positively ionizable region in the pharmacophore (8.9 Å). The volume of the channel's central cavity in closed and open conformations was, respec-

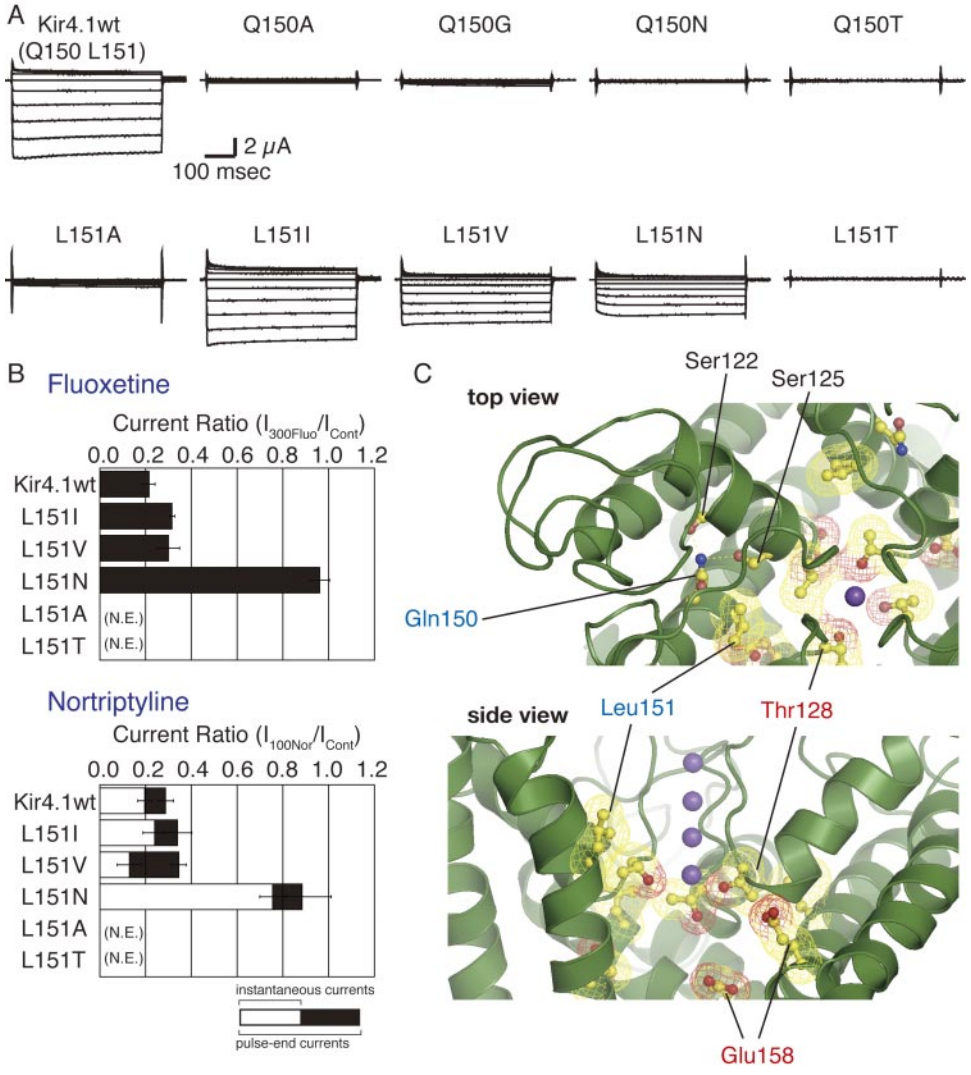

Fig. 3. Effects of mutations at Gln150 and Leu151 on Kir4.1 channel currents and block. A, representative currents from Kir4.1 wild-type (wt) channel and various mutant channels are shown. The oocytes expressing Kir4.1 wt or mutant channels were voltage-clamped at −20 mV (~E_K) in 45 mM $[K^+]_o$, and then currents were recorded during a voltage pulse from −120 to 60 mV (20-mV increments). B, effects of fluoxetine (300 μM, top) and nortriptyline (100 μM, bottom) on mutated Kir4.1 channels. The drug response was expressed as the current ratio (at −80 mV) $I_{Drug}/I_{Control}$. N.E., mutant channel that lacked functional expression. Data represent the mean ± S.E.M. of three to four separate experiments. C, Top (top) and side (bottom) view of Kir4.1 pore homology model highlighting residues Gln150 and Leu151 in TM2, as well as Ser122 and Ser125 in pore helix.

tively, 220 and 360 Å^3, which could accommodate fluoxetine (213 Å^3) and nortriptyline (196 Å^3). Therefore, Thr128 and Glu158 can stabilize drug molecules within the pore cavity.

In this study, we also found that the potency of both fluoxetine- and nortriptyline-block was reduced in L151N mutants but was not changed in L151I and L151V mutants. Homology models suggest that the side chain of Leu151 in the TM2 is located to fill the space behind selectivity filter and partially exposes to the central cavity. Leu151 of Kir4.1 corresponds to Ser165 of Kir2.1 and Ser177 of Kir3.2, a position reported to be involved in block of Kir2.1 by Rb^+, Cs^+, and Mg^{2+} (Thompson et al., 2000; Fujiwara and Kubo, 2002) and Kir3.2 channel gating and ion selectivity (Yi et al., 2001; Bichet et al., 2004). How does the mutation at this residue affect drug-affinity? Given that multiple hydrophobes are common features of antidepressants, the side chain of Leu151 may provide hydrophobic interaction with drugs. Alternatively, it may be also possible that Leu151 rather somehow structurally support the drug-binding site in its correct configuration.

On the other hand, the side chain of Gln150 seems to be away from the cavity and direct the two serine residues (Ser122 and Ser125) in the pore helix in the same subunit. Moreover, the mutations at Gln150 did not express the functional Kir currents. We found that Q150A mutant current was not inhibited by fluoxetine and nortriptyline, although the currents expressed were too small (data not shown). Alanine mutation at Gln150 might interfere with the conformation of the pore domain and thus disrupt the drug-binding site. A similar idea has been proposed in the study of vernakalant that blocks Kv1.5 (Eldstrom et al., 2007).

There are some technical limitations in our in silico analyses. First, our modeling by 3D QSAR generation for Kir4.1 blockers was performed based on only nine drugs. The number of the drugs may be insufficient for definition of their property. Second, as for the template of docking simulation, because the structure of Kir4.1 channel remains unsolved, we used the Kv1.2- and KirBac1.1-based homology models of Kir4.1 pore domain. Finally, we selected the energy-minimized docking models for presentation, although other solutions are also possible. It still remains controversial how to handle these outcomes. Even under these limitations, there is good agreement among the results of three distinct approaches (i.e., experimental mutagenesis assays of the channel, pharmacophore analyses of the drugs, and the docking simulation between the channel and the drugs). Accordingly, our strategy using the multiple methods is efficient and reliable to examine the mechanism underlying drug-channel interaction. This technical approach is more improved as the pharmacological profile of Kir4.1 is examined more extensively in the future, enabling the quantitative comparison of drug actions.

Molecular Determinants of Differential Kir Channel Sensitivity to Antidepressants. Our models can explain the mechanism of differential effects of antidepressants on Kir channels (Ohno et al., 2007; Su et al., 2007). The rank order of the effects of antidepressants on Kir channels is Kir4.1, Kir3.1, Kir2.1, and Kir1.1 (Su et al., 2007; Ohno et al., 2007; K. Furutani, personal communication). The residue corresponding to Glu158 in Kir4.1 is Asp173 in Kir3.1, Asp172 in Kir2.1, and Asn171 in Kir1.1 (Fig. 5A). Here we demonstrated that it was possible to construct a high-affinity drug-binding site at position 171 in Kir1.1 by single amino acid substitutions with the same order of efficacy, glutamic acid > aspartic acid > asparagine (Kir1.1 WT) (Fig. 5C). Therefore, the differential affinity of Kir channels for these drugs is primarily due to a single amino acid at this position.

The substitution of Asn171 of Kir1.1 to glutamic acid did not fully confer the same degree of blockade by the drugs as Kir4.1 channel (Fig. 5), suggesting that other unidentified residues in Kir4.1 involved in the effect of antidepressants. Hydrophobic drug-channel interaction might be possible be-

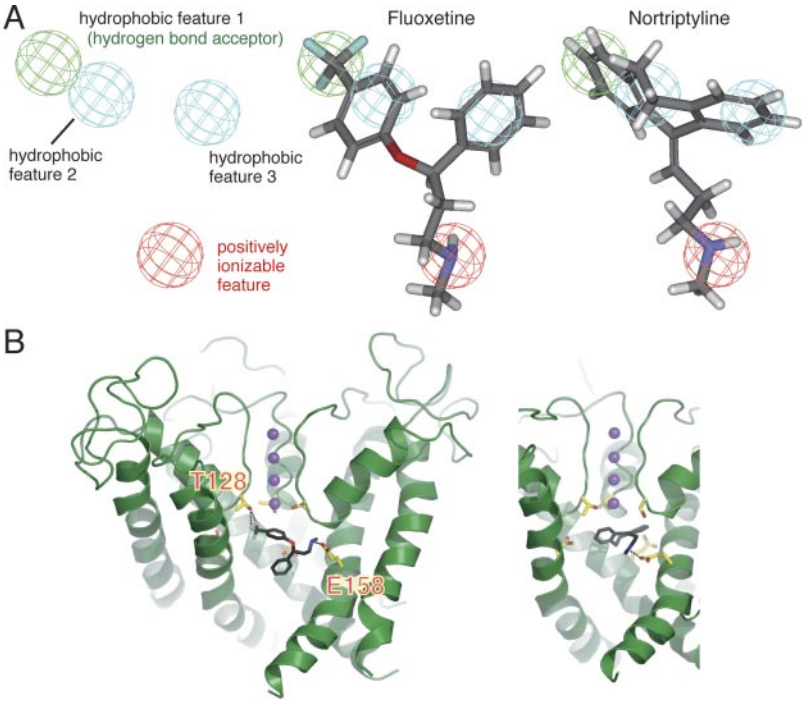

Fig. 4. In silico analysis for common pharmacophores of Kir4.1 blockers and interaction between Kir4.1 and its blockers. A, construction of a pharmacophore for Kir4.1 blockers. Quantitative structure-activity relationship-based pharmacophores were generated by the Catalyst program. The four-point pharmacophore hypothesis comprises one hydrogen bond acceptor (green), two hydrophobic (pale blue), and one positively ionizable (red) features. The hydrogen bond acceptor could also be characterized as a hydrophobic feature. The structures of fluoxetine and nortriptyline are fitted to the hypothesis. B, docking conformations of compounds in the Kir4.1 pore region. Open conformation models of Kir4.1 pore region docked with either fluoxetine (left) or nortriptyline (right). The two fluorine atoms and an amine in fluoxetine are close enough to interact, respectively, with side chains of Thr128 and Glu158. The secondary amine in nortriptyline could contact the side chain of Glu158.

cause the effect of mutations at Leu151 might be related to the hydrophobicity, and hydrophobic features are common between Kir4.1 blockers. In addition, based on our homology models, the side chain of Thr127 located at the base of pore helix and/or Thr154 or Thr155 located at one helical turn above Glu158 might interact with the drug.

Possible Mechanism of Antidepressants Block within the Central Cavity. How can we understand the mechanism underlying the antidepressants block of the K^+ channel pore function? It is clear that blocking antidepressants enter the channel pore and finally reach the central cavity. A central cavity of K^+ channels is water-filled and lowers electrostatic barriers to permeant ions by surrounding an ion with polarizable waters without creating deep energy wells in the low dielectric membrane, facilitating a high K^+ throughput (Doyle et al., 1998). Our docking simulation showed that the drugs were positioned between diagonal two-channel subunits (Fig. 4B). In addition, given their volumes, when antidepressants suspend in the cavity, the drugs occupy substantial space of the cavity and most likely plug the pore. We therefore propose the blocking mechanism as the physical constraint on the ion diffusion across the cavity by the accommodated drugs.

Apparent Voltage-Dependence of Nortriptyline Block. The voltage-independent blocker fluoxetine uses the same binding sites as the apparently voltage-dependent blocker nortriptyline. The effect of nortriptyline shares many features with classic intracellular pore blockers such as ammonium derivatives and polyamines (Armstrong and Binstock, 1965; Hille and Schwarz, 1978; Pearson and Nichols, 1998; Spassova and Lu, 1998; Guo et al., 2003; Su et al., 2007): these phenomena are better described as strong block at depolarized potential and unblock at hyperpolarized potential. The interaction sites for nortriptyline are located within the ion conduction pathway, and one of these (Glu158) is that for polyamine and/or Mg^{2+} binding in the Kir4.1 channel (Fakler et al., 1994). Although the location of the drug-interaction site may affect the apparent voltage-dependence, it is clear that the location of the drug-interaction site is not the only determinant for the voltage-dependent behav-

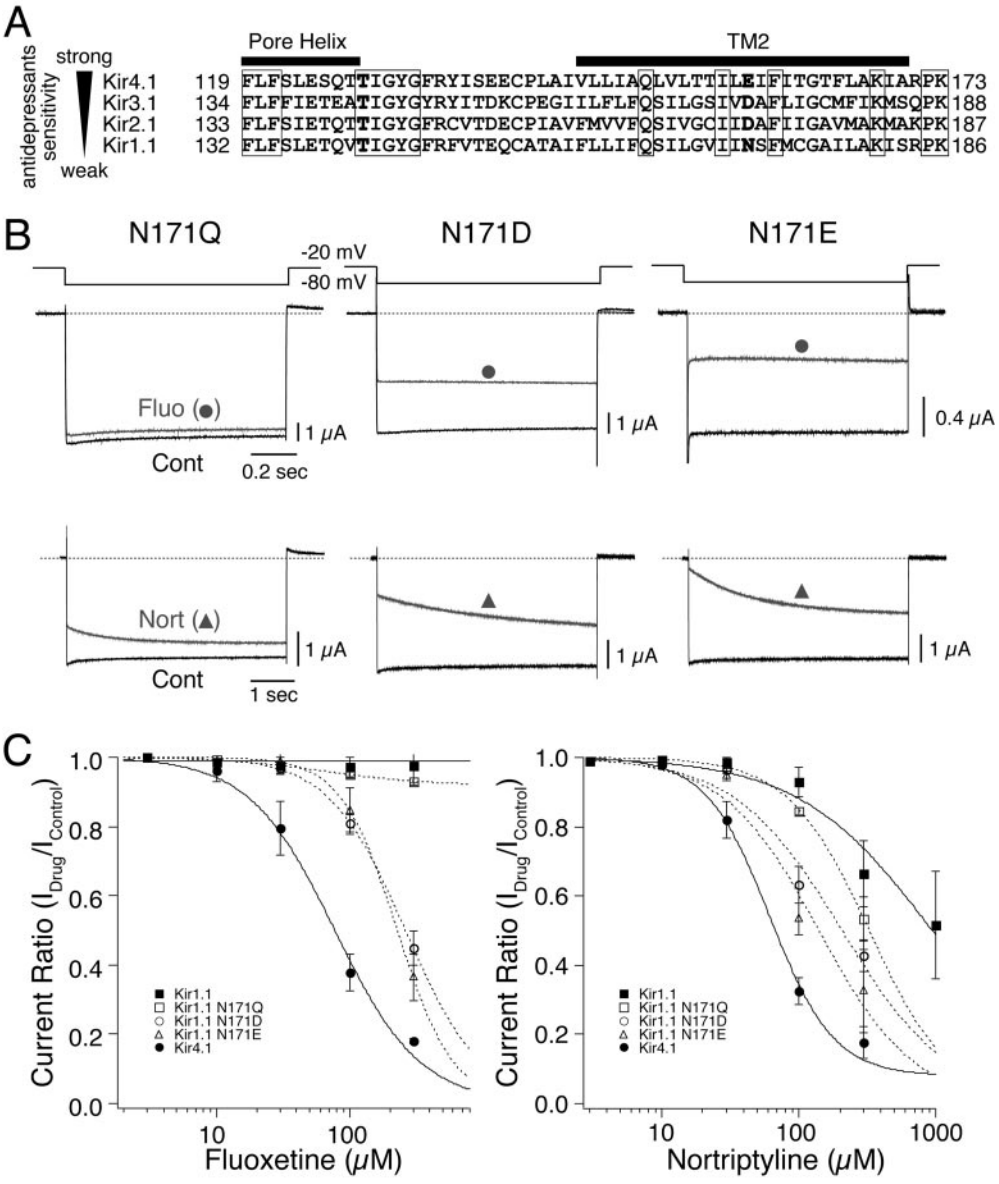

Fig. 5. Effects of substitution of Asn171 of Kir1.1 on its inhibition by fluoxetine and nortriptyline. A, amino acid sequence alignment of Kir4.1, Kir 3.1, Kir2.1, and Kir1.1 channels. Thr128 and Glu158 are the putative drug interaction sites in Kir4.1 (see *Results* and *Discussion*). B, representative responses of Kir1.1 mutants (i.e., N171Q, N171D, and N171E) to 300 μM fluoxetine (Fluo, top row) and 100 μM nortriptyline (Nort, bottom row). The oocytes expressing channels were voltage-clamped at −20 mV ($\sim E_K$) in 45 mM $[K^+]_o$ and stepped to −80 mV for 1 or 5 s. Superimposed traces were obtained before and after (steady state) drug application. C, dose-dependent effects of fluoxetine and nortriptyline on Kir1.1 mutants and wild-type Kir4.1 and Kir1.1. The drug response was expressed as the current ratio (at −80 mV) $I_{Drug}/I_{Control}$ (Fig. 1D). Symbols represent the mean ± S.E.M. of three to five separate experiments.

ior. The apparent voltage-independent action of fluoxetine at negative voltages may be due to its extremely slow kinetics of unblock.

Phe160, which is conserved in the homologous site of all Kir channels, might regulate the slow unblock of the antidepressants (Fig. 2). Our homology models of Kir4.1 in open and closed states show that Phe160 cannot directly interact with the drugs. Rather, the orientation of this residue raises the possibility of involvement of Phe160 in interactions between TM1 and TM2 domains of the Kir4.1 channel. Rapedius et al. (2007) has reported the importance of hydrogen-bonding interactions between TM1 and TM2 domains of Kir channels in channel gating. Therefore, the enhanced voltage-dependence of nortriptyline in F160A may result from changes in gating properties of the mutant.

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