

# Structural Requirements of Human Ether-a-go-go-related Gene Channels for Block by Bupivacaine

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**Background:** Local anesthetics interact with human ether-a-go-go-related gene (HERG) channels via the aromatic amino acids Y652 and F656 in the S6 region. This study aimed to establish whether the residues T623, S624, and V625 residing deeper within the pore are also involved in HERG channel block by bupivacaine. In addition, the study aimed to further define the role of the aromatic residues Y652 and F656 in bupivacaine inhibition by mutating these residues to threonine.

**Methods:** Alanine and threonine mutants were generated by site-directed mutagenesis. Electrophysiologic and pharmacologic properties of wild-type and mutant HERG channels were established using two-electrode voltage-clamp recordings of *Xenopus laevis* oocytes expressing HERG channels.

**Results:** Tail currents at  $-120$  mV through HERG wild-type channels were inhibited with an  $IC_{50}$  value of  $132 \pm 22$   $\mu$ M ( $n = 33$ ). Bupivacaine ( $300$   $\mu$ M) inhibited wild-type tail currents by  $62 \pm 12\%$  ( $n = 7$ ). Inhibition of HERG tail currents by bupivacaine ( $300$   $\mu$ M) was reduced by all mutations ( $P < 0.001$ ). The effect was largest for F656A (inhibition  $5 \pm 2\%$ ,  $n = 6$ ) in the lower S6 region and for T623A (inhibition  $13 \pm 4\%$ ,  $n = 9$ ) near the selectivity filter. Introducing threonine at positions 656 and 652 significantly reduced inhibition by bupivacaine compared with HERG wild type ( $P < 0.001$ ).

**Conclusions:** The authors' results indicate that not only the aromatic residues Y652 and F656 but also residues residing deeper within the pore and close to the selectivity filter of HERG channels are involved in inhibition of HERG channels by the low-affinity blocker bupivacaine.

INADVERTENT intravascular injection of local anesthetics may lead to prolongation of the cardiac action potential<sup>1</sup> and to severe cardiac side effects such as sudden cardiac arrest.<sup>2,3</sup> Human ether-a-go-go-related gene (HERG) channel block is supposed to be the mechanism underlying the acquired long QT syndrome caused by various drugs<sup>4</sup> including local anesthetics.<sup>5–8</sup> HERG channels constitute the molecular correlate of the rapidly activating delayed rectifier current ( $I_{Kr}$ ) in human heart.<sup>9</sup> Mutations in HERG channels may lead to a prolongation of the cardiac action potential detectable as a

prolonged QT interval in the electrocardiogram due to repolarization deficiency.<sup>9,10</sup>

In contrast to most voltage-dependent  $K^+$  channels, HERG channels lack the conserved Pro-Val-Pro motif that is supposed to produce a kink in the S6 helix.<sup>11</sup> This may lead to a larger pore-forming cavity of HERG channels, allowing preferential trapping of many structurally unrelated drugs.<sup>4</sup> Two aromatic amino acids, Y652 and F656, in the S6 helix were identified by alanine scanning analysis to be important for high-affinity drug binding to HERG channels.<sup>12–14</sup> In a previous study, we established that these two aromatic residues are also involved in low-affinity block of HERG channels by amino-amide local anesthetics.<sup>8</sup> Mutating the aromatic amino acids Y652 and F656 to alanine reduces the inhibition of HERG channels by bupivacaine, ropivacaine, and mepivacaine 4- to 30-fold.<sup>8</sup>

The residues G648 in the S6 helix and T623, S624, and V625 residing deeper within the cavity and close to the selectivity filter were—in addition to the aromatic amino acids—shown to be involved in the interaction of HERG channels with high-affinity blockers such as MK-499, vesnarinone, ibutilide, dofetilide, and E-4031.<sup>12–15</sup> Low-affinity block has been suggested to involve interaction with different residues of HERG channels than high-affinity block.<sup>16</sup> However, low-affinity block of HERG channels by local anesthetics shares several properties with high-affinity block.<sup>8</sup> We therefore extended our analysis of molecular determinants of bupivacaine interaction with HERG channels to residues T623, S624, and V625 residing in the pore region near the selectivity filter as well as to G648 in the S6 helix. In addition, the mutations Y652T and F656T were introduced to further define the role of the aromatic amino acids in local anesthetic action on HERG channels. Understanding the molecular determinants of local anesthetic interaction with HERG channels may hasten the future development of long acting local anesthetics with a wider margin of cardiac safety.

## Materials and Methods

All animal experiments were conducted in accordance with institutional guidelines and approved by local authorities (Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz, Freie und Hansestadt Hamburg, Germany).

### Molecular Biology and cRNA Preparation

The HERG mutants T623A, S624A, V625A, G648A, Y652A, Y652T, F656A, and F656T were created by site-

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Received from the Department of Anesthesiology, University Medical Center Hamburg-Eppendorf, Germany. Submitted for publication August 11, 2006. Accepted for publication November 30, 2006. Supported by a Research Grant Award (to Dr. Friederich) from the European Society of Anaesthesiology, Brussels, Belgium; and the Department of Anesthesiology and the Institute for Neural Signal Transduction, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. Presented in part at the Annual Meeting of the American Society of Anesthesiologists, Chicago, Illinois, October 14–18, 2006.

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directed mutagenesis.<sup>17</sup> All constructs were cloned in the pGEM expression vector for complementary RNA (cRNA) synthesis. After linearization of the plasmid with *NotI* (Fermentas, St. Leon-Rot, Germany), the cRNA was synthesized *in vitro* with the mMACHINE Kit (Ambion, Austin, TX) according to the manufacturer's protocol. The cRNA was purified with a phenol/chloroform extraction. The integrity of the cRNA was analyzed in a denaturing gel. The concentration was determined with the RiboGreen method (RiboGreen RNA Quantification Reagent, Molecular Probes, Eugene, OR).

#### Oocyte Preparation and Injection

For preparation of oocytes, female *Xenopus laevis* frogs (Nasco, Fort Atkinson, WI) were anesthetized with tricaine solution (0.6 g/500 ml) for approximately 20 min, and ovarian lobes were removed surgically. The oocytes were separated mechanically, and the connective tissue was digested by collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) for 2–4 h. Afterward, good oocytes (stages V and VI) were selected and incubated at 17°C in gentamicin containing oocyte ringer solution (75 mM NaCl, 2 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM Na-pyruvate, and 5 mM HEPES, pH adjusted to 7.5 with NaOH, 50 µg/ml gentamicin; all from Sigma, Deisenhofen, Germany). The cRNA was injected (50 nl per oocyte) by using a Nanoliter injector (World Precision Instruments, Sarasota, FL). The total concentration of cRNA was 80 ng/µl. Two electrode voltage clamp experiments were performed 2–5 days after the cRNA injection.

#### Electrophysiology

Two-electrode voltage clamp recordings were performed with an Oocyte-Clamp OC-725C amplifier (Warner Instrument Corporation, Hamden, CT) and Pulse software version 8.50 (HEKA Elektronik, Lambrecht, Germany). Sharp electrodes were pulled from borosilicate glass capillary tube (World Precision Instruments) on a vertical puller (Narishige, Tokyo, Japan) and filled with 3 M KCl and 3% agar. The standard extracellular recording solution contains 79.5 mM NaCl, 2 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES (all from Sigma, Deisenhofen, Germany), pH adjusted to 7.5 with NaOH. For some mutants that showed small currents under standard conditions, an extracellular solution with 20 mM KCl (containing 97 mM NaCl, 20 mM KCl, 1.8 mM CaCl<sub>2</sub>, and 10 mM HEPES) was used. Different pulse protocols were used for characterization of the channels and to establish their pharmacologic sensitivities. The holding potential was –80 mV for all experiments. For the activation protocol, depolarizing pulses were applied from –80 to +60 mV in 10-mV steps. The duration of the depolarization was 1 s, and tail currents were recorded at –120 mV. The steady state inactivation was

assessed by a three-step protocol: First, the membrane was depolarized for 2 s from –80 to +40 mV; then, pulses from –120 to +60 mV in 10-mV steps were applied for 30 ms; and finally, the membrane potential was +40 mV for 0.5 s. For pharmacologic experiments, single square pulses to +0 or +40 mV for 2 s were used. Tail currents were recorded at –40 or –120 mV. Repetitive pulses were applied to determine that steady state inhibition was reached.

Bupivacaine HCl (Sigma) was dissolved in the respective extracellular recording solution. A hydrostatically driven perfusion system was used to apply the drugs onto the oocytes and to exchange the extracellular solutions. All experiments were performed at room temperature.

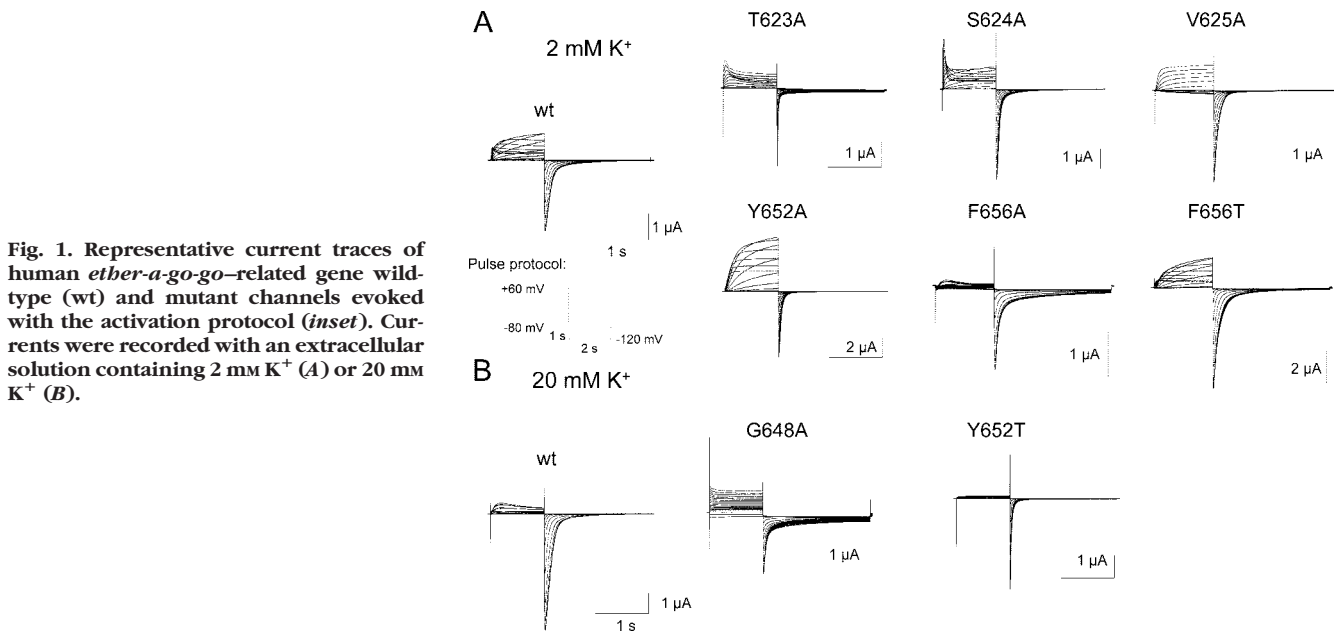
#### Data Analysis

Data were analyzed with Pulse Fit software (HEKA Elektronik) and with KaleidaGraph software (Synergy Software, Reading, PA). The normalized tail currents during the activation protocol were fitted by a modified Boltzmann equation:  $I = I_{\max}/[1 + \exp((V_{0.5} - V_m)/k)]$ , where  $V_{0.5}$  is the voltage of half-maximal activation,  $V_m$  is the membrane potential, and  $k$  is the slope factor. For the analysis of the steady state inactivation, maximal current amplitudes during the tail were corrected for the closing during the hyperpolarizing step at very negative potentials as described previously.<sup>18</sup> The corrected curves were fitted with a Boltzmann function. The time course of inactivation was fitted with a monoexponential function after test pulse to –120 mV:  $y = C + A_i(-t/\tau_{\text{inact}})$ , with the time constants of inactivation  $\tau_{\text{inact}}$  and the amplitude  $A_i$ . The inhibition of currents by local anesthetics was quantified by the reduction of the maximal current during the test pulse. The fractional block  $f$  was calculated by the following formula:  $f = 1 - (I_{\max, \text{drug}}/I_{\max, \text{control}})$ . Concentration–response curves were fitted by a Hill function:  $f = 1/[1 + (IC_{50}/c)^h]$ , where  $IC_{50}$  is the concentration of half-maximal inhibition,  $c$  is the concentration of the local anesthetic, and  $h$  is the Hill coefficient. The time course of the onset of block was analyzed using the following equation:  $y = I_{\text{bupi}}/I_{\text{con}}$ , where  $I_{\text{con}}$  is the current at any given time under control conditions and  $I_{\text{bupi}}$  the current at the same time after application of bupivacaine. An exponential function with one or two time constants was used to fit the time course of the onset of block:  $y = C + A_1 \exp(-t/\tau_1) + A_2 \exp(t/\tau_2)$ , with the time constants  $\tau_1$  and  $\tau_2$ , and the amplitudes  $A_1$  and  $A_2$ . Statistical significance was tested using a two-sided Student  $t$  test (Excel, Microsoft, Redmond, WA). Data are presented as mean  $\pm$  SD unless stated otherwise;  $n$  values indicate the number of experiments.

## Results

#### Electrophysiologic Properties of HERG Mutants

Before evaluating the effect of the mutations on local anesthetic sensitivity of HERG channels, the electro-



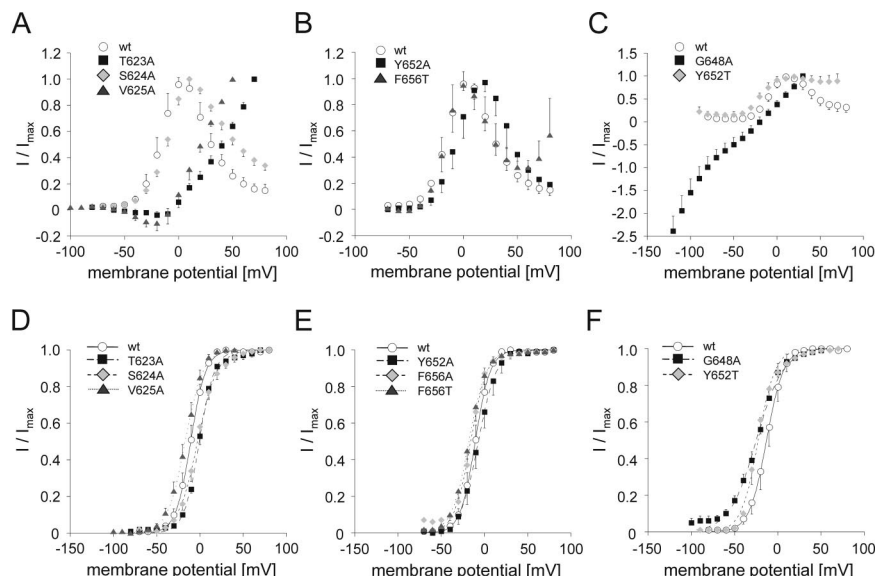
**Fig. 1.** Representative current traces of human *ether-a-go-go*-related gene wild-type (wt) and mutant channels evoked with the activation protocol (*inset*). Currents were recorded with an extracellular solution containing 2 mM  $K^+$  (A) or 20 mM  $K^+$  (B).

physiologic properties of the mutant channels had to be characterized first. Representative current traces evoked with the activation protocol are shown in figure 1. Tail currents were recorded at a potential of  $-120$  mV because some of the mutants (HERG<sub>T623A</sub>, HERG<sub>V625A</sub>, and HERG<sub>F656A</sub>) gave rise to only small tail currents at  $-40$  mV. The mutants HERG<sub>G648A</sub> and HERG<sub>Y652T</sub> were recorded at an extracellular potassium concentration of 20 mM because small currents possibly due to poor membrane expression did not allow measurements under standard conditions. This potassium concentration allowed recording larger inward tail currents at negative potentials without profoundly affecting inhibition of HERG channels.<sup>19,20</sup>

The mutations S624A, Y652A, and F656T had only a small influence on voltage-dependent activation (figs.

2A and B). The current-voltage relation determined at the end of the activating test pulse exhibited the typical bell-shaped relation that was similar to the current-voltage relation of HERG<sub>wt</sub> channels (figs. 2A and B). In contrast, the mutations T623A and V625A caused a loss of the inward rectification and showed a linear current-voltage relation (fig. 2A). Outward currents of HERG<sub>F656A</sub> channels were too small to be analyzed. The current-voltage relation of HERG<sub>Y652T</sub> channels reached a plateau between membrane potentials of 0 to  $+50$  mV (fig. 2C). The mutation G648A resulted in a linear current-voltage relation between membrane potentials of  $-120$  and  $+40$  mV (fig. 2C). The current-voltage relation of tail currents were described with Boltzmann functions (figs. 2D-F). Apart from HERG<sub>Y652A</sub>, all mutations significantly shifted the

**Fig. 2.** Analysis of the activation behavior of human *ether-a-go-go*-related gene wild-type (wt) or mutant channels. Normalized maximal current ( $I/I_{max}$ ) during the test pulse (A-C) or during the tail (D-F) were plotted against the membrane voltage. Parameters of Boltzmann fits are summarized in table 1. Recordings were performed with an extracellular solution containing 2 mM  $K^+$  (A, B, D, and E) or 20 mM  $K^+$  (C and F).



**Table 1. Parameters for Boltzmann Fits of Activation and Inactivation of Human *ether-a-go-go*-related Gene Wild-type and Mutant Channels**

	Activation (Tail -120 mV)			Inactivation			
	$V_{0.5}$ , mV	k, mV	n	$V_{0.5}$ , mV	k, mV	$\tau_{inact}$ , ms	n
Wt	$-9.4 \pm 3.3$	$8.8 \pm 0.9$	7	$-29.2 \pm 11.0$	$-17.5 \pm 1.5$	$9.0 \pm 3.9$	5
Wt (20 mM $K^+$ )	$-13.0 \pm 4.4$	$9.5 \pm 0.7$	8	$-69.8 \pm 2.2\ddagger$	$-24.7 \pm 1.0\ddagger$	$9.0 \pm 1.0$	6
T623A	$-1.2 \pm 1.5\ddagger$	$8.3 \pm 0.9$	8	$-57.4 \pm 4.9\ddagger$	$-17.7 \pm 2.4$	$3.0 \pm 0.9^*$	7
S624A	$-3.3 \pm 2.5^*$	$10.7 \pm 0.6\ddagger$	6	$-46.0 \pm 6.6^*$	$-22.8 \pm 2.3\ddagger$	$16.8 \pm 4.3^*$	9
V625A	$-16.9 \pm 2.8\ddagger$	$10.2 \pm 0.2\ddagger$	10	No inactivation			
G648A (20 mM $K^+$ )	$-22.6 \pm 3.1\ddagger$	$13.7 \pm 1.3\ddagger$	11	$-82.1 \pm 3.7\ddagger$	$-26.9 \pm 4.8$	$4.9 \pm 0.7\ddagger$	6
Y652A	$-7.2 \pm 4.0$	$10.1 \pm 1.3^*$	9	$-14.0 \pm 10.9^*$	$-21.8 \pm 1.5\ddagger$	$4.0 \pm 0.8^*$	6
Y652T (20 mM $K^+$ )	$-23.4 \pm 3.0\ddagger$	$8.8 \pm 0.4^*$	8	$-67.5 \pm 1.8$	$-19.2 \pm 1.7\ddagger$	$3.0 \pm 0.3\ddagger$	7
F656A	$-16.2 \pm 1.2^*$	$9.2 \pm 0.4$	5	$-95.2 \pm 5.5\ddagger$	$-19.4 \pm 2.4$	$3.4 \pm 0.8^*$	8
F656T	$-18.3 \pm 3.4\ddagger$	$9.7 \pm 0.4^*$	6	$-47.8 \pm 5.0^*$	$-22.1 \pm 2.9\ddagger$	$9.4 \pm 1.3$	7

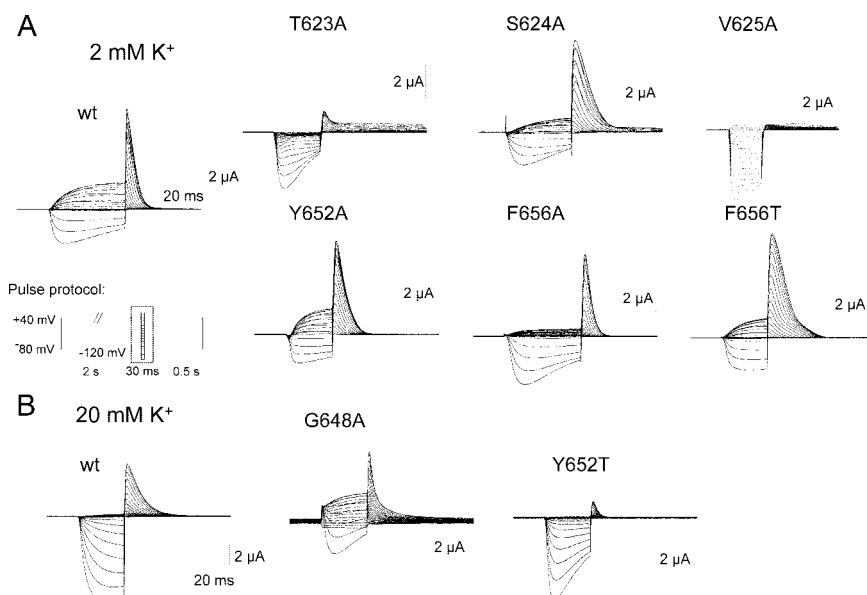
t test vs. wild-type (wt) (for G648A and Y652T vs. wt with 20 mM  $K^+$ ): \*  $P < 0.05$ ; †  $P < 0.001$ .

k = slope factor; n = number of experiments;  $\tau_{inact}$  = time constant of inactivation;  $V_{0.5}$  = voltage of half-maximal activation/inactivation.

midpoint of current activation either in the depolarizing or in the hyperpolarizing direction (figs. 2D-F). The parameters of the Boltzmann functions are summarized in table 1. The influence of the mutations on voltage dependence of inactivation was analyzed next (fig. 3). The mutant HERG<sub>T623A</sub> gave rise to only small outward currents after recovery from inactivation, and the mutation V625A completely abolished inactivation (fig. 3A). The voltage dependence of inactivation was described with Boltzmann functions (fig. 4). Parameters of the Boltzmann functions are given in table 1. Whereas Y652A shifted the inactivation midpoint into the depolarizing direction and Y652T did not influence the inactivation midpoint, all other mutations shifted the midpoint of current inactivation into the hyperpolarizing direction. Time constants of inactivation at -120 mV remained unchanged by F656T, were slowed by S624A, and were accelerated by all other mutations (table 1).

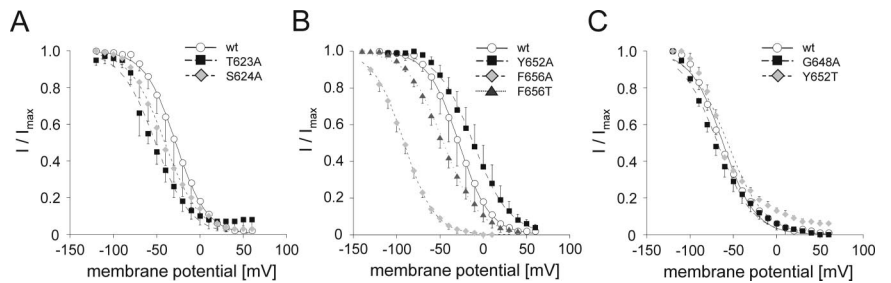
### Inhibition of HERG Wild-type Channels by Bupivacaine

Concentration dependence of HERG<sub>wt</sub> inhibition was established first to determine a concentration suitable for comparing the sensitivities of mutant and wild-type channels. Inhibition was analyzed as inhibition of the current elicited by the depolarization to 0 mV ( $I_{test}$ ) and as inhibition of the tail current elicited by a step to -40 mV ( $I_{tail} -40$ ) and to -120 mV ( $I_{tail} -120$ ), respectively (fig. 5A). The concentration response data were fitted with Hill functions (fig. 5B). The parameters of the Hill functions are given in table 2. Deactivating tail currents at -120 mV were less sensitive to inhibition by bupivacaine than tail currents elicited at -40 mV as well as currents elicited by the depolarizing pulse to 0 mV. The extent of HERG<sub>wt</sub> channel inhibition by 300  $\mu$ M bupivacaine was significantly altered by extracellular  $K^+$  when analyzed at a test potential of 0 mV (2 mM  $K^+$ :  $84 \pm 3\%$ , n = 7; 20 mM  $K^+$ :  $76 \pm 5\%$ , n = 6;  $P < 0.01$ ). However,



**Fig. 3. Representative current traces of human *ether-a-go-go*-related gene wild-type (wt) and mutant channels evoked with the inactivation protocol (inset). Currents were recorded with an extracellular solution containing 2 mM  $K^+$  (A) or 20 mM  $K^+$  (B).**

**Fig. 4.** Current–voltage relation for human *ether-a-go-go*-related gene channel inactivation for wild-type (wt) and mutant channels. (A and B) 2 mM K<sup>+</sup>; (C) 20 mM K<sup>+</sup>. Parameters of Boltzmann fits are presented in table 1.



inhibition remained unchanged when analyzed as inhibition of tail currents at a membrane potential of  $-120$  mV (2 mM K<sup>+</sup>:  $62 \pm 12\%$ ,  $n = 7$ ; 20 mM K<sup>+</sup>:  $58 \pm 7\%$ ,  $n = 6$ ;  $P > 0.05$ ).

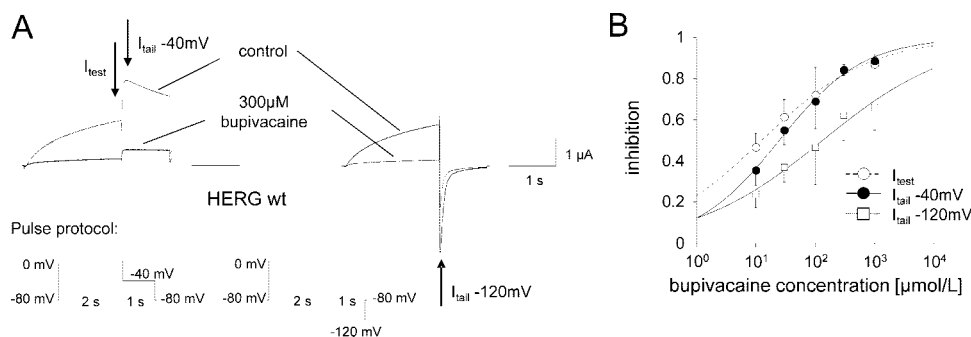
#### Inhibition of HERG Mutants by Bupivacaine

Because the  $IC_{50}$  value for inhibition of HERG<sub>wt</sub> channels was dependent on the test protocol (table 2), a concentration of bupivacaine (300  $\mu$ M) was chosen that allowed comparing inhibitory effects on mutated and wild-type channels with all protocols (fig. 6). For the mutant channels HERG<sub>S624A</sub>, HERG<sub>Y652A</sub>, and HERG<sub>F656T</sub>, inhibition of currents elicited during pulses to 0 mV (figs. 6A and E) and inhibition of tail currents at  $-40$  and  $-120$  mV was analyzed (figs. 6A, E, and F). All three mutations reduced inhibition of HERG channels at all protocols, with the effect being most pronounced for HERG<sub>F656T</sub>. The mutant channels HERG<sub>T623A</sub> and HERG<sub>V625A</sub> exhibited only small outward currents. Therefore, inhibition of outward currents was analyzed at  $+40$  mV, and tail current inhibition was analyzed at  $-120$  mV (figs. 6B and F). The mutations T623A and V625A significantly reduced the inhibitory effect of bupivacaine at both test potentials. The mutation F656A only allowed measuring inhibition of tail currents at  $-120$  mV (figs. 6C and F). The mutation significantly reduced the inhibitory effect of bupivacaine. Inhibition of HERG<sub>G648A</sub> and HERG<sub>Y652T</sub> had to be analyzed at an extracellular K<sup>+</sup> concentration of 20 mM (figs. 6D and F). Inhibition of HERG<sub>wt</sub> channels was analyzed under these conditions as well. Both G648A and Y652T significantly reduced inhibition when compared with wild-type channels (figs. 6D and F).

The onset of block was analyzed for HERG<sub>wt</sub> and all mutants except for HERG<sub>F656A</sub>. Outward currents conducted by this mutant were too small for analysis. Exemplary plots are shown in figure 7. Onset of block developed biexponentially for HERG<sub>wt</sub> under either extracellular K<sup>+</sup> concentrations (2 mM K<sup>+</sup>:  $\tau_{onset 1} = 42 \pm 11$  ms,  $\tau_{onset 2} = 300 \pm 64$  ms,  $n = 7$ ; 20 mM K<sup>+</sup>:  $\tau_{onset 1} = 44 \pm 13$  ms,  $\tau_{onset 2} = 374 \pm 54$  ms,  $n = 6$ ). The slower time constant  $\tau_{onset 2}$  differed significantly between both conditions ( $P < 0.05$ ). Both time constants were significantly slowed for HERG<sub>S624A</sub> (fig. 8A). For HERG<sub>V625A</sub>,  $\tau_{onset 1}$  decreased and  $\tau_{onset 2}$  remained unchanged compared with HERG<sub>wt</sub>. For the mutants T623A, G648A, Y652A, Y652T, and F656T, the onset of block was best described with only a single time constant (fig. 8A). The onset of block was accelerated for HERG<sub>Y652A</sub> and slowed for HERG<sub>T623A</sub>, HERG<sub>G648A</sub>, and HERG<sub>Y652T</sub>. It remained unchanged for HERG<sub>F656T</sub>. Whereas the onset of block did not significantly differ between HERG<sub>Y652A</sub> and HERG<sub>F656T</sub>, it was slower for HERG<sub>Y652T</sub> than for HERG<sub>Y652A</sub> and HERG<sub>F656T</sub>. After the initial onset of block HERG<sub>T623A</sub>, HERG<sub>Y652A</sub>, HERG<sub>Y652T</sub>, and HERG<sub>F656T</sub> partially recovered from block (fig. 7). Time constants for partial recovery from block were not significantly different between these mutations (fig. 8B).

## Discussion

The aromatic residues F656 and Y652 in the S6 region of HERG channels have been identified to be important



**Fig. 5.** Inhibition of human *ether-a-go-go*-related gene (HERG) wild-type channels by bupivacaine. (A) Exemplary current traces of square pulses to 0 mV under control conditions and after application of 300  $\mu$ M bupivacaine. Tail currents were recorded at  $-40$  and  $-120$  mV. (B) Concentration–response curves of HERG wild-type channel inhibition by bupivacaine for the different protocols. Parameters of Hill fits are summarized in table 2.

**Table 2. Parameters for Hill Fits of Inhibition of Human *ether-a-go-go*-related Gene Wild-type Channels Recorded with the Different Protocols**

	$I_{\text{test}}$ 0 mV	$I_{\text{tail}}$ -40 mV	$I_{\text{tail}}$ -120 mV
$IC_{50}$ , $\mu\text{M}$	$12 \pm 2$	$25 \pm 2$	$132 \pm 22$
Hill	$0.48 \pm 0.03$	$0.61 \pm 0.04$	$0.40 \pm 0.04$
n	33	33	33

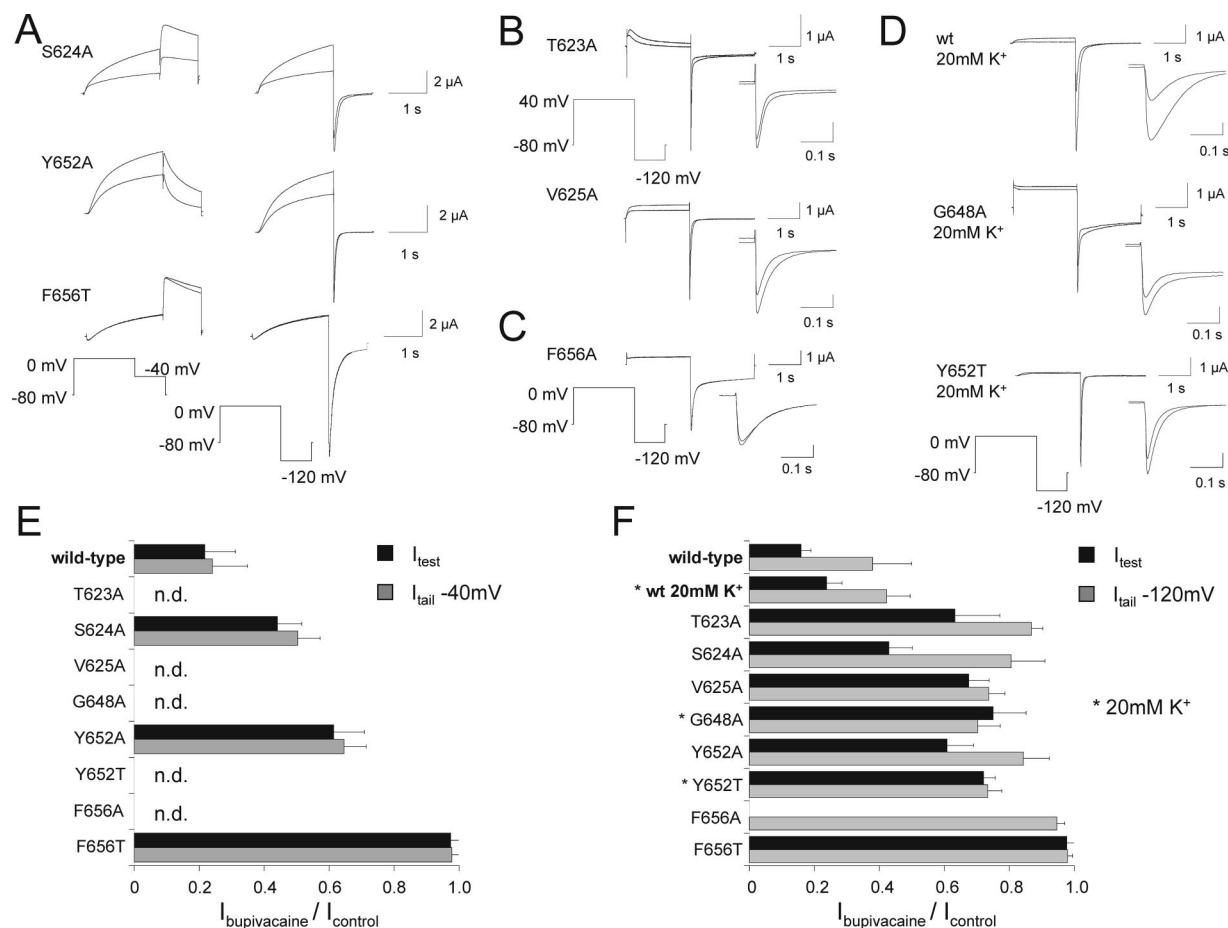
Inhibition was analyzed during the test pulse to 0 mV ( $I_{\text{test}}$  0 mV) and during the tail at -40 mV ( $I_{\text{tail}}$  -40 mV) or at -120 mV ( $I_{\text{tail}}$  -120 mV).

Hill = Hill coefficient;  $IC_{50}$  = concentration of half-maximal inhibition; n = number of experiments.

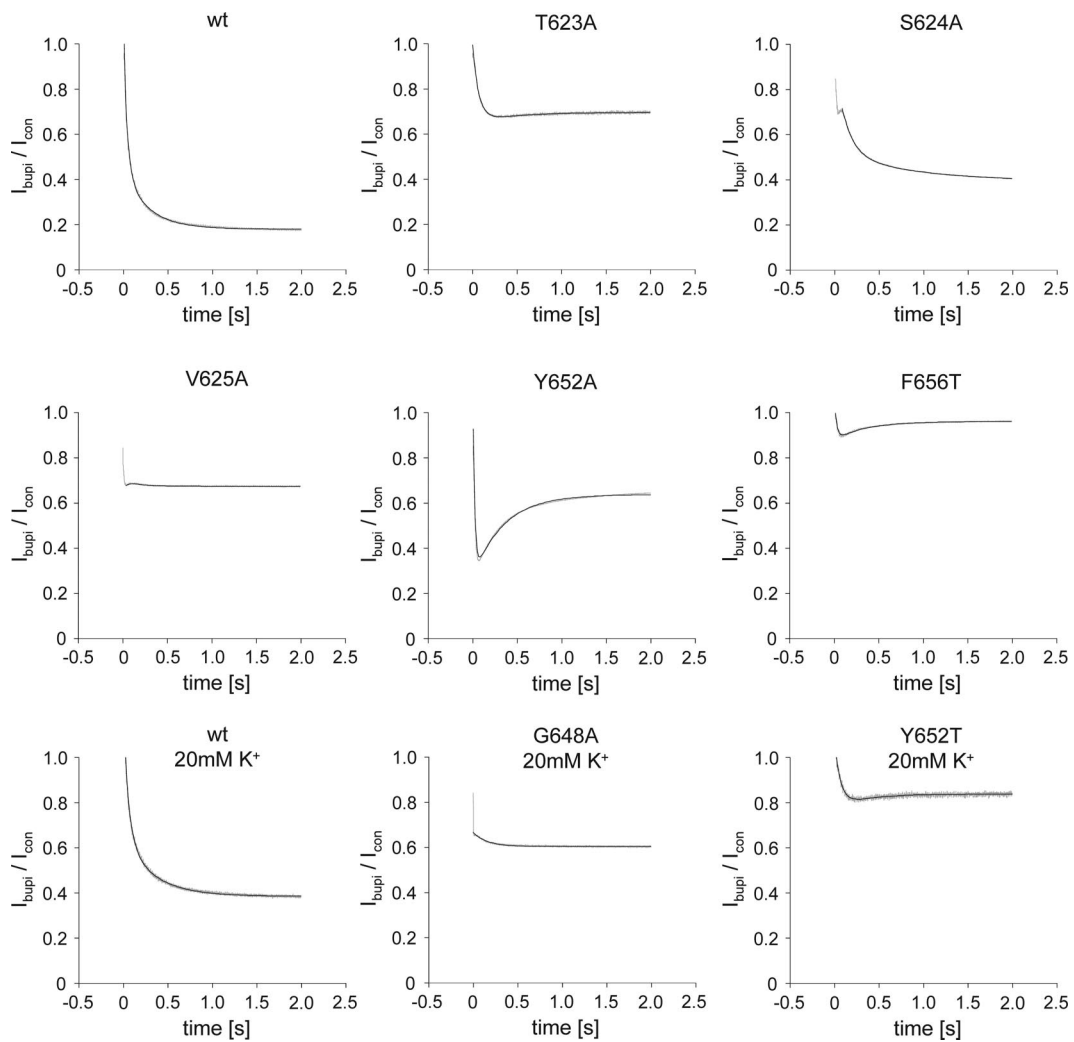
for local anesthetic interaction.<sup>8</sup> The results of this study confirmed the role of these aromatic amino acids in local anesthetic block and extended the analysis of molecular determinants of HERG channel block to residues residing deeper within the pore. The contribution of three residues near the selectivity filter, T623, S624, and V625, was analyzed in this work by mutating these residues to alanine. All three mutations altered the gating behavior of the channels. T623A and V625A eliminated inward rectification of HERG channels and altered the typical

bell-shaped voltage dependence of outward currents into a linear current-voltage relation. The mutation V625A furthermore abolished inactivation of HERG channels. The mutation S624A shifted the voltage dependence of activation and inactivation without larger changes of the macroscopic current behavior. Although it cannot be excluded that the reduced inhibition by bupivacaine is related to the changes in channel gating, this seems less likely because the changes in gating are qualitatively different, but the extent of reduction in inhibition is similar for all three mutations.

The role of inactivation in HERG channel block is controversial: Lipka *et al.* demonstrated that abolishing C-type inactivation by introducing the double mutant HERG<sub>G628C/S631C</sub> reduces inhibition by bupivacaine<sup>5</sup> similar to other HERG channel blockers such as dofetilide<sup>21</sup> and E-4031.<sup>19</sup> In contrast, other mutations affecting HERG inactivation yield inconsistent results concerning the inhibition by high-affinity<sup>4,22</sup> as well as by low-affinity blockers.<sup>23</sup> Also, inhibition of HERG channels by cocaine is only affected by some but not all mutations influencing inactivation.<sup>24</sup> The results of this study dem-



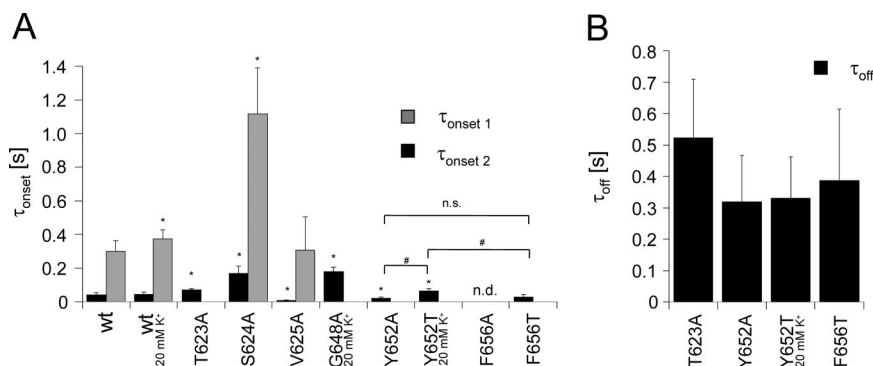
**Fig. 6. (A–D)** Currents through human *ether-a-go-go*-related gene (HERG) wild-type (wt) and mutant channels under control conditions and after application of 300  $\mu\text{M}$  bupivacaine. The first 250 ms of the deactivating tail currents are shown as *blow-up*. The respective pulse protocols are shown as *insets*. **(E and F)** Comparison of the residual current  $I_{\text{bupivacaine}}/I_{\text{control}}$  after application of 300  $\mu\text{M}$  bupivacaine for HERG wild-type and the different mutant channels. Inhibition by 300  $\mu\text{M}$  bupivacaine was reduced by all mutations ( $P < 0.001$ ). n.d. = no data.



**Fig. 7.** Onset of block. The onset of block was analyzed as the fractional current  $I_{\text{bupivacaine}}/I_{\text{control}}$  ( $I_{\text{bupi}}/I_{\text{con}}$ ) for every time point of the depolarization. The time course of onset was fitted by an exponential function with one or two time constants. No analysis was possible for the outward currents of the mutant F656A. wt = wild-type.

onstrate that inhibition of HERG channels by bupivacaine is reduced to a similar extent by a mutation that accelerates inactivation (T623A) and by a mutation that

abolishes inactivation (V625A). Reduced bupivacaine affinity of noninactivating HERG mutant channels (Lipka *et al.*,<sup>5</sup> this study) may thus result from inactivation gating-



**Fig. 8.** (A) Comparison of the time constants ( $\tau_{\text{onset}}$ ) derived from the analysis of the onset of block (fig. 7). Onset of human *ether-a-go-go*-related gene (HERG) wild-type (wt) was best described with two time constants. However, for some mutants, the onset of block followed a monoexponential process. \* Significant difference ( $P < 0.05$ ) compared with  $\text{HERG}_{\text{wt}}$  under the respective conditions. Time constants for the onset were compared between the mutations of the aromatic amino acids Y652 and F656 as well (#  $P < 0.05$ ; n.d. = no data; n.s. = not significant). (B) Time constants for the partial recovery from block ( $\tau_{\text{off}}$ ) did not significantly differ between  $\text{HERG}_{\text{T623A}}$ ,  $\text{HERG}_{\text{Y652A}}$ ,  $\text{HERG}_{\text{Y652T}}$ , and  $\text{HERG}_{\text{F656T}}$  ( $P > 0.05$ ).

associated reorientation of residues in the S6 domain that comprise the bupivacaine binding site rather than resulting from a direct effect of the inactivation. Taken together, our findings may therefore indicate that inactivation is neither a sufficient nor a necessary prerequisite for low-affinity block of HERG channels by bupivacaine. The results furthermore suggest that these three residues at the base of the pore helix immediately adjacent to the GFG signature sequence of the selectivity filter are potential binding sites not only for some high-affinity blockers<sup>4,14,25</sup> but also for the low-affinity blocker bupivacaine. Mutations from threonine to alanine at position 623 and serine to alanine at position 624 both remove the hydroxyl group of the amino acid side chain. This group may be responsible for mediating polar interactions with hydrophilic parts of the bupivacaine molecule. Because loss of the hydroxyl group at the position T623 caused a more pronounced reduction of inhibition by bupivacaine than loss of the same group at position S624, it may be argued that position T623 is more important for interaction with bupivacaine. On the other site, it may be argued that the small difference in inhibition between these mutation points to a nonpolar rather than a polar interaction of the drug molecule with the ion channel protein that does not depend on the hydroxyl group of the amino acids.

The mutation G648A in the S6 helix poorly expresses under standard conditions<sup>12</sup> and was thus analyzed with an extracellular solution containing 20 mM potassium. These channels were continuously open, even at very negative membrane potentials. Voltage dependence of activation and inactivation were shifted to negative potentials. Because glycine does not possess a side chain that could account for bupivacaine interaction, it is surprising that introducing a methyl group at this position by the mutation G648A did not increase but decreased anesthetic sensitivity. The reduced inhibition by bupivacaine for this mutant may thus also be related to gating-associated reorientation of residues in the S6 domain rather than to a direct involvement of this amino acid in bupivacaine binding.

The aromatic amino acids Y652 and F656 have been implicated in the interaction of local anesthetics with HERG channels.<sup>8</sup> Mutating either aromatic amino acid to alanine significantly reduced local anesthetic sensitivity of HERG channels. However, these mutations also introduce gating changes that may explain altered local anesthetic sensitivity.<sup>8</sup> These gating changes are qualitatively and quantitatively different, and they are much more pronounced for F656A than for Y652A. The introduction of threonine in either position had the opposite effects on channel gating than the introduction of alanine. Albeit being again qualitatively and quantitatively different, they were more pronounced for Y652T than for F656T. The consequences of these mutations therefore allowed comparing the effects on bupivacaine sensitivity of two

different mutations at either position causing different effects on channel gating. Regardless of the effects on channel gating, however, all four mutations at positions 652 and 656 reduced the inhibition by bupivacaine. These results, therefore, confirm and extend our previous observations<sup>8</sup> that aromaticity at positions 652 and 656 is an important prerequisite for local anesthetic sensitivity of HERG channels.

Analysis of the onset of block revealed a partial recovery from block for mutants at positions 623, 652, and 656. Partial recovery from block may explain why mutating the aromatic residues Y652 and F656 as well as T623 reduced inhibition of HERG channels by bupivacaine the most. This observation supports the notion that these three amino acids are pivotal in the interaction of local anesthetics with HERG channels. It may, furthermore, be suggested that—after a first contact with the pore—stable interactions with the bupivacaine molecule cannot be established without these amino acids. It may be hypothesized that interaction with only one of these amino acids is required for initial bupivacaine binding. Interaction with all three amino acids would, however, be required for establishing a stable drug-protein interaction. The time constants both for the onset of block and for the partial recovery from block did not differ between the mutants Y652A and F656T, the mutation that altered channel gating the least. This observation may suggest that local anesthetic interaction with either aromatic amino acid is mechanistically similar and does not depend on channel gating. A partial recovery from block for the mutation Y652A has been described for the inhibition of HERG channels by chloroquine, quinidine, and vesnarinone.<sup>26,27</sup> In contrast to Y652A, a partial recovery from block was not detectable for the mutant Y652F.<sup>26,27</sup> This result is compatible with the idea that aromaticity at this position is important for establishing a stable interaction between HERG channels and the drug molecule. In addition to this aromatic amino acid at position 652 the residues F656 as well as T623 near the selectivity filter seem a contributing factor for stable interaction of local anesthetics with HERG channels.

In summary, the results of this study support the hypothesis that aromaticity in positions 652 and 656 is an important feature in bupivacaine binding to HERG channels. The results furthermore define a significant contribution of amino acids T623 and S624 as well as V625 near the selectivity filter to the interaction of the low-affinity blocker bupivacaine with HERG channels. Moreover, our results indicate that inactivation is neither a sufficient nor a necessary prerequisite for inhibition of HERG channels by bupivacaine. Therefore, despite being low-affinity blockers, amino-amide local anesthetics seem to share a common mode of interaction with several high-affinity blockers of HERG channels.



The authors thank Olaf Pongs, Ph.D. (Director of the Institute of Neural Signal Transduction, University Medical Center Hamburg-Eppendorf, Germany), for his continuous support, and Alexander Schworer, M.D. (Department of Vegetative Physiology and Pathophysiology, University Medical Center Hamburg-Eppendorf), for critically reading the manuscript.

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