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Original Article

Tricyclic antidepressant amitriptyline inhibits 5-hydroxytryptamine 3 receptor currents in NCB-20 cells

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ABSTRACT Amitriptyline, a tricyclic antidepressant, is commonly used to treat depression and neuropathic pain, but its mechanism is still unclear. We tested the effect of amitriptyline on 5-hydroxytryptamine 3 (5-HT_3) receptor currents and studied its blocking mechanism because the clinical applications of amitriptyline overlapped with 5-HT_3 receptor therapeutic potentials. Using a whole-cell voltage clamp method, we recorded the currents of the 5-HT_3 receptor when 5-HT was applied alone or co-applied with amitriptyline in cultured NCB-20 neuroblastoma cells known to express 5-HT_3 receptors. To elucidate the mechanism of amitriptyline, we simulated the 5-HT_3 receptor currents using Berkeley Madonna® software and calculated the rate constants of the agonist binding and receptor transition steps. The 5-HT_3 receptor currents were inhibited by amitriptyline in a concentration-dependent, voltage-independent manner, and a competitive mode. Amitriptyline accelerated the desensitization of the 5-HT_3 receptor. When amitriptyline was applied before 5-HT treatment, the currents rose slowly until the end of 5-HT treatment. When amitriptyline was co-applied with 5-HT, currents rose and decayed rapidly. Peak current amplitudes were decreased in both applications. All macroscopic currents recorded in whole cell voltage clamping experiments were reproduced by simulation and the changes of rate constants by amitriptyline were correlated with macroscopic current recording data. These results suggest that amitriptyline blocks the 5-HT_3 receptor by close and open state blocking mechanisms, in a competitive manner. We could expand an understanding of pharmacological mechanisms of amitriptyline related to the modulation of a 5-HT_3 receptor, a potential target of neurologic and psychiatric diseases through this study.

INTRODUCTION

Unlike other 5-hydroxytryptamine (5-HT) receptors, which are G protein-coupled receptors, 5-HT_3 receptor is a ligand-gated ion channel and belongs to a Cys-loop receptor family which includes nicotinic acetylcholine receptors, γ-aminobutyric acid (GABA) type A receptors and glycine receptors [1,2]. The 5-HT_3 receptor is permeable to cations, so it can induce a fast inward current in the neurons [3]. It mainly locates in presynaptic terminals, thus it could modulate the release of neurotransmitters such as dopamine, GABA, acetylcholine, substance P, and also 5-HT itself [4,5]. 5-HT_3 receptors are widely distributed in the brain, relatively high in the area postrema and nucleus tractus solitarius. Also, it is found in the hippocampus, frontal cortex, cingulate cortex, amygdala, nucleus accumbens, substantia nigra and ventral tegmental area. In outsides of the brain, dorsal horn and dorsal root ganglia of spinal cord, lung, stomach, colon, kidney, and inflammation cells express the 5-HT_3 receptor [6].

Clinically, 5-HT_3 receptor antagonists like ondansetron are widely used as antiemetics [7]. Also, 5-HT_3 receptor antagonist

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could be effective for the treatment of morphine and ethanol addiction because it reduced dopamine release induced by ethanol and morphine in animal studies [8,9]. Other researches revealed that ethanol potentiated 5-HT_3 receptor function [10,11], which suggest that the 5-HT_3 receptor could be related to ethanol addiction. Schizophrenia, depression, anxiety disorder, irritable bowel syndrome, anorexia, cognitive disorder, Alzheimer's disease, inflammatory pain, neuropathic pain, and migraine are also suggested to be potential therapeutic targets of 5-HT_3 receptor modulators [6,12,13]. However, the pathophysiologic and pharmacologic role of the 5-HT_3 receptor for the chronic pain and analgesic action are still need to be elucidated. Amitriptyline, one of the tricyclic antidepressant, has an affinity for serotonin-norepinephrine reuptake transporters, muscarinic acetylcholine receptors, 5-HT_{1A} and 5-HT_{2A} receptors, α-adrenergic receptors, histamine receptors [14]. It is effective to treat migraine, fibromyalgia, neuropathic pain, and irritable bowel syndrome, but its pharmacologic mechanism is still unclear [15-17]. The clinical uses of amitriptyline overlapped with the potential therapeutic targets of 5-HT_3 receptor modulators. However, a direct effect of amitriptyline on 5-HT_3 receptor was not tested yet, although the past study showed amitriptyline inhibited chimeric 5-HT_3 receptor and reduced 5-HT_3 receptor-mediated cGMP formation [18,19].

Thus, we studied the effect of amitriptyline on the 5-HT_3 receptor and its mechanism using a whole cell patch clamp recording with a fast drug application system and compared the recorded data with the simulated macroscopic current. Based on our study, we could suggest an expanding of pharmacologic action of amitriptyline on various neurologic and psychiatric diseases.

METHODS

Cell culture

NCB-20 neuroblastoma cells, which used for studying 5-HT_3 receptor [11,20,21], were provided by Dr. Lovinger (National Institute on Alcohol Abuse and Alcoholism, USA). Cells were incubated in the culture flask filled with culture medium which contained 89% Dulbecco modified Eagle's medium, 10% fetal bovine serum, and 1% hypoxanthine aminopterin thymidine supplement. Cells were grown in an incubator maintained by 5% CO_2 at 37°C, harvested by 0.25% trypsin-EDTA, and seeded to a treated culture dish 24-48 h prior to recording. Culture medium was exchanged to extracellular solution 1-2 h before recording and cells were transferred on cover glasses and moved to the recording chamber.

Electrophysiology

Extracellular solution was made by (in mM) 150 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 0.1 $MgCl_2$, 10 D-glucose and 10 *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethansulfonic acid (HEPES) and pH was adjusted to 7.4 with NaOH and osmolality adjusted to 340 mOsm/kg with sucrose. Intracellular solution consisted of (in mM) 140 CsCl, 2 $MgCl_2$, 5 ethylene glycol bis(2-aminoethylether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA), 10 HEPES and pH adjusted to 7.2 with CsOH, osmolality adjusted to 310 mOsm/kg with sucrose.

Whole-cell patch clamp technique was performed at room temperature (24°C) by using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) with a Carl Zeiss Axiovert 135M microscope (Jena, Germany). Pipettes were made by borosilicate glass capillaries (1B150-4, World Precision Instruments, Sarasota, FL, USA) which pulled with a horizontal micropipette puller (P-97, Sutter Instrument, Novato, CA, USA). Pipette tip resistance was formed around 2.5 MΩ when it was filled with an intracellular solution.

Signals were filtered at 2 kHz, digitized at 10 kHz, and saved using Digidata 1322 and pClamp 10.0 software (Molecular Devices). Pipette capacitance was compensated before making whole cell configuration and whole cell capacitance and series resistance were compensated before drug application. All compensations were done by Multiclamp 700B Commander software (Molecular Devices). Currents were recorded at the –50 mV holding potential except when measured the current-voltage relationship.

Drug preparation and application

After achieving whole-cell configuration at the –50 mV holding potential, cells were lifted and moved toward the one side of theta glass pipette (Clark Borosilicate Theta, Warner Instruments, 2 mm outer diameter, 1.4 mm inner diameter, 0.2 mm septum thickness) which was pulled to an outer diameter of ~300 μm and continuously perfused with extracellular solution. The solution flow was driven by gravity from the reservoirs. It was controlled by a perfusion valve control system (VC-8, Warner Instruments, Hamden, CT, USA). While the cell was placed on the side of the extracellular solution flow, the agonist or tested drug were perfused on the other side of theta glass tubing. We turned on the VC-8 valve control system 5 s prior to every drug application to wash the space of theta glass pipette. Theta glass pipette was mounted with a piezoelectric translator (P-601 PiezoMove Z Acturator, PhysikInstrumente, Karlsruhe, Germany), which moved theta pipette laterally in ms time resolution. Therefore the cell could rapidly move to the drug side from the extracellular solution side. After the cell was exposed to the agonist for a programmed time, the cell was returned to the original side of the theta pipette perfused with the extracellular solution.

Data analysis, statistics, and simulation

Peak amplitudes of current were measured by Clampfit software (Molecular Devices). All time constants and slopes were cal-

culated by the built-in statistical tools in Clampfit software. Peak current data were normalized to the 10 μM 5-HT current for statistical analysis. Desensitized currents were fitted by first order exponential function by using Clampfit software. Normalized concentration-peak amplitude data were fitted to sigmoid curve calculated by the four-parameter logistic equation using Prism 6.0 (GraphPad Software, San Diego, CA, USA).

For calculating EC_{50}, equation (1) was used.

$$Y=bottom+(top-bottom)/(1+10^{((LogEC_{50}-X)Hillslope)}) \quad (1)$$

Bottom of the equation (1) is minimal response, top is maximal response, X is the logarithm of 5-HT concentration, and EC_{50} is concentration of half between top and bottom.

For calculate IC_{50}, equation (2) was used.

$$Y=bottom+(top-bottom)/(1+10^{((LogIC_{50}-X)Hillslope)}) \quad (2)$$

Bottom of the equation (2) is minimal response, top is maximal response, X is the logarithm of amitriptyline concentration, and IC_{50} is concentration of half between top and bottom.

The mean and S.E.M value were calculated by Prism 6.0. Statistical significance was determined by p value <0.05, calculated by the Paired student's *t*-test, ANOVA, and Tukey's multiple comparisons test using Prism 6.0.

We formulated a differential equation of the 5-HT$_3$ receptor kinetic models derived from a Michaelis-Menten equation, and it was solved by the fourth order Runge-Kutta method using our recorded data. Thus we could obtain rate constants of the kinetic model and simulated current. All this process was done by the Berkeley Madonna® software developed by Robert Macey and George Oster of the University of California at Berkeley.

RESULTS

5-HT$_3$ receptor currents

First, we studied the agonist concentration-response relationship using whole-cell patch clamp technique. Fig. 1A shows our representative 5-HT$_3$ receptor currents of the NCB-20 cells depending on 5-HT concentrations of 0.3, 1, 3, 10, 30 μM. 5-HT was applied for 5 s, in 1 min interval at the –50 mV holding potential. As seen in Figs. 1A and B, the peak current was increased in a 5-HT concentration-dependent manner. A maximal current was obtained at 10 μM of 5-HT. Using equation (1), the calculated EC_{50} of peak currents by 5-HT was 1.73±0.08 μM with a Hill coefficient of 2.44±0.08 (n=10). The shape of current trace, EC_{50}, and Hill coefficient values are all similar with our previous investigations [22,23]. The currents induced by the application of 5-HT in NCB-20 cells were known to be mediated by the 5-HT$_3$ receptor activation, because these currents were completely blocked by the GR 38032F and IC 205-930, the potent 5-HT$_3$ receptor antagonists [24].

Effect of amitriptyline on 5-HT$_3$ receptor currents

Figs. 2A and B present the 5-HT$_3$ receptor current traces depending on amitriptyline concentration at the fixed 5-HT concentrations. To test the concentration-inhibition relationship, 3 μM or 10 μM 5-HT was applied concurrently with various concentration of amitriptyline (0.3, 1, 3, 10, 30 μM). Amitriptyline inhibited the 5-HT$_3$ receptor currents in a concentration-dependent manner. The peak current was significantly blocked by 30 μM of amitriptyline (82.18±1.39% inhibition at 10 μM of 5-HT, p<0.001, n=10; 91.34±1.90% inhibition at 3 μM of 5-HT, p<0.001, n=10). In Fig. 2C, IC_{50} of amitriptyline on the 3 μM 5-HT-induced currents was 1.78±0.37 μM, and on the 10 μM 5-HT was 6.36±0.45 μM. The Hill coefficient of amitriptyline in 3 μM 5-HT was 1.00±0.08, and in 10 μM 5-HT was 0.91±0.04 (n=10 cells). IC_{50} and Hill coefficient were calculated by equation (2). Because the IC_{50} of

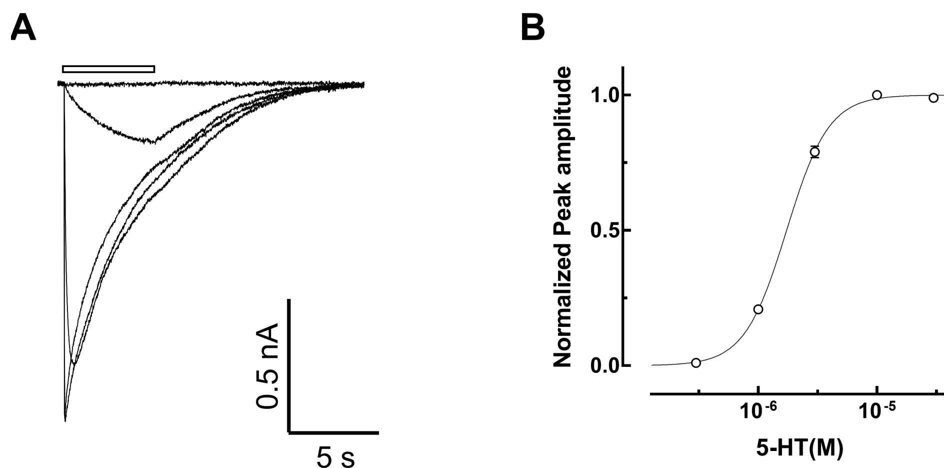

Fig. 1. Characteristics of 5-HT$_3$ receptor currents in NCB-20 neuroblastoma cells. (A) Representative 5-HT$_3$ receptor currents induced by 0.3, 1, 3, 10, 30 μM of 5-HT. 5-HT was applied for 5 s indicated by the open horizontal bar. 5-HT$_3$ receptor current amplitudes were increased depending on 5-HT concentrations. (B) Averaged concentration-response curve of 5-HT$_3$ receptor currents. Data were normalized to the peak amplitude induced by 10 μM of 5-HT, which was taken as 1. EC_{50} value was 1.73±0.08 μM with Hill coefficient of 2.44±0.08 (n=10). Data are expressed as mean±S.E.M.

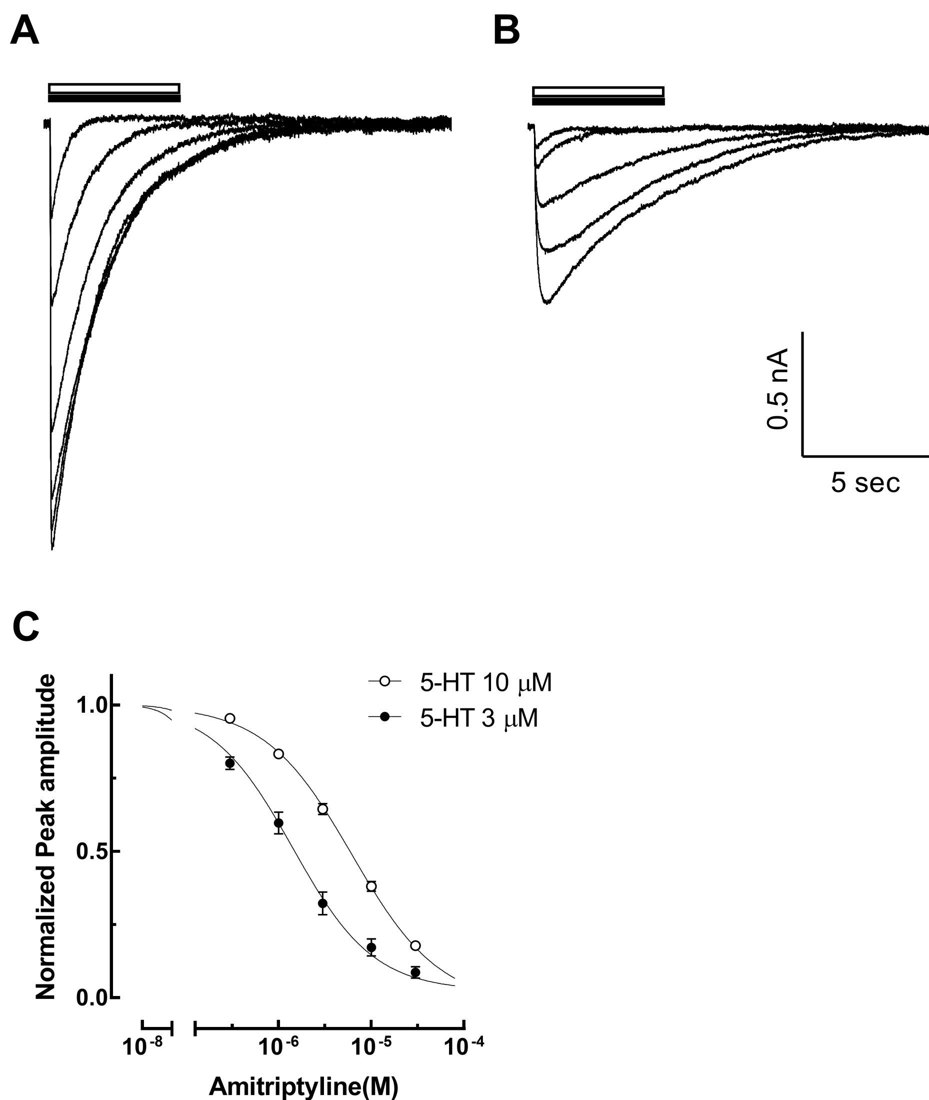

Fig. 2. Concentration-dependent inhibition of 5-HT_3 receptor currents by amitriptyline. (A) Representative current traces induced by 5-HT 10 μM (open horizontal bar) co-applied with 0.3, 1, 3, 10, 30 μM of amitriptyline (closed horizontal bar). (B) Representative current traces induced by 5-HT 3 μM (open horizontal bar) co-applied with 0.3, 1, 3, 10, 30 μM of amitriptyline (closed horizontal bar). Inhibitory effect of the amitriptyline was profound at the 3 μM of 5-HT induced currents. (C) Averaged concentration-inhibition curve of 5-HT_3 receptor currents by amitriptyline. Data were normalized to the peak amplitude induced by 10 μM of 5-HT (○) or 3 μM of 5-HT (●). IC_{50} of amitriptyline on 3 μM 5-HT-induced currents was 1.78±0.37 μM with Hill coefficient of 1.00±0.08 (n=10 cells), and IC_{50} of amitriptyline on 10 μM 5-HT was 6.36±0.45μM with Hill coefficient of 0.91±0.04 (n=10 cells). IC_{50} of amitriptyline on 3 μM 5-HT was significantly lower than that of 10 μM 5-HT (unpaired *t*-test, p<0.001). Data are expressed as mean±S.E.M.

the 3 μM 5-HT was smaller than 10 μM 5-HT (unpaired t-test, p<0.001, n=10), we expected that amitriptyline could block more effectively at a low concentration of 5-HT, which suggested that 5-HT concentration influence the inhibitory effect of amitriptyline.

Amitriptyline acts as a competitive blocker on 5-HT_3 receptor

To elucidate how amitriptyline blocks the 5-HT_3 receptor-mediated current, we tested whether amitriptyline acts as a non-competitive or competitive blocker on the 5-HT_3 receptor. Because the IC_{50} of amitriptyline at 3 μM 5-HT was 1.78 μM, we used 3 μM of amitriptyline, above the IC_{50}. We tested the effect of 3 μM amitriptyline co-applied with the 0.3, 1, 3, 10, 30 μM of 5-HT, and compared the peak current amplitudes with the 5-HT alone. Figs. 3A-E shows representative traces of the 5-HT_3 receptor currents with or without amitriptyline. EC_{50} of 5-HT alone was 1.73±0.08 μM and Hill coefficient was 2.44±0.08 from the Fig. 1B. However, EC_{50} of the co-application of 5-HT and amitriptyline was 9.21±0.06 μM, and the Hill coefficient was 2.00±0.13 (Fig. 3F). Although EC_{50} was increased from 1.73 to 9.21 μM by amitriptyline (paired t-test, p<0.001, n=7), amitriptyline could not decrease the maximal peak current amplitudes induced by 30 μM of 5-HT (paired t-test, p=0.13, n=7). The normalized agonist concentration response curve was shifted to the right. Therefore, we could expect that amitriptyline acts as a competitive inhibitor on the 5-HT_3 receptor.

Voltage independence of amitriptyline effect

Voltage dependency of the 5-HT_3 receptor current was performed to test the inhibitory mechanism of amitriptyline. We measured the 5-HT_3 receptor-mediated current of 3 μM 5-HT with or without 3 μM amitriptyline at the holding potentials of –50, –30, –10, 0, 10, 30 mV, respectively. Figs. 4A and B show the current traces when we changed the holding potential from –50 mV to 30 mV. In Fig. 4C, the reversal potential in the absence of

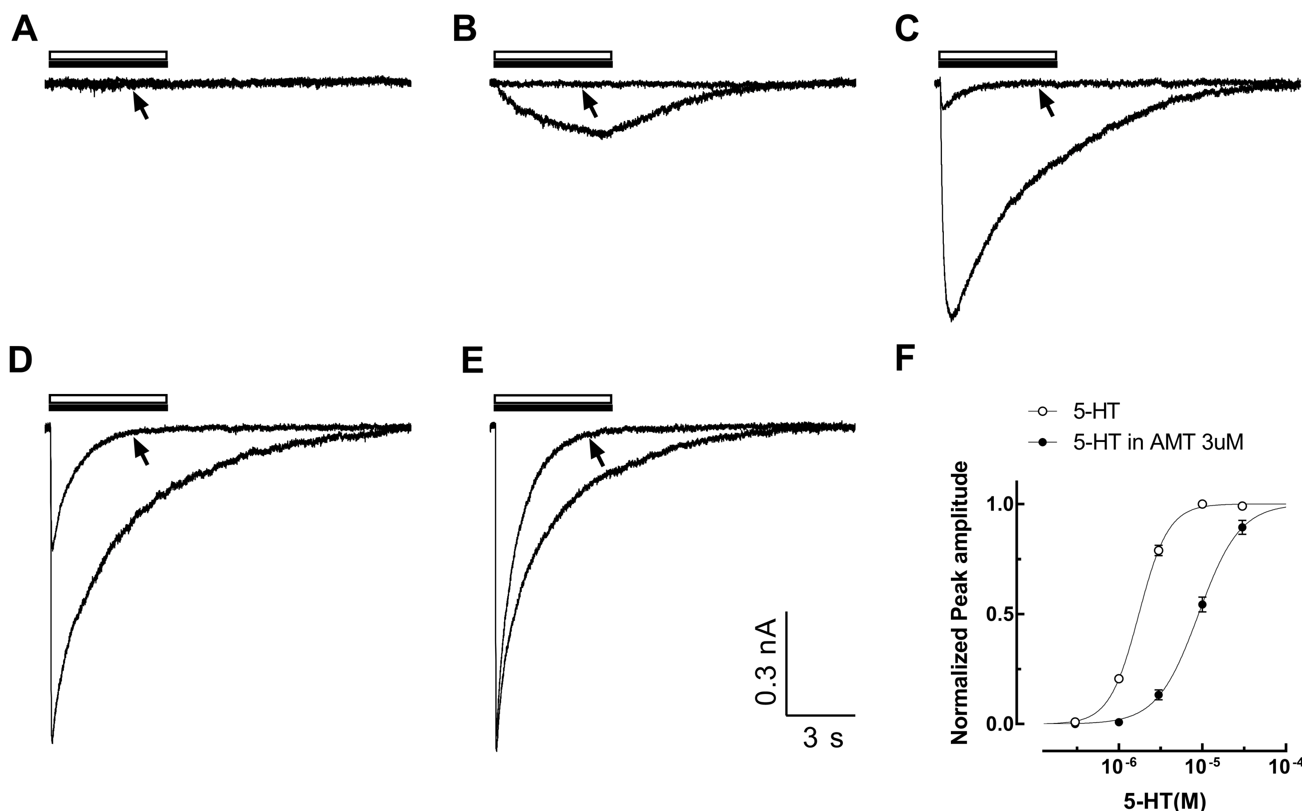

Fig. 3. Change of 5-HT concentration-response relationship by amitriptyline. (A-E) Representative traces of 5-HT$_3$ receptor currents induced by 0.3, 1, 3, 10, 30 μM of 5-HT (open horizontal bar) in the presence (indicated by arrow) or absence of 3 μM amitriptyline (closed horizontal bar). Amitriptyline inhibited 5-HT induced currents more effectively at the lower concentration of 5-HT. (F) Averaged concentration-response curve of 5-HT$_3$ receptor currents in the presence (●) or absence (○) of amitriptyline. Data were normalized to the peak amplitude induced by 10 μM of 5-HT. EC_{50} of 5-HT was increased from 1.73±0.08 μM to 9.21±0.06 μM by amitriptyline (p<0.001, n=10). Amitriptyline did not decrease the peak amplitudes induced by 30 μM of 5-HT (paired *t*-test, p=0.13, n=7). Data are expressed as mean±S.E.M.

amitriptyline was 8.54±0.32 mV, and the reversal potential with amitriptyline was 8.88±0.44 mV. The difference between these reversal potentials were not statistically significant (p=0.4899, n=10).

Fig. 4D shows that the calculated inhibition ratios were not changed significantly over all the tested holding potential ranges. Because the result represents that amitriptyline works in a voltage independent manner, we could expect that amitriptyline does not act at the open pore of the receptor.

Comparison between pre-application and co-application of amitriptyline

In order to test whether amitriptyline acts as a closed state blocker or not, we compared the effects of amitriptyline in different modes of application, because previous reports suggested that pre-application of a drug is effective when antagonist dominantly acts as a close state blocker [25-27]. During co-application of amitriptyline (3 μM) with 3 μM 5-HT, the peak amplitude was decreased with shape of fast inward current which was similar with trace of 3 μM 5-HT alone (Fig. 5A). However, at the pre-application mode of amitriptyline, the 5-HT$_3$ receptor-mediated current by 3 μM 5-HT raised with small rising slope to the end of 5-HT application without current decay (Fig. 5B). It was similar with the current of a lower concentration of 5-HT (i.e., ≤1 μM, see Fig. 1A). However, we could not exclude the possibility that the slow activation observed in the pre-application experiments likely caused by washout of amitriptyline, which gradually increase the number of available receptors. When amitriptyline was pretreated and followed by co-application, the 5-HT$_3$ receptor current was completely blocked (Fig. 5C). Taken together with these results and previous reports, we suggest that amitriptyline could block the 5-HT$_3$ receptor before channel opening via binding to the closed ion channel.

Effects of amitriptyline on receptor desensitization

To find out whether amitriptyline could accelerate desensitization of 5-HT$_3$ receptor or not, we compared the currents of 5-HT alone and 5-HT with amitriptyline for 15 second application (Fig. 6A), which protocol was already used to study desensitization [27]. In Fig. 6B, the desensitization time constant was decreased from

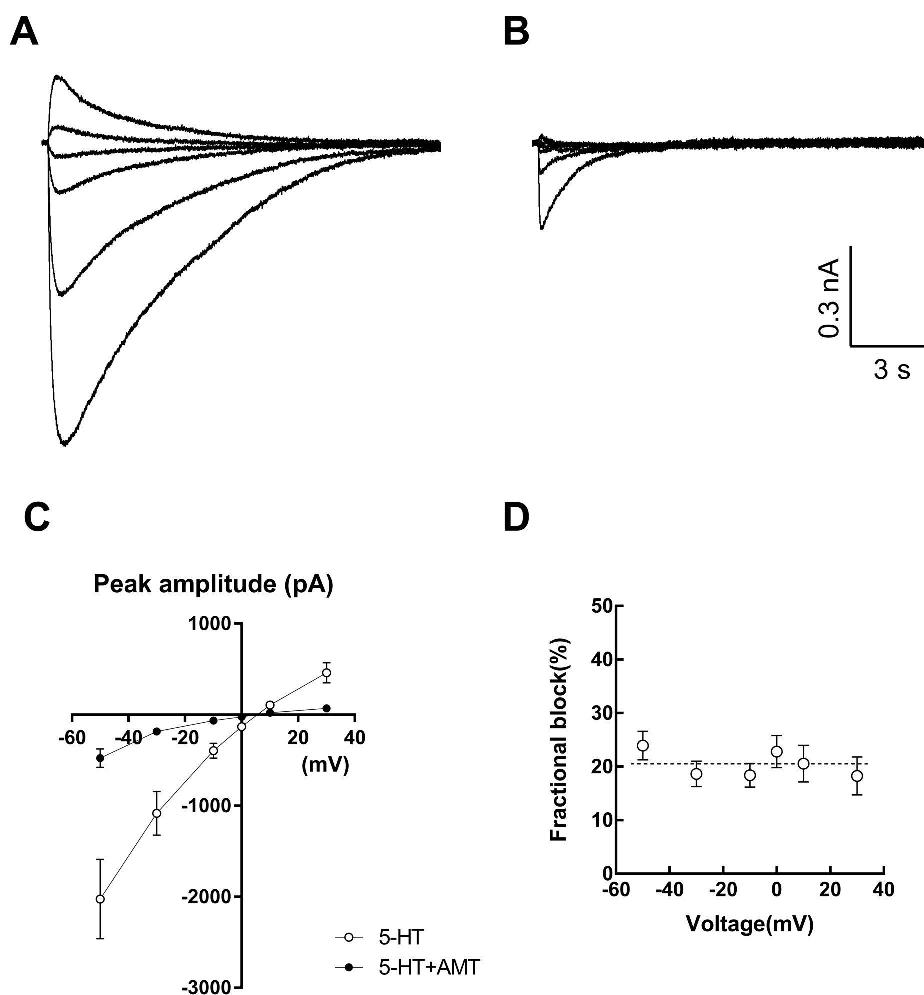

Fig. 4. Effect of amitriptyline on current-voltage (I-V) relationship of $5\text{-}HT_3$ receptor currents. (A) Representative traces of $5\text{-}HT_3$ receptor currents induced by 3 μM of 5-HT alone at the holding potentials of –50-+30 mV. (B) $5\text{-}HT_3$ receptor currents induced by 3 μM of 5-HT at the holding potentials of –50-+30 mV with amitriptyline 3 μM. (C) I-V plots of averaged peak amplitude induced by 5-HT with (●) or without (○) amitriptyline (AMT). The reversal potentials were 8.54±0.32 mV at 5-HT alone, and 8.88±0.44 mV at 5-HT with amitriptyline. Reversal potential was not significantly changed by co-application of amitriptyline (p=0.4899, n=10). (D) Fractional block of amitriptyline (peak amplitude of 5-HT with amitriptyline/peak amplitude of 5-HT alone) as a function of holding potentials. Inhibitory effect of amitriptyline showed a voltage independency. Data are expressed as mean±S.E.M.

3838±1972 ms at 5-HT alone to 1304±506 ms at co-application of amitriptyline and 5-HT, and it was statistically significant (paired t-test, p<0.01, n=9). Decreased desensitization time constant at the 15 s long application could be understood that amitriptyline forces an opened $5\text{-}HT_3$ receptor to the desensitization, so it acts as an open channel blocker.

Simulation

To determine which step of receptor kinetic process amitriptyline acts on, a reaction scheme of agonist, receptor and antagonist were formulated (Fig. 7A). Binding of the agonist and channel, channel opening, channel closing, and channel desensitization were included in the reaction scheme. All reaction rate constants for each reaction steps were calculated and used to simulate macroscopic currents. Past studies used two or five binding of agonists to $5\text{-}HT_3$ receptor in the kinetic model [28-30], however in this study, we formulated an one agonist binding model to estimate each rate constant since we simply supposed to which step was the target of the amitriptyline among the various channel states (close state, channel opening, and open state). Rate constants were calculated by the Berkeley Madonna® software and its results were shown in Table 1. The k_f and k_r were association and dissociation rate constants, respectively. The β and α were channel opening and closing rate constant, and the k_{d+} and k_{d-} were desensitization and re-open from desensitization rate constant, respectively. Because we intended to elucidate the drug target based on current rising, decay and channel opening steps, we just compared k_f, β, k_{d+}, respectively. In Fig. 7B, the simulated currents generated by 5-HT alone, co-application and pre-application with amitriptyline were compared with experimental data. Simulated currents were closely overlapped with recorded the whole cell macroscopic data. The k_f was significantly decreased by pre-application of the amitriptyline than other application mode (p<0.001, ANOVA and Tukey's multiple comparisons test, n=6). These results were matched to our macroscopic current data because k_f were used to generate the rising slope (Table 1). So, currents slowly increased to the end of the 5-HT application in the pre-application mode. The β was not significantly changed by amitriptyline even in both application modes (p=0.89 by ANOVA, n=6), while k_{d+} was greatly increased in a co-application manner (p<0.001, ANOVA and Tukey's multiple comparisons test, n=6). From this study, we could expect that amitriptyline has less effect on the channel conformational change from a close to

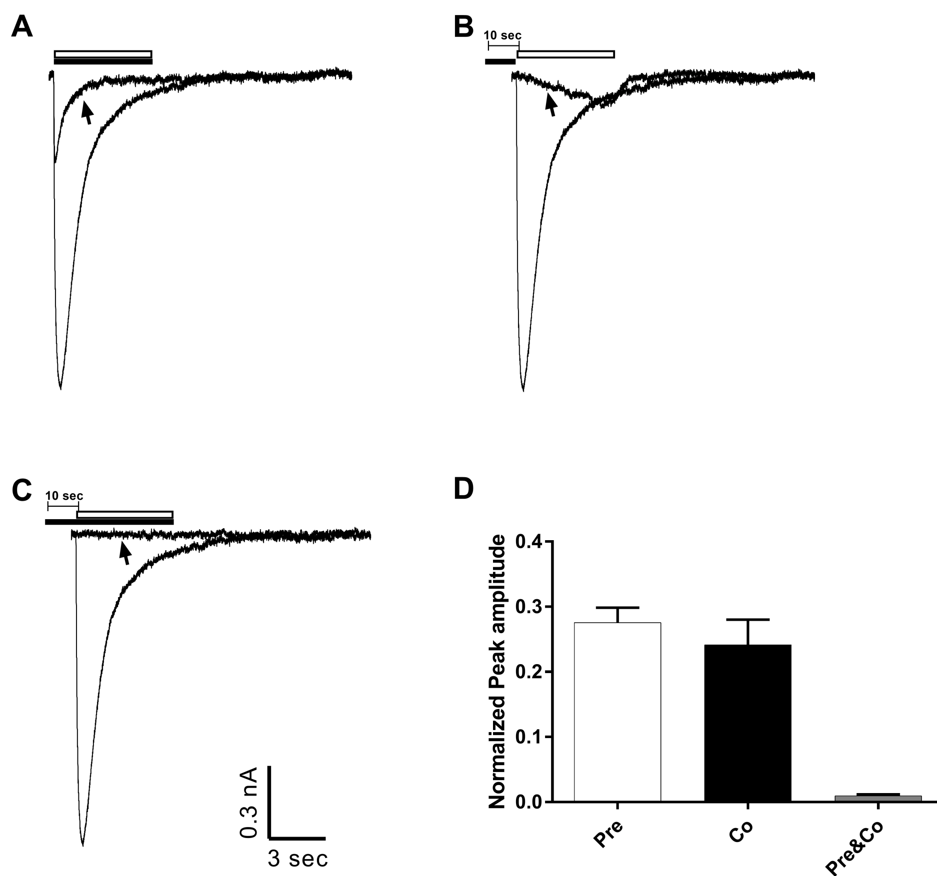

Fig. 5. 5-HT$_3$ receptor currents induced by different modes of amitriptyline application. (A) Representative trace of 5-HT$_3$ receptor currents induced by 5-HT (3 μM) alone (open horizontal bar) and co-application of 5-HT and amitriptyline (3 μM, closed horizontal bar, indicated by arrow). (B) Representative traces of 5-HT$_3$ receptor currents induced by 5-HT alone and pre-application of amitriptyline for 10 s (indicated by arrow). (C) Representative traces of 5-HT$_3$ receptor currents induced by 5-HT alone and co-application of 5-HT and amitriptyline after 10 s pre-application of amitriptyline (indicated by arrow). (D) Averaged inhibitory ratio (peak amplitude of 5-HT with amitriptyline/peak amplitude of 5-HT alone) at each application mode of amitriptyline. 5-HT induced current was blocked by amitriptyline in both of pre-application (Pre) and co-application (Co) modes, and completely blocked by combination of pre- and co-application (Pre & Co). Data are expressed as mean±S.E.M.

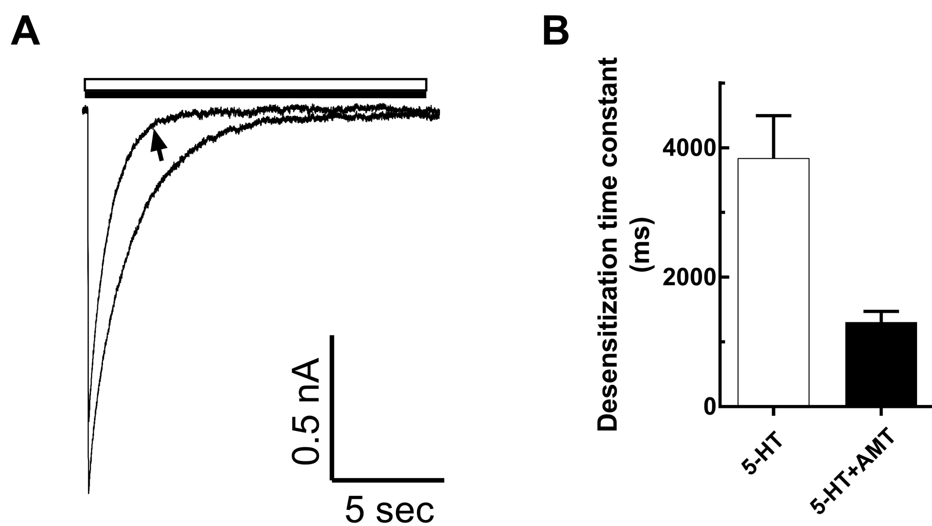

Fig. 6. Effect of amitriptyline on desensitization of 5-HT$_3$ receptor. (A) Representative 5-HT$_3$ receptor currents induced by 10 μM of 5-HT (open horizontal bar) for 15 s with or without 3 μM of amitriptyline (closed horizontal bar, indicated by arrow). (B) Averaged desensitization time constant of the 5-HT alone (5-HT) and with amitriptyline (5-HT+AMT). Amitriptyline decreased the time constant of 5-HT$_3$ receptor current decay (paired *t*-test, *p<0.01, n=9) suggesting the desensitization was accelerated by amitriptyline. Data are expressed as mean±S.E.M.

an open. And it is effective to force the channel to stay in the close state or change the channel state from open to close by accelerating 5-HT$_3$ receptor desensitization.

DISCUSSION

In our study, amitriptyline inhibited the peak amplitude of 5-HT$_3$ receptor current in a competitive manner and accelerated receptor desensitization. Its inhibitory effect was voltage independent and the currents were blocked by both pre-application and co-application modes.

By the pre-application of amitriptyline, currents evoked by 3 μM of 5-HT, which rapid rise and then fast decay, were changed to be similar with the currents induced by a low concentration of 5-HT, which the currents rose slowly to the end of 5-HT applica-

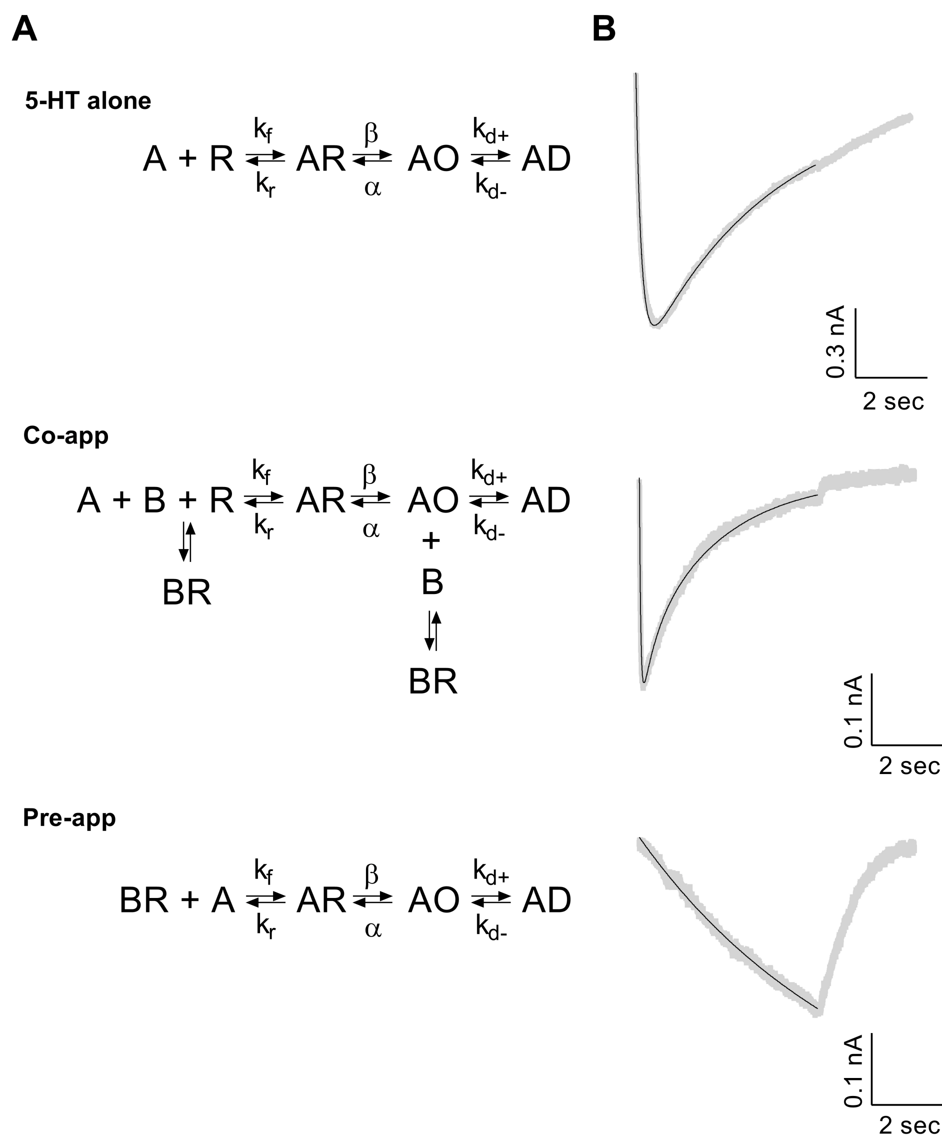

Fig. 7. Kinetic schemes and simulated 5-HT$_3$ receptor currents. (A) Ion channel kinetic scheme of 5-HT$_3$ receptor (R), 5-HT (A), and amitriptyline (B) in each application mode, 5-HT alone, co-application of amitriptyline with 5-HT (Co-app), and pre-application of amitriptyline with 5-HT (Pre-app). (B) Recorded current trace (gray) and simulated 5-HT current (black) in each application mode. The simulated 5-HT current was well matched with the recorded current trace.

Table 1. Change of rate constants of reaction steps by amitriptyline in 5-HT$_3$ receptor channel kinetics

	5-HT	5-HT+Amitriptyline	
		Pre-application	Co-application
K_f (s^{-1})	2727.287±405.048	18.041±5.973*	2269.873±399.793
Beta ($M{\cdot}s^{-1}$)	338.137±73.374	322.154±67.689	289.826±77.202
K_d ($M{\cdot}s^{-1}$)	0.779±0.214	0.328±0.111	27.052±5.869*

k_f was the largest in the 5-HT alone, and decreased by amitriptyline, especially in the pre-application mode. β was not significantly changed by amitriptyline even in both application modes. k_{d+} was greatly increased at the co-application manner. 5-HT, 5-hydroxytrytamine; k_f, association rate constant; β, channel opening rate constant; k_{d+}, desensitization rate constant. Data are expressed as mean±S.E.M.
*p<0.0002 by ANOVA and Tukey's multiple comparisons test.

tion (Fig. 5). However, in the co-application, the peak currents were decreased with the fast rising and fast desensitization. The inhibitory effect by the pre-application of a drug usually represents the close state blocking mechanism [25,27]. In contrast, the inhibitory effect by the co-application or the use dependency represents the open state blocking mechanism of a drug [26]. If both of co-treatment and pre-treatment show inhibitory effect, the antagonist inhibits an ion channel via both of close state and open state blocking mechanisms [31]. Therefore our results suggest that amitriptyline acts as a close and open state ion channel blocker to

the 5-HT$_3$ receptor. Also we showed that the amitriptyline accelerated the receptor desensitization, which could support amitriptyline bound to the open state of ion channel and then forced the channel toward a desensitized state. Although voltage-dependent blocking of the ion channel is strong evidence of an open channel blocker, not all open channel blockers show a voltage dependence [32]. Because voltage dependency is properly related with a channel pore blocking mechanism, if antagonist binds at the outside of opened pore, it could show voltage independency. Taken together, we could suggest that amitriptyline acts as an open state blocker via binding at the outside of opened pore, and this binding site closely connected to the 5-HT binding on the 5-HT$_3$ receptor, and act as a competitive blocker.

To compare each rate constant of recorded data when treating 5-HT alone, co-application with amitriptyline, and pre-application of the amitriptyline, the association rate constant k_f, opening constant β, and desensitization constant k_{d+} were estimated by using the simulation (see Materials and Methods). Because the current trace was rising rapidly at the both of co-treatment with amitriptyline and 5-HT alone, there was no significant difference between the k_f values. However, in the pre-application of amitriptyline, k_f is significantly lower than 5-HT alone or with co-application. This current change by pre-application of amitriptyline is reasonable because 5-HT could force the dissociation of bounded amitriptyline from the receptor through a competitive manner, thus antagonist free 5-HT$_3$ receptors are increased during the 5-HT application and generate slow rising inward current. The change of opening rate constant β caused by amitriptyline co- and pre-applications was statistically insignificant (Table 1). However, the desensitization rate constant k_{d+} was increased especially in the co-application method. The increase in k_{d+} due to amitriptyline in simulated data was consistent with our macroscopic current data, which showed desensitization was accelerated by amitriptyline only in the co-application mode (Fig. 6). In the pre-application mode, receptor desensitization might be occurred not much because current rise slowly and did not decay to the end of 5-HT application, thus k_{d+} was not significantly changed (Table 1). Although a macroscopic current recording technique useful to study the change of channel kinetics by a drug, it is still limited because it could not clarify the drug targeted-reaction step in the ion channel kinetics. Thus, a simulation study has frequently been used to interpret the ion channel kinetics change in combination with the macroscopic current recording [30]. In this study, we suggest that amitriptyline did not affect the channel opening conformational change in simulation because β was not changed greatly (Table 1). This result could not be proven by the analysis of macroscopic current. So we expect that our approach is useful to interpret a drug action mechanism on the 5-HT$_3$ receptor.

Amitriptyline has been also used to treat migraine, fibromyalgia, neuropathic pain, and irritable bowel syndrome [15-17]. However in the dorsal horn (the first synaptic site in nociception), an analgesic mechanism of amitriptyline is still unclear. From our study, we would suggest that 5-HT$_3$ receptor is a potential target of the amitriptyline. 5-HT$_3$ receptor is distributed at the dorsal horn of the spinal cord [33], connecting to the descending tracts from raphe-spinal neuron for the modulation of nociception [34]. However its pathophysiologic role in these chronic pain syndromes is still controversial. 5-HT$_3$ receptor antagonist is known to block the GABA release at the dorsal horn, thus lack of GABA signals contributes a hypersensitivity of the peripheral nerve injury [35]. In contrast to this pro-nociceptive effect of the 5-HT$_3$ receptor antagonist, the intra-thecal injection of ondansetron showed an analgesic effect in a spinal cord injury model, while agonist enhanced allodynia in the same experiment [36]. These results were interpreted that the 5-HT$_3$ receptor regulated a pro-nociceptive neurotransmitter level at the synapse of dorsal horn [37]. This interpretation was also supported by the reports that 5-HT$_3$ receptor activated the primary afferent fibers, including A-delta and C fiber in a patch clamp study [38], and 5-HT$_3$ receptors is involved in the behavioral hypersensitivity activating the neuro-inflammatory signaling cascade [39]. Since amitriptyline is commonly used for the treatment of certain types of neuropathic pain, including diabetic neuropathy, post-herpetic neuralgia and other neuropathic pain [16,40], our results, showing an inhibitory effect of amitriptyline on the 5-HT$_3$ receptors, could be helpful to interpret the previous studies exploring the pathophysiologic role of 5-HT$_3$ receptor in the neuropathic pain [18,19].

Inhibition of the 5-HT$_3$ receptor was expected to be related an antidepressive mechanism via increased monoamine contents in synapses, increased availability of other 5-HT receptor, and reducing inhibitory neurotransmission [1,5,6,12,41]. To understand the pathophysiologic role of 5-HT$_3$ receptor in the depressive disorders, the effect of antidepressants on the 5-HT$_3$ receptor and the effects of 5-HT$_3$ receptor antagonist on depression models have been studied in various ways. Functional antagonism of the antidepressants on 5-HT$_3$ receptor was reported in previous studies [21,27,42]. Also ondansetron was known to improve a mood disturbance in the depressive patients and reduce anxiety behaviors in the mice models of depression [41,43-45]. Although these reports suggest the important role of 5-HT$_3$ receptor in the depressive disorders, few antidepressants have been studied to test whether they modulated this receptor directly. Therefore we studied the direct effect of amitriptyline, even a classic tricyclic antidepressant, on the 5-HT$_3$ receptor to expand our understanding the pathophysiology of depressive disorders.

In summary, we showed that amitriptyline directly inhibits the 5-HT$_3$ receptor in close and open states ion channel in a competitive blocking mechanism. We could expand our understanding about the pharmacological mechanisms of amitriptyline in the treatment of depression and neuropathic pain, and the pathophysiologic role of 5-HT$_3$ receptor, a potential target for a wide variety of neurologic and psychiatric diseases.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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