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# Protective Effects of Duloxetine against Cerebral Ischemia-Reperfusion Injury via Transient Receptor Potential Melastatin 2 Inhibition[S]

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## ABSTRACT

Activation of transient receptor potential melastatin 2 (TRPM2), an oxidative stress–sensitive $Ca^{2+}$-permeable channel, contributes to the aggravation of cerebral ischemia-reperfusion (CIR) injury. Recent studies indicated that treatment with the antidepressant duloxetine for 24 hours (long term) attenuates TRPM2 activation in response to oxidative stress in neuronal cells. To examine the direct effects of antidepressants on TRPM2 activation, we examined their short-term (0–30 minutes) treatment effects on $H_2O_2$-induced TRPM2 activation in TRPM2-expressing human embryonic kidney 293 cells using the $Ca^{2+}$ indicator fura-2. Duloxetine exerted the strongest inhibitory effects on TRPM2 activation among the seven antidepressants tested. These inhibitory effects appeared to be due to the inhibition of $H_2O_2$-induced TRPM2 activation via an open-channel blocking-like mechanism, because duloxetine reduced the sustained phase but not the initial phase of increases in intracellular $Ca^{2+}$ concentrations. In a whole-cell patch-clamp study, duloxetine reduced the TRPM2-mediated inward current during the channel opening state. We also examined the effects of duloxetine in a mouse model of CIR injury. The administration of duloxetine to wild-type mice attenuated CIR injury, similar to that in *Trpm2* knockout (KO) mice. The administration of duloxetine did not reduce CIR injury further in *Trpm2* KO mice, suggesting that it exerts neuroprotective effects against CIR injury by inhibiting TRPM2 activation. Regarding drug repositioning, duloxetine may be a useful drug in reperfusion therapy for ischemic stroke because it has already been used clinically in therapeutics for several disorders, including depression.

## Introduction

Ischemic stroke is a common cause of mortality and long-term disability. Cerebral ischemia alters normal cellular function, which is followed by neuronal cell death. Although reperfusion is critical for the rescue of neuronal cells from ischemia, it also results in the progression of neuronal cell damage because of the production of a large amount of reactive oxygen species (ROS) and inflammation with the infiltration of inflammatory cells (Chan, 2001; Granger and Kvietys, 2015; Zhang et al., 2016). Accumulating evidence has shown that the increase in intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$) during reperfusion is a common key mechanism for the induction of neuronal cell death and aggravation of inflammation (Wu et al., 2010; Granger and Kvietys, 2015; Belrose and Jackson, 2018).

Transient receptor potential melastatin 2 (TRPM2) is a $Ca^{2+}$-permeable nonselective cation channel that is activated by oxidative stress induced by ROS, such as $H_2O_2$ (Hara et al., 2002). TRPM2 possesses a NudT9-H domain in its C terminus that binds intracellular ADP-ribose, leading to activation of TRPM2 (Perraud et al., 2001). $H_2O_2$-induced TRPM2 activation is mediated by ADP-ribose production via the poly(ADP-ribose) polymerase-1/poly(ADP-ribose) glycohydrolase pathway (Fonfria et al., 2004; Buelow et al., 2008). TRPM2 expression has been detected in the brain, lungs, pancreas, and inflammatory cells such as monocytes, macrophages, and neutrophils (Perraud et al., 2001; Hara et al., 2002; Wehage et al., 2002; Nazıroğlu et al., 2012). TRPM2-evoked $[Ca^{2+}]_i$ increases have been shown to induce cell death in various types of cells, including neuronal cells (Hara et al., 2002; Kaneko et al., 2006; Verma et al., 2012). Another major physiologic role of TRPM2 is to regulate the signal cascades leading to chemokine production in monocytes and macrophages. We previously reported that activation of TRPM2 in monocytes stimulated secretion of chemokine (C–X–C motif) ligand CXCL8/CXCL2 and enhanced inflammation

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**ABBREVIATIONS:** $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; CIR, cerebral ischemia-reperfusion; G418, geneticin disulfate; HEK, human embryonic kidney; KO, knockout; ROS, reactive oxygen species; TRPA, transient receptor potential ankyrin; TRPM, transient receptor potential melastatin; TRPV, transient receptor potential vanilloid; TTC, 2,3,5-triphenyltetrazolium chloride; WT, wild type.

(Yamamoto et al., 2008; Shimizu et al., 2015). Furthermore, recent studies implicated activation of TRPM2 channels in the aggravation of cerebral ischemia-reperfusion (CIR) injury (Jia et al., 2011; Gelderblom et al., 2014; Shimizu et al., 2016; Huang et al., 2017).

Akpinar et al. (2014) suggested that the antidepressants agomelatine and duloxetine exert inhibitory effects on $H_2O_2$-induced TRPM2 activation in PC12 cells in a long-term treatment (24 hours). Demirdaş et al. (2017) recently demonstrated that the ADP-ribose–induced TRPM2 current was attenuated in the dorsal root ganglion by a long-term treatment (24 hours) with duloxetine. Thus, duloxetine exerts inhibitory effects on TRPM2 channels in cells exposed to long-term treatments. However, the direct effects of duloxetine on TRPM2 channels remain unknown.

The purpose of this study was to clarify the direct inhibitory effects of antidepressants, including duloxetine, on $H_2O_2$-induced TRPM2 activation and to investigate whether antidepressants, which inhibit TRPM2 activation, attenuate CIR injury in a mouse model. The goal of this study was to show that antidepressants have potential as a useful drug in reperfusion therapy for ischemic stroke.

## Materials and Methods

**Materials.** Agomelatine, duloxetine, imipramine, milnacipran, fura-2 acetoxymethyl ester, and $H_2O_2$ (30% solution) were purchased from Wako Pure Chemical Corporation (Osaka, Japan). Paroxetine, fluvoxamine, and 2,3,5-triphenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich (Tokyo, Japan). Nortriptyline was purchased from MP Biomedicals (Illkirch, France). Isoflurane was purchased from Mylan (Osaka, Japan). Alteplase was purchased from Tanabe Mitsubishi Pharma (Tokyo, Japan). All other reagents were of the highest grade and commercially available.

**Reagent Preparation.** In in vitro studies, all reagents were dissolved in dimethylsulfoxide in ×1000 stock solution. The stock solutions were diluted in HEPES-buffered saline containing 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 11.5 mM glucose, and 20 mM HEPES, which was adjusted to pH 7.4 using NaOH, before use. In in vivo studies, antidepressants were dissolved in saline and administered intraperitoneally to mice used in this study. The administration volume was 10 ml/kg body weight.

**Cell Culture.** Human embryonic kidney (HEK) 293 cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin.

**Stable Expression of Transient Receptor Potential Channels in HEK 293 Cells.** Transfection of recombinant plasmid human TRPM2/pCMV-TAG4, transient receptor potential ankyrin 1 (TRPA1)/pCI-neo, transient receptor potential vanilloid 1 (TRPV1)/pCI-neo, and TRPM8/pCI-neo into HEK cells was performed using the Amaxa electroporation system with the Cell Line Nucleofector Kit V (Amaxa Inc., Gaithersburg, MD) as described previously (Ishii et al., 2007). After electroporation, cells were incubated in culture medium containing 500 $\mu$g/ml geneticin disulfate (G418). Surviving cells were cloned and cultured, and stable TRPM2-, TRPA1-, TRPV1-, and TRPM8-expressing clones were generated (TRPM2/HEK, TRPA1/HEK, TRPV1/HEK, and TRPM8/HEK cells, respectively).

**Measurement of $[Ca^{2+}]_i$.** Changes in $[Ca^{2+}]_i$ were measured at 30°C using fura-2 as a $Ca^{2+}$ indicator, as described previously (Shimizu et al., 2006; Toda et al., 2016). Briefly, cells were transferred to coverslips precoated with poly(L-lysine) (BD Biosciences, Bedford, MA), and they were loaded with 5 $\mu$M fura-2 acetoxymethyl ester in culture medium containing 10% fetal bovine serum at 37°C for 40 minutes. Cells were then washed with HEPES-buffered saline. Coverslips were placed in a perfusion chamber mounted on an inverted microscope. Fura-2 fluorescence images of cells were then recorded at 10-second intervals and were analyzed using a video image analysis system (Mete Fluor; Nippon Roper, Tokyo, Japan). Fluorescence at 510 nm (bandwidth: 50 nm) was observed after the alternate excitation of fura-2 at 340 nm (bandwidth, 10 nm) and 380 nm (bandwidth, 13 nm). Results are presented as a measure of the F340/F380 ratio or ΔF340/F380 ratio.

**Electrophysiology.** Currents were recorded using whole-cell patch-clamp techniques (Hamill et al., 1981) with an EPC-10 patch-clamp amplifier (HEKA, Pfalz, Germany) at room temperature. Patch pipettes were made from borosilicate glass capillaries (outer diameter, 1.5 mm; Hilgenberg, Malsfeld, Germany) using a model P-87 Flaming-Brown micropipette puller (Sutter Instrument, San Rafael, CA). Pipette resistance ranged between 2 and 3.5 MΩ when filled with the pipette solution described below. Cell capacitance was 23.9 ± 3.7 pF ($n$ = 8). Currents were sampled at 1 kHz. The pipette solution contained 105 mM CsOH, 105 mM aspartic acid, 40 mM CsCl, 2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5 mM EGTA, 2 mM $Na_2ATP$, and 5 mM HEPES, adjusted to pH 7.2 with CsOH. The calculated free $Ca^{2+}$ concentration was 50 nM. The 2 mM $Ca^{2+}$ external solution contained 125 mM NaCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 26 mM mannitol, and 11.5 mM HEPES, adjusted to pH 7.4 with NaOH. The 0 mM $Ca^{2+}$ external solution contained 121.7 mM NaCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2 mM EGTA, 10 mM glucose, 24 mM mannitol, and 11.5 mM HEPES, adjusted to pH 7.4 with NaOH (calculated free $Ca^{2+}$ concentration, 90 nM). The rapid exchange of external solutions surrounding the cell was performed within 1 second using a modified "Y-tube" method (Hara et al., 2002).

**Animals.** Generation of *Trpm2* KO mice was described previously (Yamamoto et al., 2008). Male *Trpm2* KO and littermate wild-type (WT) mice (C57BL/6J background, 6–9 weeks of age) were used in this study and handled in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Experimental Animal Committee of Teikyo Heisei University approved the experimental protocol (no. 29-053).

**CIR Model.** CIR was induced as described previously (Shibata et al., 2010; Hashimoto et al., 2014). In this thrombus stroke model, the thrombus has been shown to travel up to the middle cerebral artery. Briefly, mice were anesthetized with 2% isoflurane. Under anesthesia, the left common carotid artery was exposed carefully from the surrounding tissue. The left common carotid artery was temporarily clamped using an aneurysm clip, and acetic acid was then applied to generate a thrombus. After 10 minutes, the clip was removed, and the thrombus was transferred to the brain for embolization by the bloodstream. Three hours after embolization, mice were administered alteplase (10 mg/kg, i.v.) for reperfusion. Twenty-one hours after reperfusion, neurologic dysfunction was measured, and the blood and brain were then collected under anesthesia with 2% isoflurane.

**Assessment of Cerebral Infarct Sizes.** The brain was sectioned coronally into four 2-mm sections. All slices were stained with 2% TTC in saline at 37°C for 30 minutes. Each slice was then photographed on the posterior surface of each section, and the TTC-unstained white area of the whole brain was measured as the infarct area. Infarct sizes calculated from all slices were expressed as the percentage of the whole brain area (infarction area of the whole brain/area of the whole brain).

**Evaluation of Neurologic Dysfunction.** Neurologic scores were obtained by a modified method described previously (Longa et al., 1989; Shibata et al., 2010). The indexes used were as follows: 0, no deficit; 1, decreased resistance to a lateral push; 2, unilateral circling; 3, failing to the right; and 4, no spontaneous walking with a depressed level of consciousness.

**Statistical Analysis.** Results are expressed as the mean ± S.E.M. In $Ca^{2+}$ imaging experiments, $n$ indicates the experimental number (average of 30–55 cells/experiment) in each measurement. Mean data were compared using an unpaired $t$ test for two groups, and multiple

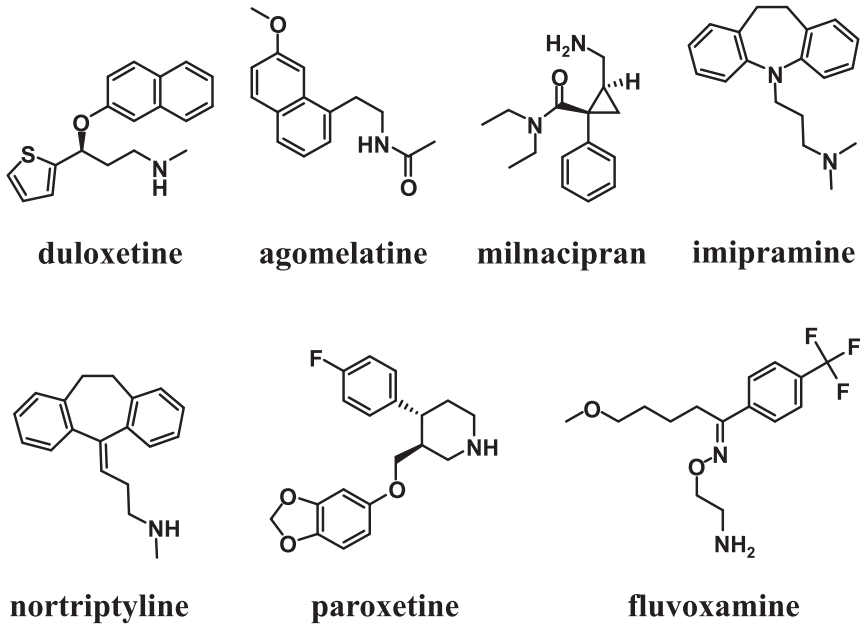


**Fig. 1.** Chemical structures of antidepressants tested in this study.

comparisons were made using an analysis of variance with the Bonferroni $t$ test. Differences were considered to be significant when $P < 0.05$.

## Results

**Effects of Antidepressants on $H_2O_2$-Induced $[Ca^{2+}]_i$ Increases in TRPM2/HEK Cells.** The chemical structures of the seven antidepressants tested are shown in Fig. 1. The addition of 100 $\mu$M $H_2O_2$ induced sustained $[Ca^{2+}]_i$ increases in TRPM2/HEK cells, but not in HEK cells (Fig. 2A), indicating that these $[Ca^{2+}]_i$ increases were mediated by TRPM2. The addition of ionomycin after $H_2O_2$ treatment further increased $[Ca^{2+}]_i$ in TRPM2/HEK cells, and $H_2O_2$-induced $[Ca^{2+}]_i$ increases accounted for 63% of ionomycin-induced $[Ca^{2+}]_i$ increases (Supplemental Fig. 1, A and B). The addition of carbachol after $H_2O_2$ treatment slightly increased $[Ca^{2+}]_i$, but it was not statistically significant

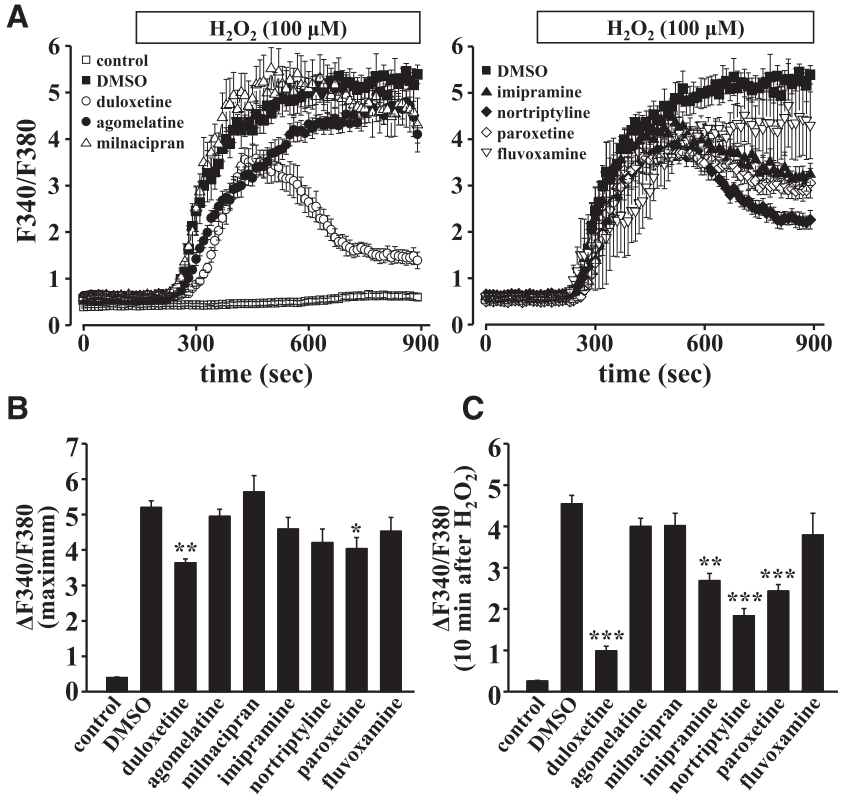


**Fig. 2.** Effects of antidepressants on $H_2O_2$-induced $[Ca^{2+}]_i$ increases in TRPM2/HEK cells. (A) HEK cells were pretreated with 0.1% DMSO (control). TRPM2/HEK cells were pretreated with 0.1% DMSO or 10 $\mu$M of each antidepressant. Cells were then treated with $H_2O_2$ (100 $\mu$M) in the presence of these compounds. (B) Delta ratios ($\Delta$F340/F380) at maximum responses were calculated from (A). (C) Delta ratios ($\Delta$F340/F380) 10 minutes after the addition of $H_2O_2$ were calculated from (A). Results are shown as the mean ± S.E.M. of four experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the DMSO group. DMSO, dimethylsulfoxide.

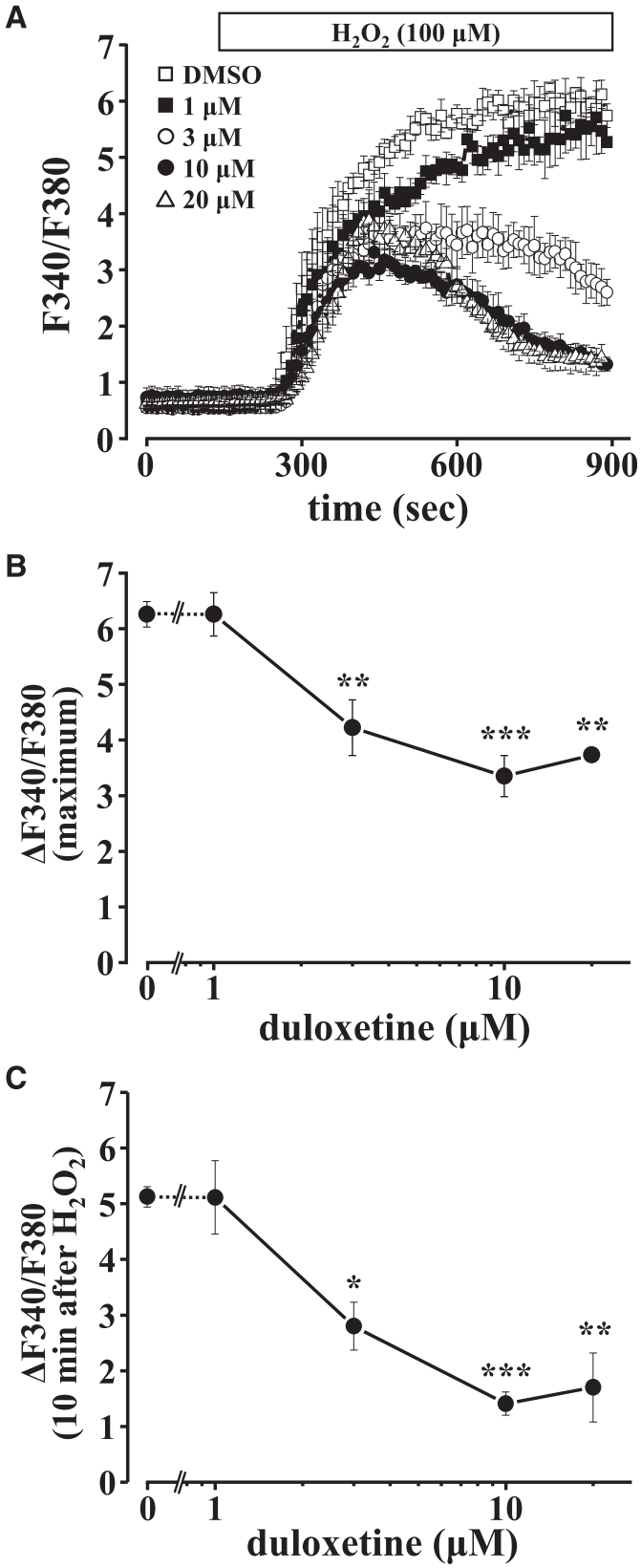


**Fig. 3.** Concentration-dependent inhibition of $H_2O_2$-induced TRPM2 activation by duloxetine. (A) TRPM2/HEK cells were pretreated with 0.1% DMSO or various concentrations of duloxetine (1–20 $\mu$M) for 10 minutes, and $H_2O_2$ (100 $\mu$M) was then added in the presence of duloxetine. (B) Delta ratios ($\Delta$F340/F380) at maximum responses were calculated from (A). (C) Delta ratios ($\Delta$F340/F380) 10 minutes after the addition of $H_2O_2$ were calculated from (A). Results are shown as the mean ± S.E.M. of four experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the DMSO group. DMSO, dimethylsulfoxide.

(Supplemental Fig. 1, C and D). To examine the effects of antidepressants on TRPM2 activation, TRPM2/HEK cells were pretreated with antidepressants for 10 minutes, and $H_2O_2$-induced $[Ca^{2+}]_i$ increases were then measured in the presence of antidepressants (Fig. 2). $[Ca^{2+}]_i$ increases peaked approximately 5 minutes after the application of $H_2O_2$ in duloxetine-treated cells, and these increases then decreased (Fig. 2A). As shown in Fig. 2, B and C, treatment with duloxetine weakly reduced $[Ca^{2+}]_i$ increases at the maximum response after the application of $H_2O_2$ (Fig. 2B) and strongly reduced them 10 minutes after the application of $H_2O_2$ (Fig. 2C). Although paroxetine also reduced $H_2O_2$-induced $[Ca^{2+}]_i$ increases similar to duloxetine, the inhibitory effects observed were weaker than those of duloxetine (Fig. 2). Imipramine and nortriptyline had no effects on $[Ca^{2+}]_i$ increases at the maximum response after the application of $H_2O_2$. On the other hand, imipramine and nortriptyline reduced $[Ca^{2+}]_i$ increases 10 minutes after the application of $H_2O_2$. The inhibitory effects of imipramine and nortriptyline were weaker than those of duloxetine (Fig. 2). Agomelatine, milnacipran, and fluvoxamine had no effects on $H_2O_2$-induced $[Ca^{2+}]_i$ increases (Fig. 2). Thus, duloxetine exerted the strongest inhibitory effects on $H_2O_2$-induced TRPM2 activation among the antidepressants tested. Therefore, we focused on the effects of duloxetine, particularly its concentration-dependent effects (Fig. 3). Duloxetine concentration-dependently reduced $H_2O_2$-induced $[Ca^{2+}]_i$ increases in the maximum response and sustained phase 10 minutes after the application of $H_2O_2$ (Fig. 3A). Maximum inhibitory effects were observed at 10 $\mu$M in the maximum response and sustained phase (Fig. 3, B and C). To further characterize the inhibitory effects of duloxetine, we investigated the effects of pretreatment times on $H_2O_2$-induced TRPM2 activation. As shown in Fig. 4, pretreatment times did not affect the inhibitory effects of duloxetine.

**Reductions in the ADP-Ribose–Induced Current Amplitude in TRPM2/HEK Cells by Duloxetine.** Conventional whole-cell patch-clamp recording was used to record the ionic currents generated by TRPM2 channels. As shown in Fig. 5A, 20 $\mu$M ADP-ribose in the pipette solution gradually evoked the TRPM2 channel current at a holding potential of −60 mV (Yamamoto et al., 2008), while 10 $\mu$M duloxetine slowly inhibited the current (Fig. 5A). During the long recording, nonspecific leak currents sometimes appeared and interfered with the objective current. We were able to discriminate between the leak current and TRPM2 channel current based on the response to extracellular $Ca^{2+}$. The TRPM2 channel current disappeared in $Ca^{2+}$-free (0 mM $Ca^{2+}$-containing) external solution (Hara et al., 2002), whereas nonspecific leak currents increased. We confirmed that the inward current was reduced in the absence of extracellular $Ca^{2+}$ (Fig. 5A). Experiments in which the amplitude of the leak current was more than 10% of the maximum current were discarded. The inhibitory effects of duloxetine on the current are summarized in Fig. 5B. The remaining current through the TRPM2 channel during the application of 10 $\mu$M duloxetine was 53.8% ± 3.4% ($n$ = 8). These electrophysiological results indicated that duloxetine inhibited TRPM2 channels.

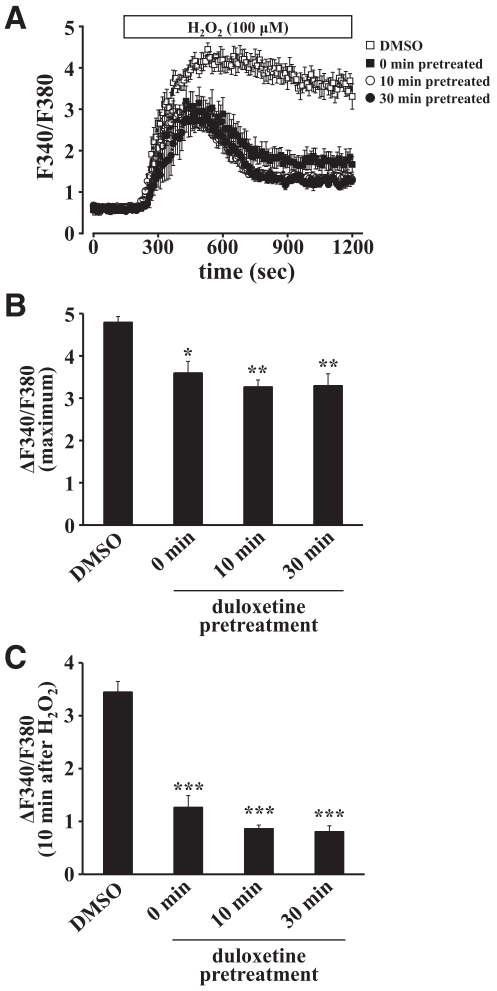


**Fig. 4.** Effects of the pretreatment time with duloxetine on $H_2O_2$-induced TRPM2 activation. (A) TRPM2/HEK cells were pretreated with 0.1% DMSO for 10 minutes (DMSO) or 10 $\mu$M duloxetine for 0, 10, or 30 minutes, and $H_2O_2$ (100 $\mu$M) was then added in the presence of duloxetine. (B) Delta ratios (ΔF340/F380) at maximum responses were calculated from (A). (C) Delta ratios (ΔF340/F380) 10 minutes after the $H_2O_2$ treatment were calculated from (A). Results are shown as the mean ± S.E.M. of four experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. the DMSO group. DMSO, dimethylsulfoxide.

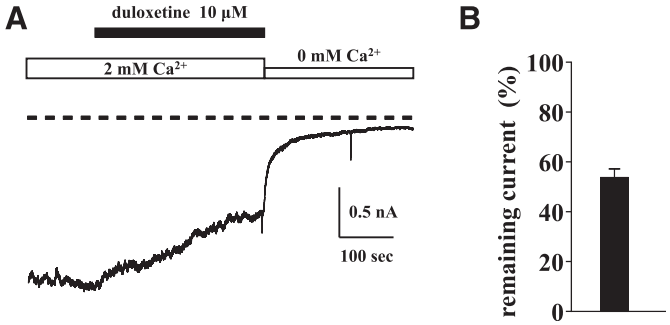


**Fig. 5.** Duloxetine-induced reductions in the ADP-ribose–induced current amplitude in TRPM2/HEK cells. (A) Typical trace of the TRPM2 channel current induced by 20 $\mu$M ADP-ribose in the pipette solution at a holding potential of −60 mV. After the maximum current amplitude was reached in 2 mM $Ca^{2+}$-containing external solution, 10 $\mu$M duloxetine was applied. The external solution was changed to $Ca^{2+}$-free external solution. The bars above the current trace depict the application periods of external solutions and duloxetine. (B) TRPM2 currents after the inhibition by 10 $\mu$M duloxetine are summarized. Results are shown as the mean ± S.E.M. ($n = 8$).

**Effects of Duloxetine on Other Transient Receptor Potential Channels.** We examined the effects of duloxetine on other transient receptor potential channels, which are activated by oxidative stress or possess a similar pore region to TRPM2 channels.

TRPA1 and TRPV1 are oxidative stress–sensitive channels that are activated by $H_2O_2$ (Andersson et al., 2008; Takahashi et al., 2008; Chuang and Lin, 2009; Shimizu et al., 2014). The addition of $H_2O_2$ to TRPA1/HEK or TRPV1/HEK cells increased $[Ca^{2+}]_i$. In contrast to TRPM2 channels, duloxetine did not affect $H_2O_2$-induced $[Ca^{2+}]_i$ increases through TRPA1 channels (Supplemental Fig. 2, A and B) or TRPV1 channels (Supplemental Fig. 2, C and D), respectively.

The TRPM8 channel possesses a similar pore region to the TRPM2 channel (Peier et al., 2002; Kühn et al., 2007) and is activated by menthol and cold temperatures (McKemy et al., 2002; Andersson et al., 2004; Mahieu et al., 2007). The addition of menthol to TRPM8/HEK cells increased $[Ca^{2+}]_i$, whereas duloxetine strongly reduced the menthol-induced $[Ca^{2+}]_i$ increases mediated by TRPM8 channels (Supplemental Fig. 3, A and B). The exposure of TRPM8/HEK cells to cold temperatures also increased $[Ca^{2+}]_i$, and these cold temperature–induced $[Ca^{2+}]_i$ increases were attenuated by treatment with duloxetine (Supplemental Fig. 3, C and D).

**Protective Effects of Duloxetine on CIR Injury via TRPM2 Inhibition.** The effects of duloxetine on CIR injury were examined in the mouse model (Fig. 6). In WT and *Trpm2* KO mice, no significant differences were observed in infarct sizes between the 3-hours ischemia group and each sham-operation group. The infarct size in WT mice was markedly increased by reperfusion after ischemia, and this increase was smaller in *Trpm2* KO mice. The administration of duloxetine (10 mg/kg, i.p.) 1 hour before ischemia decreased the infarct size induced by CIR in WT mice, similar to that in *Trpm2* KO mice. Duloxetine did not induce further decreases in the infarct size in *Trpm2* KO mice (Fig. 6A). Neurologic scores also showed that reperfusion after cerebral ischemia induced neurologic dysfunction in WT mice, and induction of neurologic dysfunction was reduced by duloxetine treatment or in *Trpm2* KO mice (Fig. 6B). However, the administration of duloxetine did not further improve neurologic dysfunction in *Trpm2* KO mice (Fig. 6B).

In order for duloxetine to be applied to the treatment of cerebral ischemia to reduce reperfusion injury, it is important for it to also exert protective effects against CIR injury, even when it is administered just before reperfusion therapy. Therefore, duloxetine was administered (10 mg/kg, i.p.) just before reperfusion, and its effects were investigated (Fig. 7). Similar to the treatment with duloxetine before ischemia, the administration of duloxetine reduced infarct sizes (Fig. 7A) and neurologic scores (Fig. 7B) in WT mice but not in *Trpm2* KO mice.

**Effects of Milnacipran on CIR Injury.** Duloxetine is a serotonin-norepinephrine reuptake inhibitor. To confirm that the protective effects of duloxetine against CIR injury are not mediated by the inhibition of serotonin-norepinephrine reuptake, the effects of another serotonin-norepinephrine reuptake inhibitor (milnacipran) on CIR injury were examined. Milnacipran did not affect infarct sizes induced by CIR (Supplemental Fig. 4).

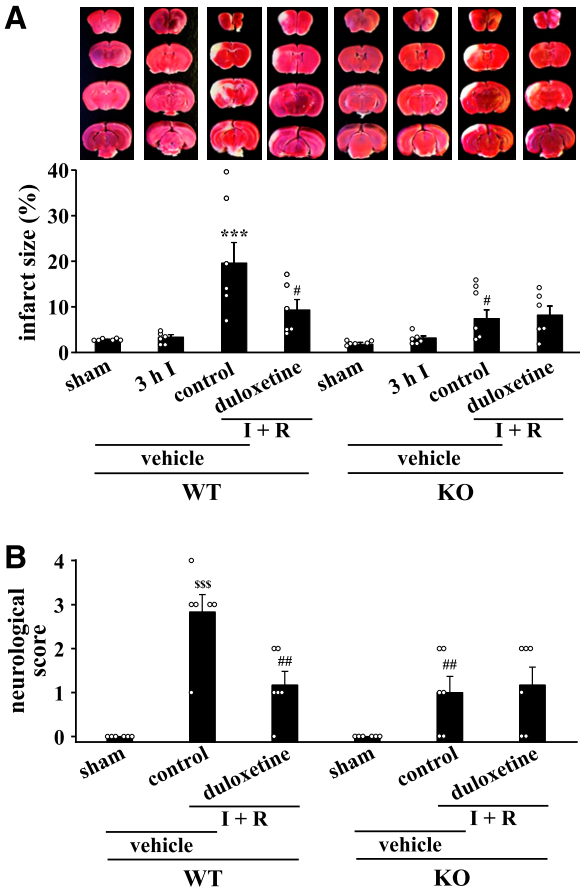


**Fig. 6.** Protective effects of duloxetine administered before ischemia against CIR injury via TRPM2 inhibition. (A) Coronal sections (thickness of 2 mm) showing TTC-stained cerebral tissue of the sham-operation (sham), 3-hour ischemia (3 h I), and vehicle (control) or duloxetine-treated ischemia reperfusion (I + R) groups in WT and *Trpm2* KO mice. In the sham group and each I + R group, vehicle or duloxetine (10 mg/kg, i.p.) was administered 1 hour before ischemia, and infarct sizes were measured 21 hours after reperfusion. In the 3-hour ischemia group, vehicle was administered 1 hour before ischemia, and infarct sizes were then assessed at 3 hours after ischemia. (B) Neurologic scores of the sham and I + R groups in WT and *Trpm2* KO mice were assessed 21 hours after reperfusion. Results are shown as the mean ± S.E.M. ($n$ = 6). $^{***}P < 0.001$ vs. the WT 3-hour ischemia group; $^{\#}P < 0.05$ vs. the WT control group; $^{\#\#}P < 0.01$ vs. the WT control group; $^{\$\$\$}P < 0.001$ vs. the WT sham group. Open circles show the individual values.

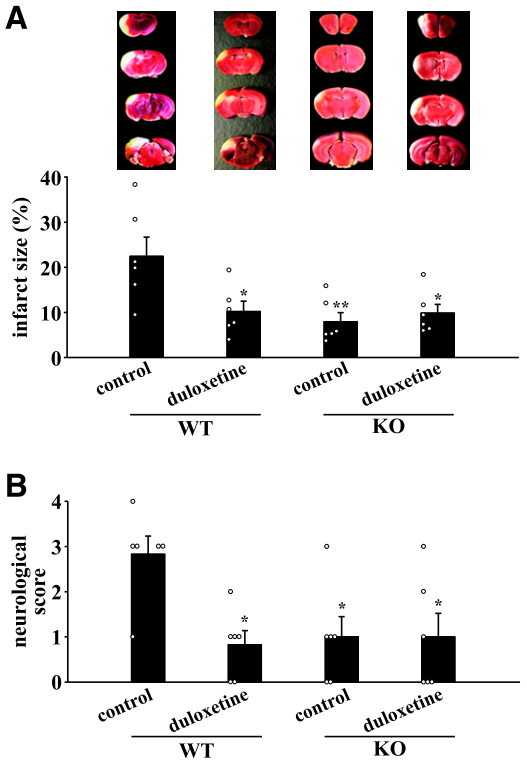


**Fig. 7.** Protective effects of duloxetine administered just before reperfusion against CIR injury via TRPM2 inhibition. (A) Coronal sections (thickness of 2 mm) showing TTC-stained cerebral tissue with ischemia reperfusion in WT and *Trpm2* KO mice. Vehicle (control) or duloxetine (10 mg/kg, i.p.) was administered just before reperfusion, and infarct sizes were then assessed 21 hours after reperfusion. (B) Neurologic scores of each group were assessed 21 hours after reperfusion. Results are shown as the mean ± S.E.M. ($n$ = 6). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. the WT control group. Open circles show the individual values.

## Discussion

Duloxetine is a dual inhibitor of serotonin and norepinephrine reuptake and drives antidepressive efficacy. Akpinar et al. (2014) reported that increases in $[Ca^{2+}]_i$ in response to $H_2O_2$ were attenuated in PC12 cells exposed to long-term treatment with duloxetine, and they suggested that the attenuation of $Ca^{2+}$ responses is mediated by inhibition of TRPM2 channels and voltage-gated $Ca^{2+}$ channels. Demirdaş et al. (2017) more recently demonstrated that ADP-ribose–induced TRPM2 currents were reduced in neuronal cells treated with duloxetine for a long time (24 hours). Moreover, these findings indicated that treatment of neuronal cells with duloxetine for 24 hours increased antioxidative enzyme levels and decreased the production of ROS that lead to oxidative stress–induced cell damage. However, it currently remains unclear whether duloxetine directly or indirectly inhibits TRPM2 channels by reducing oxidative stress. We showed that among the seven antidepressants tested in this study, duloxetine exerted direct inhibitory effects on TRPM2 channels, with the strongest inhibitory effects being observed on $H_2O_2$-induced TRPM2 activation. Moreover, we demonstrated that duloxetine exerted neuroprotective effects against CIR injury by inhibiting TRPM2 channels.

The inhibitory effects of duloxetine on $H_2O_2$-induced TRPM2 activation were independent of the pretreatment time (0–30 minutes), and they strongly reduced the sustained phase but not the initial phase of TRPM2-mediated $[Ca^{2+}]_i$ increases. These results prompted us to hypothesize that duloxetine reduces increases in $[Ca^{2+}]_i$ through TRPM2 channel inhibition by an open-channel blocking-like mechanism. The results showing that duloxetine reduced ADP-ribose–induced TRPM2 current during the channel open state support our hypothesis. TRPM8 is also a $Ca^{2+}$-permeable channel that is activated by cold temperatures and menthol (McKemy et al., 2002; Andersson et al., 2004; Mahieu et al., 2007). The pore region of TRPM8 was previously shown to be similar to that of TRPM2 (Peier et al., 2002; Kühn et al., 2007). Duloxetine suppressed menthol- or cold temperature–evoked TRPM8 activation (Supplemental Fig. 3), suggesting that it attenuates $H_2O_2$-induced TRPM2 activation by affecting its pore region. TRPA1 and TRPV1 are also oxidative stress–sensitive $Ca^{2+}$ permeable channels (Andersson et al., 2008; Takahashi et al., 2008; Chuang and Lin, 2009; Shimizu et al., 2014). However, duloxetine did not affect $H_2O_2$-induced

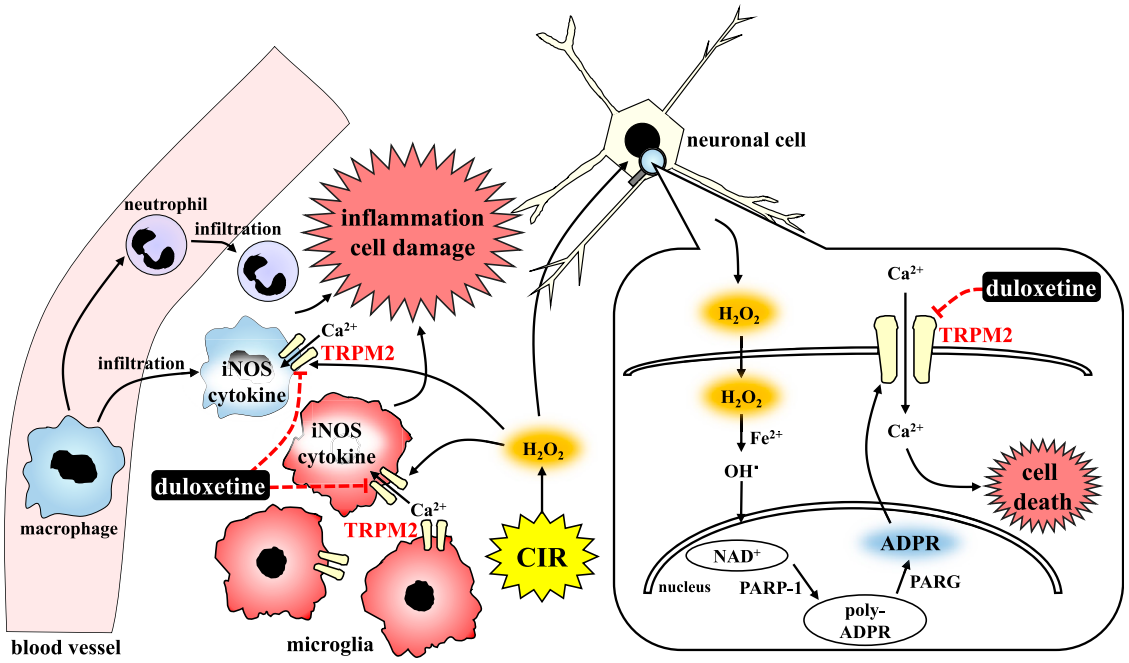


**Fig. 8.** Prospective mechanisms of duloxetine against CIR injury via TRPM2 inhibition. ADPR, ADP-ribose; iNOS, inducible nitric oxide synthase; PARG, poly(ADP-ribose) glycohydrolase; PARP, poly(ADP-ribose) polymerase.

TRPA1 or TRPV1 activation (Supplemental Fig. 2). The inhibitory effects of duloxetine on $H_2O_2$-induced TRPM2 activation do not appear to be mediated by reductions in oxidative stress, which strongly supports our hypothesis that the attenuation of $H_2O_2$-induced TRPM2 activation by duloxetine may be related to its pore region during the channel open state.

$H_2O_2$-induced TRPM2 activation is enhanced by around body temperature, comparing to 37°C (Shimizu et al., 2015). The mechanisms by which body temperature enhances TRPM2 activation are thought to accelerate $H_2O_2$-induced ADP-ribose production. The temperatures in our $[Ca^{2+}]_i$ measurement and whole-cell patch-clamp study were 30°C and room temperature, respectively. The inhibitory effect of TRPM2 inhibitors, which reduce the production of ADP-ribose, may be influenced by temperature. However, duloxetine inhibits TRPM2 activation by an open-channel blocking-like mechanism. Therefore, temperature does not seem to affect the inhibitory effect of duloxetine.

Reperfusion of the ischemic brain is essential for the rescue of neuronal cells; however, this causes secondary neuronal damage, a phenomenon termed "reperfusion injury," which is characterized by the production of ROS and inflammation leading to neuronal damage (Chan, 1996, 2001). Therefore, the development of drugs to prevent reperfusion injury is important and useful. Accumulating evidence has implicated activation of TRPM2 channels in the aggravation of CIR injury (Jia et al., 2011; Gelderblom et al., 2014; Shimizu et al., 2016; Huang et al., 2017). TRPM2 expression is widely observed in the brain, including neuronal cells (Kaneko et al., 2006; Olah et al., 2009; Verma et al., 2012; Ye et al., 2014; Li et al., 2017) and microglial cells (Kraft et al., 2004; Fonfria et al., 2006; Mortadza et al., 2017), and in macrophages (Yamamoto et al., 2008; Gelderblom et al., 2014). The underlying mechanisms by which TRPM2 channels aggravate brain injury during reperfusion may involve induction of necrotic neuronal cell death by a $Ca^{2+}$ overload (Ye et al., 2014; Zhan et al., 2016), activation of microglia (Wu et al., 2010; Gelderblom et al., 2014; Shirakawa et al., 2014), and infiltration of macrophages (Gelderblom et al., 2014; Shirakawa et al., 2014). The activated microglia and the infiltrated macrophages secrete inflammatory cytokines and inducible nitric oxide synthase–derived nitric oxide, leading to neuronal cell death and aggravated inflammation (Gelderblom et al., 2009; Wu et al., 2010; Shirakawa et al., 2014). Consistent with these findings, we observed that CIR injury was attenuated in *Trpm2* KO mice. Furthermore, duloxetine exerted neuroprotective effects against CIR injury. Duloxetine may reduce CIR injury by inhibiting TRPM2 activation in neuronal cells, microglia, and infiltrated macrophages during CIR (Fig. 8). Although duloxetine is a serotonin-norepinephrine reuptake inhibitor, another serotonin-norepinephrine reuptake inhibitor (milnacipran) did not reduce CIR injury, suggesting that duloxetine protects the brain against CIR injury independent of its inhibition of serotonin and norepinephrine reuptake. Treatment with duloxetine for 24 hours has been shown to attenuate activation of TRPV1 channels and voltage-gated $Ca^{2+}$ channels in addition to TRPM2 channels. Importantly, duloxetine did not further reduce CIR injury in *Trpm2* KO mice, indicating that it exerts a neuroprotective effect against CIR injury mainly through the inhibition of TRPM2 activation. Treatment of neuronal cells for 24 hours with duloxetine has been reported to increase antioxidative systems, including reduced glutathione/glutathione peroxidase and Cu,Zn-superoxide dismutase (Akpinar et al., 2014; Lee et al., 2016). Therefore, the induction of antioxidative systems by duloxetine may contribute to inhibition of TRPM2 activation during CIR followed by CIR injury.

Lee et al. (2016) reported that pretreatment with duloxetine for 3 days attenuated ischemia-induced glial activation,

decreased oxidative stress, and exerted neuroprotective effects against transient global cerebral ischemia. On the other hand, in this study, we showed that CIR injury was reduced by treatment with duloxetine not only 1 hour before ischemia but also just before reperfusion. Therefore, duloxetine has potential as a useful drug in reperfusion therapy for ischemic stroke.

In conclusion, we demonstrated herein that duloxetine exerts inhibitory effects on TRPM2 activation. The mechanisms underlying TRPM2 inhibition appear to be open-channel blocking via an association with its pore region. Moreover, we found that even when duloxetine was administered just before reperfusion, it exerted neuroprotective effects against CIR injury through the inhibition of TRPM2 activation. Therefore, duloxetine has potential as a useful drug in reperfusion therapy for ischemic stroke, and duloxetine may be easily applied to prevent or treat CIR injury from the viewpoint of drug repositioning because it has already been clinically used to treat depressive disorders, diabetic neuropathy, and urinary incontinence.

#### Acknowledgments

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#### Authorship Contributions

*Participated in research design:* Toda, Yamamoto, Shimizu.

*Conducted experiments:* Toda, Yamamoto, Umehara, Wakamori.

*Performed data analysis:* Toda, Yamamoto, Umehara, Wakamori, Shimizu.

*Wrote or contributed to the writing of the manuscript:* Toda, Yamamoto, Umehara, Mori, Wakamori, Shimizu.

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# Protective effects of duloxetine against cerebral ischemia-reperfusion injury via TRPM2 inhibition

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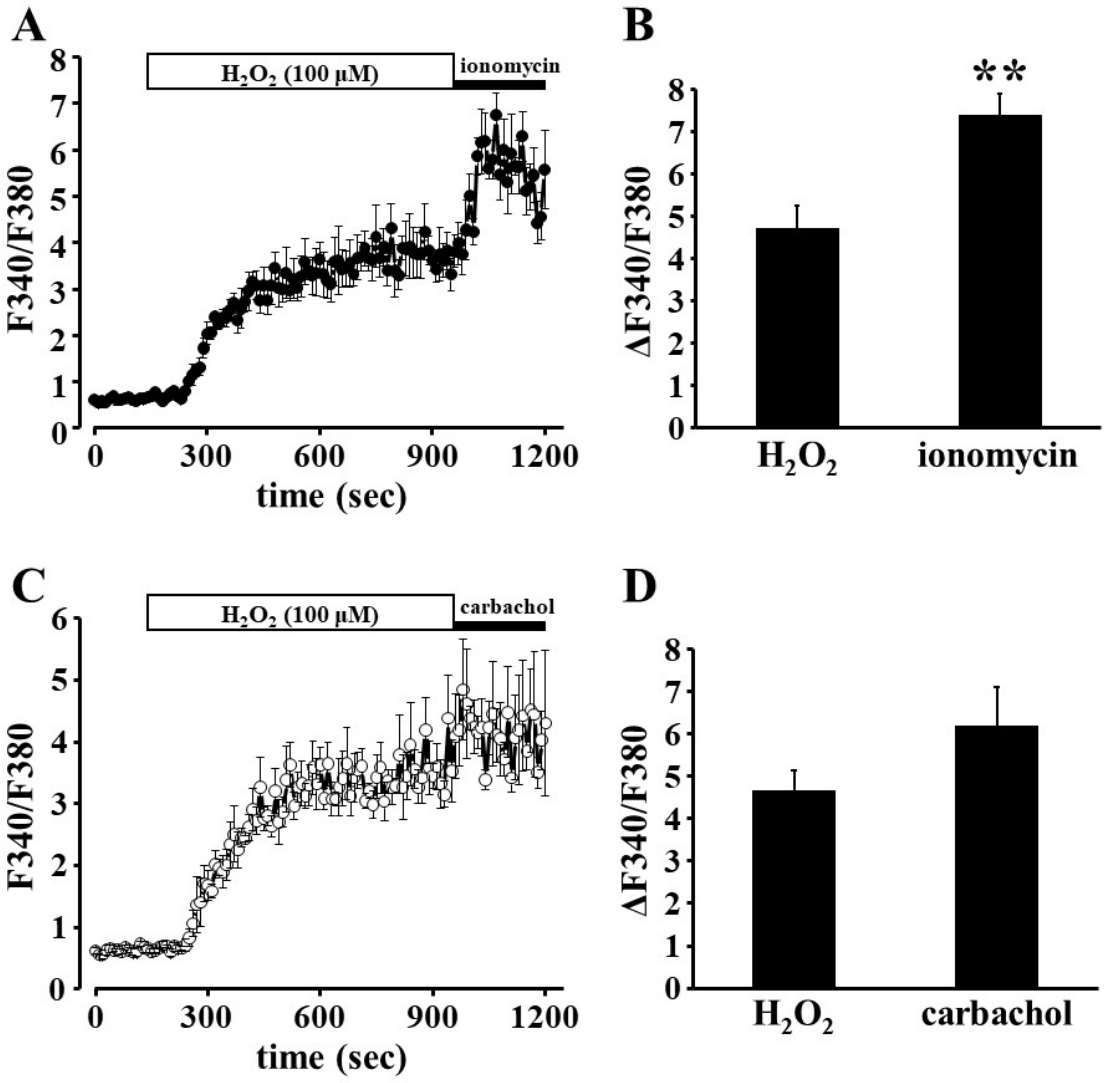
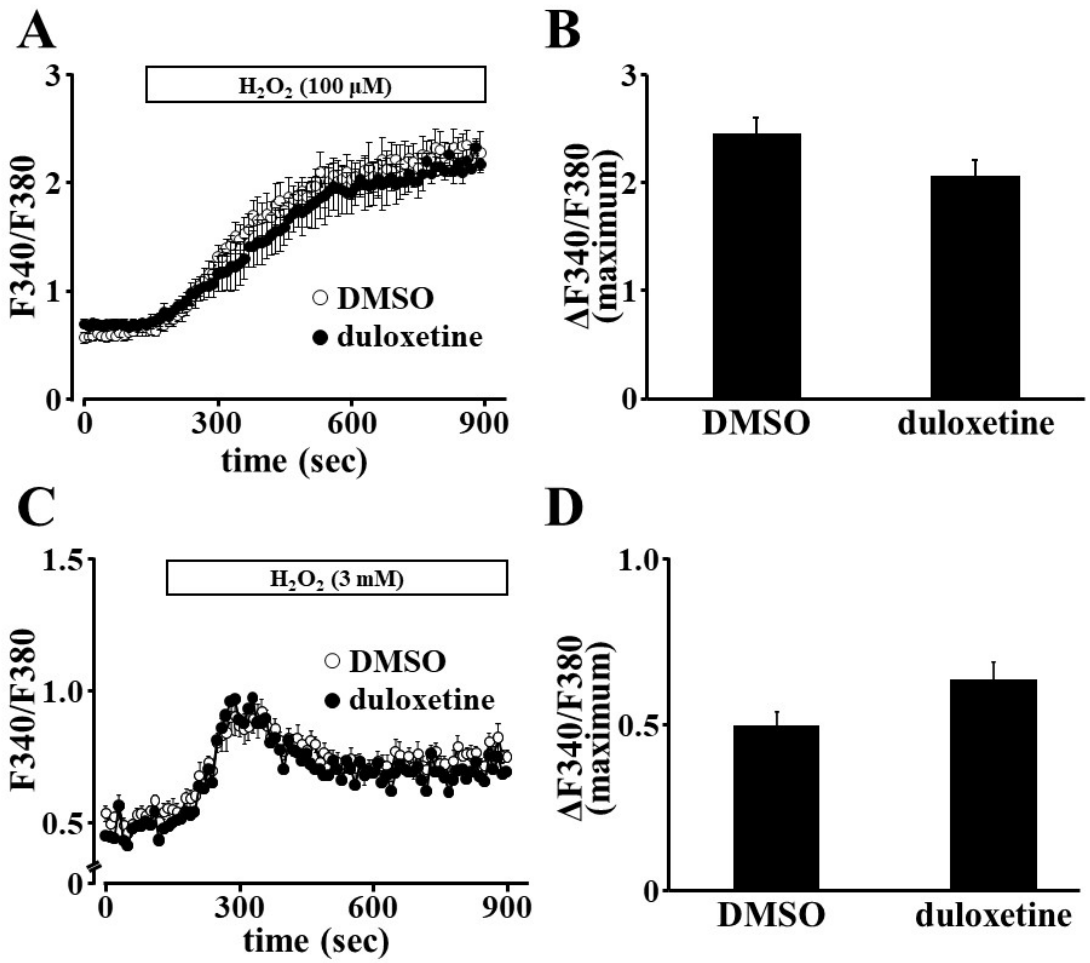
**Supplementary Figure 1**




Supplementary Figure 1. Comparison of $H_2O_2$-induced $[Ca^{2+}]_i$ increases with $Ca^{2+}$ ionophore- and carbachol-induced responses.

TRPM2/HEK cells were treated with $H_2O_2$ (100 μM), and then treated with 2 μM ionomycin (A) or 100 μM carbachol (C). Delta ratios (ΔF340/F380) at maximum responses during $H_2O_2$, ionomycin (B), and carbachol (D) treatments were calculated from A and C, respectively. Results are shown as the mean ± S.E.M. of 4 experiments. **$P < 0.01$ vs. $H_2O_2$.

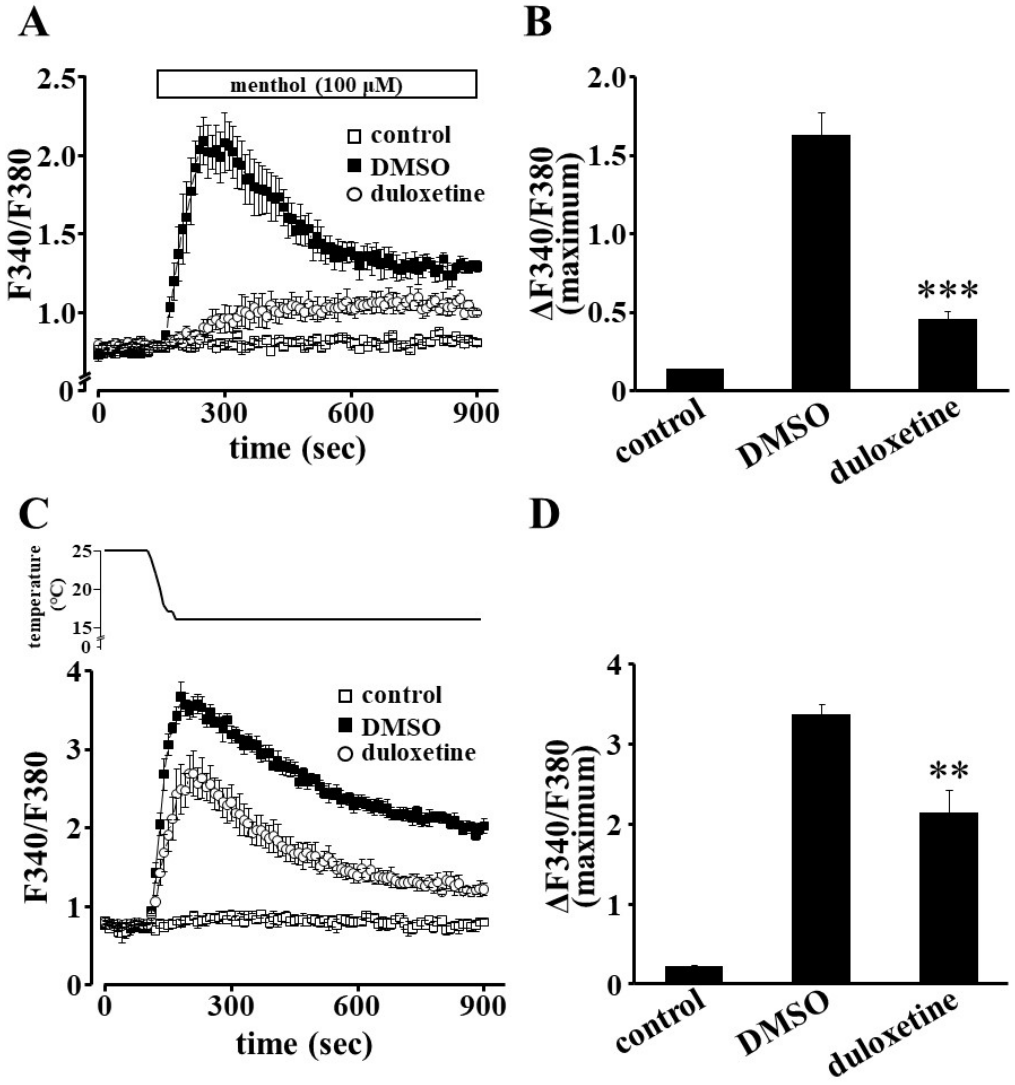
**Supplementary Figure 2**




Supplementary Figure 2. Effects of duloxetine on $H_2O_2$-induced $[Ca^{2+}]_i$ increases in TRPA1/HEK, TRPV1/HEK.

TRPA1/HEK cells (A) and TRPV1/HEK cells (C) were treated with 0.1% DMSO or 10 μM duloxetine, and then $H_2O_2$ (100 μM) was added in the presence of duloxetine. (B and D) Delta ratios (ΔF340/F380) at maximum responses were calculated from A and C, respectively. Results are shown as the mean ± S.E.M. of 4 experiments.

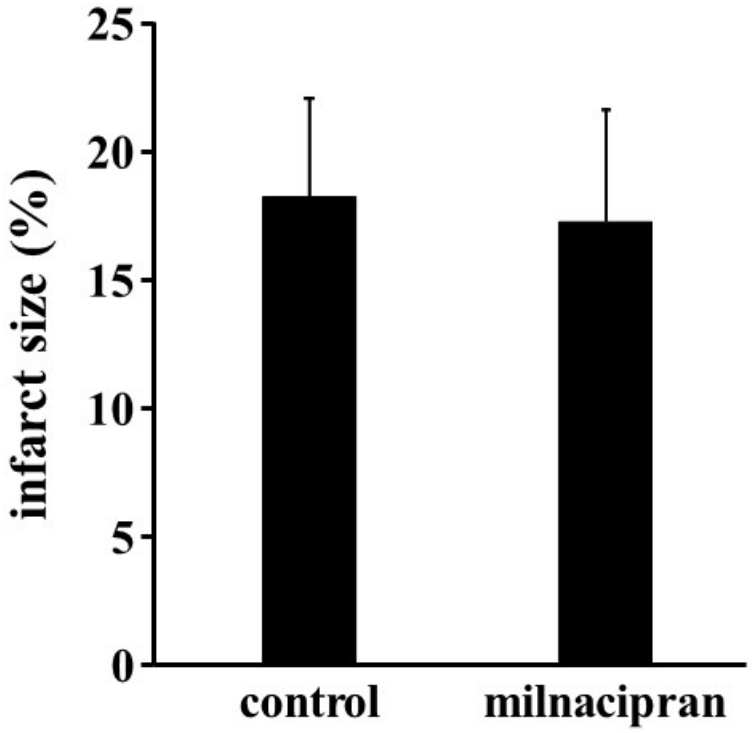
**Supplementary Figure 3**




Supplementary Figure 3. Effects of duloxetine on menthol- and cold temperatures-induced $[Ca^{2+}]_i$ increases in TRPM8/HEK.

(A) HEK cells were pretreated with 0.1% DMSO (control). TRPM8/HEK cells were treated with 0.1% DMSO (DMSO) or 10 μM duloxetine, and then menthol (100 μM) was added in the presence of duloxetine. (B) Delta ratios (ΔF340/F380) were calculated from A. (C) HEK cells were pretreated with 0.1% DMSO (control). TRPM8/HEK cells were pretreated with 0.1% DMSO (DMSO) or 10 μM duloxetine at 25°C. The temperature was then decreased to 16 °C in the presence of duloxetine. (D) Delta ratios (ΔF340/F380) were calculated from C. Results are shown as the mean ± S.E.M. of 4 experiments. **P < 0.01, ***P < 0.001 vs. DMSO group.

**Supplementary Figure 4**




Supplementary Figure 4. Effects of milnacipran on CIR injury.
Vehicle (control) or milnacipran (10 mg/kg, i.p.) were administered 1 h before ischemia, and then infarct size was determined at 21 h after reperfusion. Results are shown as the mean ± S.E.M., n = 6.