Supplemental Data

Screening of TRPC Channel Activators Identifies Novel Neurotrophic Piperazine Compounds

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Molecular pharmacology

Supplemental Materials and Methods

General Materials and Methods of Synthesis

All the solvents and the reagents were purchased from commercially suppliers and used without further purification. NMR spectra were performed on JEOL JMN-ECS-400. Chemical shifts are expressed in ppm, and the splitting patterns were described using the following abbreviations: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), and broad singlet (brs). Mass spectra were performed on a Waters Acquity UPLC/MS system equipped with a UPLC binary pump, a SQD 3100 mass spectrometer with electrospray ionization (ESI) source, a PDA detector (220, 254 nm), and an evaporative light scattering detector (ELSD), eluting with 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile.

Synthesis

[4-(5-chloro-2-methylphenyl)piperazin-1-yl](3-fluorophenyl)methanone (PPZ1)

To a solution of 1-(5-chloro-2-methylphenyl)piperazine (15.0 g, 71.2 mmol) and triethylamine (12.9 mL, 92.6 mmol) in THF (150 mL) was added dropwise 3-fluorobenzoyl chloride (9.52 mL, 75.3 mmol) over a period of 10 minutes at 0°C, and the resulting mixture was stirred for 2 hours at the same temperature. Then water and ethyl acetate were added to the reaction mixture, and the layers were separated. The

organic layer was washed with water, dried over $MgSO_4$, and concentrated under reduced pressure. Recrystalization of the resulting solid from ethyl acetate/n-hexane afforded the product as a white solid (19.7 g, 59.3 mmol, 83% yield); ^{1}H NMR ($CDCl_3$): δ 7.42-7.36 (m, 1H), 7.20 (d, J = 7.3 Hz, 1H), 7.14-7.08 (m, 3H), 6.99-6.94 (m, 2H), 3.90 (brs, 1H), 3.56 (brs, 1H), 2.92-2.86 (m, 2H), 2.26 (s, 3H) ppm. ^{13}C NMR ($CDCl_3$): δ 168.9, 168.9, 163.7, 161.3, 151.7, 137.7, 137.6, 132.1, 131.8, 130.9, 130.4, 130.3, 123.7, 122.7, 122.7, 119.7, 116.9, 116.7, 114.4, 114.2, 51.6, 48.0, 42.5, 17.3 ppm. ESI MS m/z calcd for $[M+H]^+$ 333.1, found 332.9.

2-[4-(2,3-dimethylphenyl)piperazin-1-yl]-*N*-(2-ethoxyphenyl)acetamide (PPZ2)

To a solution of 2-ethoxyaniline (15.9 g, 116 mmol) in dichloromethane (130 mL), 2 M sodium hydroxide aqueous solution (87 mL) was added. After the mixture was cooled to 0°C, bromoacetyl chloride (11.6 mL, 139 mmol) was added dropwise over a period of 10 minutes with keeping the temperature at 0°C, and then warmed to room temperature. After a stirring of 2 hours at room temperature, water was added and the resulting mixture was extracted with dichloromethane. The organic layer was dried over $MgSO_4$, and concentrated under reduced pressure. Purification of the residue by silica gel column chromatography (ethyl acetate/*n*-hexane) afforded 2-bromo-*N*-(2-ethoxyphenyl)acetamide as a white solid (29.2 g, 114 mmol, 98% yield). To a solution of 2,3-dimethylphenylpiperazine (15.7 g, 82.5 mmol), potassium carbonate (17.1 g, 124 mmol) and 2-bromo-*N*-(2-ethoxyphenyl)acetamide (21.3 g, 82.5 mmol) were added subsequently, and the mixture was stirred overnight at room temperature. And then water was added and the resulting mixture was extracted with ethyl acetate. Toluene was added to the organic layer and the solution was washed with water, dried over $MgSO_4$, and concentrated under reduced pressure. Recrystalization of the resulting solid from ethyl acetate/n-hexane afforded the product as a white solid (25.6 g, 69.7 mmol, 84% yield) ; ^{1}H NMR ($CDCl_3$): δ 9.91 (brs, 1H), 8.44 (d, J = 8.0 Hz, 1H), 7.08 (t, J = 8.0 Hz, 1H), 7.03-6.85 (m, 5H), 4.09 (q, J = 6.4 Hz, 2H), 3.23 (s, 2H), 2.98 (s, 4H), 2.80 (s, 4H), 2.27 (s, 3H), 2.22 (s, 3H), 1.52 (t, J = 6.4 Hz, 3H) ppm. ^{13}C NMR ($CDCl_3$): δ 168.3, 151.2, 147.5, 138.1, 131.4, 127.4, 125.8, 125.1, 123.5, 120.9, 119.3, 116.4, 110.7, 63.9, 62.3, 53.9, 52.4, 20.6, 15.5, 13.9 ppm. ESI MS m/z calcd for $[M+H]^+$ 368.2, found 368.0.

Recordings of Unitary TRPC6 Currents in Cell-Attached Configuration. Cell-attached mode of patch clamp recordings and data analysis were performed as described previously (Shi et al., 2004). HEK293 cells were co-transfected with pCI-neo vector or mouse TRPC6 together with pEGFP-N1 and human muscarinic type 1 receptor using FuGENE^{TM} HD transfection reagent (Promega, Madison, WI) 24–36 hours before recordings (Itsuki et al., 2014). Solutions had the following compositions, with the concentrations in mM unless otherwise indicated. Bath solution: 140 K^+, 2 Mg^{2+}, 144 Cl^-, 1 EGTA, 10 HEPES (pH 7.4 adjusted with Tris base); Pipette solution: 140 Na^+, 5 Tetraethylammonium$^+$, 1.2 Mg^{2+}, 0.1 Ca^{2+}, 149.4 Cl^-, 10 glucose, 10 HEPES (pH 7.4 adjusted with Tris base). Voltage generation and current signal acquisition were accomplished using a high-impedance low-noise patch clamp amplifier (AxoPatch 200B, Axon Instruments, Foster City, CA) in conjunction with an A/D, D/A-converter (Digidata 1440A, Axon Instruments). Sampled data were low-pass filtered at 2 kHz, digitized at 20 kHz and analyzed using Clampfit v. 9.2 (Axon Instruments). The currents were recorded at a holding potential of −60 mV. All experiments were performed at room temperature. From the single channel events list, the NP_o (N, number of channels; P_o, open probability) of single channels was calculated by dividing the total time spent in the open state by the total time of continuous recording (10–60 seconds) in the patches. For calculation of unitary conductances, single channel amplitudes from indivisual patches at different membrane potentials were pooled together and the unitary conductance were determined by linear data fitting.

Quantitative RT-PCR. Total RNA of cultured rat CGNs and hippocampal neurons were extracted using ISOGEN following the manufacturer's instructions (Wako). Reverse transcription of total RNA to cDNA was performed using the RNA LA PCR Kit (TaKaRa-Bio). This cDNA was used as template for real-time PCR to detect genes expression using Power SYBR^{TM} Green PCR Master Mix according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Following primers are used to detect target genes: TRPC3, 5'-TGCTAATTATGGTCTGGGTTCTC-3' (forward) and 5'-CCACAGCTGCACGATGTACT-3' (reverse); TRPC6, 5'-GCAGCTGTTCAGGATGAAAAC-3' (forward) and 5'-ACATTCAGCCCATATCATTCCTA-3' (reverse); TRPC7,

5’-CGGAAGTGGCATACTTCACC-3’ (forward) and 5’-CGAGATGATCTGGGGGTCT-3’ (reverse); TRPV1, 5’-TCCTGACGGCAAGGATGAC-3’ (forward) and 5’-TGATGATACCCACATTGGTGTTC-3’ (reverse); rpl27 5’-CCAAGCGATCCAAGATCAAG-3’ (forward) and 5’-TCTGAACACATCCTTGTTGAC-3’ (reverse). Temperature cycle was as follows: initial 94°C for 10 minutes was followed by 40 cycles at 95°C for 15 seconds, 60°C for 20 seconds. The identity of the PCR product was confirmed by generating a melting curve ranging from 65°C to 95°C. Values are expressed relative to housekeeping gene ribosomal protein 27 (rpl27) (de Jonge et al., 2007).

Luciferase Reporter Assay. CGNs were co-transfected with 3 μg of pNFAT-Luc (Stratagene) and 0.06 μg of pRL-SV40 (Promega), and seeded on 96-well plates at a density of 5×10^4 cells/cm^2. Two to four hours after transfection, cells were treated with 0.1% DMSO, 50 ng/ml BDNF, 10 nM PPZ1 or 10 nM PPZ2 alone or in combination with 10 μM CsA or 1 μM FK506. After 24 hours treatment, cells were lysed and luciferase activity was measured with Infinite M200 plate reader (Tecan, GmbH, Austria) using Dual-Luciferase™ Reporter Assay System according to the manufacturer’s instructions (Promega).

Supplemental References

de Jonge HJ, Fehrmann RS, de Bont ES, Hofstra RM, Gerbens F, Kamps WA, de Vries EG, van der Zee AG, te Meerman GJ and ter Elst A (2007) Evidence based selection of housekeeping genes. *PLoS One* **2**:e898.

Itsuki K, Imai Y, Hase H, Okamura Y, Inoue R and Mori MX (2014) PLC-mediated PI(4,5)P_2 hydrolysis regulates activation and inactivation of TRPC6/7 channels. *J Gen Physiol* **143**:183-201.

Shi J, Mori E, Mori Y, Mori M, Li J, Ito Y and Inoue R (2004) Multiple regulation by calcium of murine homologues of transient receptor potential proteins TRPC6 and TRPC7 expressed in HEK293 cells. *J Physiol* **561**:415-432.

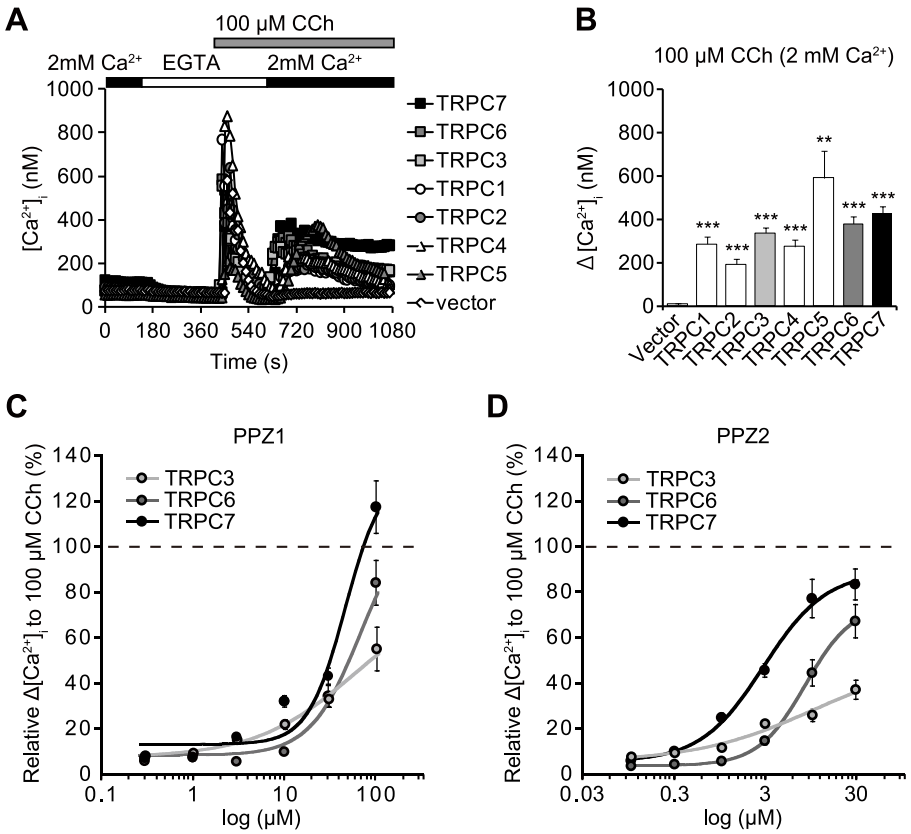

Fig. S1. Normalization of the PPZ1- or PPZ2-activation of TRPC3, TRPC6, and TRPC7 to CCh-induced activation. (A and B) $[Ca^{2+}]_i$ changes induced by 100 μM CCh in Ca^{2+}-free, 0.5 mM EGTA-containing and 2 mM Ca^{2+}-containing external solutions in HEK 293 cells expressing TRPC subtypes TRPC1–C7, respectively. Averaged time courses (A) and $\Delta[Ca^{2+}]_i$ after re-addition of extracellular Ca^{2+} (B) (n = 22–43). $^{**}P < 0.01$; $^{***}P < 0.001$ compared with vector. (C and D) Dose-response relationships for $\Delta[Ca^{2+}]_i$ induced by PPZ1 (C) and PPZ2 (D) in HEK 293 cells expressing TRPC3, TRPC6, and TRPC7. The $\Delta[Ca^{2+}]_i$ values shown in Fig. 3, C and D were normalized to those obtained by 100 μM CCh in B above (n = 66–166). The plots were fitted to the Hill equation: $f(x) = A_0 + (A_{max} - A_0)/[1 + (EC_{50}/x)^n]$, where A_0 is the basal response, A_{max} is the maximum response, x is the PPZ concentration, and n is Hill coefficient. For PPZ1, EC_{50} values were 57.0, 67.3, and 45.9 μM and Hill coefficients were 0.80, 1.59, and 2.14, for PPZ2, EC_{50} values were 10.20, 8.37, and 2.90 μM and Hill coefficients were 0.67, 1.67, and 1.25 for TRPC3, TRPC6, and TRPC7, respectively. Data points show the means ± S.E.M.

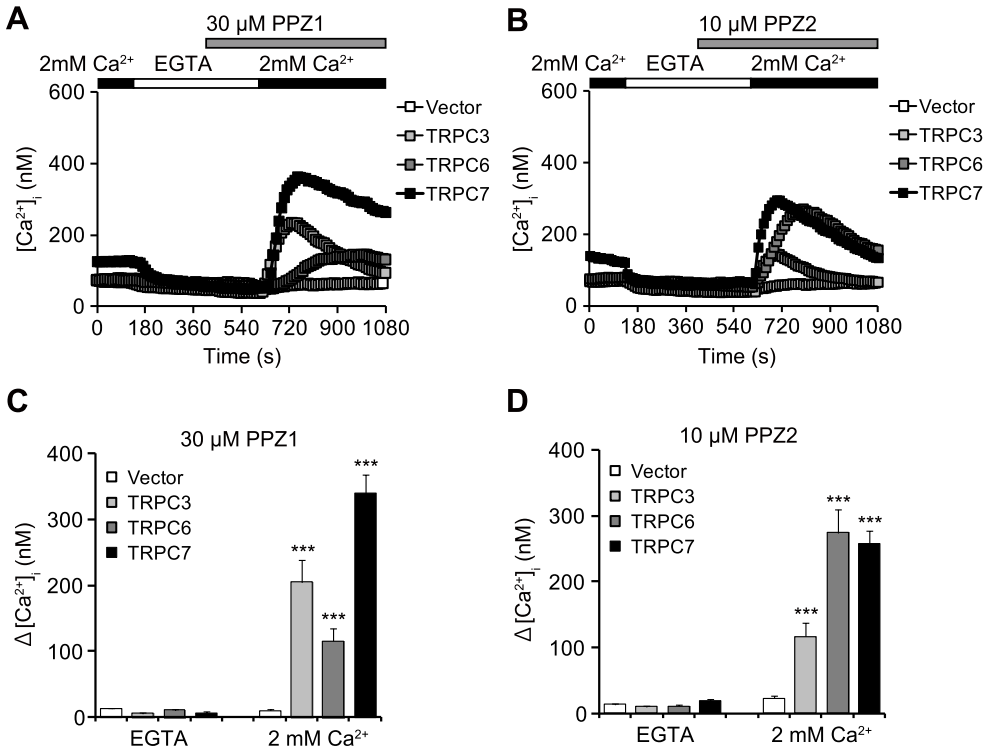

Fig. S2. PPZ1- and PPZ2-induced activation of TRPC3, TRPC6, and TRPC7 in the absence and presence of extracellular Ca^{2+}. $[Ca^{2+}]_i$ changes induced by 30 μM PPZ1 and 10 μM PPZ2 in Ca^{2+}-free, 0.5 mM EGTA-containing and 2 mM Ca^{2+}-containing external solutions in HEK 293 cells transfected with vector, TRPC3, TRPC6, or TRPC7. Averaged traces (A, B) and $\Delta[Ca^{2+}]_i$ induced by PPZs before and after re-addition of extracellular Ca^{2+} (C, D) (n = 38–43). $^{***}P < 0.001$ compared with vector. Data points show the means ± S.E.M.

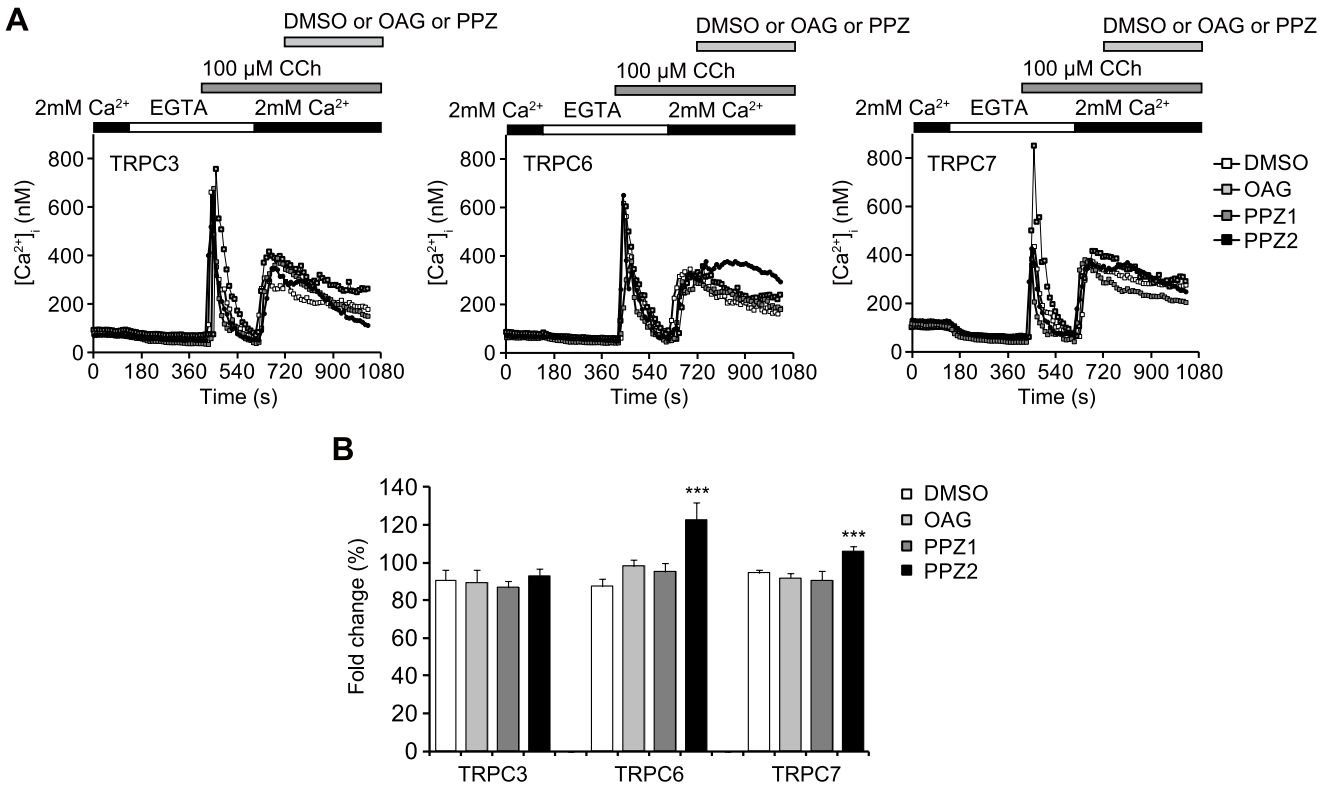

Fig. S3. The effects of PPZ1 and PPZ2 on CCh-induced activation of TRPC3, TRPC6, and TRPC7. (A) Averaged traces of $[Ca^{2+}]_i$ changes induced by 100 μM CCh in Ca^{2+}-free, 0.5 mM EGTA-containing and 2 mM Ca^{2+}-containing external solutions in TRPC3-, TRPC6-, or TRPC7-expressing HEK 293 cells. Two min after re-addition of extracellular Ca^{2+} under CCh stimulation, 0.1% DMSO, 30 μM OAG, 10 μM PPZ1, or 10 μM PPZ2 was administered. (B) Fold changes of $[Ca^{2+}]_i$ after addition of DMSO, OAG, PPZ1 or PPZ2 are indicated as percentage ratios of maximum $[Ca^{2+}]_i$ rises after the application of the stimulants (during 720–840 sec) to the $[Ca^{2+}]_i$ rises immediately prior to their application (at 710 sec) (n = 22–41). $^{***}P < 0.001$ compared with DMSO. Data points show the means ± S.E.M.

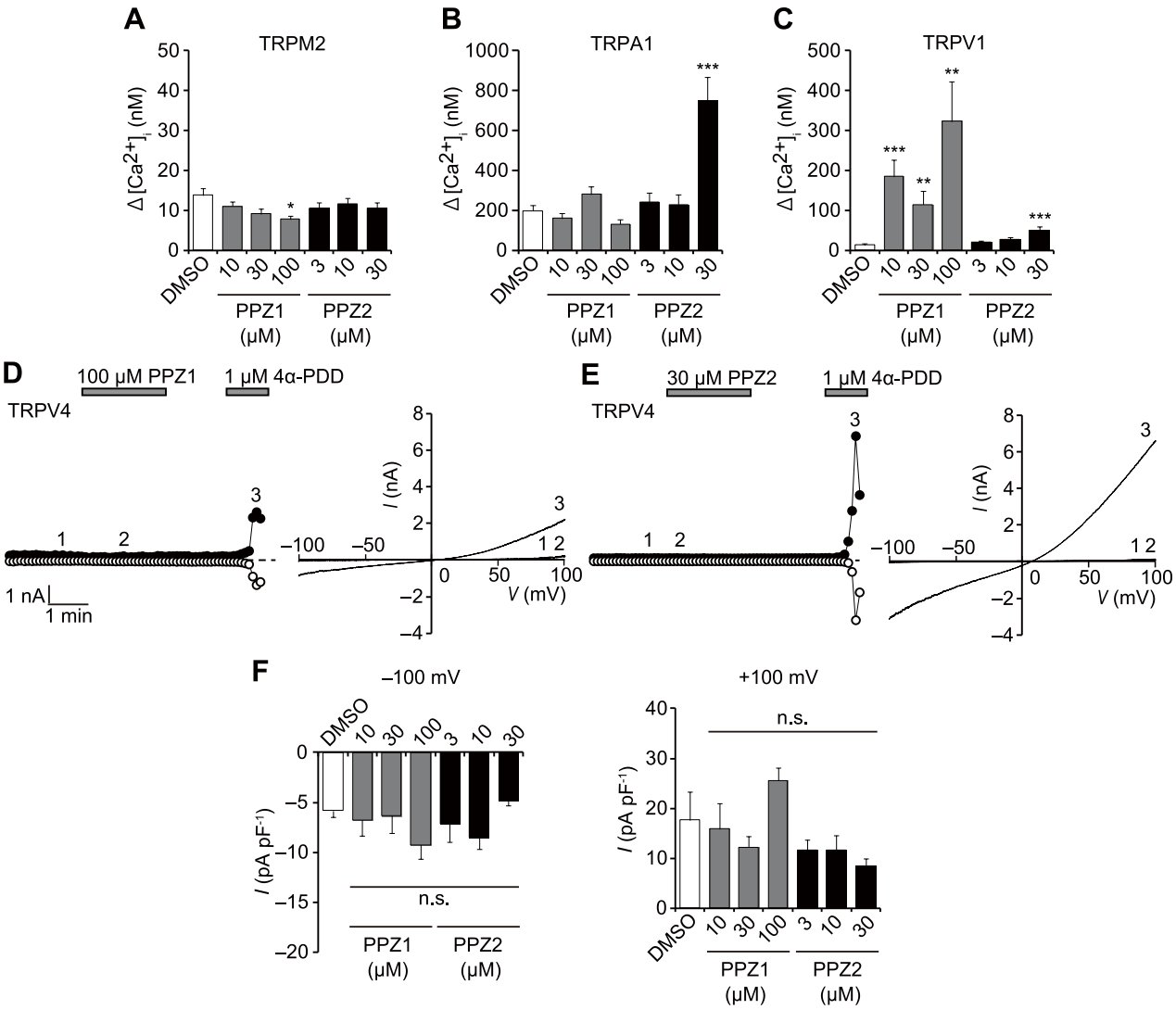

Fig. S4. The effects of PPZ1 and PPZ2 on TRPM2, TRPA1, TRPV1 and TRPV4. (A–C) $\Delta[Ca^{2+}]_i$ induced by PPZ1 and PPZ2 in TRPM2- (A), TRPA1- (B), TRPV1-expressing HEK 293 cells (C) (n = 25-128). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with DMSO. (D–F) Whole-cell currents during application of PPZ1 and PPZ2 in TRPV4-expressing HEK 293 cells. One μM 4α-phorbol-12,13-didecanoate (4α-PDD) was used as a positive control. Representative traces of inward and outward currents during application of PPZ1 (D) and PPZ2 (E) recorded at –100 and +100 mV respectively, under ramp clamp (left). Corresponding I-V relationships at the time points 1, 2 and 3 (Right). Peak current densities at –100 and +100 mV during the application of PPZ1 and PPZ2 (F) (n = 5). n.s.; not significant compared with DMSO. Data points show the means ± S.E.M.

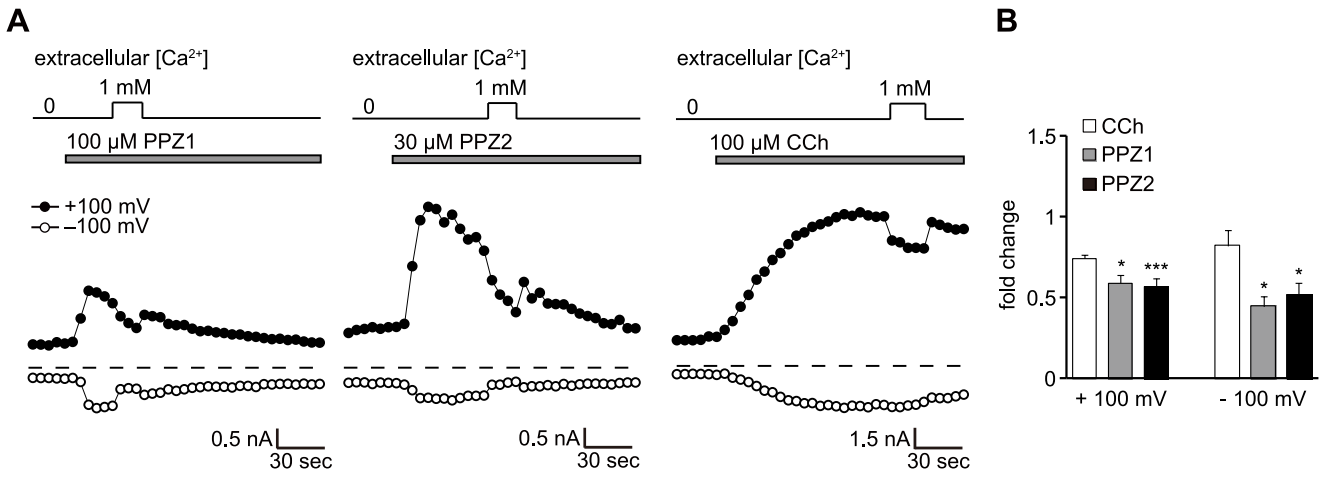

Fig. S5. Extracellular Ca^{2+}-dependence of PPZ1-, PPZ2- and CCh-induced TRPC6 currents. Whole-cell patch clamp recordings of ionic currents evoked by 100 μM PPZ1, 30 μM PPZ2, and 100 μM CCh in HEK 293 cells expressing TRPC6. (A) Representative traces of inward and outward currents recorded at –100 and +100 mV, respectively, under ramp clamp. TRPC6 was first activated in the absence of extracellular Ca^{2+}, and was then exposed to 1 mM extracellular Ca^{2+}. (B) Relative amplitudes of TRPC6 currents in 1 mM extracellular Ca^{2+} at –100 and +100 mV (n = 5–6). Fold changes of TRPC6 currents are indicated as ratios of minimum amplitudes obtained in 1 mM extracellular Ca^{2+} to the amplitudes immediately prior to application of extracellular Ca^{2+}. $^{*}P < 0.05$; $^{***}P < 0.001$ compared with CCh. Data points show the means ± S.E.M.

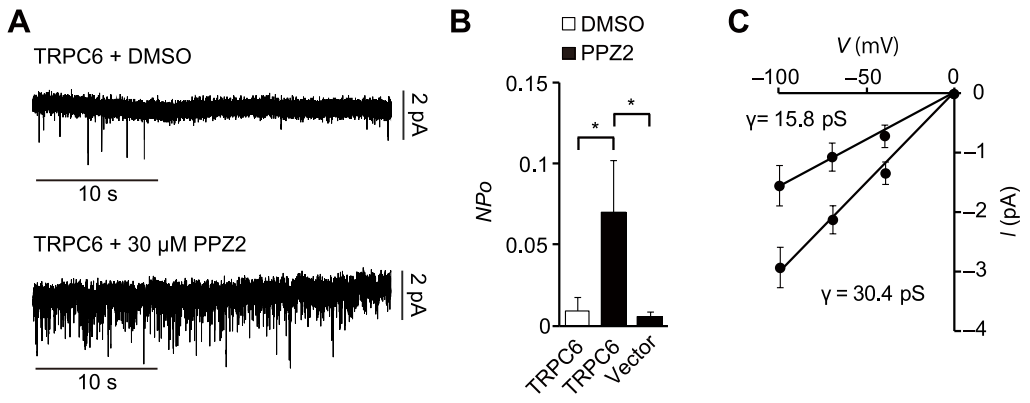

Fig. S6. Unitary TRPC6 channel activity induced by PPZ2 in a cell-attached patch. (A) Representative recordings obtained using the cell-attached pipette containing DMSO (upper) or 30 μM PPZ2 (bottom) in HEK 293 cells transfected with TRPC6 at a membrane potential of –60 mV**.** Resting membrane potential of HEK293 cell was almost nulled by excess K^+ (140 mM) in the bath. (B) Averaged NP_o of the cell-attached recordings (n = 6–10). $^*P < 0.05$. TRPC6 activity was increased by 30 μM PPZ2 in the recording pipette in TRPC6-expressing cells. (C) *I-V* relationship of PPZ2-activated TRPC6 channel currents. The unitary conductances (γ) were evaluated by linear data fitting at membrane potentials from –100 to 0 mV (n = 5). Data points show the means ± S.E.M.

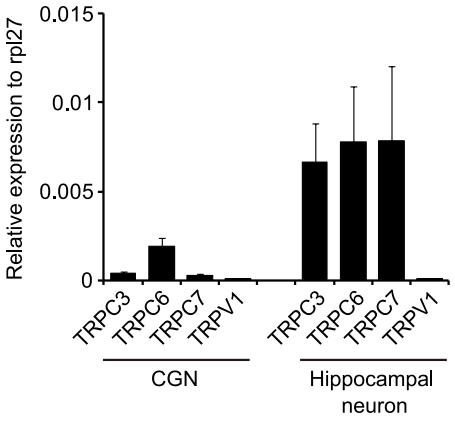

Fig. S7. Expression of TRPC3, TRPC6, TRPC7 and TRPV1 in cultured CGNs and hippocampal neurons. Quantitative PCR analysis for mRNA expression of TRPC3, TRPC6, TRPC7 and TRPV1 channels in CGNs and hippocampal neurons at 1 DIV (n = 3–4). The gene expression levels were calculated as a relative ratios to the value of housekeeping gene, rpl27. Data points show the means ± S.E.M.

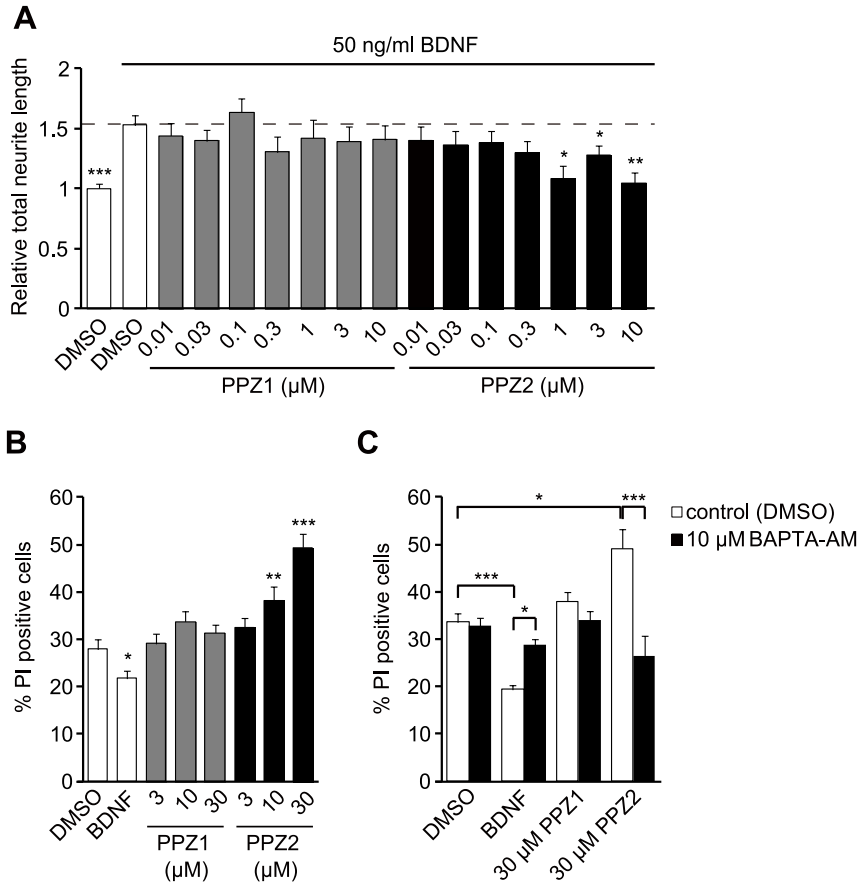

Fig. S8. PPZ2 at high concentrations exerted suppressive effects on neuronal growth and survival. (A) The effects of PPZ1 and PPZ2 in BDNF-induced neurite growth promotion in CGNs at 1 DIV. PPZ1 and PPZ2 at various concentrations were co-treated with 50 ng/ml BDNF for 24 hours. Mean values of total neurite length were normalized to that in DMSO-treated control cells (n = 34–103). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with BDNF + DMSO. (B) PPZ2 at relatively high concentrations induced cell-death in CGNs. Percentage numbers of PI-positive CGNs treated with 0.1% DMSO, 50 ng/ml BDNF, PPZ1 and PPZ2 at indicated concentrations for 24 hours at 1 DIV (n = 12–14). $^{*}P < 0.05$; $^{**}P < 0.01$ compared with DMSO. (C) Ca^{2+} chelation rescued PPZ2-induced cell death in CGNs. CGNs were treated with 0.1% DMSO, 50 ng/ml BDNF, 30 μM PPZ1 and 30 μM PPZ2 alone or in combination with 10 μM BAPTA-AM for 24 hours at 1 DIV (n = 5–8). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with DMSO. Data points show the means ± S.E.M.

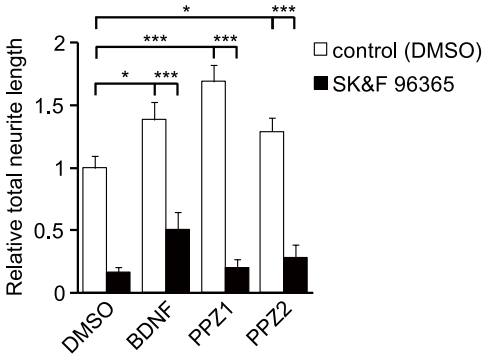

Fig. S9. The effects of 20 μM SK&F 96365 on 100 nM PPZ1- or 100 nM PPZ2-induced neurite outgrowth in hippocampal neurons. Hippocampal neurons were treated with 0.1% DMSO, 50 ng/ml BDNF, 100 nM PPZ1 or 100 nM PPZ2 alone or in combination with 20 μM SK&F 96365 for 48 hours at 1 DIV (n = 17–32). Mean values of total neurite length were normalized to that in DMSO-treated control cells without SK&F 96365. $^{*}P < 0.05$; $^{***}P < 0.001$ compared with DMSO. Data points show the means ± S.E.M.

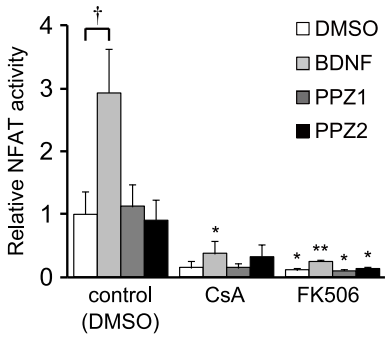

Fig. S10. The effects of cyclosporine A and FK506 on NFAT activity in CGNs. NFAT-mediated transcription was measured by using luciferase reporter vector containing NFAT-binding sequence. Reporter vector-transfected CGNs were treated with 0.1% DMSO, 50 ng/ml BDNF, 10 nM PPZ1 or 10 nM PPZ2 alone or in combination with 10 μM CsA or 1 μM FK506 for 24 hours at 1 DIV. Luciferase expression in CGNs was quantitated using dual-luciferase assay system. Relative NFAT activity was calculated by normalizing the obtained values to that of DMSO-control (n = 4–8). $^{*}P < 0.05$; $^{**}P < 0.01$ compared with control. $^{\dagger}P < 0.05$ compared with DMSO. Data points show the means ± S.E.M.