Supplemental Data

Expanded Materials and Methods

Generation of Transgenic Mice

The over-expression construct was generated by ligating the full-length β3 subunit coding sequence into the pCXN2 plasmid, in which the *Hin*dII site had been substituted with a *Not*I site (gift from Dr. Miyazaki, Osaka University School of Medicine), under the CAG promoter (a combination of CMV and β-actin promoters), followed by IRES (intra-ribosomal entry site) and EGFP (enhanced green fluorescent protein) sequences, and completed with a rabbit β-globin poly(A) signal (Fig. 1Ai). The CAG–β3–IRES–EGFP construct was cleaved with *Sal*I and *Not*I, and the resulting CAG–β3 fusion cDNA fragment was purified and microinjected into the male pronucleus of fertilized zygotes from superovulated C57BL6 mice. Surviving zygotes were implanted into pseudopregnant foster mothers. Transgenic (β3-Tg) founder mice were identified through genomic DNA analysis by Southern blotting.

The β3-deficient mouse strain ($\beta3^{-/-}$) was constructed from genomic DNA clones, as reported previously (16). The CaV2.2-deficient mice were generated by gene-targeting methods (11). Genetically modified mice were back-crossed into a C57BL/6NCrj (Charles River Japan, Tokyo, Japan) genetic background to the N14 generation. The wild-type and all mutant mice were experimentally naive and 12-16 weeks old. They were maintained at 22 ± 0.5°C in a 12/12 h light/dark cycle. All experiments were conducted in accordance with the Guidelines for the Use of Laboratory Animals of the Akita University School of Medicine.

RNA Isolation and RT-PCR

Total RNA isolation, reverse transcription reactions, and PCR amplifications were performed using standard methods. The β1, β2, β3, and β4 subunit-specific sequences were amplified by PCR with the following primers: MB1S and MB1A, which correspond to the V_{163}RKLDSLR$_{169}$ and K_{240}TSVSSVP$_{246}$ sequences, respectively, of the murine β1 subunit, MB2S and MB2A, which correspond to the L_{131}ENMRLQ$_{137}$ and P_{198}KPSANS$_{204}$ sequences, respectively, of the murine β2 subunit, MB3S and MB3A, which correspond to the L_{129}KQEQKAR$_{135}$ and P_{186}SLKGYE$_{192}$ sequences, respectively, of the murine β3 subunit, and MB4S and MB4A, which correspond to the L_{154}RLENIR$_{160}$ and Y_{213}DVVPSM$_{219}$ sequences, respectively, of the murine β4 subunit. The CaV2.1 (α_{1A}), CaV2.2 (α_{1B}), CaV2.3 (α_{1E}), CaV1.2 (α_{1C}), and CaV1.3 (α_{1D}) subunit-specific sequences were amplified by PCR with the following primers: A1A1 and A1A2, which

correspond to the T_{403}PENSLIVT$_{411}$ and E_{463}KKEEEEAD$_{471}$ sequences, respectively, of the murine α_{1A} subunit, A1B1 and A1B2, which correspond to the G_{319}DKETRNHQ$_{327}$ and G_{403}NMNLEGQA$_{411}$ sequences, respectively, of the murine α_{1B} subunit, A1E1 and A1E2, which correspond to the R_{754}AHHMSMW$_{761}$ and P_{885}RSCHGN$_{892}$ sequences, respectively, of the murine α_{1E} subunit, A1C1 and A1C2, which correspond to the L_{510}AIAVDNL$_{517}$ and T_{580}KIHMDALQ$_{588}$ sequences, respectively, of the murine α_{1C} subunit; and A1D1 and A1D2, which correspond to the A_{819}NSDNKV$_{825}$ and K_{893}KTNPIRV$_{899}$ sequences, respectively, of the murine α_{1D} subunit. To avoid contamination of genomic DNA in RT-PCR analysis, each primer set was designed to span at least one intron.

Western Blot Analysis

Partially purified supracervical ganglion (SCG) membranes from wild-type and β3-Tg mice were prepared and suspended in 50 mM Tris–HCl buffer (pH 7.4) containing protease inhibitors. Aliquots of homogenate (100 μg) from each mouse were resolved by 6% SDS-polyacrylamide gel electrophoresis. Commercially available polyclonal antibodies specific for β1, β3, and β4 (Abcam PLC, Cambridge, UK); β2 (Sigma-Aldrich, St. Louis, MO, USA); and CaV2.2 (α1B) and CaV1.2 (α1C) (Alomone, Jerusalem, Israel) were used for immunodetection. An anti-GFP antibody (MBL, Woods Hole, MA, USA) was also used to confirm integrated transgene expression. As a control, an anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used to confirm comparable sample loading.

ECG Telemetry

ECG telemetry was performed as described previously (14). During the recording period (60 min), animals were at liberty in their home cage. Analog ECG signals were transferred to a receiver device and directly digitized by an analog-to-digital converter system (Mini-Digi-1A; Axon Instruments, Foster City, CA, USA). The effect of ω-conotoxin GVIA (30 μg/kg) was obtained at 30 min after its intraperitoneal injection.

Blood Pressure Measurement

Mice were anesthetized with urethane (1.5 g/kg, i.p.). The mean arterial blood pressure was obtained using arterial catheters surgically inserted into the right carotid artery (11). Following surgery, each animal was allowed to stabilize for 30 min, and mean arterial blood pressure was measured with a microtip catheter pressure transducer connected to a carrier amplifier (AP-601G; Nihon Koden, Tokyo, Japan). The left carotid artery was

ligated for 30 s to observe the baroreflex responses. ω-Conotoxin GVIA (30 μg/kg) was administered intravenously to block N-type channels.

Langendorff Perfusion Experiments

The isolated heart was mounted on a Langendorff apparatus and perfused at a constant hydrostatic pressure of 75 mmHg with an oxygenated Tyrode's solution (37°C) composed of 136.9 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 10.0 mM glucose, and 5.0 mM HEPES (12). Atropine (5.5 μM) was added to the perfusate, and a fluid-filled balloon catheter connected to a pressure transducer (CD200; Nihon Koden) was inserted into the left ventricle via the left atrium. The sinoatrial node region was removed, and the heart was paced electrically at 400 beats/min (bpm). The electrical field stimulation (EFS) was applied in conjunction with the pacing stimulation (delay, 4 ms; duration, 1 ms for 5 s).

Plasma Catecholamines

The animals were anesthetized with α-chloralose (100 mg/kg; Wako, Tokyo, Japan). This anesthesia is thought to have a minimal depressant effect on peripheral autonomic nervous activity (29).

After one hour, blood was removed via cardiac puncture by using a 1-ml syringe containing 100 μl of heparin. Plasma was separated by centrifugation and stored at –80^{o}C. Catecholamines (epinephrine and norepinephrine) were determined after alumina extraction by high-performance liquid chromatography combined with electrochemical detection.

Isolation of SCG Neurons and Whole-Cell Recordings

Electrophysiological measurements (whole-cell mode) were performed on the supracervical cervical ganglion (11). Currents were recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments)(16). The pipettes (2–5 MΩ) were filled with a solution containing 140 mM CsCl, 2.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM ATP, and 10 mM HEPES. The pH was adjusted to 7.4 with 1 N KOH. The bath solution contained 133 mM TEA-Cl, 10 mM $BaCl_2$, 2.5 mM CsCl, 10 mM HEPES, and 1 μM TTX, pH 7.4 (with NaOH). The holding potential for most recordings was –80 mV. To examine channel components, ω-agatoxin IVA (0.1 μM), ω-conotoxin GVIA (1 μM), nimodipine (10 μM), and nickel (100 μM) were used. The analysis was carried out with the pCLAMP software (Axon Instruments). All values are given as the mean ± S.E.

Material and Methods (Supplemental Data)

Echocardiography

Mice were anesthetized with 2% inhaled sevoflurane. An echocardiography machine equipped with a 10-MHz transducer (Aspen, Acuson, Stockton, CA, USA) was used to record the B- and M-mode echocardiogram of the left ventricle. Echocardiographic recordings were analyzed to determine the left ventricular inner diameter at the end of diastole (LVED) and at the end of systole (LVES), and the left ventricular ejection fraction, using the software supplied with the system (12).

Spontaneously Beating Atria

Right atria were dissected, and appendages were tied with 4-0 silk sutures. The atria were placed in an oxygenated 37°C tissue bath containing Krebs solution (118 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 0.57 mM $MgSO_4$, 1.0 mM Na_2PO_4, 2.5 mM $NaHCO_3$, 11.1 mM glucose) for 30 min to stabilize the basal contraction. Isometric contractile force in response to different doses of isoproterenol (0.1-100 nM) was measured with a force transducer (CD200; Nihon Koden).

Isolation of Single Cardiomyocytes and Electrophysiological Recording

Single ventricular cells were isolated from the hearts of 8- to 10-week-old mice by an enzymatic dissociation protocol using collagenase (Wako, Osaka, Japan). The L-type Ca^{2+} channel currents were recorded using a whole-cell patch clamp method.

Expression Analysis of CaV2.2 (α1B) and β Subunits in HEK-293T Cells

The full-length CaV2.2 (α1B)-specific sequence was amplified using primers B1s (5'-ATGGTCCGCTTCGGGGACGAGCTA-3') and B1as (5'-CTAGCACCAGTGGTCCTGATCGGG-3'), which amplify the full-length ORF of the murine CaV2.2 subunit. The sequenced cDNA fragment was inserted into the pcDNA3 (Invitrogen, Carlsbad, CA, USA) plasmid for expression analysis. The pcDNA3 expression vector constructs of all the reported β subunits (β1–4) were prepared as described previously.

Cell Culture

For transient expression analysis, human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HEK-293 cells were transfected with pcDNA3 expression vectors that carried the cloned CaV2.2 sequence with/without β subunits, which were subcloned into pcDNA3,

using LipofectAMINE Plus reagent (Invitrogen).

References

29. Kass, DA., Hare, JM., and Georgakopoulos, D. (1998) *Circulation Research,* 82, 519-522.

Additional Figures and Supporting Information

Figure 5

Increased cardiac pump function (ejection fraction in echocardiography)

(A) Representative M-mode results in wild-type (WT, upper panel) and transgenic (TG, lower panel) mice. Arrows indicate the left-ventricular diameter at the end of systole (LVES, red arrows) and at the end of diastole (LVED, black arrows).

(B) Summarized results of basal ejection fractions (EF) in WT (white bar), β3-null (blue bar), and Tg (red bar) mice. Compared with the WT ejection volume, the ejection fraction in β3-null mice was significantly decreased, and that of Tg mice was increased (asterisk).

(C) Effect of intraperitoneal administration of ω-conotoxin GVIA (30 μg/kg) on the ejection fraction (EF) in WT (white bar), β3-null (blue bar), and β3-Tg (Tg, red bar) mice. Tg mice showed a greater response to ω-conotoxin GVIA (asterisk). Data are expressed as the means ± S.E. of at least seven animals. * $p < 0.05$.

Figure 6

Unmodified cardiac contractility

(A) Representative contractile traces induced by isoproterenol (30 nM) in wild-type (WT, left panel) and β3-Tg (Tg, right panel) mice.

(B) Summarized results of contractile responses to various concentrations of isoproterenol in WT (open circle), β3-null (blue triangle) and Tg (red square) mice. No significant difference in contractile response was observed. The data represent the means ± S.E.M. of at least eight animals.

Figure 7

Calcium channel currents recorded in ventricular myocytes

(A) Representative traces of voltage-dependent Ca^{2+} channel currents in ventricular myocytes from wild (WT, top panel, black), β2-deficent (B2, middle panel, red), and β3-deficent (B3, bottom panel, blue) mice. Myocytes were voltage-clamped at the holding potential of -80 mV. To inactivate Na^{+} currents, conditioning pulses to -40 mV with a duration of 50 ms preceded the test pulse to 0 mV for 300 ms.

(B) The *I-V* curves of Ca^{2+} channel current in wild (wt, black, n = 15), β2-deficent (B2, middle panel, red, n = 12), and $\beta3^{-/-}$ (bottom panel, blue, n = 12) mice. The current density was estimated by dividing the peak amplitude by the cell capacitance (pA/pF).

(C) Effects of isoproterenol on Ca^{2+} channel current in ventricular myocytes. The amplitude of Ca^{2+} current recorded in the presence of 1 μM isoproterenol was normalized

to that of the control. Data are expressed as means ± S.E. of at least five cells.

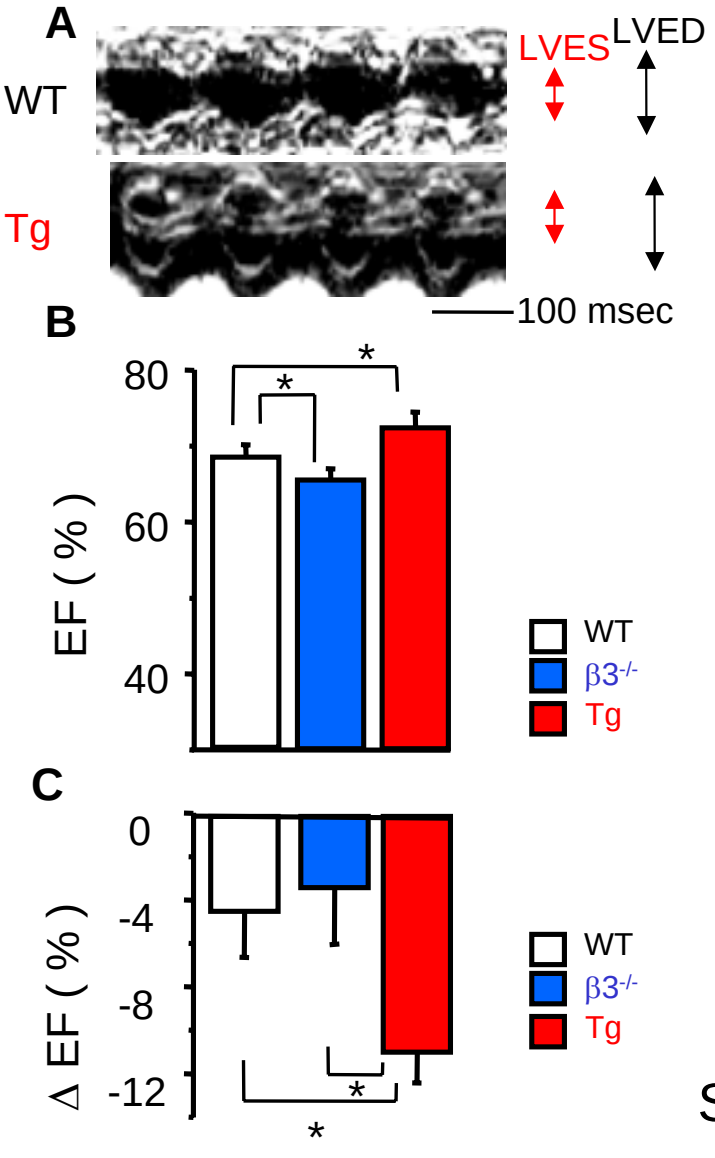
Figure 8.

Effect of the β subunit on the appearance of N-type calcium channels in the microsomal fraction.

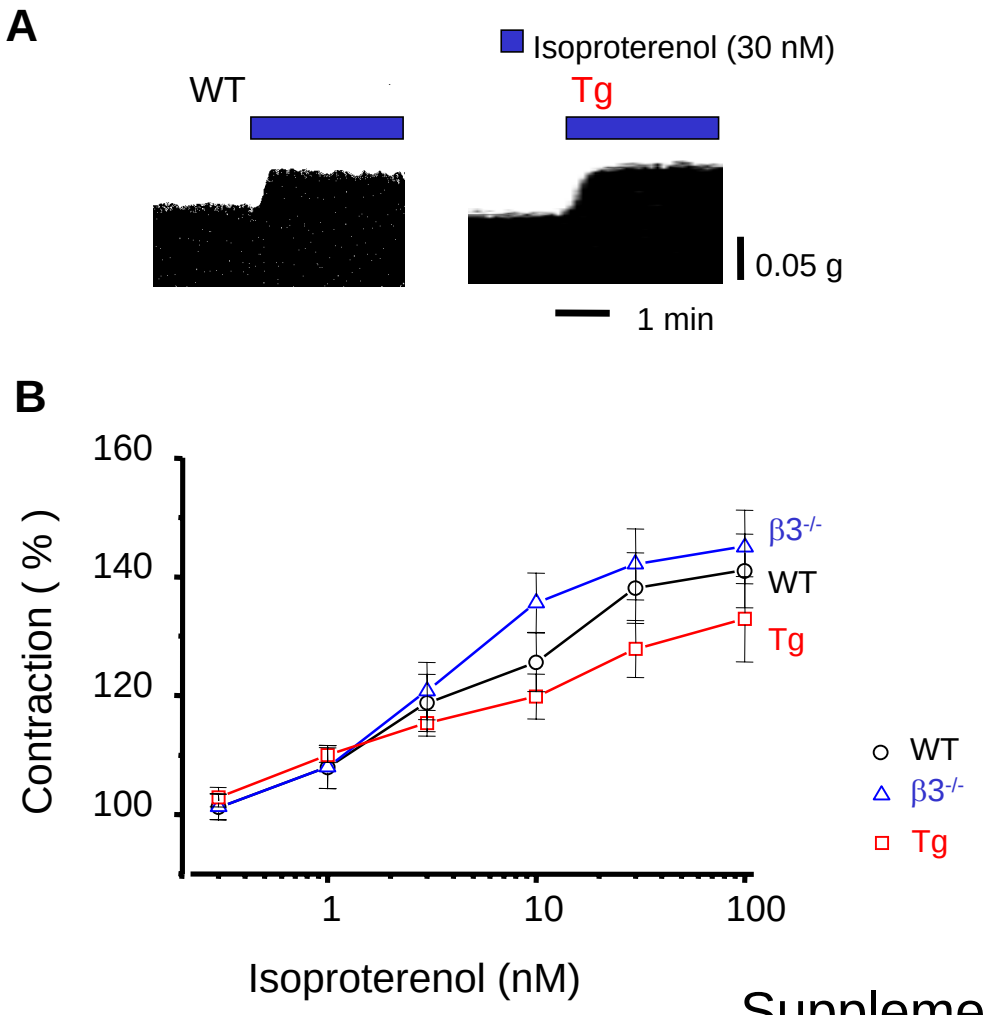
(A) cDNA expression constructs for the CaV2.2, β1, β2, β3, and β4 subunits.

(B) Representative immunoblots of transiently transfected HEK293T cells.

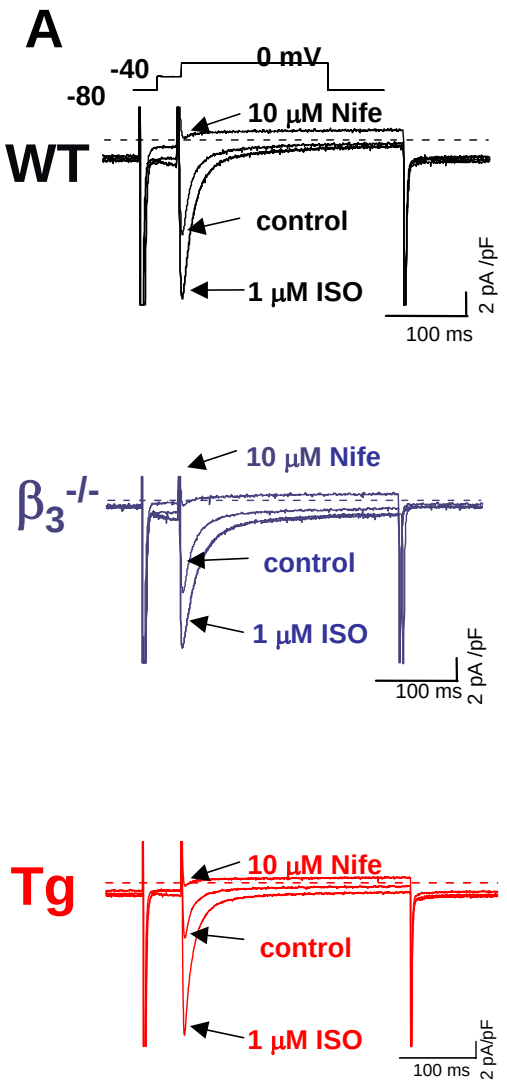
(i) After 48 h of transient expression in HEK293T cells, protein levels of the β3 subunit were dependent on the amount of transfected cDNA (0, 50, 150, and 300 ng/1.9 cm^2, ~5 × 10^5 cells). (ii) Expression of each β subunit was confirmed after 48 h of transient expression. (iii) Effect of the β subunit on the protein level of N-type-forming CaV2.2 in the microsomal fraction of HEK293T cells. The amount of the CaV2.2 gene product at 24–72 h was clearly dependent on the amount of the co-transfected β3 gene plasmid. After 48 h of transfection, β1, β2, and β4 also showed associated bands of CaV2.2 (bottom panel), although the band intensities were weaker than that with the β3 subunit (300 ng/1.9 cm^2 after 48 h).

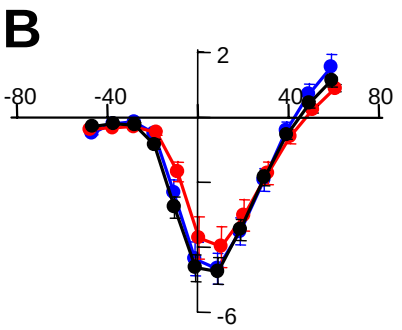


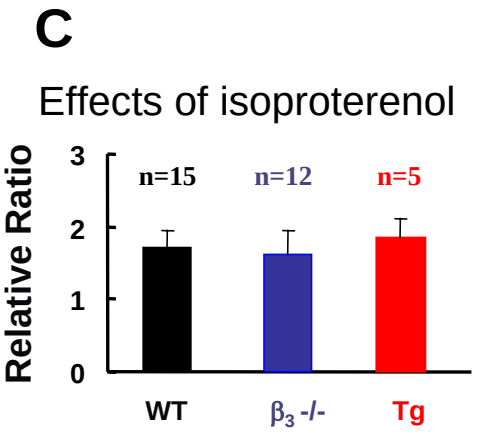
Supplemental Data Figure 5



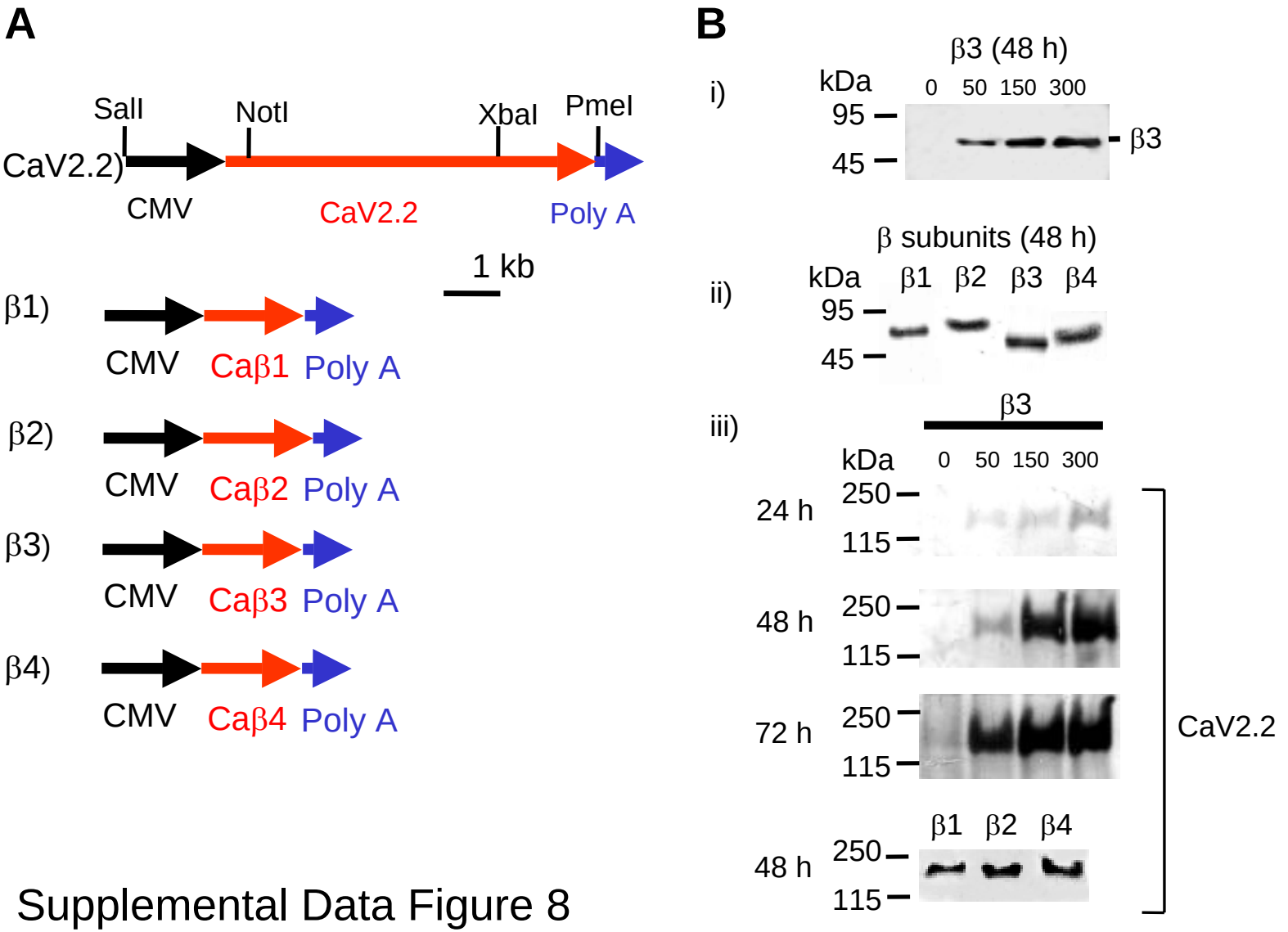
Supplemental Data Figure 6







Supplemental Data Figure 7



Supplemental Data Figure 8