## Supplementary Information for

# ER-resident STIM1/2 couples Ca2+ entry by NMDA receptors to pannexin-1 activation.

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**This PDF file includes:**

Supplementary text
Figures S1 to S7 (not allowed for Brief Reports)
SI References

## Materials and Methods

### *Animals*

CD1 mice were obtained from Central Animal Care Services, University of Manitoba. Panx1 knockout (KO) first mice (Panx1$^{tm1a(KOMP)Wtsi}$), in which the Panx1$^{tm1a}$ allele is rendered non-expressive due to insertion of a gene trapping cassette, were purchased from KOMP at UCDavis. Residual Panx1 transcripts have been detected in Panx1 KO first mice (1), albeit without detectable Panx1 protein (1–4), on the basis of which the mice may be considered hypomorphs. Genotyping is achieved by extracting genomic DNA from ear punch samples followed by PCR using KOMP defined genotyping primers (CSD-loxF 5'-GAGATGGCGCAACGCAATTAATG-3' and CSD-Panx1-R 5'-CTGGCTCTCATAATTCTTGCCCTGG-3' for the 381bp transgene band, and CSD-Panx1-F 5'-CTAGTTCCAGATCACCCTCCACTGC-3' and CSD-Panx1-ttR 5'-ACTGAAAAGCAAGAGCCAGTCATGG-3' for 691bp WT band). All mice were housed and used with approval from the University of Manitoba Animal Care Committee, in accordance with the Canadian Council on Animal Care guidelines.

### *Cell culture*

Primary cultures of mouse hippocampal neurons were prepared as follows. Hippocampi were dissected from embryonic day 17-18 CD1, Panx1 KO or WT mice under sterile conditions. Dissected hippocampi were minced and placed in 0.25% trypsin-EDTA before mechanical dissociation by trituration. Cells were counted and plated onto poly-D-lysine coated 35 mm culture dishes (for electrophysiology or biochemistry) or glass-bottom dishes (MatTek Corp, MA) (for

imaging) at a density of 180,000 cells per dish in plating media (composed of Neurobasal Media (Thermo) with 2% GS21 supplement (GlobalStem), 0.5 mM GlutaMAX (Thermo) and 3.7 µg/ml glutamate (MilliporeSigma)). The cultures were incubated at 37 ˚C with 5% $CO_2$ and half media changes were started on day 4 using BrainPhys Media (StemCell) supplemented with 2% GS21, done every 3-4 days. Neurons were used between days in-vitro (*DIV*) 18-23 for all experiments. HEK 293T cells (ATCC CRL-3216) were cultured and maintained using Dulbecco's Modified Eagle Medium containing glutamine (DMEM, Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS) in 100 mm cell culture dishes at 37 ˚C with 5% $CO_2$.

***Transient transfections***

HEK 293T cells were transfected using jetPRIME reagent (Polypus transfection, France) according to manufacturer's guidelines. For imaging or electrophysiology, cells were transfected with a Flag-Panx1 construct (0.1-0.4 µg), with or without STIM1 or STIM2 (0.1-0.2 µg). Additionally, for electrophysiology, a plasmid expressing eGFP or mCherry was added to the transfection mix, which acted as a fluorescent marker for transfection efficiency and as a target for cell selection. HEK cells that were mock-transfected with carrier plasmid (pcDNA3.1) only were used as negative controls and simply referred to as empty vector in Fig. 2, Fig. S2 and Fig. S6. 24 hours post transfection, the cells were re-seeded onto 35 mm culture dishes (for electrophysiology) or onto poly-D-lysine coated imaging grade plastic bottom dishes (Ibidi, WI). Dishes were used within 24 hours of re-seeding. For biochemistry, cells were incubated for 48 hours post transfection and then lysed for western blot and immunoprecipitation assays.

***Whole-cell recordings from cultured neurons and HEK 293T cells***

Whole-cell voltage-clamp recordings were performed from *DIV18-23* neurons or HEK 293T cells at room temperature (20-22 ˚C) using an Axon MultiClamp 700A patch clamp amplifier and Digidata 1322A data acquisition system (Molecular Devices, Sunnyvale, CA, USA). We used thin-walled borosilicate glass (TW150-F3; WPI, Sarasota, FL, USA) as patch pipettes. These were pulled using a Narishige two-stage puller (PP-83; Narishige, Greenvale, NY, USA) to get recording electrodes. Electrodes had a final resistance of 4-5 MΩ when filled with intracellular fluid (ICF) containing (in mM): 142 cesium gluconate, 10 HEPES, 2 $MgCl_2$, 8 NaCl, pH 7.2 (adjusted with 1M Cs-OH) and osmolality between 295-305 mOsmol/kg. For experiments with anti-Panx1 (CST), vehicle or anti-Panx1 (CST) was added to the ICF with dilution (in µl) at 1:400 (final dilution= 0.325 ng/µl). Standard extracellular fluid (ECF) was composed of (in mM): 140 NaCl, 5.4 KCl, 25 HEPES, 33 glucose, 2 $CaCl_2$, 1 $MgCl_2$, pH of 7.4 (adjusted with 10N NaOH) and osmolality between 305-315 mOsmol/kg. TTX (0.1 µM) was only added to the standard ECF when recording from neurons. For the ion substitution experiments, the NMDG-Cl and $CaCl_2$ solutions were composed of (in mM): 140 NMDG-Cl, 5.4 KCl, 25 HEPES, 33 glucose, 2 $CaCl_2$, 1 $MgCl_2$, 0.1 µM TTX, and 120 $CaCl_2$, 5.4 KCl, 25 HEPES, 33 glucose, 1 $MgCl_2$, 0.1 µM TTX, respectively, with pH of 7.4 (adjusted with 6N HCl) and osmolality between 305-315 mOsmol/kg. Additionally, for experiments in HEK cells examining the mechanosensitivity of Panx1, the composition of the hypotonic solution was (in mM): 110 NaCl, 5.4 KCl, 25 HEPES, 33 glucose, 2 $CaCl_2$, 1 $MgCl_2$, pH of 7.4 (adjusted with 10N NaOH) and osmolality between 245-250

mOsmol/kg. For this experiment, the salt composition of the isotonic solution was identical with osmolality increased to 305-310 mOsmol/kg by addition of 60 mM D-Mannitol (inert).

Cells were voltage-clamped at -60 mV and a stream of ECF (with flow rate of 0.5 ml/min) was applied using a computer-controlled, multibarrelled perfusion system (SF-77B; Warner Institute, Hamden, CT, USA) to achieve rapid exchange ($\tau$ of exchange ~3 ms (5)) with solutions containing either ECF, thapsigargin (3 μM), NMDA (100 μM), hypotonic ECF, $La^{3+}$ (100 μM) or other Panx1 blockers as mentioned in Fig. 1 (details in chemicals and reagents section provided in SI appendix). Currents that showed less than 50% inhibition by application of Panx1 blockers were excluded. For experiments where specified, thapsigargin applications were also done in $Ca^{2+}$ free ECF supplemented with 1 mM EGTA. For experiments with NMDA, the ECF used for recording was devoid of $MgCl_2$ and supplemented with 3 μM D-serine and NMDA was applied (with or without the specified NMDAR blockers) after switching the amplifier from voltage- to current-clamp mode (6). Following NMDA application, amplifier was returned to voltage clamp mode and currents were recorded for duration of 5-10 minutes. Cells that did not recover post NMDA washout were excluded. For experiments with Dan and Xe-C+Dan, dishes were first pretreated with 10 μM Dan for 10 minutes at 37 ˚C in media. Following incubation, the media was replaced with ECF and dish was used for experiment. The current-voltage (IV) characteristics of baseline, thapsigargin, NMDA or hypotonic treatment-induced currents were monitored by the application of voltage-ramps (±100 mV, 500 ms) every 60 s for neurons and every 10 s for HEK 293T cells. The IV curves shown in figures are averaged from respective set of recordings. All data were filtered at 2

kHz, digitized and acquired at 10 kHz using pCLAMP 9.2 software (Molecular Devices). All data were acquired from a minimum of 3 independent experiments and analysed using Clampfit 10.7 software (Molecular Devices). All data shown in bar graphs for peak currents at -60mV consists of baseline subtracted currents from each individual recordings (when no baseline bar graph is shown). For experiments in which hypotonic solutions were applied, normalized currents (Fig. S6, A and B) are shown where peak current at +100mV in each condition was divided by its baseline current at 310 mOsmol/kg for each recording.

***Constructs***

Messenger RNA was obtained from mouse brain using a kit (Qiagen). This was followed by RT-PCR using oligo(dT) as primers to produce mouse brain cDNA, which was used as a template for cloning. Oligos spanning mouse genes of interest, including Panx1 (Accession #NM_019482.2), STIM1 (Accession #NM_009287.4) and STIM2 (Accession #NM_001081103.2 using the upstream AUG codon with a putative N-terminal extension), were designed with restriction sites on either side for insertion into pcDNA3.1(+). Once the full-length genes were obtained by PCR, inserted into plasmid, and sequence-confirmed with identical match to reported NCBI accession numbers, we proceeded with the following modifications to create 4 distinct constructs: Flag tag (DYKDDDDK) was inserted onto the N-terminus of Panx1 (Flag-Panx1), eGFP tag was fused to the C-terminus of Panx1 (Panx1-eGFP), and mCherry was inserted between the signal sequence for STIM2 (amino acid 1-86; mCherry-STIM2) and the rest of the protein. All terminal deletion constructs of Panx1 were created by PCR, using oligos designed with restriction sites allowing

replacement of full-length Panx1 (pcDNA3.1-Flag-Panx1) with Panx1 deletion PCR product. Site directed internal deletions or mutations were produced by primer extension, where oligos targeting the site to be mutated generated two PCR products on either side of the mutation site that overlapped and allowed the two PCR products to be stitched and filled in. Deletion or mutation constructs were as follows (with deleted amino acids preceded by a Δ): Flag-Panx1Δ300-426, Flag-Panx1Δ379-426, Flag-Panx1Δ1-35, Flag-Panx1Δ1-18, Flag-Panx1Δ19-35, Flag-Panx1Δ1-6, Flag-Panx1Δ1-12, Flag-Panx1Δ13-18, Flag-Panx1(13-18/6A) and Flag-Panx1($^{15}$FLL$^{17}$/3N). All Panx1, STIM1 and STIM2 constructs were sequence-confirmed.

To create lentivirus transfer plasmids for ectopic gene expression used for protein localization or in rescue experiments, two types of constructs were created. 1) The gene of interest (i.e. Flag-Panx1, Flag-Panx1Δ379-426, Flag-Panx1Δ1-18) was fused to eGFP with a T2A self-cleaving peptide sequence in between, and subcloned into a lentiviral expression plasmid (FUGW; Addgene plasmid #14883). Once transduced into cells, this plasmid leads to a full protein being produced (Flag-Panx1-T2A-eGFP) which is then subsequently cleaved to release the Flag-Panx1 and eGFP proteins. This allows neurons to be identified for electrophysiology or imaging as having received the lentivirus package (i.e. eGFP positive), with no possibly confounding large fluorophore tag on the Panx1 mutant C-term. 2) The gene of interest (Panx1-eGFP, mCherry-STIM2 as described above) were subcloned into FUGW. These were used for cell protein localization, as well as for electrophysiology.

***shRNA***

To optimize the knockdown efficiency, we designed and screened multiple shRNA expression constructs each for STIM1 (4 shRNAs) and STIM2 (8 shRNAs). Each was ligated into an expression vector (pLB; gift from Stephan Kissler (Addgene plasmid #11619)) in which a U6 promoter drives shRNA expression, as well as a CMV promoter for the expression of eGFP (used for visual selection of infected cells). For the STIM2 shRNAs, we modified the pLB plasmid by replacing the eGFP with mCherry. This allowed for visual identification and selection, for electrophysiological recording, of neurons transduced with both STIM1 and STIM2 shRNAs. Sequences were based on the RNAi Consortium analysis using mouse sequences for STIM1 and STIM2. Each shRNA was first screened in HEK 293T cells expressing either STIM1 or STIM2 followed by screening in neurons. Cells were lysed and equal amounts of protein (37.8 µg/ lane) was loaded onto SDS-PAGE and separated. Using western blotting we identified the shRNA for each protein that was knocked down by >95%, which we are calling STIM1-hp1 (5'-gcagtactacaacatcaagaa-3') and STIM2-hp1 (5'-cctctgtcataatggtgagaa-3'), with a scrambled sequence serving as control (5'-aattctccgaacgtgtcacgt-3').

***Lentivirus preparation***

Lentiviral particles were prepared by following a modified version of the Trono Lab protocol (7). Briefly, HEK 293T cells were transfected with packaging vectors pSL3, pSL4, and pSL5, as well as the transfer plasmid (pLB or FUGW constructs previously described), using lipofectamine 2000 (Invitrogen). Cells were allowed to incubate for 24-30 hours after which the media was collected

into an Amicon Ultra-15ml 100k MWCO centrifugal filter device and centrifuged in a swinging bucket at 4,000xg for 15 minutes at 4 ˚C to concentrate the viral particles. The lentiviruses were used to transduce CD1 neurons in culture between *DIV 3-6* at 0.5-1µl/dish. To evaluate expression levels of STIM1 and STIM2 knockdown in neurons, transduced cells were lysed at *DIV 19* using RIPA buffer. Transduced sister cultures were used for electrophysiology experiments between *DIV 18-23.* For rescue experiments in Panx1 KO neurons, lentiviruses were used to transduce cultures at *DIV 8-11* and used between *DIV 18-23* for electrophysiology or immunostaining experiments.

***Western blotting***

Equal amounts of total protein (inputs) or equal volume (immunoprecipitations) were loaded onto 10% tris-glycine gels, separated, and transferred to BioTrace NT nitrocellulose transfer membrane (Pall Corp). Membrane was blocked with blotto (5% nonfat milk in tris-buffered solution containing 0.1% tween-20 (TBS-T)) for 1 hour at room temperature, followed by incubation in primary antibody diluted in blotto overnight at 4 ˚C. The next day the membrane was washed 3 times with TBS-T, followed by incubation for 1 hour with appropriate peroxidase-labelled secondary antibody diluted in blotto. Following the last incubation, the membrane was washed with TBS-T and then exposed to ECL reagent (Pierce Supersignal West Pico Plus, Thermo). Western blotting was performed using the following antibodies: FlagM2-HRP (MilliporeSigma, A8592, 1:4000), STIM1 (ProteinTech, 11565-1-AP, 1:800), STIM2 (Alomone, ACC-064, 1:250), Panx1CT395 (gift from Drs. Dale Laird and Silvia Penuela, Western University, 1:1000) and β-actin-HRP (MilliporeSigma, A3854, 1:7,000). Chemiluminescence was detected and captured by

Bio-Rad ChemiDoc and analysis was done using Bio-Rad's ImageLab software. Inputs were measured as a total flag signal (all glycosylation states together) or the single Gly-2 state of Panx1. Total protein in each lane was measured using 2,2,2-trichloroethanol at a 0.5% concentration within the gel which becomes activated upon UV exposure. Transfer to the membrane retains this fluorescence and can be measured accurately. For analysis of lysates from STIM knockdowns, signal intensity for proteins of interest was normalized to that of ß-actin.

***Immunoprecipitation***

Thapsigargin treatments (3 µM, for 15 min at 37 ˚C) for HEK cells co-expressing Panx1 or Panx1 deletions with STIM1, were done in media or ECF ($Ca^{2+}$ free, composition as stated previously). After treatments, transfected cells were washed with ECF, scraped and pelleted. The pellet was resuspended in ComplexioLyte No. 48 lysis buffer (CL-48-LB; LogoPharm, Germany) and mechanically lysed by passing through a 25G needle 8-10 times. The lysate was placed on a nutator mixer at 4 ˚C for 30 min, followed by centrifugation at 21,000xg at 4 ˚C for 10 min. The supernatant contained the soluble fraction to be used for immunoprecipitation. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo). 400 µg total protein was used per immunoprecipitation (IP) sample, diluted in final solution of 50% CL-48-LB/50% D-PBS. Lysates were pre-cleared at 4 ˚C for 90 min with mouse IgG-Agarose (25 µl 50% slurry/IP; MilliporeSigma, A0919). At the same time, anti-FlagM2-Affinity Gel (40 µl 50% slurry/IP; MilliporeSigma, A2220) was pre-cleared with 5% BSA in D-PBS. Pre-cleared lysates were then incubated with pre-cleared anti-FlagM2-Affinity Gel for 3 hours at 4 ˚C on nutator

mixer. Samples were centrifuged and pellets were washed 3 times with ice-cold CL-48 Dilution Buffer (LogoPharm). Pellets were resuspended in 2X-SDS loading buffer. Equal volume of immunoprecipitation sample (20 µl/lane) was loaded onto 10% tris-glycine gel and subjected to Western blotting. To determine expression levels of Panx1 and STIM constructs (inputs), 10 µg total protein was loaded per lane. The immunoprecipitation analysis was done using Bio-Rad's ImageLab software, wherein STIM1 pulldown intensity was expressed as a ratio of the Flag-Panx1 (or Panx1 deletion/substitution mutant) immunoprecipitated intensity. The resulting values were then normalized to the corresponding signal intensity ratio (STIM1 pulldown/Flag-Panx1 IP) from untreated or thapsigargin treated Flag-Panx1+STIM1 expressing HEK cells.

***Immunocytochemistry***

Transfected HEK 293T cells cultured on ibidi dishes (ibidi, WI) and neurons cultured on glass bottom dishes (MatTek) were used for immunocytochemistry. Thapsigargin treatments (3 µM, for 15-20 min at 37 ˚C) for HEK cells co-expressing Panx1 and STIM1 or STIM2 were done in $Ca^{2+}$ free ECF supplemented with 1 mM EGTA. Control HEK cells were treated with same ECF and duration at 37 ˚C. In neurons, NMDA treatments (100 µM, for 5 min at 37 ˚C) were done in ECF used for NMDA recordings. After NMDA treatment, dishes were washed and incubated for additional 10 min in ECF at 37 ˚C. Control neurons were treated with same ECF and duration at 37 ˚C. After treatments, the cells were fixed using Periodate-Lysine-Paraformaldehyde (PLP) fixative (8). First, the dishes were washed with respective ECF thrice, followed by incubation in PLP fixative for 10 minutes at room temperature (RT). After incubation the dishes were washed

thrice for 5 mins using D-PBS. The fixed cells were then permeabilized using 0.2% Triton-X in D-PBS (for 10 mins). The cells were blocked for 30 mins in D-PBS containing 5% normal serum based on the host of the secondary antibody used. After blocking, the cells were incubated with primary antibodies in 0.5% BSA in D-PBS for duration of 2 hours followed by three washes of D-PBS for 5 mins each. The primary antibodies used were: FlagM2 (MilliporeSigma, F1804, 1:400), STIM1 (Abnova, H00006786-M01, 1:100), STIM2 (Protein Tech, 21192-1-AP, 1:250), Panx1 (D9M1C, Cell Signaling Technology, 91137, 1:200), Panx1CT395 (1:100) and calnexin (Novus, NBP1-97485, 1:400). Cells were then incubated with fluorophore conjugated secondary antibodies in 0.5% BSA in D-PBS for 1 hour in the dark, followed by three washes of D-PBS for 5 mins each. The following secondary antibodies were used at 1:1000 dilution: anti-mouse Alexa 488 (Invitrogen, A21202), anti-mouse Alexa 555 (Invitrogen, A31570), anti-rabbit Alexa 488 (Invitrogen, A21206) and anti-rabbit Alexa 555 (Invitrogen, A21429). Stained cells were additionally incubated with DAPI for 5 mins to stain the nuclei and washed with D-PBS thrice before preserving the dishes at 4 ˚C in D-PBS. For wheat germ agglutinin (WGA conjugated to Alexa488, Invitrogen, W11261, 1:200) staining, cells were treated with WGA in ECF for 10 minutes at RT followed by 3 washes with ECF before fixing. Fixed, labelled cells were imaged within two weeks of immunostaining using super resolution inverted confocal microscope Zeiss LSM 880, AxioObserver.Z1 with AiryScan (Carl Zeiss Microscopy GmbH, Jena, Germany). All images were acquired with a Plan-Apochromat 63x magnification/1.4 numerical aperture differential interference contrast (DIC M27) oil immersion objective. The microscope was

controlled using Zeiss Zen 2.3 black edition (Zen) software and 405 nm, 488 nm and 561 nm lasers were used to acquire images. The Zen software was also used for AiryScan processing of acquired raw images which enhanced signal to noise ratio and resolution. The processed images were then exported as Bitmap (BMP, 16 bit) or TIF format using Zen software for representative images or analysis respectively.

***Imaging analysis***

Percent area of colocalized particles at the plasma membrane for Panx1+STIM1 or Panx1+STIM2 was analysed using ImageJ (NIH) software. Green or red channel images were first scaled and background subtracted. After converting images to 8-bit color, colocalization analysis was done using the JACoP plugin (9). Images were thresholded and a mask of colocalized signal was created. This mask image was then thresholded to perform particle analysis function. Regions were drawn around the perimeter of each cell using freehand tool and colocalized particles within these regions were analysed. The same setting (size: 0-infinity, circularity: 0-1) was applied to all images.

***Deglycosylation assay***

Protein lysates were deglycosylated with PNGase F or Endoglycosidase H (Endo H) as per New England Biolabs protocols. Briefly, lysate samples were diluted to 1 µg total protein per µl lysate and denatured for 10 min at 100 ˚C, then buffer and enzymes (as well as NP-40 for PNGase F) were added and incubated for 60 minutes at 37 ˚C. Following the incubation, 6X SDS-loading buffer was added and samples were run on 12% tris-glycine gels for Western blotting to determine glycosylation states of Panx1 using Panx1CT395 antibody (1:1000).

***Chemicals and reagents***

All salts used for making extracellular fluid (ECF) and intracellular fluid (ICF) were purchased from either Sigma-Aldrich or Bioshop Canada except tetrodotoxin (TTX, 0.1 μM) which was purchased from Alomone labs. NMDA (final concentration, 100 μM) and NMDAR antagonists, D-APV (100 μM) and MK-801 (20 μM) as well as the RyR blocker Dantrolene (Dan, 50 μM or 10 μM applied intracellularly) and NMDG (140 mM) were from Sigma-Aldrich. The IP3R blocker Xestospongin-C (Xe-C, 2.5 μM applied intracellularly) was purchased from Tocris Bioscience whereas the NMDAR blocker, 7-chlorokynurenic acid (7-KYNA, 10 μM) was from Abcam. The Panx1 blockers lanthanum ($La^{3+}$, 100 μM), carbenoxolone (CBX, 100 μM) and probenecid (Prb, 2 mM) were purchased from Sigma-Aldrich. $^{10}$Panx mimetic peptide inhibitor (100 μM) was purchased from Tocris. The anti-Panx1 (CST, 1:400 applied intracellularly, final dilution= 0.325 ng/μl) was purchased from Cell Signaling Technology (D9M1C, cat #91137, rabbit monoclonal). Thapsigargin (3 μM) was purchased form Invitrogen. First aliquots of drugs were made in DMSO or water followed by dilution in ECF or ICF to achieve final concentration as stated.

**Supplementary figures**

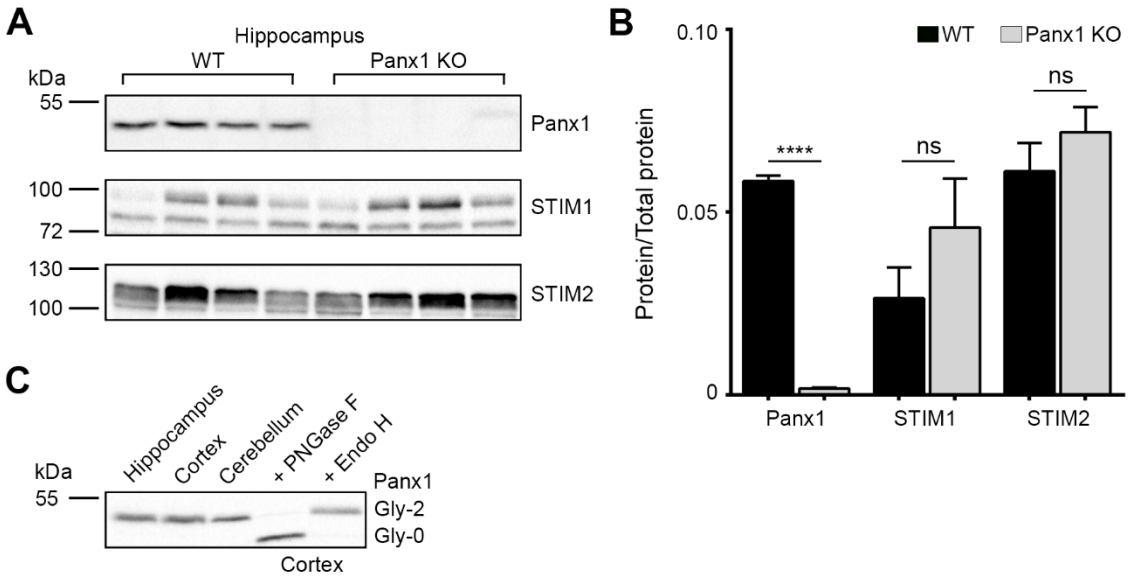


**Fig. S1. Panx1 is expressed as Gly-2 species in brain lysates and not detected in Panx1 KO mice.**

**(A-B)** Representative blot **(A)** and quantified data **(B)** shows detection of Panx1 in hippocampal brain lysates prepared from WT ($n$ = 4), but not Panx1 KO ($n$ = 4) mice. No significant change seen for expression of STIM1 or STIM2 between WT and Panx1 KO mice. ns $P$>0.05, ****$P$<0.0001, unpaired $t$ test. **(C)** Deglycosylation assay using PNGase F and Endo H shows Panx1 in hippocampus, cortex and cerebellum exists as a Gly-2 species. Data represented as mean ± SEM (B).

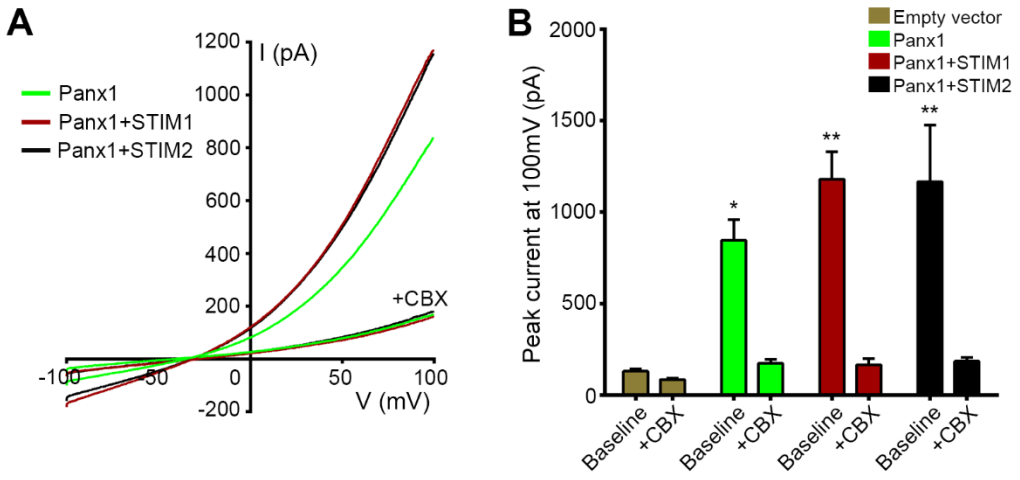


**Fig. S2. Outward rectifying Panx1 currents are not altered by co-expression of STIM1 or STIM2 in untreated HEK 293T cells.**

**(A-B)** Averaged IV curves (**A**) and summary of peak current recorded at 100 mV (**B**), before and after application of carbenoxolone (CBX, 100 μM), from a series of recordings, in untreated HEK 293T cells transfected with empty vector ($n$ = 9), Panx1 alone ($n$ = 12), Panx1+STIM1 ($n$ = 9) or Panx1+STIM2 ($n$ = 10). *$P$<0.05, **$P$<0.01, one-way ANOVA with post hoc Bonferroni test when baseline currents were compared with empty vector. No significant difference seen between Panx1, +STIM1 and +STIM2 baseline currents. Data represented as mean ± SEM (B).

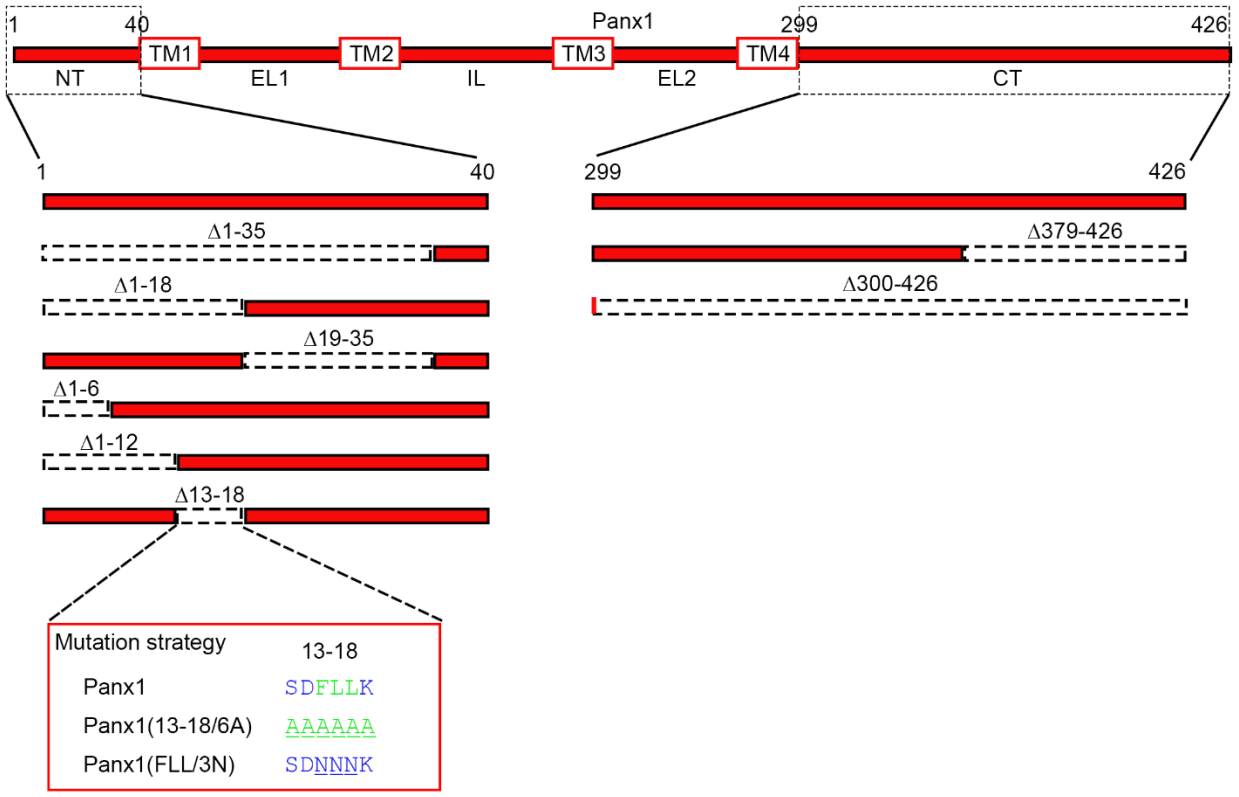


**Fig. S3. Strategy for characterizing functional importance of Panx1 N- and C-term.**

Schematic linear representation of Panx1 channel with 4 transmembrane domains (TM), extracellular loop (EL), intracellular loop (IL), N-term (NT) and C-term (CT). CT deletions used were Panx1Δ300-426 and Panx1Δ379-426. NT deletions used were Panx1Δ1-35, Panx1Δ1-18 and Panx1Δ19-35. Further, sequential deletions of 1-18 NT were made as follows: Panx1Δ1-6, Panx1Δ1-12 and Panx1Δ13-18. Within 13-18 NT, hydrophilic (in blue) and hydrophobic (in green) amino acids are highlighted. Two substitution mutations targeting 13-18 NT were made as

follows: Panx1(13-18/6A)- 13-18 amino acid region substituted to hydrophobic alanine; and Panx1(FLL/3N)- hydrophobic amino acids ($^{15}$FLL$^{17}$) substituted to hydrophilic asparagine (N).

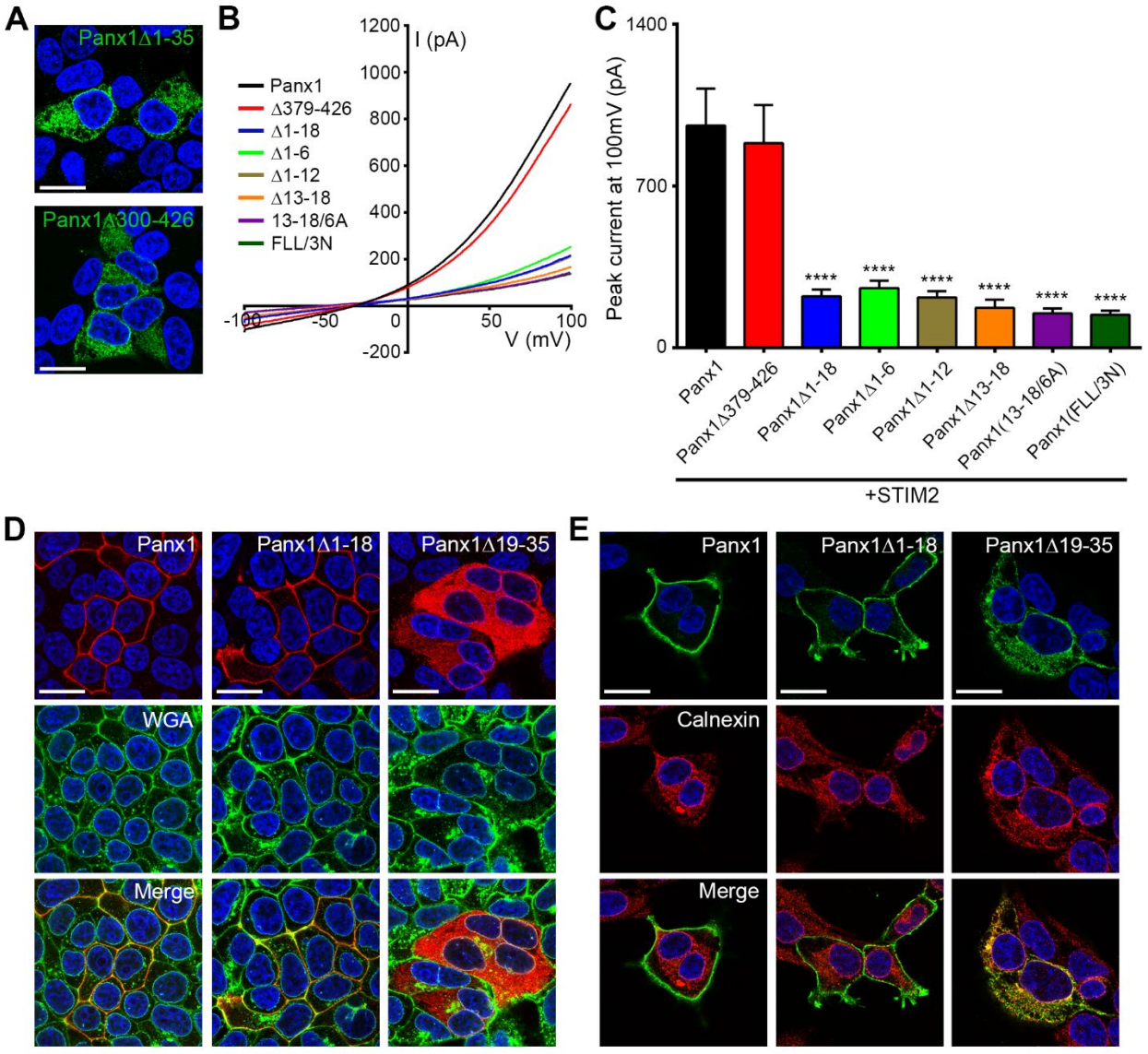


**Fig. S4. Characterization of Panx1 outward rectifying currents and subcellular localization of Panx1 and N- or C-term deletion or substitution mutants in untreated HEK 293T cells.**

**(A)** Representative images showing localization of Flag-tagged Panx1 N-term (Panx1Δ1-35) and C-term (Panx1Δ300-426) deletion mutants expressed in HEK 293T cells. Cell nuclei were stained

using DAPI; scale bars, 20 μm. **(B-C)** Averaged IV curves (**B**) and summary of peak current at 100 mV (**C**) for baseline currents in untreated HEK 293T cells co-expressing STIM2 with Panx1 ($n$ = 15), Panx1Δ379-426 ($n$ = 10), Panx1Δ1-18 ($n$ = 10), Panx1Δ1-6 ($n$ = 10), Panx1Δ1-12 ($n$ = 10), Panx1Δ13-18 ($n$ = 10), Panx1(13-18/6A) ($n$ = 14) or Panx1(FLL/3N) ($n$ = 11). ****$P$<0.0001 one-way ANOVA with post hoc Bonferroni test compared to Panx1+STIM2. **(D-E)** Representative images comparing localization of Panx1, Panx1Δ1-18 or Panx1Δ19-35, in relation to labeling of the plasma membrane by wheat germ agglutinin (WGA, **D**) or of the ER by calnexin (**E**). Cell nuclei were stained using DAPI; Scale bars, 20 μm (D-E). Data represented as mean ± SEM (C).

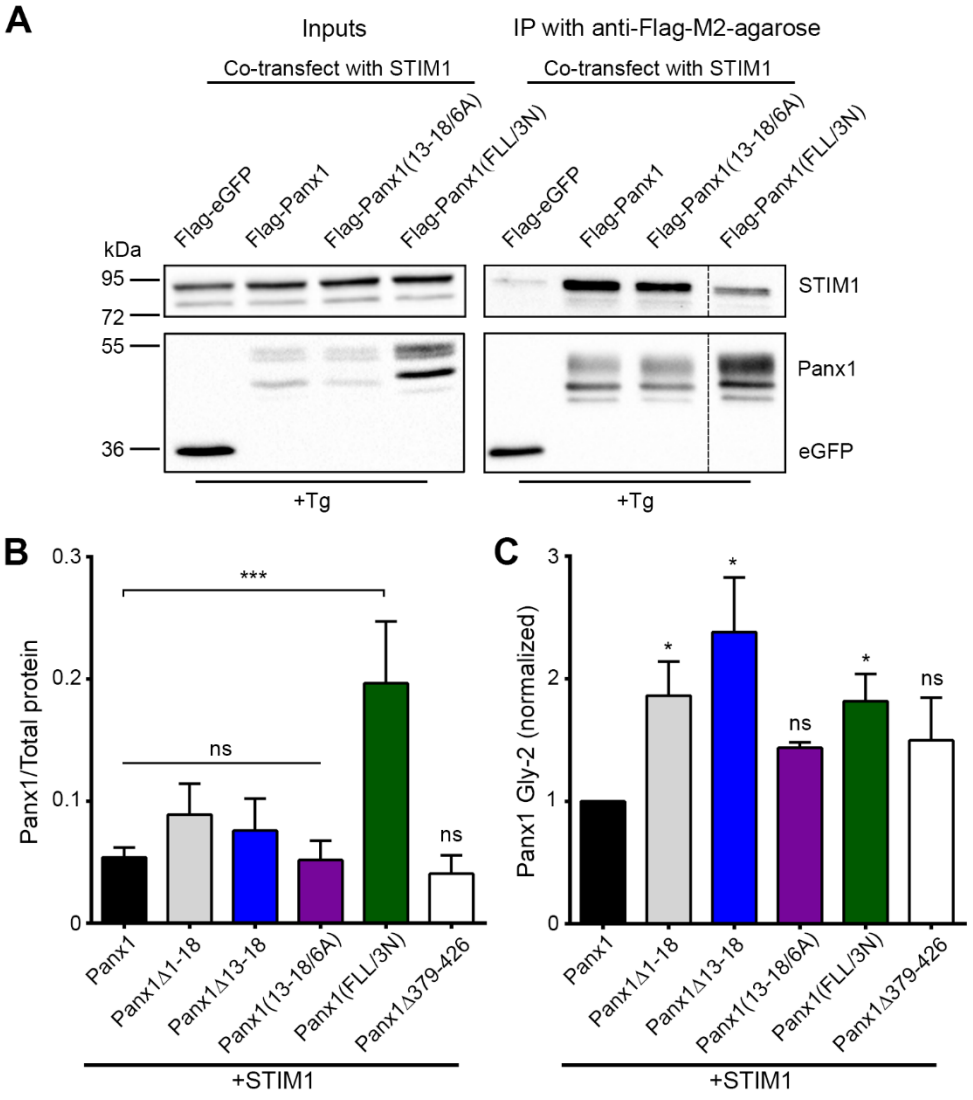


**Fig. S5. Panx1(FLL/3N) shows reduced STIM1 pull down in co-IP but is highly expressed in HEK 293T cells.**

**(A)** Representative blot from lysates (left) and anti-Flag immunoprecipitates (right) in thapsigargin treated (3 μM, 15 min) HEK 293T cells co-expressing STIM1 with either Flag-Panx1, Flag-

Panx1(13-18/6A), Flag-Panx1(FLL/3N) or Flag-eGFP (negative control). Note, stippled lines indicate non-contiguous lanes from the same blot. **(B-C)** Quantified data for Panx1 expression displayed as a ratio to total protein **(B)** and normalized data for Panx1 Gly-2 species **(C)** in untreated HEK 293T cells co-expressing STIM1 with Panx1 ($n$ = 14), Panx1Δ1-18 ($n$ = 11), Panx1Δ13-18 ($n$ = 3), Panx1(13-18/6A) ($n$ = 3), Panx1(FLL/3N) ($n$ = 8) or Panx1Δ379-426 ($n$ = 7). ns $P>0.05$, *$P<0.05$, ***$P<0.001$, one-way ANOVA with post hoc Bonferroni test when compared with Panx1. Data represented as mean ± SEM (B-C).

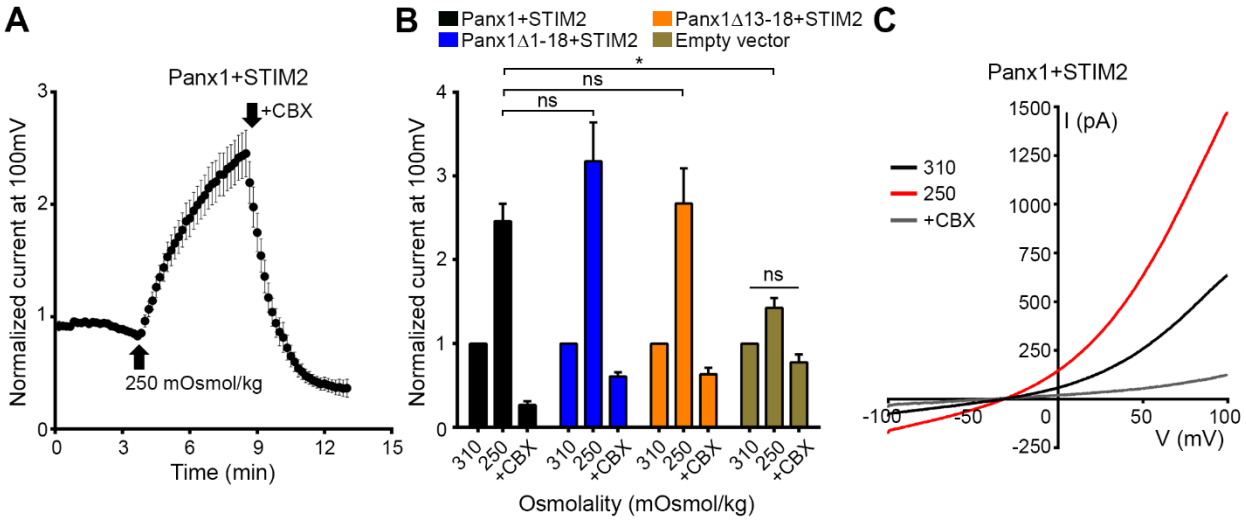


**Fig. S6. Summary of mechanosensitive properties of Panx1 channels.**

**(A)** Normalized currents observed in Panx1+STIM2 expressing HEK 293T cells where baseline (310 mOsmol/kg) was recorded for 3 minutes followed by application of hypotonic solution (250 mOsmol/kg) for 5 minutes. Mediation by Panx1 was confirmed by application of carbenoxolone (CBX, 100 μM). **(B)** Summary of normalized currents recorded in Panx1+STIM2 ($n$ = 11), Panx1Δ1-18+STIM2 ($n$ = 10), Panx1Δ13-18+STIM2 ($n$ = 11) and empty vector ($n$ = 10). ns $P$>0.05, *$P$<0.05, two-way ANOVA with post hoc Bonferroni test when response at 250 mOsmol/kg of Panx1+STIM2 was compared with empty vector. **(C)** Averaged IV curves for activity of Panx1+STIM2 ($n$ = 11) expressing HEK 293T cells in solutions with different osmolarity. Data represented as mean ± SEM (A-B).

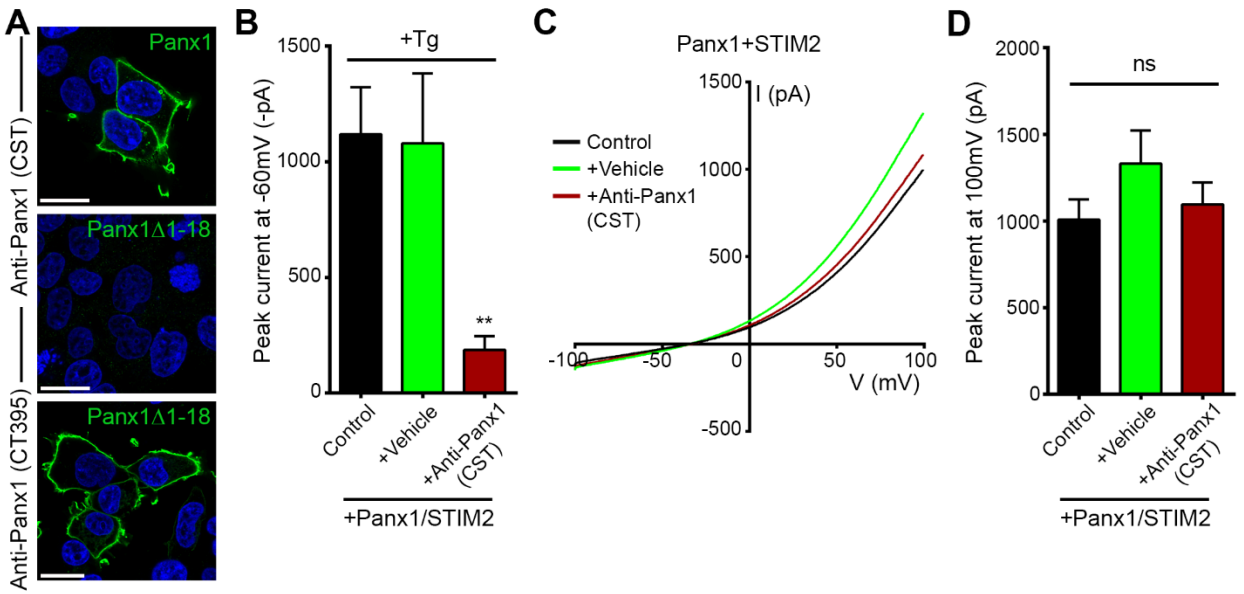


**Fig. S7. Anti-Panx1 (CST) blocks STIM-dependent Panx1 activation, but not outward rectifying currents.**

**(A)** Representative images for HEK 293T cells expressing Panx1 or Panx1Δ1-18 and immunostained with anti-Panx1 (CST; top and middle) or anti-Panx1 (CT395; bottom). Anti-Panx1 (CST) can detect Panx1, with N-term intact, but not Panx1Δ1-18. Antibody targeting C-term (anti-Panx1 CT395) readily detects Panx1Δ1-18 and confirms its expression and localization at the plasma membrane. Cell nuclei were stained using DAPI; scale bars, 20 µm. **(B)** Summary of recordings from Panx1/STIM2 co-expressing HEK 293T cells shows that anti-Panx1 (CST, 1:400 dilution) inhibits thapsigargin (+Tg, 3 µM)-induced Panx1 activation. $^{**}P<0.01$, one-way ANOVA with post hoc Bonferroni test when +Anti-Panx1 (CST) ($n$ = 12) was compared with control ($n$ = 14). No difference seen between vehicle ($n$ = 10) and control. **(C-D)** Averaged IV

curves (**C**) and summary of peak current at 100 mV (**D**) recorded in untreated Panx1/STIM2 co-expressing HEK 293T cells. No difference seen in control ($n$ = 14), +vehicle ($n$ = 10) and +Anti-Panx1 (CST) ($n$ = 12). ns $P$>0.05, one-way ANOVA with post hoc Bonferroni test. Data represented as mean ± SEM (B and D).

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