Supplemental Material

Detailed Methods

1. Left coronary artery ligation and transverse aorta constriction

Permanent left coronary artery (LAD) ligation was performed and cardiac function determined by in vivo echocardiography at the indicated time point after ligation as previously described[1]. To perform transverse aorta constriction (TAC), a 27 gauge needle was placed in the transverse aorta between the innominate and left carotid arteries to produce an increased blood pressure proximal to the constriction site as previously described[2,3].

2. Creation of conditional cardiac specific knockout of P2X4 receptors.

P2X4 gene targeting and production of targeted ES cells were carried out as described [4]. The first loxP site was inserted into intron 4 and the second loxP site together with the Frt-PGKneo-Frt cassette was inserted in intron 1 in a direction opposite to the transcription of *P2X4*. The final targeting vector was then electroporated into ES cells, which was derived from F1 (129Sv/C57BL6j) blastocyst. Chimeric animals were generated by aggregating targeted ES cells with CD1 morula. The PGKneo cassette was removed by breeding chimeric males with ROSA26-Flpe females (Jax stock no: 009086) to generate $P2X4^{floxed/+}$ F1 pups. F1 mice were backcrossed with C57BL6 mice for over ten generations to produce $P2X4^{floxed/floxed}$ mice in C57BL6 background. Subsequent to this repeated backcrossing, homozygous $P2X4^{floxed/floxed}$ mice were obtained. Homozygous $P2X4^{floxed/floxed}$ mice were then bred with Tg mice (Myh6-cre/Esr1*, Jax stock no: 005650 in BL6 background) containing a tamoxifen responsive Cre-estrogen receptor chimeric recombinase driven by the α-myosin heavy chain promoter to yield $P2X4^{floxed/floxed}$ */Myh6Tg* mice. Deletion of the floxed *P2X4* allele in $P2X4^{floxed/floxed}$ */Myh6Tg* was induced by oral gavage of 1 mg tamoxifen (50 mg/kg/day) for 5 days at 7 weeks of age [5]. Deletion efficiency was verified by immunoblotting for P2X4Rs and by absence of an increase in

cardiac myocyte and intact heart contractility in response to receptor agonist 2-meSATP plus P2X4 specific allosteric enhancer ivermectin. Genotyping of mice was carried out using primers listed in Supplemental Table 1.

3. Fibrosis

Whole hearts were fixed in a solution of 10% neutral buffered formalin at room temperature overnight, sectioned in 1mm thickness from apex to base, embedded in paraffin, and then cut at a thickness of 5 μm for staining with Trichrome or Picrosirius red. Picrosirius red staining was carried out using a staining kit (Polyscientices, Inc. Warrington, PA) and scanned with ArtrixScan 400tf (Microtek, Santa Fe Springs, CA)[6]. Collagen content, determined by Picrosirius red or Trichrome, was quantified with Image ProPlus as % of collagen positive area (% collagen area / area of total tissue).

4. Immunostaining, immunohistochemistry, immunoblotting, and histology

Cardiomyocytes were isolated as described previously and fixed with 4% paraformaldehyde in PBS for 20 min. Cells were then cytospun onto slides for staining. Cardiomyocytes were permeabilized with 0.5% Triton, and then blocked with 10% normal goat serum plus 3% BSA in PBS for 1 hour at RT or 4C overnight. Primary antibodies eNOS (BD Transduction) and P2X4 (Alomone Labs) were applied both at 1:150 dilution for 1 hour, followed by incubation with secondary antibodies (goat-α-mouse-FITC from Santa Cruz and goat-α-rabbit- Texas Red from Invitrogen) at 1:500 for 45min at RT. For immunostaining of isolated cardiac myocytes to evaluate co-localization of P2X4R and eNOS, cardiomyocytes were fixed with 4% paraformaldehyde in PBS for 20 min. Cells were then cytospun onto slides for staining. Cardiomyocytes were permeabilized with 0.5% Triton, and then blocked with 10% normal goat

serum plus 3% BSA in PBS at 4ºC overnight. Primary antibodies to eNOS (BD Transduction, San Jose, CA) and P2X4R (Alomone Labs, Jerusalem, Israel) were applied both at 1:150 dilution for 1 hour, followed by incubation with secondary antibodies (goat-α-mouse-FITC from Santa Cruz and goat-α-rabbit-Texas Red, Invitrogen, Life Technologies, Grand Island, NY) at 1:500 for 45min at RT. Confocal fluorescence images were acquired on a Carl Zeiss LSM780 with 488 nm and 516 nm laser excitation using a 63X oil lens (Carl Zeiss Microscopy, LLC, Thornwood, NY). For histology, whole hearts were fixed in a solution of 10% neutral buffered formalin at room temperature overnight, sectioned in 1mm thickness from apex to base, embedded in paraffin, and then cut at a thickness of 5 μm for staining with Trichrome or Hematoxylin and Eosin staining. Data summarized in Supple Fig. 6 used antibodies from Cell Signaling for immunoblotting of eNOS (#9572) and phospho-eNOS Ser1177 (#9571). There were no discernible bands on immunoblots of WT and P2X4 Tg heart homogenates using antibodies for phospho-eNOS Thr495 (#9574) or iNOS (#2982, all were from Cell Signaling). Using another phospho-eNOS Thr495 antibody (Millipore #04-811), there was also no band detected. A faint band for nNOS (#4236 from Cell Signaling) was seen in WT and P2X4 Tg hearts (not shown). The slight fainting precluded comparison.

5. Determination of NO formation and cyclic GMP levels

NO formation was measured by real-time imaging with diaminofluorescein-FM diacetate (DAF-FM DA; 5 μM for 30 min at 32ºC, Molecular Probes, Eugene, OR) using laser confocal microscopy (Zeiss LSM510meta) as previously described[7]. Rod shaped striated myocytes were randomly selected. Fluorescence was excited at 488 nm and imaged through a 525-nm long path filter. The fluorescence intensities were quantified using MetaMorph software (Molecular Devices, Inc, Sunnyvale, CA). Some fluorescein-like photobleaching was observed (Molecular

Probe® Handbook, 11th edition). Myocytes were incubated with vehicle normal saline or 2-meSATP and fluorescence determined at baseline and at each min after for 10 min. Determination of cGMP levels was performed in homogenates of isolated P2X4R overexpressing Tg and WT hearts following the manufacturer's acetylated protocol (the cGMP determination kit GE Healthcare). In demonstrating responsiveness of cGMP levels to changes, isolated hearts were perfused with buffer or NO donor SNAP (S-nitroso-N-acetylpenicillamine, 100 μM) for 10 minutes. SNAP caused a large increase in cGMP levels (buffer-perfused Tg: 2.61 ± 0.33 fmol/mg, n=5 vs. SNAP-perfused Tg: 5.61 ± 0.40 fmol/mg, n=5, $P<0.05$). Buffer-perfused WT vs. Tg hearts were compared as described in Results of main text.

6. *Biotin Switch for S-nitrosylated Proteins*

Heart tissues were obtained from five P2X4R overexpressing Tg and five WT control mice. Heart tissues of 8 μm thickness was subjected to biotin switch staining following manufacturer protocol (Cayman Chemicals, Ann Arbor, MI). Slides were visualized on a Zeiss LSM 510 Meta confocal microscope at 20X magnification. Five 12-bit images of non-overlapping, tissue rich regions were acquired for each slide. For each image, the intensity distribution was separated into 256 bins. The counts for each intensity bin was summed across all five images to produce one distribution for each tissue slide.

Supplemental Table 1 Primer sequences for genotyping

P2X4RTg mice:

αMHC LINKER F	GTCGACTGACTAACTAGAAGCT
$P2X_4$ LINKER R	CTGAGCTGGTATCACATAATCC

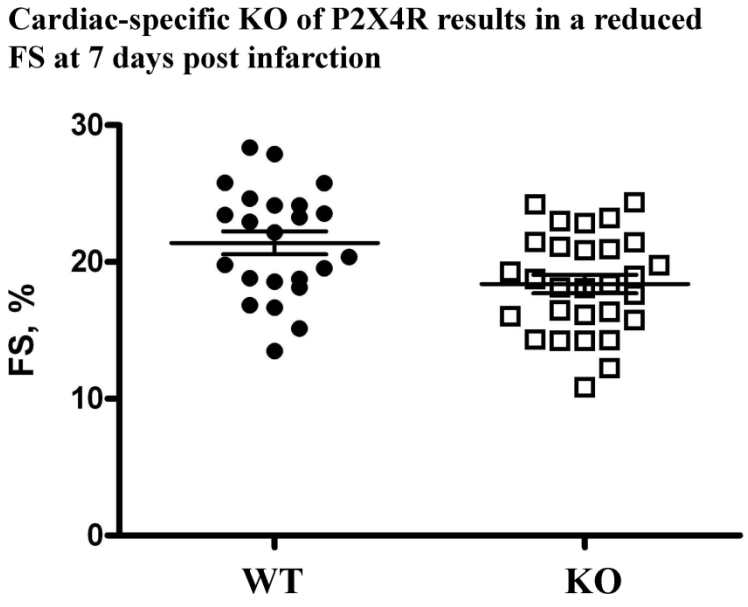
Myh6-cre/Esr1* mice:

3797 Tg F	ATA CCG GAG ATC ATG CAA GC
3798 Tg R	AGG TGG ACC TGA TCA TGG AG

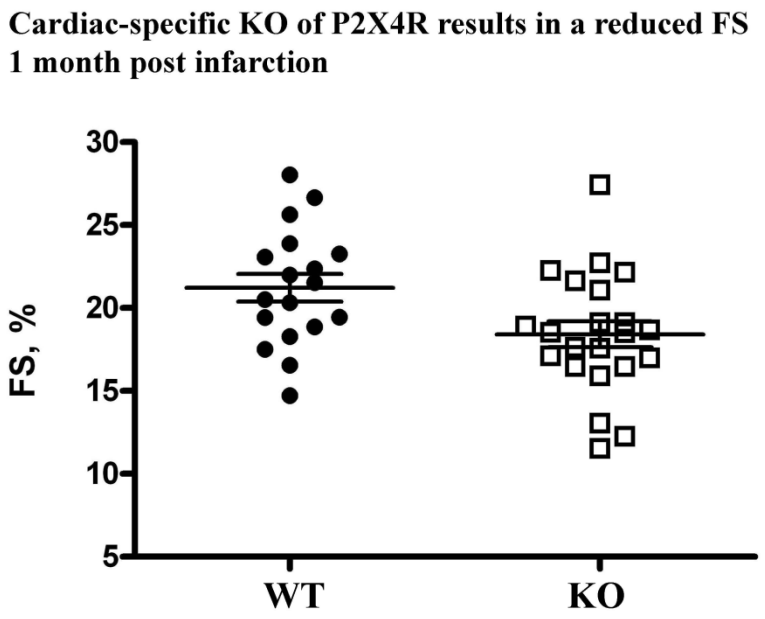
P2X4$^{floxed/floxed}$ mice:

loxP F	TTG CGA TTC AGA CGC CAA CT
loxP R	TCT ATT GCA GAC ATG CTA CC
Frt-loxP F	TTC TGG CTT GGG TTC AAA TC
Frt-loxP R	AGC CCC TTA CCC AGC TAC TC

Supplemental figures and legends



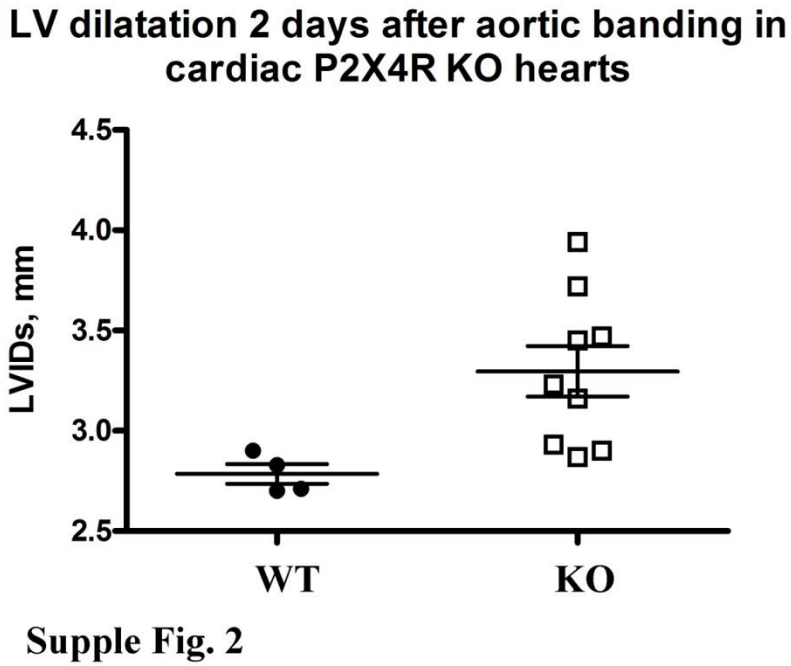
Supple Fig 1a



Supple Fig 1b

Figure 1. Cardiac KO of P2X4 receptors showed reduced FS as early as 7 days after LAD

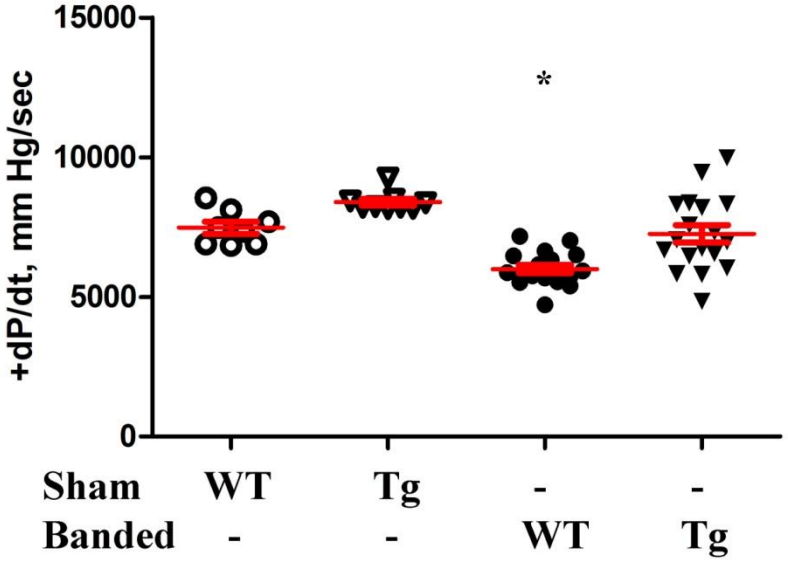
ligation. (a) Seven days after ligation, KO hearts (n=29) showed a more depressed FS than WT control hearts (n=23, P=0.0064). (b) At 1 month after ligation, KO hearts (n=22) had a lower FS than WT control hearts (n=18, P=0.019). There was no difference in infarct size at any time point between the control and KO hearts.



Supple Fig. 2

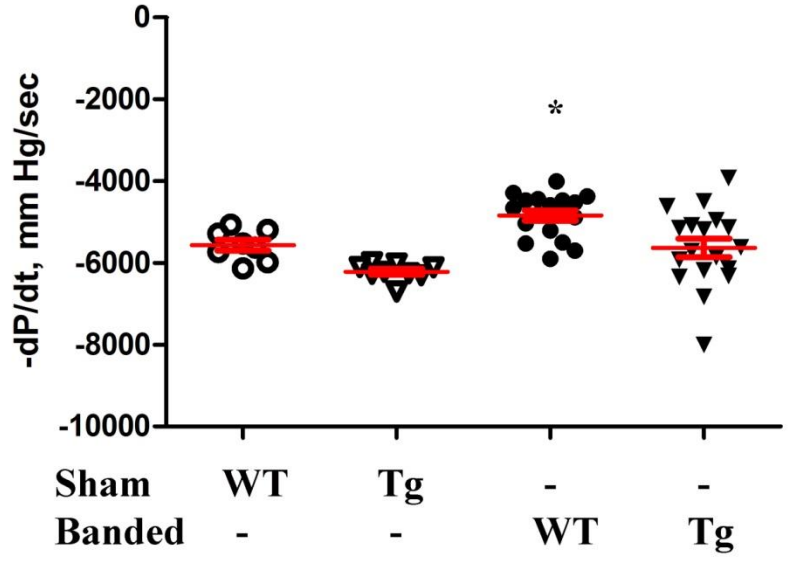
Figure 2. Cardiac KO of P2X4R showed cardiac dilatation as early as 2 days after TAC. Two days after TAC, KO hearts (n=9) exhibited a more dilated LVID@S than WT hearts (n=4, P=0.019).

Cardiac P2X4R overexpression improved +dP/dt during aortic banding



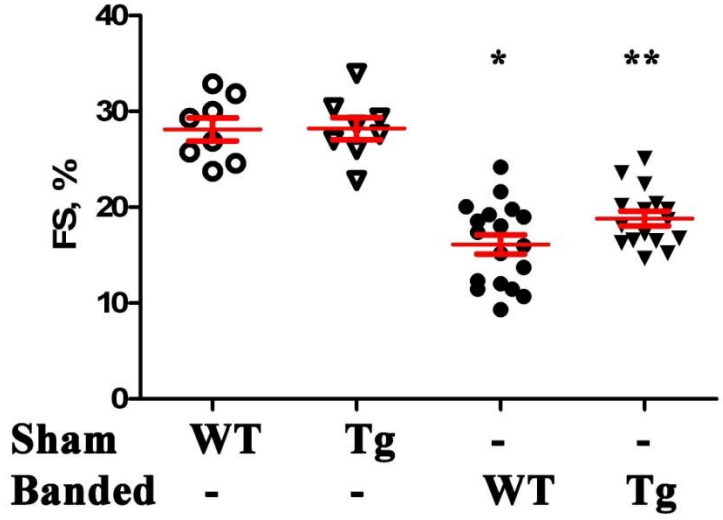
Supple Fig 3a

Cardiac P2X4R overexpression improved -dP/dt during aortic banding



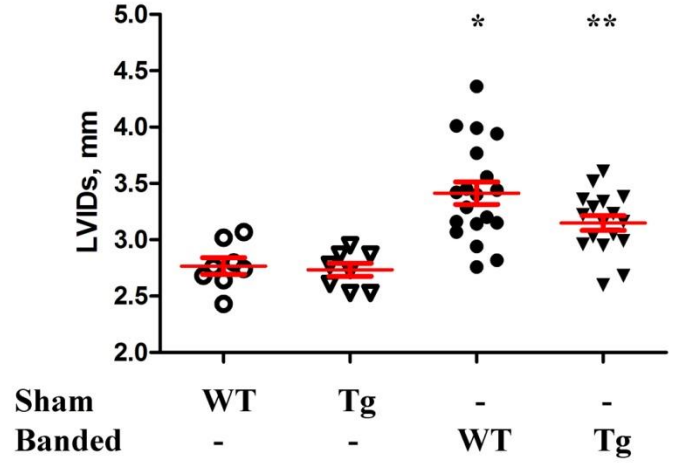
Supple Fig 3b

Cardiac P2X4R overexpression caused better FS during aortic banding



Supple Fig 3c

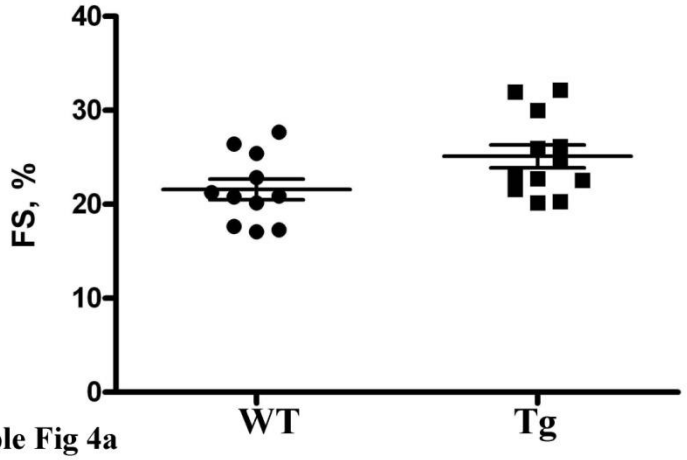
Cardiac P2X4R overexpression reduced LV dilatation at systole during aortic banding



Supple Fig. 3d

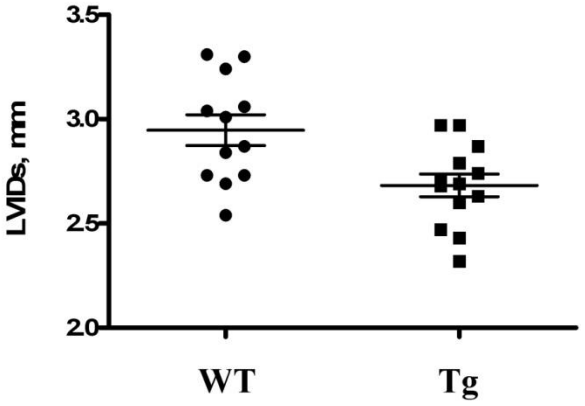
Figure 3. Mice with cardiac-specific Tg overexpression of P2X4R had better cardiac function 3 weeks after TAC. Tg mice (n=18) had higher +dP/dt (a) and -dP/dt (b) than WT mice (n=18). Tg hearts (n=16) also had a higher FS (c) and a smaller LVID@S (d) than control WT mice (n=18). *P<0.05 WT subjected to TAC was lower vs. sham WT (n=8), sham Tg (n=8 and 9) or Tg subjected to TAC; **P<0.05 Tg subjected to TAC was different from sham Tg or sham WT.

Cardiac P2X4R overexpression caused better FS
7 days after aortic banding



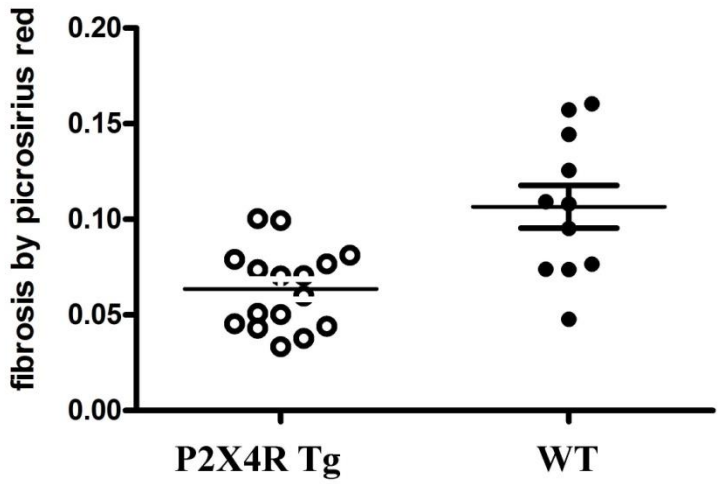
Supple Fig 4a

Cardiac P2X4R overexpression reduced
LV dilatation during systole at 7 days after aortic banding



Supple Fig. 4b

Supple Fig 4c



Supple Fig 4d

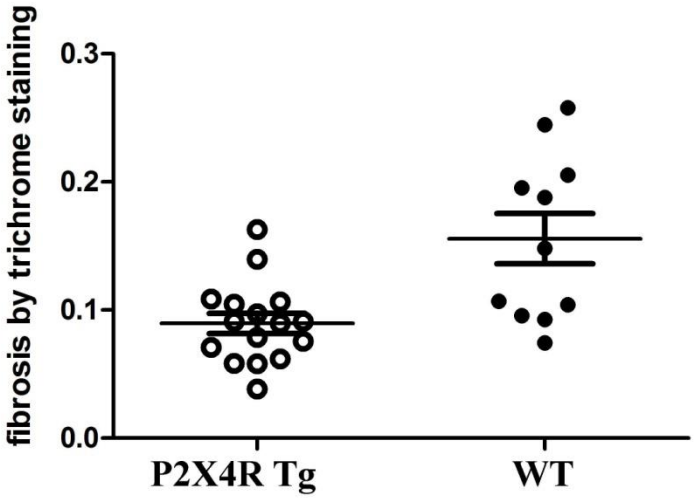


Figure 4. Cardiac overexpression of P2X4R caused a better preserved FS at 7 days after TAC. Seven days after TAC, (a) FS was greater in P2X4R Tg hearts (n=12) than in WT control hearts (n=11, P=0.046). (b) LVID@S was less in Tg than in WT control hearts (P=0.0077). At 3 weeks after TAC, P2X4 Tg hearts (n=16) showed less fibrosis by (c) picrosirius red (P=0.0008) and (d) trichrome (P=0.0017) staining as compared to WT hearts (n=11).


Supple Fig 5 a
P2X4

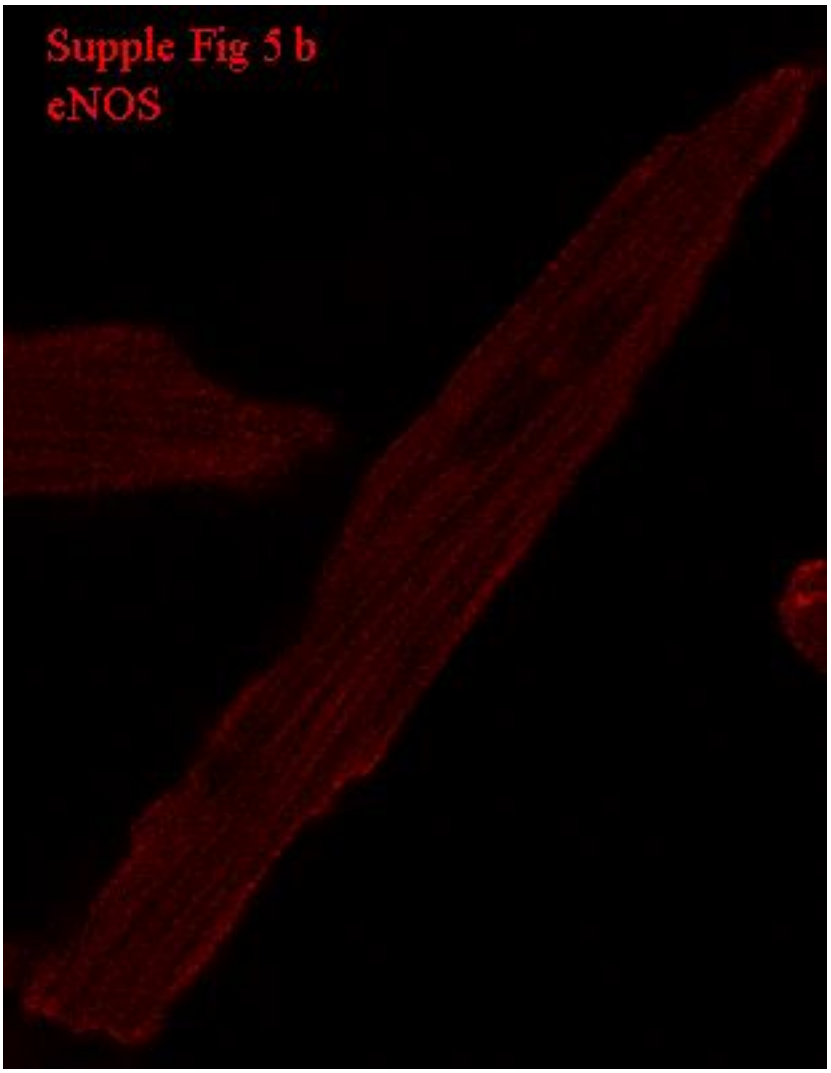
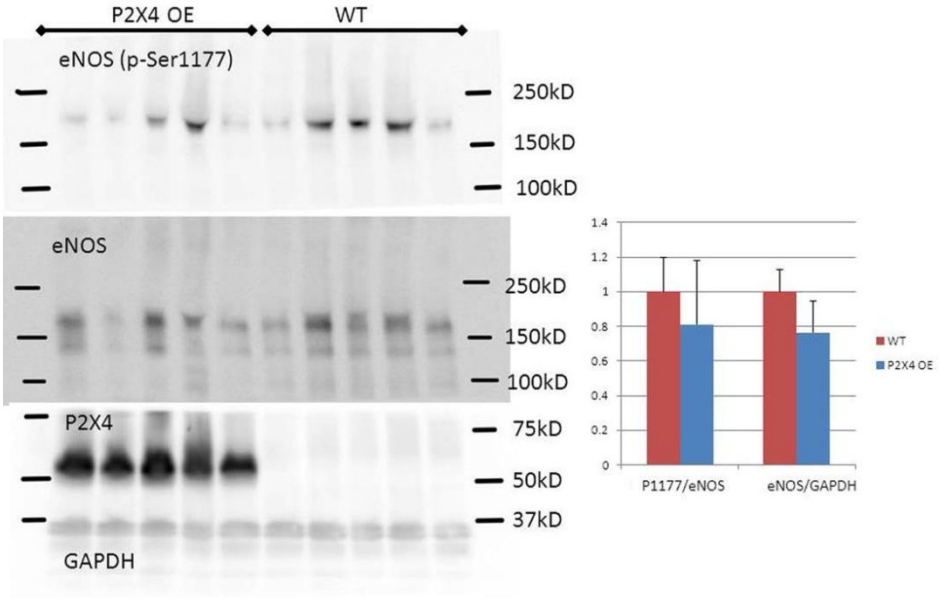

Supple Fig 5 b
eNOS

Figure 5. Immunostaining of P2X4R and eNOS in WT cardiac ventricular myocytes. Immunostaining was carried out as described in Methods. Both P2X4R (a) and eNOS (b) showed sarcolemmal staining and some diffuse pattern. With only secondary antibodies (goat-α-mouse-FITC from Santa Cruz and goat-α-rabbit- Texas Red from Invitrogen), there was a slight background staining (c). The staining was representative of 6 myocytes from three hearts.

Supple Fig. 6



Supple Fig. 6

Figure 6. Immunoblotting for eNOS and phospho-eNOS Ser1177 in P2X4R Tg and WT heart homogenates.

Immunoblotting on heart homogenates of P2X4R overexpressing (OE) Tg and WT animals (n=5 for each genotype) was carried out as described in Methods. Upper part of the blot was first probed with antibody against phospho-eNOS Ser1177 and then re-probed with antibody against eNOS after stripping. Lower part of the same blot was cut for probing with antibodies against P2X4R and GAPDH. In P2X4R OE vs. WT comparison, ratios of phospho-eNOS to total eNOS (p1177/eNOS) were similar. Ratios of total eNOS to GAPDH were also not different. Data were plotted as ratios normalized to the average of the WT ratios, as shown in the graph.

Supplemental references

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