SUPPLEMENTARY MATERIALS

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1. Action potential recordings

Spontaneous action potentials (APs) were recorded in single HL-1 cell using the patch-clamp technique in perforated-patch configuration (Amphotericin B method) [1]. Cells were placed in an experimental bath on the stage of an inverted microscope (Nikon Eclipse Ti, Japan) and superfused with normal Tyrode's solution containing: NaCl 132 mM, KCl 4 mM, $MgCl_2$ 1.2 mM, HEPES 10 mM, $CaCl_2$ 1.8 mM and Glucose 11 mM (pH 7.35, NaOH). APs were measured with a patch amplifier (Multipatch 700B; Molecular Devices Inc., USA), signals were digitized via a DAC/ADC interface (Digidata 1550; Molecular Devices Inc., USA) and data acquired by means of pCLAMP 10.1 software (Molecular Devices Inc., USA).

Patch-clamp pipettes, prepared from glass capillary tubes by means of a horizontal puller (Flaming/Brown micropipette puller, model P-1000; Sutter Instrument) had a resistance of 2.5-3.5 MΩ when filled with the internal solution containing: K Methanesulfonate 115 mM, KCl 25 mM, HEPES 10 mM, $MgCl_2$ 3 mM, Amphotericin B 0.13 mM (pH 7.00 KOH). Temperature was maintained at 36.5 ± 0.5°C throughout the experiment.

2. Optical impulse propagation

Impulse propagation was assessed optically [2] in HL-1 cell line monolayer. Briefly, the cells were seeded onto 22 mm glass coverslips until the confluence has been reached. Then the preparations were stained using a voltage sensitive dye (135 μM Di-8-ANNEPPS) for 4 min and mounted on a custom-made temperature controlled chamber. Impulse propagation was assessed optically using a fast-resolution CMOS camera (L-Ultima, Scimedia, USA) and recorded at 10X magnification (Nikon Eclipse Ti) for 8 sec. Preparations were perfused continuously with Hanks Balanced Salts Solution (Gibco, UK). Extracellular stimulation has been achieved using a glass micropipette electrode positioned 1 mm from the cell surface at the periphery of the monolayer (ca. 1 cm away from the field of view for avoiding electrical artefact). Preparation were pre-stimulated using 15 consecutive stimuli (10 V amplitude, 2 ms duration at 0.5 Hz, Grass

Apparatus) for reaching the steady state and the impulse propagation recorded from the last 8 seconds.

3. Cell viability assay

Cell viability after stimulation was assessed performing Propidium Iodide (PI) staining (at a final concentration of 1 μg/ml) according to manufacturer's instructions (Bender MedSystems, USA) or counting HL-1 cells with Trypan blue.

4. Neonatal rat ventricular cardiomyocyte (NRCM) isolation

The procedures used in this work conform to National and European directives for the care and use of laboratory animals (D.L. 116/1992; 86/609/CEE). Animal protocols were reviewed and approved by the local Institutional Review Board and by the Italian Ministry of Health. As previously described [3] hearts were collected from 48 hours Sprague Dawley (Charles River, USA) and put into ice-cold PBS, where atriums were removed and residual ventricles were cut into small pieces. Heart fragments were then digested in four 20 minutes steps at 37 °C, using an enzyme digestion solution containing 136.8 U/ml collagenase CLS I (Worthington biochemical Corp., USA), 0.6 mg/ml pancreatin (Sigma-Aldrich, USA) in ADS buffer (116.4 mM NaCl, 5.4 mM KCl, 5.5 mM Glucose, 1 mM NaH_2PO_4 H_2O, 0.8 mM $MgSO_4$ H_2O, 20 mM Hepes, pH 7.4). NRCMs were purified by using a Percoll (Sigma-Aldrich, USA) step gradient and centrifuging at 3000 rpm for 30 min at room temperature in a swinging bucket rotor, switching-off the centrifuge brakes. Fibroblast cells, in the upper phase, were removed by aspiration and NRCMs were collected and washed in ADS buffer. Cardiomyocytes were finally resuspended in plating medium (4:1 DMEM:M199) containing 10% horse serum, 5% FBS, 1% Sodium Pyruvate, 10000 U/ml Penicillin, 10000 μg/ml Streptomycin and 20 mM L-Glutamine (all from Sigma-Aldrich, USA) and plated at a density of 80000 cells/cm^2 on dishes coated with 0.1% gelatin. The following day, the plating medium was substituted with maintenance medium (4:1 DMEM:M199, 5% horse serum, 5% FBS, 1% Sodium Pyruvate, 10 mM cytosine arabinoside (Ara-C), 10000 U/ml Penicillin, 10000 μg/ml Streptomycin and 20 mM L-Glutamine (all from Sigma-Aldrich, USA). The purity of cultured NRCMs was approximately 90% when the number

of cell nuclei (DAPI-positive) was compared with the number of myocytes (Troponin-C-positive).

5. Human adult cardiac stromal cell isolation

Human adult cardiac fibroblasts were isolated as stromal cells and amplified as previously described [4] from small fragments of right auricles obtained from patients undergoing either coronary bypass or valve substitution following Local Ethic Committee approval and signed informed consent in accordance with the declaration of Helsinki.

6. Real Time RT-PCR

For gene expression analysis, total RNA was extracted using TRIzol reagent and 1 μg of RNA was reversely transcribed using SuperScript® VILO cDNA Synthesis Master Mix (Invitrogen, USA). cDNA was amplified by SYBR-GREEN quantitative PCR on CFX96™ Real-Time PCR Detection System (Bio-Rad, USA). Five candidate housekeeping genes, among several commonly used ones, were investigated in order to select the most stable in HL-1 cell model: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-Actin (ACTB), hypoxanthine guanine phosphoribosyl transferase (HPRT), TATA box binding protein (TBP) and β-2 microglobulin (B2M). NormFinder software [5] was used to identify the optimal normalization gene among the selected candidates and TBP resulted as the most suitable housekeeping gene for Connexin-43 (Cx43) expression study. The following primers for mouse Cx43 and TBP were used: TBP fw 5′-TCAAACCCAGAATTGTTCTCC-3’; TBP rev 5′-AACTATGTGGTCTTCCTGAATCC-3’; Cx-43 fw 5′-ACTCCTGTACTTGGCTCACGT -3’; Cx-43 rev 5′-CCTTGCCGTGTTCTTCAATCC -3’.
Raw expression intensities of Cx43 were normalized to the Ct value of TBP, chosen as internal control. Relative quantitation was performed using the ΔΔCt method. Fold changes in gene expression were estimated as $2^{(-\Delta\Delta Ct)}$ [6].

7. Cell immunofluorescence

HL-1 cells were fixed in 4% PFA for 10 min at room temperature and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. After blocking for 1 h at room temperature with PBS containing 5% goat serum, cells were incubated overnight at 4°C with a rabbit polyclonal anti-Cx43 antibody (Abcam Cat# ab11370, UK) (dilution 1:400) in PBS supplemented with 2% goat serum. The following day, cells were washed three times with PBS and then incubated for 1 h at 37°C with the FITC-conjugated goat anti-rabbit secondary antibody (dilution 1:200) diluted in PBS supplemented with 2% goat serum. Nuclei were counterstained with Hoescht 33342 (Sigma-Aldrich, USA). Epifluorescence images were obtained using a confocal microscope (LSM710 ConfoCor3, Zeiss, Germany) equipped with a 40X oil objective. Image acquisition was carried out at room temperature using the Zen imaging software (Zeiss, Germany). Brightness/contrast adjustment was performed in Adobe Photoshop.

BIBLIOGRAPHY

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SUPPLEMENTARY FIGURES

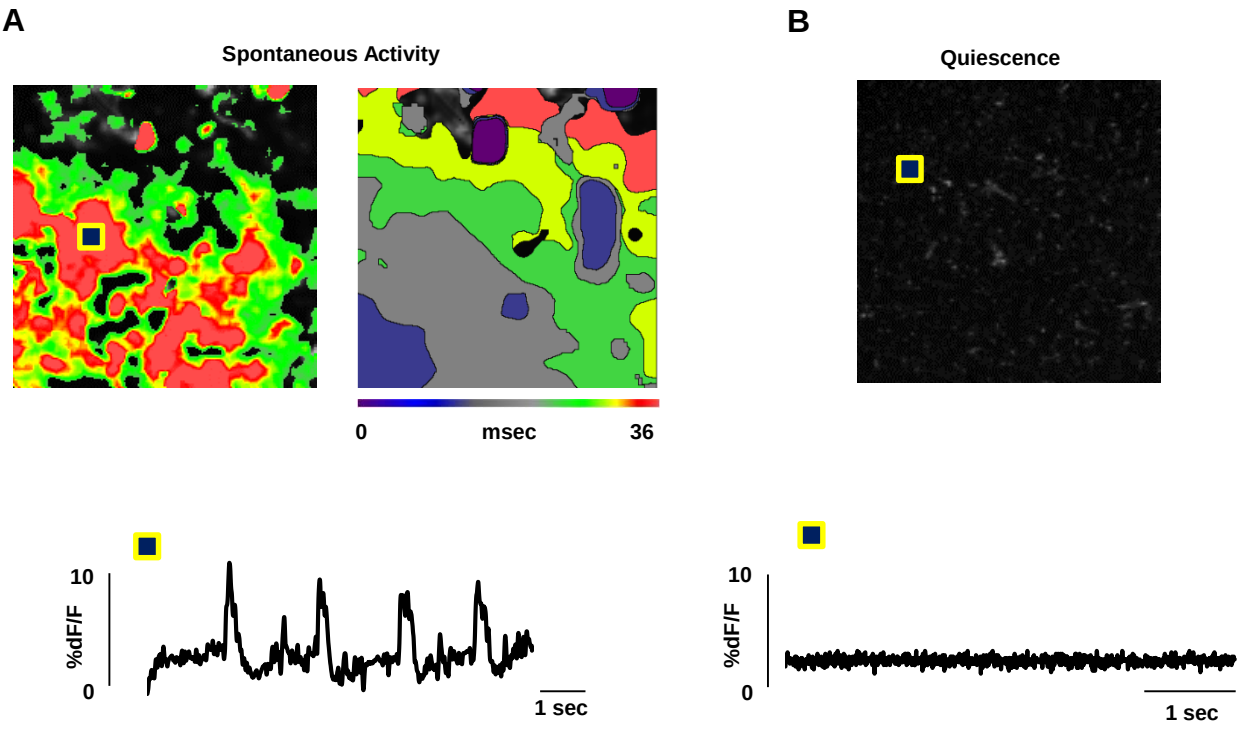
Supplementary Fig. 1: Electrical impulse propagation optically assessed in monolayers of HL-1 cells. (A) Spontaneous electrical activity originated from the lower left corner of the image and propagated throughout the monolayer (top left). Activation time map (5 ms interdistance) of the same propagation (top right). Spontaneous optical action potential recorded during 8 sec recording (ca. 0.5 Hz) (bottom). **(B)** Same as A showing a quiescent state of the monolayer during recording (no extracellular stimulation).

Supplementary Fig. 2: Effect of different chronic pacing conditions (0.5, 1 and 3 Hz) at different time points (1.5 hours, 24 hours and 4 days) on Cx43 expression and viability. Representative images of HL-1 cells stimulated at 0.5 Hz **(A)**, 1 Hz **(C)** and 3 Hz **(E)** at different time points. Western blot analysis shows Cx43 expression in NS and St HL-1 cells at 0.5 Hz **(B)** and 1 Hz **(D)** for 1.5 and 24 hours and at 3 Hz for 1.5 hours **(F)** (n=3). Cell viability evaluation using Trypan blue of NS and St HL-1 after 24 hours pacing at 1 Hz **(D)** and 3 Hz **(F)**. Data are presented as mean ± s.e.m, unpaired Student t-test: * $p < 0.05$.

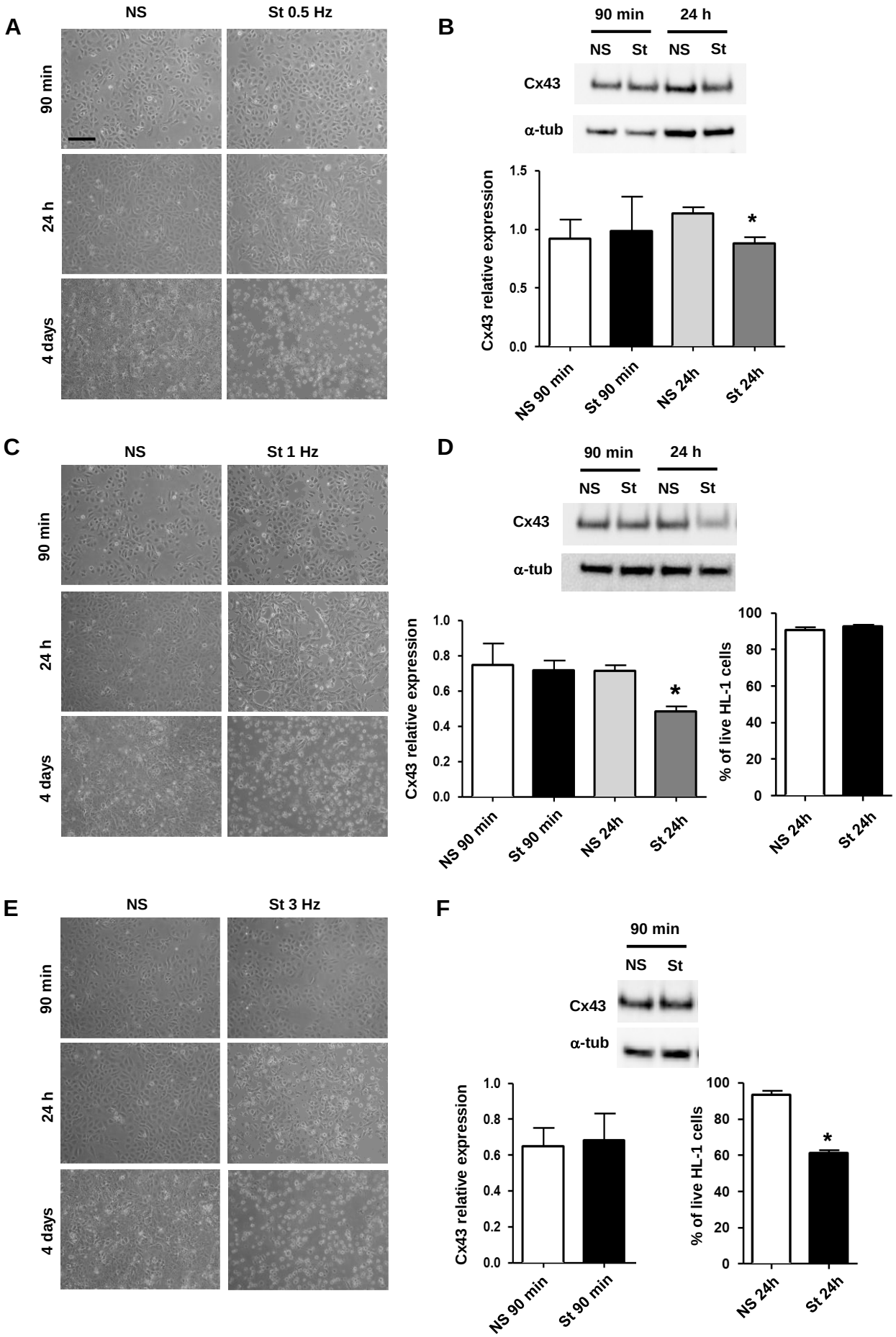
Supplementary Fig. 3: Effect of HDAC inhibitors on Cx43 expression and localization. (A) Western Blot analysis of HL-1 cells treated with 500 nM of TSA and 2.5 μM of SAHA at different time points. **(B)** Confocal analysis of Cx43 expression and distribution on HL-1 cells. Samples were analyzed before and after 4 hours treatment with 500 nM TSA. Nuclei were counterstained with Hoescht (blue). Scale bar = 25 μm.

Supplementary Fig. 4: Effect of electrical stimulation on Cx43 phosphorylation. (A) Representative Western Blot analysis images showing a typical phosphorylation banding patterns in NS and St HL-1 cells, detected by a specific total Cx43 antibody against amino acids 1–20 (n=4). **(B)** Western blot and densitometric analysis of NS and St HL-1 cells for Cx43 phosphorylation at the specific inhibitory residue Ser368 (n=6). Data are presented as mean ± s.e.m, unpaired Student t-test: * $p < 0.05$.

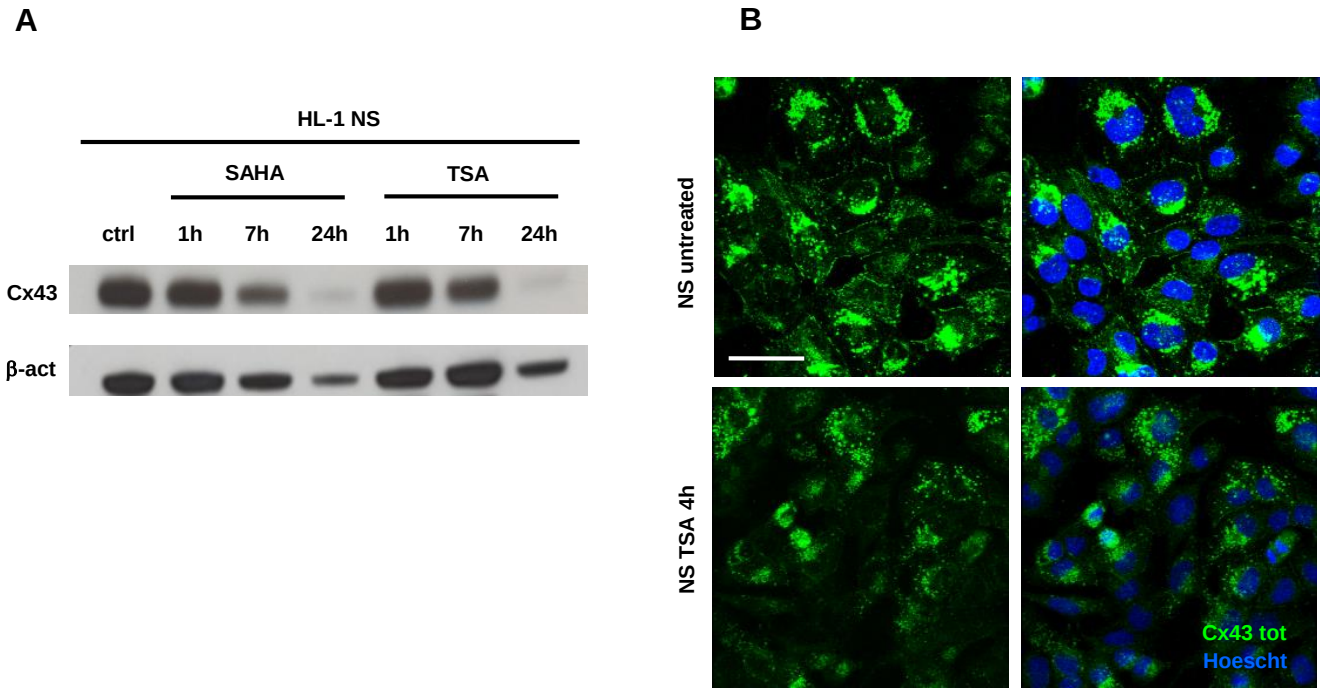
Supplementary Fig.1



Supplementary Fig.2



Supplementary Fig. 3



Supplementary Fig. 4