Supporting Information

Wang et al. 10.1073/pnas.1006785107

SI Materials and Methods

Cell Culture. Normal rat kidney (NRK) cells (ECACC 86032002) or HEK293 cells (ATCC CRL-1573) were cultured in DMEM (GIBCO, Invitrogen cell culture) supplemented with 10% FCS (PAA Laboratories GmbH) and grown at 37 °C and 5% CO_2. Human umbilical vein endothelial cells (HUVEC) (kindly provided by J. Lorens and L. Evensen, University of Bergen, Norway) were cultured in EBM-2 Basal Medium containing EGM-2 SingleQuot Kit Supplement and Growth Factors (Clonetics, Lonza) at 37 °C and 5% CO_2. For the present experiments, only cells with passage numbers 2 to 6 were used. PC12 cells (clone 251) were cultured as previously described (1). For mechanical stimulation experiments, cells were plated on 0.1 mg/mL poly-L-lysine-coated (PLL, Sigma-Aldrich Co.) Met-Tek glass-bottom culture dishes (MatTek Corporation) at low density and cultured for 20 to 24 h. For electrophysiological recording, NRK or PC12 cells were plated on 20-mm glass coverslips in 10-cm glass dishes and cultured for 20 to 24 h.

To obtain embryos for neural tube explants, fertilized quail eggs (from local supplier) were maintained at 38 °C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (HH) tables (2). The neural tube was explanted from stage HH 14 to 15, as described, with slight modifications (3). Briefly, eggs were rinsed with 70% ethanol and blastoderms were isolated. The yolk membrane around the blastoderms was removed. A rectangle of explant containing the last seven somites was cut out from the embryos. Excised explant was transferred and treated with 10 mg/mL pancreatin (Sigma-Aldrich) for 3 to 5 min at room temperature. Somites and notochord were removed form the neural tube. Then, the neural tube was transfered to Met-Tek glass-bottom culture dishes coated with 20 μg/mL fibronectin (Sigma-Aldrich) and cultured in DMEM containing 10% FCS, glutamine, 50 U/mL penicillin, and 0.05 mg/mL streptomycin (Sigma-Aldrich) at 37 °C and 5% CO_2. After 20 h in culture, the neural crest cells (NCCs) migrated from the neural tube over the substrate.

Cell Transfection. To investigate membrane continuity, the following cDNAs were used: GPI-EGFP corresponds to the previously described (4) GFP-GL-GPI fluorescent protein. GPI-mCherry cDNA was cloned by releasing the mCherry coding region from pmCherry-C2 vector (Clontech Laboratories) by restriction digestion with AgeI and BsrGI and ligated into AgeI/BsrGI-digested GFP-GL-GPI. This process resulted in the swapping of the GFP for mCherry and the generation of GPI-mCherry cDNA. NRK or HEK293 cells were transfected with GPI-EGFP or GPI-mCherry using electroporation, as described (5). After transfection, cells were mixed and plated on PLL-coated microscopy chambers (ibidi GmbH). Imaging was performed after 24 h on a Leica TCS SP5 confocal microscope (Leica Microsystems GmbH) with a 40×/1.25 NA oil-immersion objective. EGFP and mCherry were sequentially excited by the 488-nm and 561-nm/594-nm laser lines, respectively.

Mechanical Stimulation. Mechanical stimulations were performed using a FemtoJet/InjectMan NI 2 system (Eppendorf AG) in combination with an Olympus IX70 microscope (Olympus Europa GmbH). Tunneling nanotube (TNT)-connected cell pairs were identified by differential interference contrast (DIC) microscopy. Then, the tip of a glass capillary pulled with a P-87 micropipette puller (Sutter Instruments Inc.) was positioned 3 to 5 μm over the cell to be stimulated. Using the automatic function of the injection system, the capillary was rapidly advanced such that the tip penetrated the cell membrane and a bolus of PBS solution containing 0.2 mg/mL Cascade Blue (Molecular Probes, Invitrogen), a marker for gap junction-dependent intercellular diffusion, was injected.

Membrane Potential and $[Ca^{2+}]_i$ Imaging. For single membrane potential measurements, cells were incubated with 2 μM of bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] (Sigma-Aldrich) at 37 °C for 45 min. $DiBAC_4(3)$ partitions into the membrane and exhibits enhanced fluorescence when a cell is depolarized. After incubation, the medium was replaced with DMEM (37 °C) without serum for subsequent experiments performed at 37 °C and appropriate CO_2. Time-lapse fluorescence images (16 bit) were acquired before and after mechanical stimulation with a 60×/1.40 NA or a 100×/1.40 NA (for PC12 cells) oil-immersion objective, a Polychrome V monochromator (T.I.L.L. Photonics GmbH), and an Andor DU-885 camera controlled by IQ 7.0 software (Andor Technology). Cascade Blue and $DiBAC_4(3)$ were excited at 400 nm and 488 nm, respectively.

For simultaneous measurement of $[Ca^{2+}]_i$ and membrane potential, cells were loaded with 0.3 μM calcium indicator X-rhod-1 AM (Molecular Probes) by incubation in DMEM at 37 °C for 30 min. Then the medium was replaced with 2 μM $DiBAC_4(3)$ in DMEM at 37 °C for 30 min and finally replaced with DMEM containing 100 μM suramin (Sigma-Aldrich). For two-wavelength imaging, dye-loaded cells were excited at 560 nm and 488 nm and monitored under the same conditions as single-membrane potential measurements. To block low voltage-gated Ca^{2+} channels, 5 μM mibefradil (Sigma-Aldrich) was applied 30 min before start of the measurements.

Image Processing and Quantitative Analysis. For both $DiBAC_4(3)$ and X-rhod-1 images, the selection of regions of interest (ROIs) and the calculation of mean fluorescence intensity of ROIs were acquired with ImageJ software (National Institutes of Health). The average fluorescence intensity of background responses near the ROI was subtracted. To eliminate the effects of photobleaching, the fluorescence intensity of TNT-connected cell pairs was corrected by subtracting the mean value of the fluorescence intensity of control cells in the same condition. The change of fluorescence intensity of cells was calculated as $\Delta F = \frac{F_n - F_0}{F_0} - \frac{1}{m}\sum_{i=1}^{m}\left(\frac{F'_n - F'_0}{F'_0}\right)$, where F_n is the average fluorescence intensity at time frame n after mechanical stimulation, F_0 is the original average fluorescence intensity before mechanical stimulation, m is the number of control cells, and F' is the average fluorescence intensity of a given control cell. In Figs. 3*D* and 4*C*, electrically coupled cells were identified by the threshold: $\max(F_n - F_0) > 0$.

Electrophysiological Measurements. Before patch-clamp recording at room temperature (22–25 °C), cell medium was replaced with buffer (in mM): 145 NaCl, 10 Glucose, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 5 Hepes (pH 7.4). Cells were visualized with DIC videomicroscopy (6). Recording pipettes (5–7 MΩ) were filled with the following solution (in mM): 125 CsCl, 8 NaCl, 1 $CaCl_2$, 5 EGTA, 15 TEA-Cl, 4 MgATP, 10 Hepes (pH = 7.3), and dual whole-cell recordings were performed with an EPC9-dual patch-clamp amplifier controlled by PatchMaster software (HEKA Elektronik GmbH). Cells were voltage-clamped at a common membrane potential (V_{hold}) of either −60 mV or 0 mV. Signals were low-pass filtered at 2 kHz and sampled at 10 kHz. Currents caused by the recording pipette capacitance (C_{fast}) and the cell membrane capacitance (C_{slow}) were measured with the automatic capacitance

neutralization circuitry of the amplifier. For such measurements, the test pulse stimuli were sent simultaneously to both amplifiers to eliminate junctional currents between the cells. The average nominal capacitance for NRK cells was 24.8 ± 6.2 pF and the average series resistance was 25.5 ± 2.9 MΩ (n = 26).

Immunofluorescence. Cells were seeded on 12-mm PLL-coated glass coverslips or fibronectin-coated Met-Tek glass-bottom culture dishes (NCCs) and cultured for 24 h. Then cells were fixed in 2% formaldehyde with 0.2 M sucrose, 0.1 M PBS (pH 7.2) at room temperature for 20 min followed by permeabilization in 0.2% Triton X-100 in PBS at 4 °C for 5 min. After blocking with 1% normal goat serum (Sigma-Aldrich) in PBS (20 min), cells were incubated for 1 h with 1:500 rabbit polyclonal anti-Cx43 antibody (Sigma-Aldrich) followed by incubation for 1 h with 1:500 Alexa Fluor 647 goat anti-rabbit secondary antibody (Molecular Probes) at room temperature. For NCCs, 1:250 HNK-1 mouse monoclonal antibody (BD Pharmingen) and 1:500 Alexa Fluor 546 goat anti-mouse secondary antibody (Molecular Probes) were used. TNTs were stained with Alexa Fluor 488 wheat germ agglutinin (WGA, Molecular Probes) during embedding with Mowiol (Calbiochem). The samples were viewed with a Leica TCS SP5 equipped with a 63×/1.40 NA oil-immersion objective.

1. Rustom A, Saffrich R, Markovic I, Walther P, Gerdes HH (2004) Nanotubular highways for intercellular organelle transport. *Science* 303:1007–1010.
2. Hamburger V, Hamilton HL (1992) A series of normal stages in the development of the chick embryo. 1951. *Dev Dyn* 195:231–272.
3. Dupin E, Le Douarin NM (1995) Retinoic acid promotes the differentiation of adrenergic cells and melanocytes in quail neural crest cultures. *Dev Biol* 168:529–548.
4. Keller P, Toomre D, Díaz E, White J, Simons K (2001) Multicolour imaging of post-Golgi sorting and trafficking in live cells. *Nat Cell Biol* 3:140–149.
5. Kaether C, Salm T, Glombik M, Almers W, Gerdes HH (1997) Targeting of green fluorescent protein to neuroendocrine secretory granules: A new tool for real time studies of regulated protein secretion. *Eur J Cell Biol* 74:133–142.
6. Veruki ML, Hartveit E (2002) AII (Rod) amacrine cells form a network of electrically coupled interneurons in the mammalian retina. *Neuron* 33:935–946.

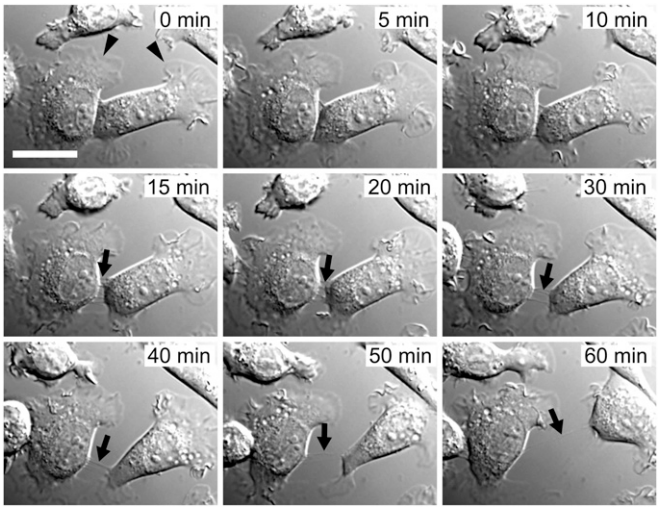

Fig. S1. The formation of TNT between NRK cells. NRK cells were plated on PLL-coated MatTek glass-bottom culture dishes and imaged 1 h after plating for a period of 15 h by DIC microscopy. Shown are selected time frames from a representative time-lapse movie (n = 54) depicting TNT formation (arrows) between dislodged NRK cells. Note that NRK cells do not exhibit filopodia, but prominent lamellipodia with frequent membrane ruffles (arrowheads). (Scale bar, 20 μm.)

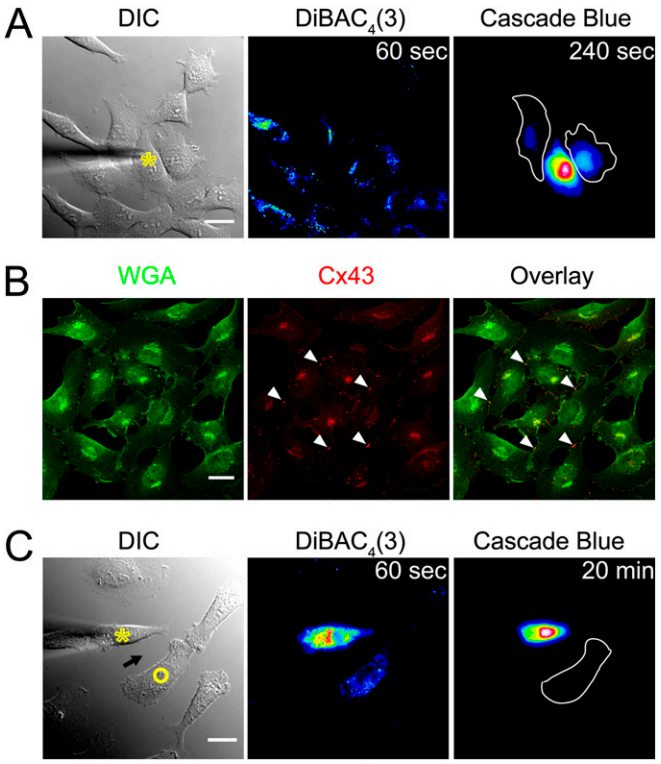

Fig. S2. The presence of functional Cx43 gap junctions in NRK cells. (*A*) Functional gap junctions are present in abutting NRK cells. The DIC image (*Left*) shows a mechanically stimulated cell (asterisk), which abuts a group of associated cells, and a control cell (top of the image) lacking physical contact to the other cells. The pseudocolored image (*Center*), generated by subtraction of the image before stimulation, depicts the increase in fluorescence of $DiBAC_4(3)$ and indicates the spread of depolarization between cells 60 s after mechanical stimulation. The intercellular spread of Cascade Blue dye 4 min after injection (*Right*, white contours) indicates the presence of functional gap junctions. (*B*) Cx43 present at contact sites of abutting NRK cells. NRK cells were double-labeled with WGA (green) and anti-Cx43 (red) and analyzed by confocal microscopy. Punctate Cx43 immmunoreactivity (arrowheads) is visible at abutting cell borders. (*C*) No dye transfer between TNT-connected NRK cells. The DIC image (*Left*) shows a mechanically stimulated cell (asterisk), the TNT-connected cell (open circle), and the TNT (arrow). The spread of depolarization to the TNT-connected cell following mechanical stimulation is indicated by the increase in fluorescence of $DiBAC_4(3)$ (*Center*). Cascade Blue dye did not spread to TNT-connected cells 20 min after injection (*Right*, white contours). (Scale bars, 20 μm.)

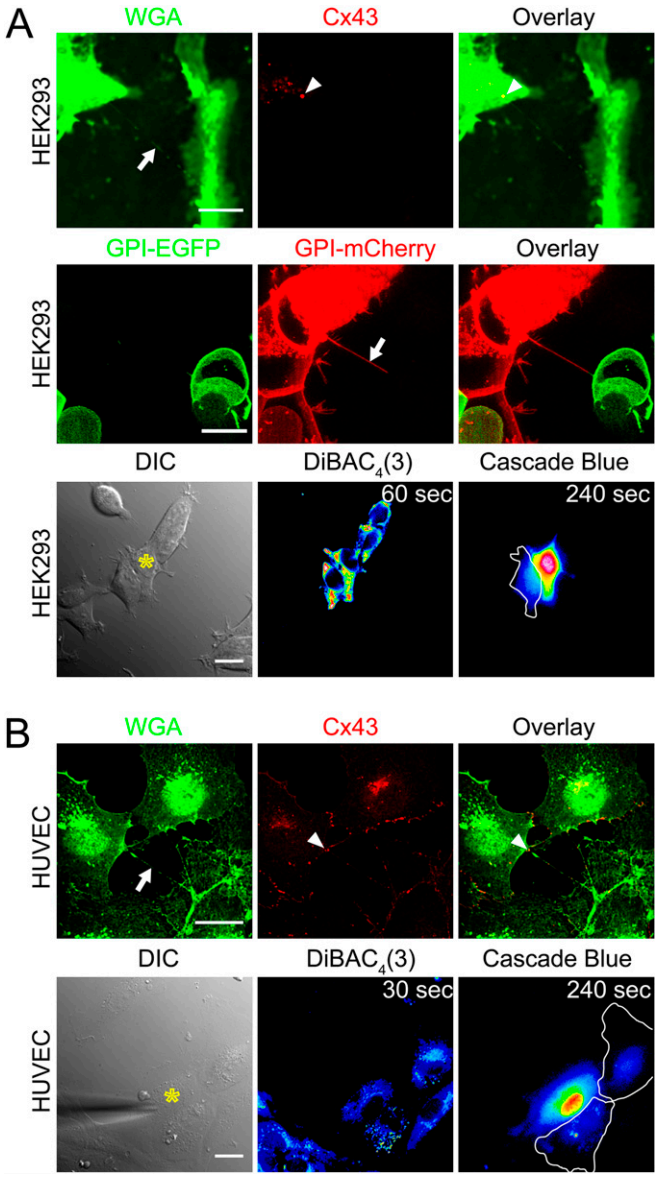

Fig. S3. The presence of gap junctions in TNTs between HEK293 or HUVEC cells. (*A*) (*Top*) Localization of Cx43 in TNTs between HEK293 cells. Confocal images of double-labeled cells using WGA (green) and anti-Cx43 (red) show punctate Cx43 immmunoreactivity (arrowhead) at one end of the TNTs (arrow) in HEK293 cells. (*Middle*) Membrane markers do not freely diffuse between TNT-connected HEK293 cells. Mixed populations of GPI-EGFP- and GPI-mCherry-labeled cells were imaged after 24 h of coculturing. A TNT (arrow) connecting GPI-EGFP (green)- and GPI-mCherry (red)-expressing cells, respectively, did not show the mixture of colors. (*Bottom*) Presence of gap junctions in abutting HEK293 cells. The DIC image shows a mechanically stimulated cell (asterisk), which is in close contact with neighboring cells, and control cells (*Bottom Right*). The spread of depolarization between abutting cells following mechanical stimulation is indicated by the increase in fluorescence of $DiBAC_4(3)$ at indicted time (*Bottom Center*). Cascade Blue dye spread to other cells 4 min after injection (*Right*, white contours). (*B*) (*Upper*) Localization of Cx43 in TNTs between HUVEC cells. Confocal images of double-labeled cells using WGA (green) and anti-Cx43 (*red*) show punctate Cx43 immmunoreactivity (arrowhead) at one end of the TNT (arrows) in HUVEC cells. (*Lower*) Presence of gap junctions between abutting HUVEC cells. The DIC image shows a mechanically stimulated cell (asterisk), which is in close contact with neighboring cells, and a control cell (top of image). The spread of depolarization between abutting cells following mechanical stimulation is indicated by the increase in fluorescence of $DiBAC_4(3)$ at indicted time (*Center*). Cascade Blue dye spread to other cells 4 min after injection (*Right*, white contours). (Scale bars, 20 μm.)

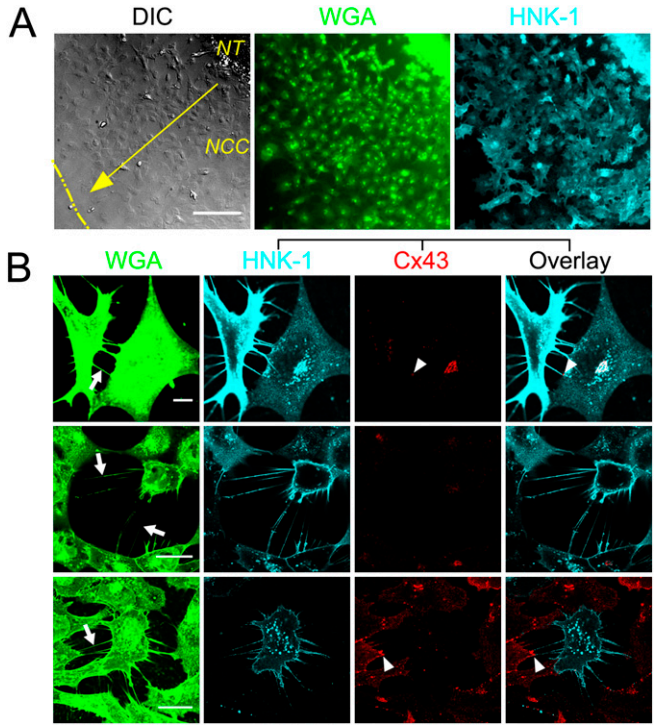

Fig. S4. Localization of Cx43 on TNTs in neural crest cultures. (*A*) Large numbers of NCC cells migrated from the neural tube after 20 h of culture. The DIC image shows the neural tube explant (*NT*), outgrowth of migratory neural crest cells (*NCC*), the direction of the migration (arrow), and the border of the culture (broken line) where TNTs were found. NCCs were labeled with WGA (green) and HNK-1 (cyan). HNK-1-positive staining indicates the presence of NCC cells. (Scale bar, 100 μm.) (*B*) Localization of Cx43 on TNTs in neural crest cultures. The cultures were triple-labeled with WGA (green), HNK-1 (cyan), and anti-Cx43 (red) and analyzed by confocal imaging. (*Top*) A TNT connecting two NCC cells (arrow) shows Cx43 immmunoreactivity at one end of the TNT (arrowhead). (*Middle*) TNTs connecting NCC cells (arrows) do not display Cx43 immmunoreactivity. (*Bottom*) TNT connecting one NCC cell (arrow) with one non-NCC cell displays Cx43 immmunoreactivity (arrowhead). [Scale bars, 10 μm (*Top*), 20 μm (*Middle* and *Bottom*).]

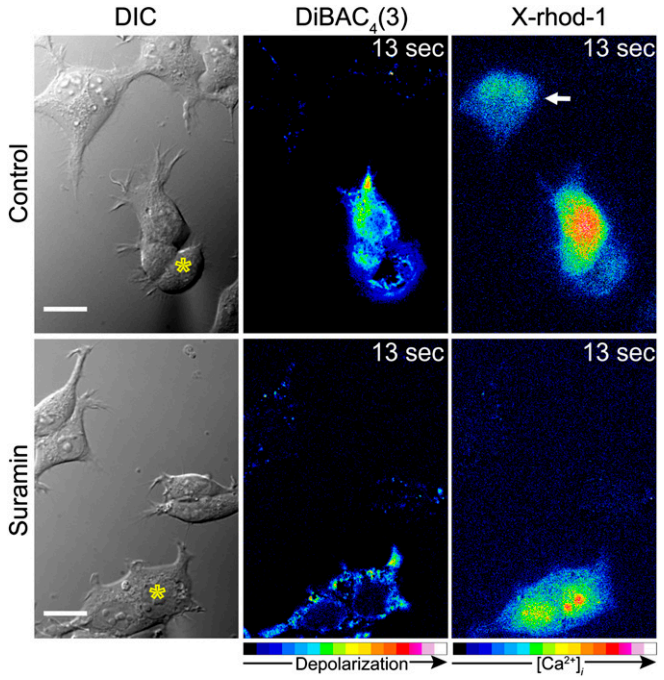

Fig. S5. Purinergic antagonists block calcium wave in isolated HEK293 cells. The DIC images (*Left*) show the mechanically stimulated cells (asterisks) and cells without physical connections to the stimulated cells (top of images). The pseudocolored intensity images, generated by subtraction of the image before stimulation, depict the fluorescence changes of $DiBAC_4(3)$ (*Center*) and X-rhod-1 (*Right*) at indicated times after mechanical stimulation. Restriction of elicited depolarization to one group of abutting cells demonstrates that there was no physical contact to the other cell groups. (*Upper*) Elevation of $[Ca^{2+}]_i$ is shown for one cell of the control group (arrow) that has no physical contact to the stimulated cell (asterisk). (*Lower*) Suramin (100 μM) abolished the spread of calcium wave between two groups of cells, which had no physical contact. Note that the bright dots of nonstimulated cells in the center panels are because of the movement of intracellular organelles. (Scale bars, 20 μm.)

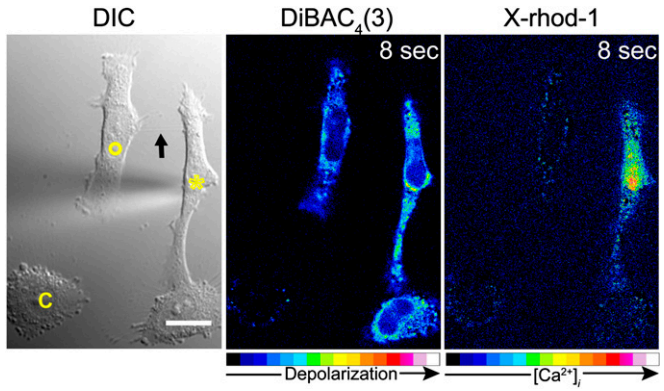

Fig. S6. Depolarization does not increase $[Ca^{2+}]_i$ in TNT-connected NRK cells. The DIC image (*Left*) shows the mechanically stimulated cell (asterisk), TNT-connected cell (open circle), control cell (c), and the location of the TNT (arrow). The pseudocolored intensity images, generated by subtraction of the image before stimulation, depict the fluorescence changes of $DiBAC_4(3)$ (*Center*) and X-rhod-1 (*Right*) at indicated time after mechanical stimulation. No $[Ca^{2+}]_i$ increase is visible in the TNT-connected cell, which received a depolarization signal. (Scale bar, 20 μm.)