Supporting Information

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SI Materials and Methods

Levels of connexin (Cx) 39, Cx43, and Cx45 as well as purinergic ionotropic $P2X_7$ receptor ($P2X_7R$) and transient receptor potential, subfamily V, member 2 (TRPV2), channels were higher in denervated than in control muscles (Fig. S1). Nonetheless, levels of Cx39, Cx43, and Cx45 of rat soleus muscles (slow muscle) at 7 d after denervation were comparable to those of the contralateral innervated one (Fig. S4*A*). Of note, levels of Cxs, $P2X_7Rs$, and TRPV channels detected in innervated muscles represent expression in cell types different from myofibers, including satellite cells and vascular cells. Moreover, myofibers of these muscles did not show Evans blue (EB^{4-}) fluorescence in their interior (Fig. S4*B*). Gastrocnemius muscles of rats at 56 d after spinal cord transection, another model of muscle paralysis known to induce muscle atrophy (1), also showed increased reactivity for all six proteins (Fig. S2) found to be elevated in denervated extensor digitorum longus (EDL) muscles.

Myofibers of Denervated Fast Muscles Are Not Coupled via Gap Junction Channels. As denervated fast muscles were found to express three Cxs and form functional hemichannels, we also tested whether they form functional gap junction channels. To this end, we performed immunofluorescence detection of these three Cxs in cryosections along the long axis of myofibers. In these experiments, the reactivity of all three Cxs was detected in the sarcolemma but did not form immunoreactive plaques at cell–cell contacts in denervated EDL muscles. In addition, we tested the intercellular transfer of ethidium (Etd^+) injected in one myofiber of freshly dissected EDL muscles. In control muscles, the injected Etd^+ stained several nuclei along the length of the injected myofiber but did not diffuse to neighboring myofibers (Fig. S3, control). In contrast, microinjection of Edt^+ into one myofiber of denervated EDL muscles stained several nuclei along the length of the microinjected cell as well as numerous nuclei of adjacent myofibers (Fig. S3). The intercellular diffusion of microinjected Etd^+ was drastically reduced by 18β-glycyrrhetinic acid, a hemichannel and gap junction channel blocker (2), and by suramin (Fig. S3), a P2Y and P2X receptor blocker (3, 4) that does not affect gap junctional communication in HeLa-Cx43 cells, indicating that the intercellular Etd^+ transfer found in denervated muscles was mediated by Cx hemichannels activated by P2 receptors and not by gap junction channels.

Reagents. Western Lightning chemiluminescence (ECL) detection reagents were obtained from PerkinElmer. Anti-rabbit IgG antibody conjugated to HRP was purchased from Pierce. Polyclonal anti-Cx39 antibody was obtained from Invitrogen, and anti-$P2X_7R$, anti-TRPV2, anti-phosphorylated p65 antibodies were from Abcam. Polyclonal anti-Cx43, anti-Cx45 (5), and anti-Pannexin1 (Panx1) antibodies have been previously described (6). Etd^+ bromide was from Gibco/BRL; Fluoromount-G was from Electron Microscopy Science; β-glycyrrhetinic acid (β-GA), oleamide, *N*-benzyl-p-toluene sulphonamide (BTS), collagenase type I rhodamine dextran (10 kDa), carbenoxolone (Cbx), EB^{4-}, 2-aminoethyl diphenylborinate (2-APB), brilliant blue G, and apyrase were obtained from Sigma; and A740003 was obtained from Tocris Biosciences.

Animals. Offspring were genotyped by using tail-snip DNA and PCR primers for Cx43 flox (7) and PCR Cx45 flox (8), or Myo-Cre. The PCR for Myo-Cre yielded an amplicon of ∼1 kb using the primers myogen-113 (5′-TGTGCAGCAACAGCTTAGAG-3′) and MH63Cre (5′-AGGCTAAGTGCCTTCTCTACAG-3′) in the presence of $MgCl_2$ (2 mM) by using the following PCR conditions: 94 °C for 4 min; 94 °C for 1 min, 67 °C for 1 min, and 72 °C for 1 min, for 35 cycles; and 72 °C for 10 min.

Muscle Denervation. Animals were anesthetized by using ketamine and xylazine. The left sciatic nerve was exposed and ∼5 to 8 mm of the nerve were removed. The right nerve was only exposed, and muscles of the same side were used as control. Wounds were sutured, and animals were fed food and water ad libitum under 12:12 h dark:light cycles. After 3, 7, or 21 d, animals were killed by decapitation under deep anesthesia, and muscles were excised after careful dissection. In all muscles used, levels of myogenic differentiation factor (MyoD) were measured by immunoblotting to verify the success of the denervation procedure.

EB^{4-} Uptake. HeLa-parental cells or HeLa cells transfected with Cx39, Cx43, Cx45, Panx1, or TRPV2 were used. Cells transfected with Cxs were previously described (5, 9) and HeLa-Panx1 was provided by Feliksas Bukaukas (Albert Einstein College of Medicine, Bronx, NY). HeLa-TRPV2 cells were generated by transient transfection (as described later). To evaluate EB^{4-} uptake, all cells were treated with conditions that increase the opening probability of the channels they express. Cx transfectants were placed in divalent cation-free solution (DCFS; containing, in mM: 145 NaCl, 5 KCl, 5.6 glucose, 0.5 EGTA, 10 Hepes-Na, pH 7.4). HeLa-Panx1 cells were treated for 2 min with 2 μM bisindolylmaleimide (Sigma), a PKC inhibitor that increases the Etd^+ uptake via Panx1 hemichannels and HeLa-TRPV2 cells were treated with the agonist 2-APB (10).

Isolation of Adult Rat and Mouse Skeletal Myofibers. Undamaged myofibers can be easily dissociated from flexor digitorum brevis (FDB) muscles (fast muscles), and inflammation triggered by ATP released by damaged cells during cell dissociation can be prevented by inclusion of suramin in the dissociation solutions. Thus, 200 μM suramin, an inhibitor of P2 receptors (3, 4), was included in the cell dissociation solution. From anesthetized rats or mice, the plantaris tendons and connective tissue were removed. Then, FDB muscles were carefully dissected and immersed in cultured medium (DMEM/F12 supplemented with 10% FBS) containing 0.2% collagenase type I, incubated for 3 h at 37 °C, and transferred to a 15-mL test tube (Falcon) containing 5 mL of culture medium, in which muscle tissue was gently triturated 10 times by using a Pasteur pipette with a wide tip to disperse single myofibers. Dissociated fibers were centrifuged at 1,000 rpm for 15 s (model 8700 centrifuge; Kubota) and washed twice by sedimentation, first with PBS solution and then with Krebs buffer (in mM: 145 NaCl, 5 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 5.6 glucose, 10 Hepes-Na, pH 7.4) containing 10 μM BTS to reduce muscle damage caused by activation of contractions during the isolation procedure. Finally, fibers were resuspended in 5 mL of Krebs Hepes buffer containing 10 μM BTS, plated in plastic culture dishes or placed in 1.5-mL Eppendorf tubes, and kept at room temperature.

TRPV2 Construct and Transfection. The rat TRPV2 cDNA was provided by David Julius (University of California, San Francisco, CA). HeLa-parental cells were transfected with pcDNA1/amp-rTRPV2 (original plasmid) or pIRES-rTRPV2 (plasmid subcloned in our laboratory) at final concentration of 2.5 ng/μL using Lipofectamine LTX and PLUS Reagents (Invitrogen Life Technologies) as described for the 24-well format according to

the manufacturer's instructions. After 48 h, cells were used for EB^{4-} uptake and calcium uptake experiments.

Dye Coupling. Control and denervated EDL muscles were isolated from rats at 7 d after denervation and maintained in Krebs buffer at room temperature for microinjection with Etd^{+} (75 mM) using glass microelectrodes. At 1 min after dye injection, the muscle was observed to determine if transfer of the dye occurred. This analysis was also performed in absence or presence of β-GA (50 μM), a Cx/Panx hemichannel blocker, or suramin (200 μM), a P2 receptor blocker.

RT-PCR Analysis. Total RNA was isolated from muscle homogenates by using TRIzol reagent following the manufacturer's instructions (Ambion). Aliquots of 2 μg of total RNA were reverse-transcribed into cDNA by using MMLV-reverse transcriptase (Fermentas), and the amplicon levels were evaluated by PCR amplification (GoTaq Flexi DNA Polymerase; Promega) as follows: 5 min at 95 °C; then, for TNF-α 32 cycles of 30 s at 95 °C, 30 s at 52 °C, 30 s at 72 °C; and for IL-1β 32 cycles of 30 s at 95 °C, 30 s at 56 °C, 30 s at 72 °C; and 10 min final extension at 72 °C. PCR reactions were performed in 25 μL containing 5 μL 5× PCR buffer, 1.5 μL 25 mM Mg^{2+}, 0.4 μL dNTPs, 0.4 μL oligonucleotide (10 μM, forward and reverse), 16.1 μL DNase-free H_2O, 0.2 μL DNA polymerase, and 1 μL of cDNA. Oligonucleotides were as follows: TNF-α 5′-CGCTCTTCTGTCTACTGAAC-3′ (forward), 5′-CAGGTTCTCTTCAAGGGACA-3′ (reverse); IL-1β 5′- CAGGAAGGCAGTGTCACTCA-3′ (forward), 5′-GGGATTTTGTCGTTGCTTGT-3′ (reverse). Expected size products were as follows: TNF-α, 301 pb; IL-1β, 339 pb.

Time-Lapse Recording of Etd^{+} Uptake. Cellular uptake of Etd^{+} was evaluated by time-lapse measurements as described previously (6). In brief, freshly isolated myofibers plated onto plastic culture dishes were washed twice with Krebs buffer solution. For time-lapse measurements, myofibers were incubated in recording medium containing 5 μM Etd^{+}. The Etd^{+} fluorescence was recorded in regions of interest that corresponded to nuclei of myofibers by using a water immersion Olympus 51W1I upright microscope (Japan). Images were captured with a Retiga 13001 fast cooled monochromatic digital camera (12-bit; QImaging) every 30 s, and image processing was performed offline with ImageJ software (National Institutes of Health).

Western Blot Analysis. Muscles were washed with ice-cold lysis buffer (in mM: 100 NaCl, 1 EDTA, 20 Hepes, and 1% Triton X-100, pH 7.4) containing protease inhibitors (2 mM PMSF, 200 μg soybean trypsin protease inhibitor, 1 mg/mL benzamidine, 1 mg/mL ε-aminocaproic acid, and 500 μg/mL leupeptin) and phosphatase inhibitors (in mM: 20 $Na_4P_2O_7$, 100 NaF, and 200 orthovanadate) and then frozen in liquid nitrogen. Tendon-free muscles were minced in small pieces by using a razor blade and then homogenized (homogenizer; Brinkmann) and sonicated (Heat Systems Microson). Tissue homogenates were centrifuged twice for 20 min at 13,000× *g* and pellets were discarded. Then, samples were processed for Western blot analyses of proteins of interest as described previously (6). Blots were incubated overnight with appropriate dilutions of primary antibodies diluted in 5% milk–PBS solution. Then, blots were rinsed with 1% PBS solution–Tween 20 and incubated for 40 min at room temperature with HRP-conjugated goat anti-rabbit IgGs (Santa Cruz Biotechnology). After five rinses, immunoreactive proteins were detected using ECL reagents according to the manufacturer's instructions (PerkinElmer).

Immunofluorescence Analysis. Muscles were fast frozen with isomethylbutane cooled in liquid nitrogen. Then, cross-sections (20 μm) were obtained by using a cryostat (CM1100; Leica) and fixed with 4% formaldehyde for 10 min at room temperature. Sections were incubated for 3 h at room temperature in blocking solution (50 mM NH_4Cl, 0.025% Triton, 1% BSA on PBS solution 1×), incubated overnight with appropriate dilutions of primary antibody, washed five times with PBS solution followed by 1 h incubation with secondary antibody conjugated to Cy2 or Cy3, and mounted in Fluoromount G. Immunoreactive binding sites were localized under a Nikon Eclipse Ti microscope equipped with epifluorescence illumination, and images were obtained by using a Clara camera (Andor).

Spinal Cord Transection. The spinal cord transection procedure was performed as previously described (11).

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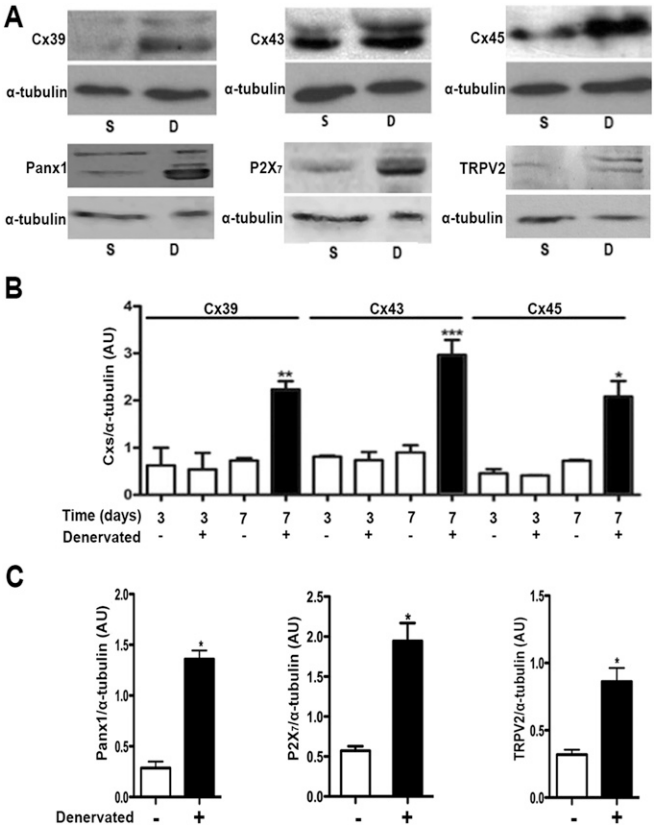

Fig. S1. Denervation increases the levels of several channels permeable to small molecules. (*A*) After 7 d of denervation (black bar), the relative levels of Cx39, Cx43, Cx45, Panx1, $P2X_7R$, and TRPV2 channel were evaluated by Western blot analysis in total homogenates of rat EDL muscles. In each animal, the contralateral muscle was used as control. Denervation increased the levels of all proteins. Quantification of relative levels of (*B*) Cxs and (*C*) Panx1, $P2X_7R$, and TRPV2 proteins. All densitometric values were normalized to the α-tubulin levels used as loading control. D, denervated; S, sham-operated (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. sham; $n = 5$ per group). Values presented as mean ± SEM.

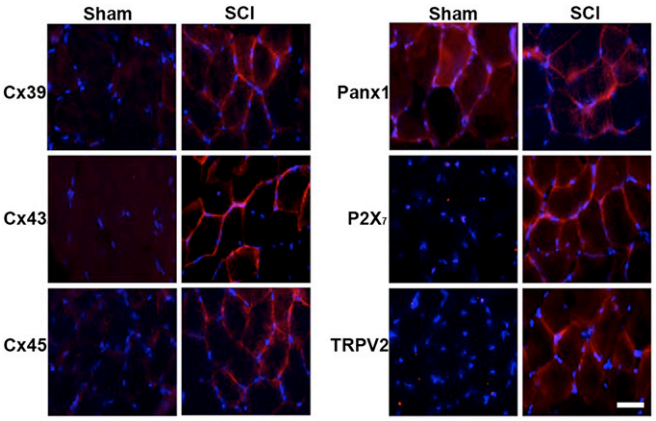

Fig. S2. Cx39, Cx43, Cx45, $P2X_7$, and TRPV2 are up-regulated in skeletal myofibers of gastrocnemius muscles of rats with spinal cord transection, and these proteins as well as Panx1 are mainly distributed to the sarcolemma. The distribution of Cx39, Cx43, Cx45, Panx1, $P2X_7$, and TRPV2 reactivity was evaluated by immunofluorescence in cross-sections of rat gastrocnemius muscles after 56 d of spinal cord injury (SCI) and sham operation ($n = 4$ per group). (Scale bar: 20 μm.)

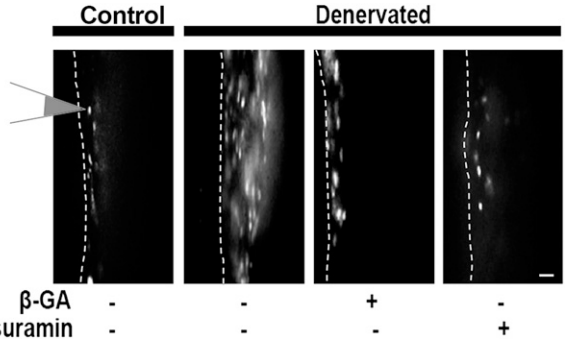

Fig. S3. Denervated skeletal muscles do not express functional gap junction channels. A single myofiber of freshly dissected control (contralateral) and 7 d denervated rat EDL muscles (fast) was injected with Etd^{+}. In parallel experiments, denervated muscles were bathed with a saline solution containing 100 μM 18β-glycyrrhetinic acid (β-GA), a hemichannel and gap junction channel blocker, or 150 μM suramin, a P2 receptor blocker. Dye transfer was only evident in denervated muscles and was blocked by β-GA and suramin ($n = 3$). (Scale bar: 100 μm.)

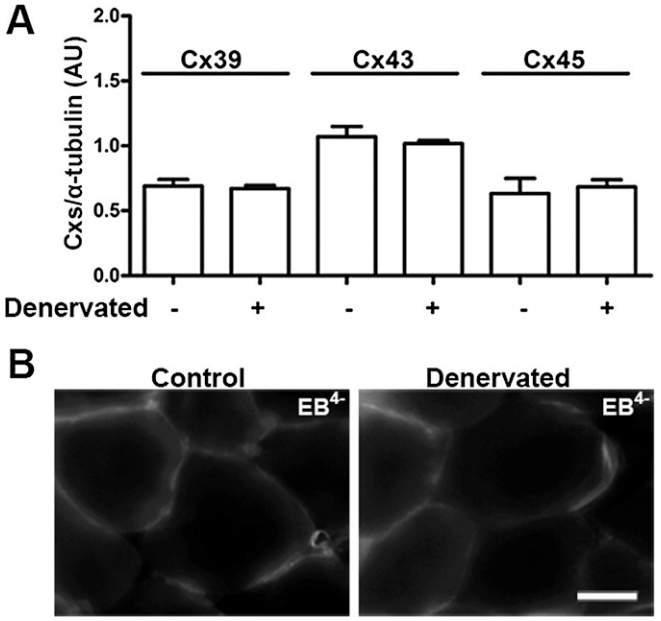

Fig. S4. Seven days of denervation does not affect levels of Cx proteins and EB^{4-} permeability of a rat slow muscle. (*A*) The relative levels of Cx39, Cx43, and Cx45 in slow (soleus) muscle were evaluated by Western blotting after 7 d of denervation and were comparable to those in control. (*B*) The membrane permeability to EB^{4-} of slow (soleus) muscles after 7 d of denervation was assessed; no intracellular fluorescence of EB^{4-} was detected in denervated slow myofibers ($n = 5$). Values presented as mean ± SEM. (Scale bar: 20 μm.)

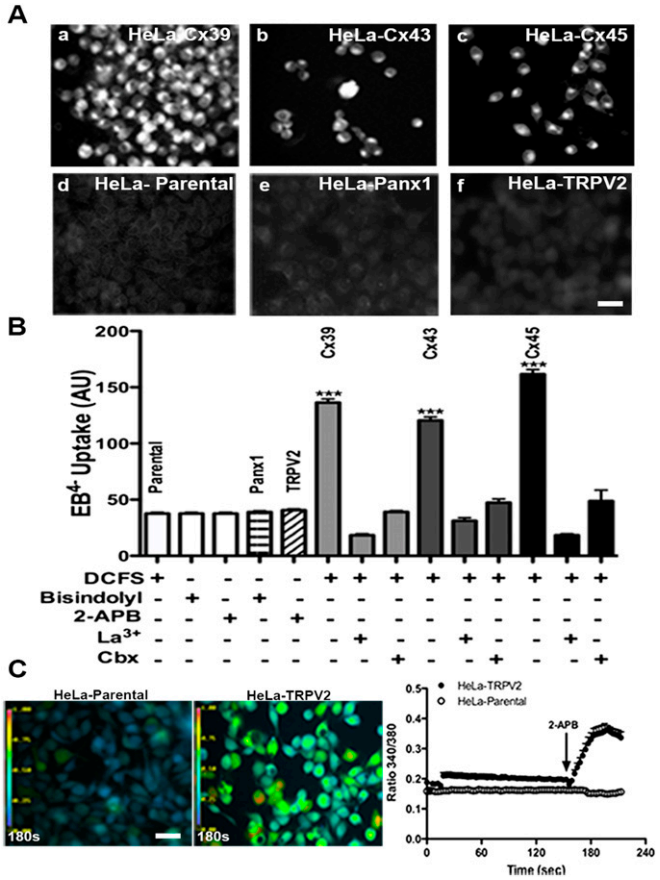

Fig. S5. Cx hemichannels but not Panx1 or TRPV2 channels are permeable to EB^{4-}. HeLa-parental cells and HeLa cells transfected with Cx39, Cx43, Cx45, Panx1, or TRPV2 were used. Cells were treated with conditions that induce opening of the corresponding channel in the presence of 1 mM EB^{4-} for 10 min. Then, extracellular EB^{4-} was washed out with saline solution containing divalent cations, and fluorescence intensity was evaluated (λ excitation, 545 nm; λ emission, 595 nm). Opening of Cx hemichannels, Panx1 channels, and TRPV2 channels was induced with extracellular DCFS, 100 μM bisindolylmaleimide, and 50 μM 2-APB, respectively. Cx HeLa transfectants (*A*, *a*–*c*, and *B*), but not HeLa-parental, -Panx1 or -TRPV2 cells (*A*, *d*, *f*, and *g*), showed EB^{4-} uptake. (Scale bar: 50 μm.) (*B*) Quantification of fluorescence intensity in parental and transfected HeLa cells under conditions that induce channel opening. The inhibitory effect of 200 μM La^{3+} or 100 μM Cbx on EB^{4-} uptake by HeLa cells transfected with Cx39, Cx43, or Cx45 was also examined. (Scale bar: 50 μm.) (*C*) Images depict fluorescent signal of cells loaded with Fura-2-acetoxymethyl ester at 180 s after 2-APB treatment to induce the Ca^{2+} signal. The 2-APB treatment increased the Ca^{2+} signal in HeLa-TRPV2 cells but not in HeLa-parental cells. The graphs shows the Ca^{2+} signal over time in HeLa-TRPV2 cells loaded with Fura-2-acetoxymethyl ester under resting conditions and after stimulation with 2-APB (arrow; $***P < 0.001$; $n = 3$ per condition). A minimum of 50 cells were recorder per condition.

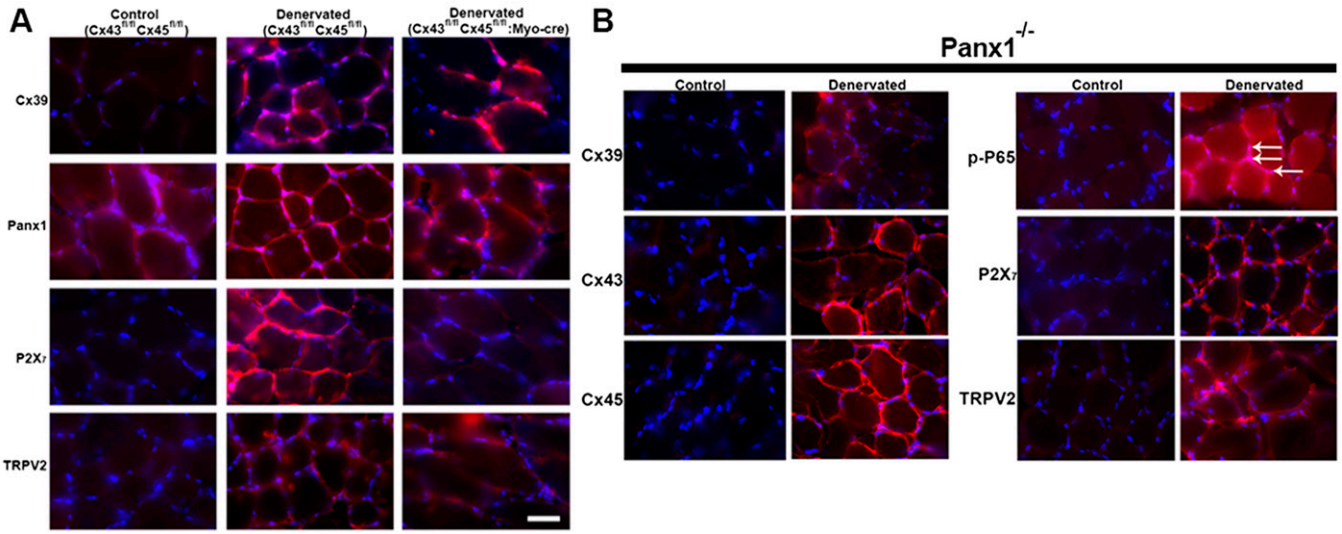

Fig. S6. Absence of Cx43 and Cx45 but not Panx1 prevents the increase of $P2X_7R$. (*A*) Absence of Cx43 and Cx45 drastically reduces the increase of $P2X_7R$ but does not significantly affect the increase in Cx39, Panx1, and TRPV2 channel reactivity induced by denervation in tibialis anterior (TA) muscle. TA muscles of $Cx43^{fl/fl}Cx45^{fl/fl}$ and $Cx43^{fl/fl}Cx45^{fl/fl}$:Myo-Cre mice were denervated. Seven days later, cross-sections were prepared of denervated and control (contralateral) muscles and used to assay the reactivity of Cx39, Panx1, $P2X_7R$, and TRPV2 channels by immunofluorescence. The reactivity of $P2X_7R$ was barely detected in denervated Cx43/Cx45-deficient muscles, whereas the reactivity of all other proteins studied increased as in denervated muscles of $Cx43^{fl/fl}Cx45^{fl/fl}$ mice. ($n = 3$). (*B*) The absence of $Panx1^{-/-}$ does not prevent the increase in immunoreactivity of Cxs, Panx1, $P2X_7R$, and TRPV2 channels induced by denervation in TA muscles. TA muscles of $Panx1^{-/-}$ mice were denervated and, after 7 d, were used for immunofluorescence detection of Cx39, Cx43, Cx45, $P2X_7Rs$, and TRPV2 channels. In each animal, the contralateral muscle was used as control ($n = 3$). (Scale bar: 20 μm.)