Supporting Online Material for:

Molecular and functional identification of a mitochondrial ryanodine receptor in neurons

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Supplementary Material and Methods

Supplementary Figs. 1 to 4.

References

Supplementary Materials and Methods

Reagents and antibodies

All reagents and antibodies were purchased from Sigma-Aldrich Corporation. (St. Louis, MO) unless otherwise indicated: Ryanodine from Calbiochem (San Diego, CA); ^{3}[H]ryanodine from GE Healthcare Life Sciences (Pittsburgh, PA); Bodipy-FL-X ryanodine (Bodipy-Ry), Bodipy TR-X ryanodine (Bodipy-TR-Ry), Bodipy-FL-X thapsigargin (Bodipy-Thap), Mitotracker Deep Red (MTR), Fura-2 and Rhod-2 from Molecular Probes (Eugene, OR); Anti-RyR (N-19, goat polyclonal IgG) anti-calnexin (rabbit polyclonal), anti-adenine nucleotide translocator (ANT) (Q-18, goat polyclonal) from Santa Cruz (Santa Cruz, Santa Cruz, CA); anti-cytochrome c oxidatse (mouse monoclonal) from Molecular Probes; and anti-voltage-dependent anion channel (VDAC) (mouse monoclonal) from Calbiochem.

Primary Cell culture

All procedures involving animal use were in accordance with *the NIH Guide for the Care and Use of Laboratory Animals*, and were approved by the University of Rochester Committee on Animal Resources. Primary striatal neurons were prepared from Sprague-Dawley rat embryos (embryonic day 18) as described previously [1,3]. Cells were grown in serum containing medium (10% Fetal Bovine Serum, 35 mM glucose, 2 mM L-glutamine, 2.5 μg/ml Fungizone, 50 μg/ml Gentamicin, in Dulbecco's Modified Eagle's Medium; final osmolality ~310 mOsm/l) up to 5 days. After treatment of 5 μM cytosine arabinoside for 2 days cells were switched to serum-free feeding medium (35 mM glucose, 0.5 mM L-Glutamine, 27.5 mM NaCl, 2.5 μg/ml Fungizone, N2 supplement, in Neurobasal Medium; final osmolality ~310 mOsm/l), to reduce the growth of glia cells. Cells were used for experiments after 10-14 days.

Isolation of rat brain mitochondria and preparation of mitochondrial subfractions

Mitochondria-enriched fractions from rat brain were isolated in isotonic ice-cold mannitol/sucrose buffer (M/S-buffer; 225 mM mannitol; 70 mM sucrose; 0.5 mM EGTA; 1 mM glutathione; 10 mM HEPES, pH 7.1) by differential centrifugation and subsequent Percoll purification as we previously described [2]. The final washes were done in EGTA free buffer.

Mitochondrial subfractions were prepared as described recently [5]. Isolated mitochondria were osmotically shocked first with 10 mM Na_2HPO_4/NaH_2PO_4 for 20 min on ice (swelling of mitochondria) and then in sucrose (20 % final sucrose concentration, shrinkage of mitochondria). Mitochondrial membranes were disrupted by sonication and eventually remaining intact mitochondria were removed by centrifugation at 7,000g. The supernatant containing the mitochondrial membranes vesicles was layered on top of a continuous sucrose gradient (60% to 30%) and centrifuged for at least 8 hr at 70,000g to separate the mitochondrial subfractions. The subfractions were then diluted at least two-fold with ice-cold M/S-buffer containing a protease inhibitor cocktail (Complete, Roche, Indianapolis, IN) and centrifuged for 90 min at 250,000g to sediment the mRyR. The pellet was resuspended in M/S-buffer and stored at -80^{o} C until used.

Western blotting

Equal amounts of proteins (100 µg of protein for RyR, 50 µg of protein for calnexin, VDAC and ANT were separated by 5 % (for RyR) or 12 % (for other proteins) SDS-PAGE and then transferred onto a nitrocellulose membrane. Blots were probed with specific primary antibodies, followed by a horseradish peroxidase conjugated secondary antibody. The signal was detected using the enhanced chemiluminescence kit (GE Healthcare Life Sciences).

Fluorescence microscopy

Localization of RyR was observed in live or fixed cultured striatal neurons using confocal microscopy (Leica, Heidelberg Germany).

For live cell images, RyR protein was labeled with Bodipy-Ry or Bodipy-TR-Ry (0.1 to 1 μM for 15 min at room temperature) in the presence 10 mM caffeine to promote high affinity ryanodine binding. For labeling of sarco/endoplasmic Ca^{2+}-ATPase (SERCA), Bodipy-Thap (1 μM for 1 min) was used. For labeling of mitochondria, Cells were co-stained with Mitotracker Deep Red (MTR) (500 nM for 15 min). All unbound fluorescent dyes were washed off with saline (in mM: 144 NaCl, 10 HEPES, 2 $CaCl_2$ 1 $MgCl_2$, 5 KCl, 10 Glucose; set to pH 7.4 and 312 mOsm) before image acquisition. Bodipy-Ry and Bodipy-Thap were excited at 488 nm and the emission was measured at 530 nm. Bodipy-TR-Ry and MTR were excited at 633 nm the emission was measured > 650 nm. No significant background fluorescence was observed before staining (data not shown). The pre-treatment of unlabeled ryanodine (10 μM for 30 min) completely abolished the bodipy-Ry staining, confirmed that Bodipy-Ry specifically binds to RyRs in live striatal neurons (Supplementary Fig.1). For time lapse studies, live cell images were collected using TILL system (TILL Photonics, München, Germany). Images were collected using a CCD camera with standard FITC optics.

For detection of RyR in fixed striatal neurons, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with ice-cold acetone for 15 min. After incubation in blocking buffer (1 % BSA, 0.05 % Tween-20, 0.005 M EGTA in phosphate buffered saline) for 1 hr, cells were then probed with specific primary antibodies followed by a fluorescence-conjugated secondary antibodies.

Scatter 2D plots of pixel intensities in red and green channels were generated by Image J software (NIH) (see also Fig. 2C) [6]. Region 1 and 2 pixels represent signal in channel 1 or 2 only, respectively and region 3 represents co-localized pixels (see Fig.

2C). Quantitative co-localization analysis was performed using Image J software (NIH) with an Intensity Correlation Analysis plug-in (The Bob and Joan Wright Cell Imaging Facility, Toronto Western Hospital). Co-localization was estimated using Pearson's correlation coefficient [6,8]. The values for Pearson's correlation are ranged from 1 to -1. A value of 1 represents perfect correlation; -1 represents perfect exclusion and zero represents random localization.

Measurements of cytosolic and mitochondrial Ca^{2+} concentration

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in striatal neurons was measured by Ca^{2+} indicator Fura-2. Cells were permeabilized with saponin and the changes in external Ca^{2+} were estimated as $[Ca^{2+}]_c$. For measurements of $[Ca^{2+}]_c$ in permeabilized striatal neurons, the recording buffer contained (in mM): KCl 120, NaCl 10, glucose 10, Na_2ATP 5, Na_2CrP 15, EGTA 0.1, HEPES 10 at pH 7.20 and 10 μM fura-2. Images were taken with a fluorescent microscope (TILL Photonics). Calibration of the measured fura-2 were performed as previously reported [1,4].

For mitochondrial Ca^{2+} measurements, cells were skinned by saponin and then perfused with recording buffer containing 100 nM Ca^{2+} with an acetoxymethyl (AM) ester form of Fura-2 or Rhod-2 (4 μM for 30 min at room temperature) [7]. To remove the extra-mitochondrial fluorescent indicator, cells were perfused with recording buffer containing 100 nM Ca^{2+} without indicators. Rhod-2 labeled mitochondria were excited at 530 ± 15 nm and fluorescence emission collected using a 565 nm long pass filter. Images were acquired and displayed as $\Delta F/F_0$ ($[(F-F_0)/F_0]$), where F is the recorded fluorescence and F_0 was obtained from the average of 15 sequential frames prior to stimulation [7]. Calibration of the measured fura-2 were performed as previously reported [1,4].

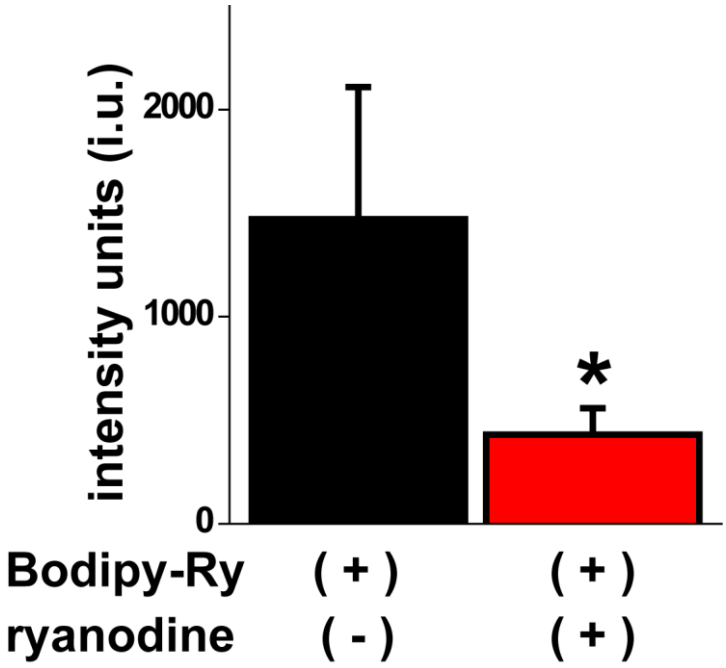
[^{3}H]ryanodine binding

For binding assays, 100 μg mitochondrial protein were incubated with different concentrations of [^{3}H]ryanodine in 0.5 ml binding buffer (in mM: 170 KCl, 0.02 $CaCl_2$, 10 MOPS, pH 7.0) for 16 h at 25°C as we described previously [2]. For non-specific binding, Ca^{2+} was replaced by 6 mM EGTA. At the end of the incubation time, the reaction mixture was filtered under reduced pressure through glass fiber filters (GE Healthcare Bio-Sciences) and washed with ice-cold buffer three times (170 mM KCl, 10 mM MOPS). The radioactivity in the dried membrane was counted with a liquid scintillation counter using a toluene-based scintillator. In some experiments, [^{3}H]ryanodine binding to isolated mitochondria was performed in the presence or absence of 20 mM caffeine in binding buffer or under the various free Ca^{2+} concentrations.

Data and Statistical Analysis

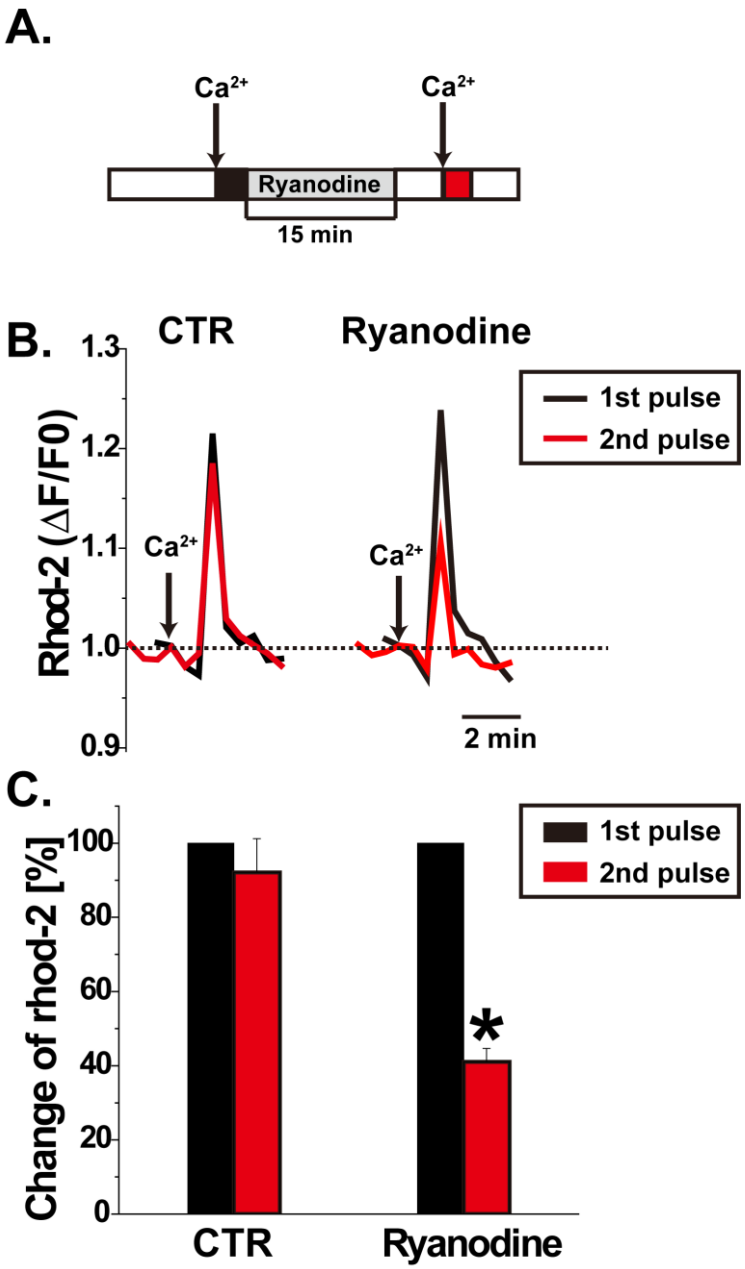
All results are shown as mean standard deviation (SD) or standard error (SE). Unpaired Student's t-test was performed. Statistical significance was set as a *p* value of <0.05.

Supplementary Figures



Supplementary Fig.1. Effect of the pretreatment of unlabeled ryanodine on Bodipy-Ry staining in live striatal neurons

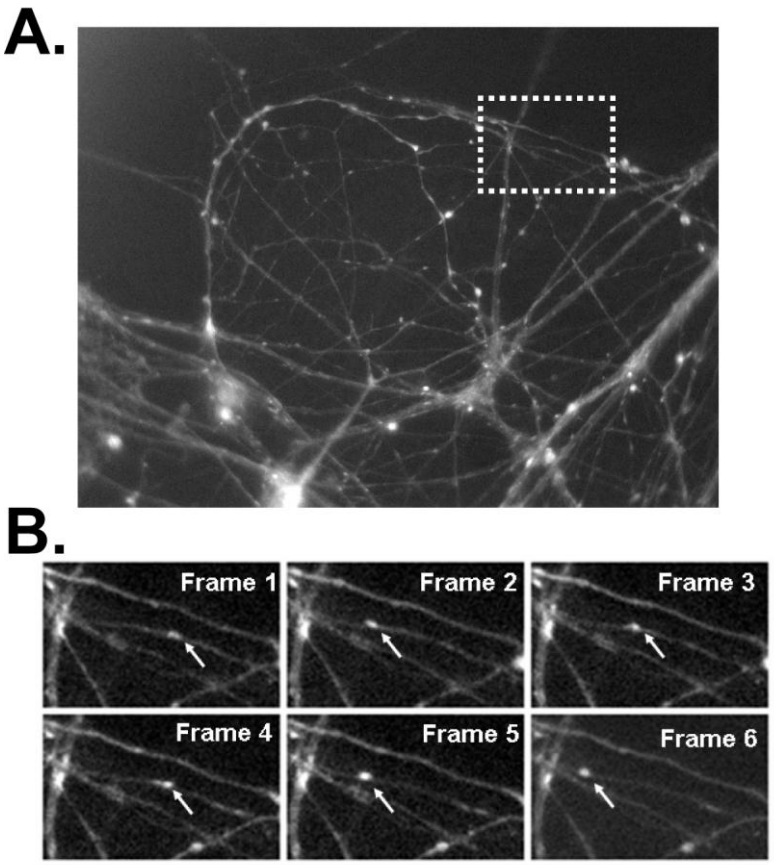
In order to confirm the specificity of the Bodipy-Rya fluorescence signal, neurons were pre-treated first with 10 μM unlabeled ryanodine for 30 minutes ('cold ryanodine') and then with 1 μM Bodipy-Rya for 30 minutes. The competition for the ryanodine binding sites by cold ryanodine decreased the binding of Bodipy-Ry significantly. Five fields were randomly chosen in each coverslip. Fluorescence intensity was determined using Vision software (TILL Photonics). Data are presented as mean ± SD. *p< 0.05, compared to Bodipy-Ry staining without pretreatment of cold ryanodine.



Supplementary Fig.2. Ryanodine and dantrolene block mitochondrial Ca^{2+} uptake in skinned striatal neurons

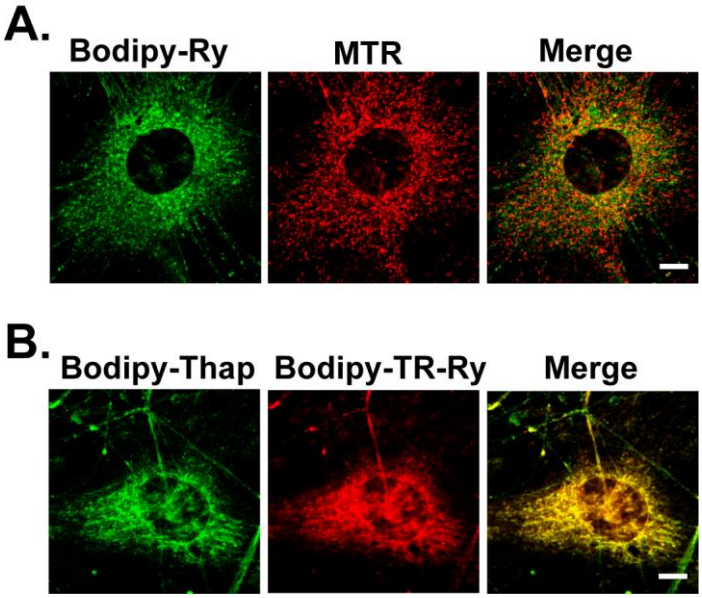
A. Schematic diagram of the protocol for the measurement of mitochondrial Ca^{2+} uptake in skinned striatal neurons. Recording buffer contains 100 nM Ca^{2+} to mimic a $[Ca^{2+}]_c$ (shown as a white belts). First Ca^{2+} pulse (the addition of 3 μM Ca^{2+} into the recording chamber) led to an increase of $[Ca^{2+}]_m$, as measured by an increase of Rhod-2

fluorescence (shown as a black belt). Then the neuron was exposed to 100 μM ryanodine for 15 min in the presence of 3 μM Ca^{2+}, to increase ryanodine binding (shown as a grey belt). After the incubation time the Ca^{2+} concentration in the buffer was reduced to 100 nM again (shown as a white belt after the grey belt) and the second Ca^{2+} pulse was added (shown as a red belt). **B.** Representative traces of Rhod-2 fluorescence in the presence (left) or in the absence (right, control: CTR) of the Ryanodine incubation. Black traces are from the first Ca^{2+} pulse and red traces are from the second Ca^{2+} pulse. Changes in $[Ca^{2+}]_m$ were displayed as $\Delta F/F_0$ ($[(F-F_0)/F_0]$), where F is the recorded fluorescence and F_0 was obtained from the average of 15 sequential frames prior to stimulation [7] **C.** The changes in Rhod-2 fluorescence intensity before (first pulse, black) and after inculcation (second incubation, red). The Rhod-2 fluorescence ($\Delta F/F_0$) was normalized by that before incubation. *$p<0.05$, compared to the Rhod-2 fluorescence intensity before incubation (first pulse, black).



Supplementary Fig.3. Time lapse images in live cultured striatal neurons stained by Bodipy-Ry

A. Representative images of live cultured striatal neurons stained by Bodipy-Ry. Dot square region were used for time lapse study in B. **B.** Typical stages of the movement of the mitochondrion along the neurite. Frame shot images show that the mitochondrion changes its direction of movement during the time course. Frame1 is the image at 40sec after recording; Frame 2, 4 min C; Frame 3, 11 min 40 s Frame 4, 12 min 40s; Frame 5, 14 min 10s; and Frame 6, 15 min.



Supplementary Fig.4. RyR is expressed in ER, but not in mitochondria in glia cells.

A. Less colocalization of the Bodipy-Ry signal and MTR in glia cells. The Pearson's value for Bodipy-Ry/ MTR staining was 0.276. Scale bar, 10 μm. **B.** Bodipy-TR-Ry was almost colocalized with the ER marker Bodipy-Thap, suggesting that RyR is expressed in ER, but not in mitochondria in glia cells. The Pearson's value for Bodipy-Ry/ MTR staining was 0.828. Scale bar, 10 μm.

References

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