SUPPLEMENTARY MATERIALS

Nitric oxide-induced activation of the type 1 ryanodine receptor is critical for epileptic seizure-induced neuronal cell death

Yoshinori Mikami[1,2], Kazunori Kanemaru[1], Yohei Okubo[1], Takuya Nakaune[1], Junji Suzuki[1], Kazuki Shibata[3], Hiroki Sugiyama[3], Ryuta Koyama[3], Takashi Murayama[4], Akihiro Ito[5,6], Toshiko Yamazawa[7], Yuji Ikegaya[3], Takashi Sakurai[4], Nobuhito Saito[5], Sho Kakizawa[8*] & Masamitsu Iino[1,9 *]

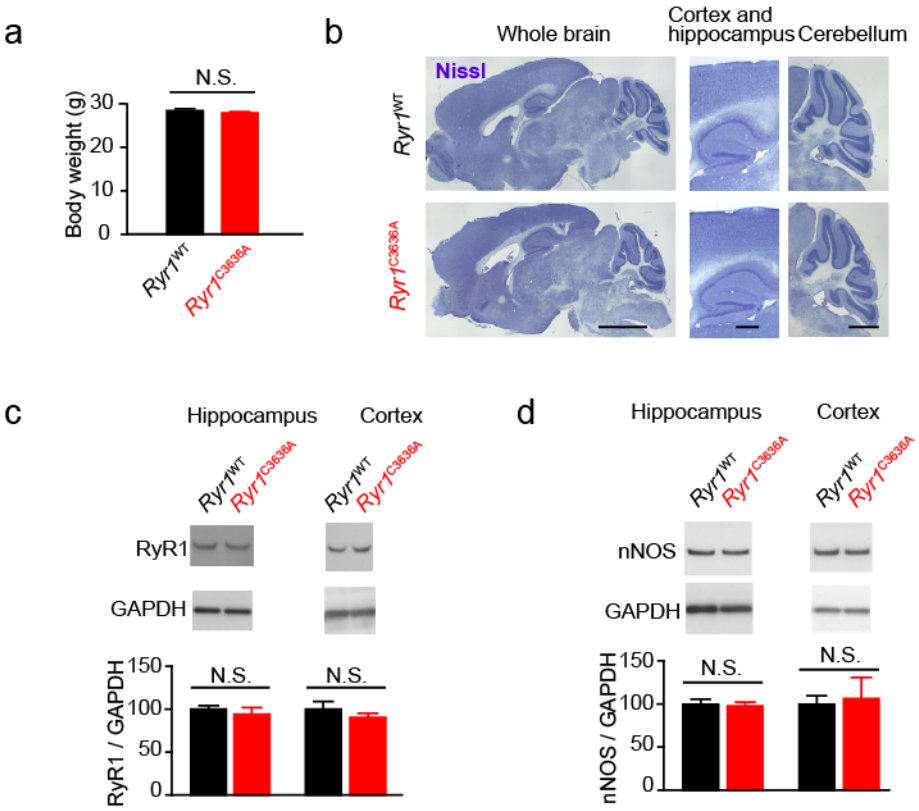

Figure S1 Characterization of *Ryr1*^{C3636A} mice and characterization of RyR1^{C3636A} channels in brain (Relates to Figure 1)

(a). Body weights of *Ryr1*^{C3636A} and *Ryr1*^{WT} littermates at 12 weeks of age. n = 7–8. Error bars indicate s.e.m. N.S., not significant, t-test compared with control. **(b)** Representative images of Nissl-stained sagittal sections from brains of *Ryr1*^{WT} littermates and *Ryr1*^{C3636A} mice at 12 weeks of age. Scale bar: 5 mm (whole brain), 500 µm (cortex and hippocampus), 1 mm (cerebellum). **(c)** Immunoblotting of cytosol and microsome-enriched fractions from hippocampus (left) and cerebral cortex (right) using antibodies against RyR1 and GAPDH (loading control). Samples were collected from *Ryr1*^{C3636A} and *Ryr1*^{WT} littermates at 12 weeks of age; n = 3–4. Error bars indicate s.e.m. N.S., not significant, t-test compared with control. **(d)** Expression levels were analyzed by immunoblotting using antibodies to nNOS and GAPDH; n = 3–4. Error bars indicate s.e.m. N.S., not significant, t-test compared with control.

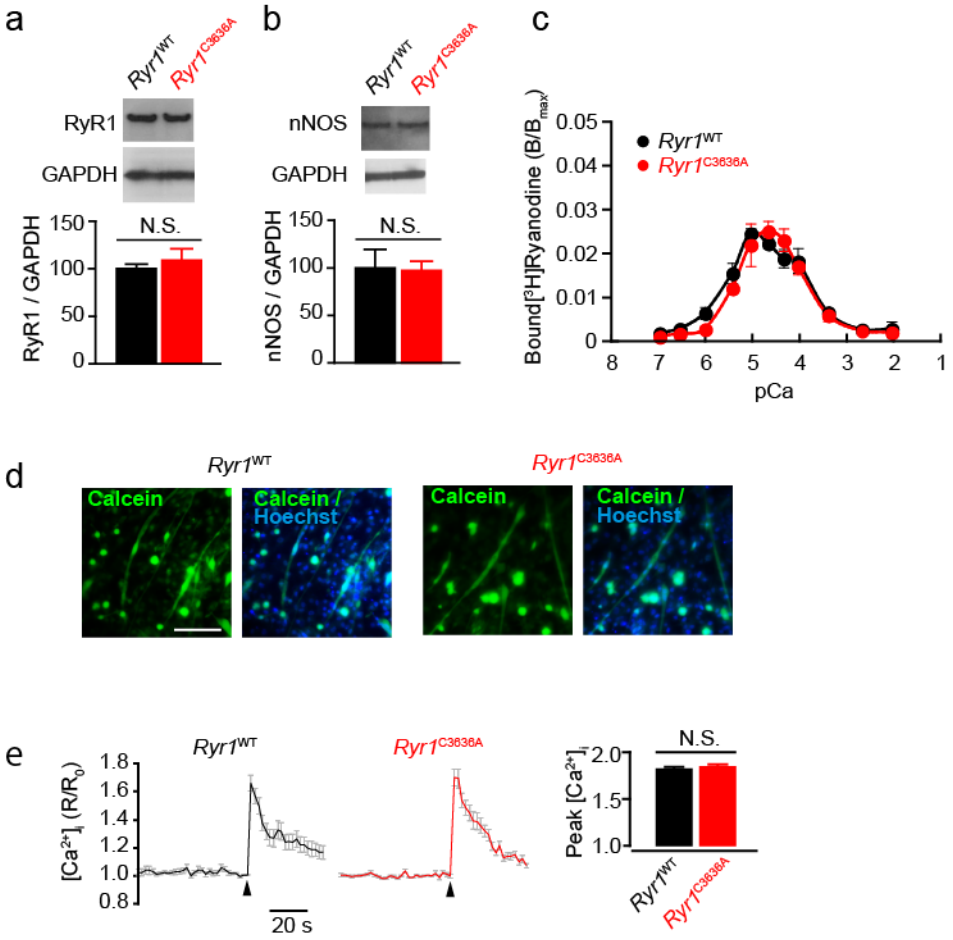

Figure S2 Characterization of RyR1^{C3636A} channels in skeletal muscle (Relates to Figure 1)

(a, b) Immunoblotting of cytosol and microsome-enriched fractions from skeletal muscle, using antibodies against RyR1 (a) or nNOS (b) and GAPDH (loading control). Samples were collected from *Ryr1*^{C3636A} and *Ryr1*^{WT} littermates at 12 weeks of age; n = 3–4**.** Error bars indicate s.e.m. N.S., not significant, *t*-test compared with control. **(c)** Ca^{2+}-dependent [^{3}H]ryanodine binding to *Ryr1*^{WT} (black) and *Ryr1*^{C3636A} (red) sarcoplasmic reticulum vesicles. **(d)** Fluorescence images of calcein-AM (green) and Hoechst 33342 (blue) stained primary cultured myocytes. Scale bar: 100 µm. **(e)** Intracellular Ca^{2+} response to field stimulation in *Ryr1*^{C3636A} and *Ryr1*^{WT} myocytes measured at 2 frames s^{-1}. R (F_{340}/F_{380} of Fura-2) was normalized by the initial R (R_0). Peak amplitudes were compared between *Ryr1*^{WT} (36 cells) and *Ryr1*^{C3636A} (42 cells) myocytes. Error bars indicate s.e.m. N.S., not significant, *t*-test compared with control.

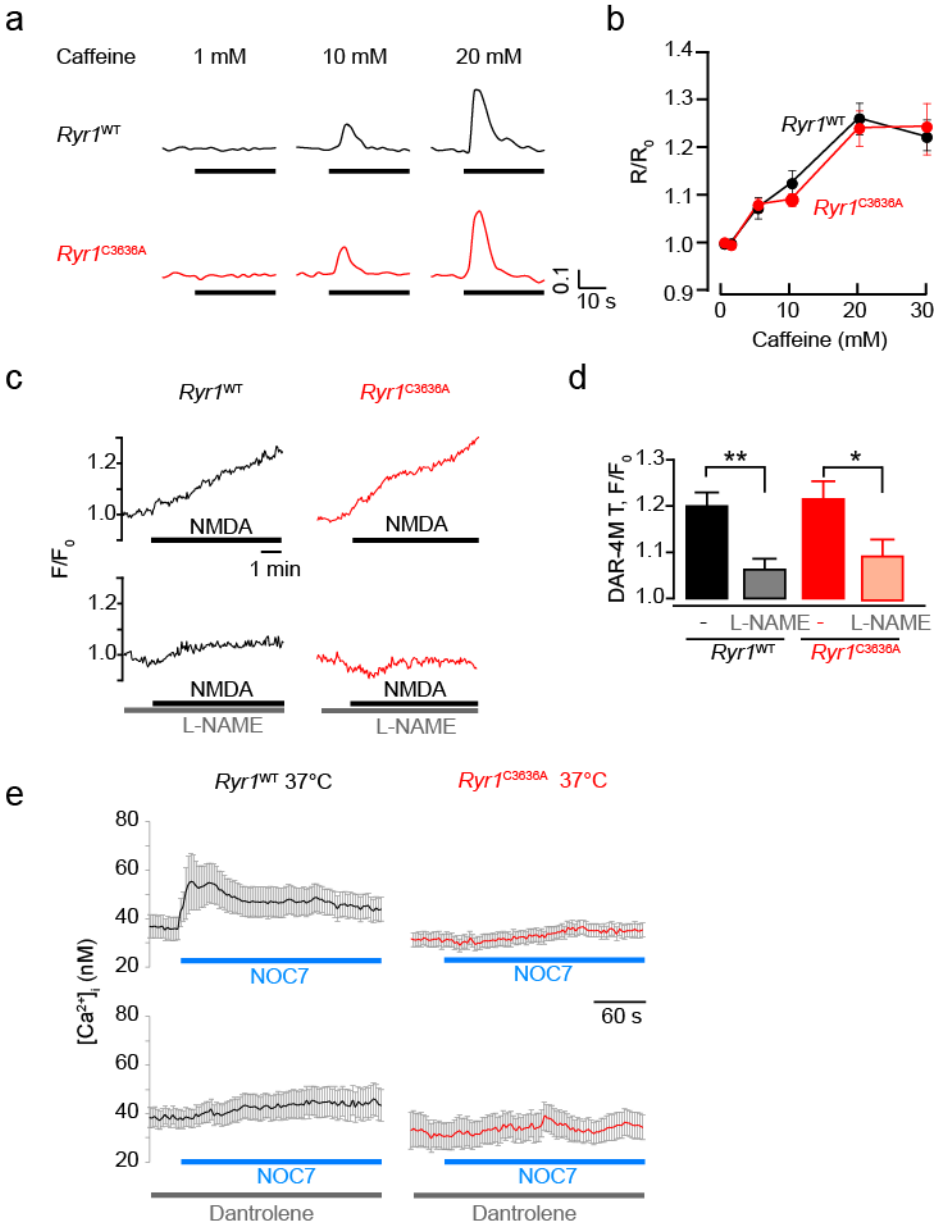

Figure S3 Characterization of RyR1^{C3636A} channels in primary cultured neurons (Relates to Figure 1)

(a) Caffeine-induced Ca^{2+} responses (expressed as R/R_0) in *Ryr1*^{C3636A} and *Ryr1*^{WT} neurons. **(b)** Peak amplitudes of $[Ca^{2+}]_i$ at varied caffeine concentrations in *Ryr1*^{WT} and *Ryr1*^{C3636A} neurons; n = 13–35 neurons. **(c)** NO production measured with DAR-4M with or without a NOS inhibitor L-NAME. Fluorescence intensity of DAR-4M was normalized by the initial value. **(d)** Magnitude of NO production at 6 min after administration of NMDA in *Ryr1*^{WT} (n = 15–19 cells) and *Ryr1*^{C3636A} (n = 18–19 cells). Error bars indicate s.e.m. t-test. * $p < 0.05$, ** $p < 0.01$. **(e)** NICR was blocked by dantrolene**.** NOC7 (500 μM)-induced intracellular Ca^{2+} increase at 37°C with or without dantrolene (10 μM) in cultured cerebral neurons of *Ryr1*^{WT} or *Ryr1*^{C3636A} mice; n = 17–30 neurons, error bars indicates s.e.m.

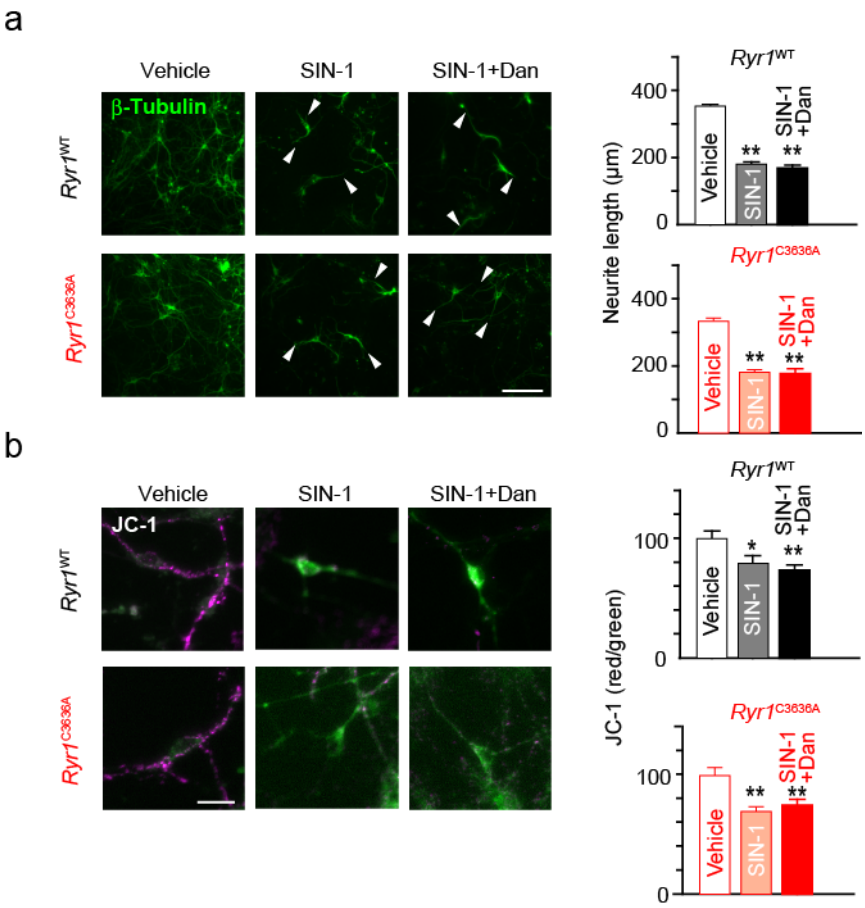

Figure S4 Peroxynitrite donor SIN-1 induces neuronal cell death in both *Ryr1*^{WT} and *Ryr1*^{C3636A} neurons to the same extent (Relates to Figure 3)

(a) Effect of the application of peroxynitrite donor SIN-1 (3-morpholino-sydnonimine; 100 μM) on neuron morphology (stained with anti-β-III tubulin antibody). Arrowheads indicate short and curly neurites. Graphs show the length of the longest neurite of each neuron; n = 95–125; error bars indicate s.e.m. Data were analyzed for significance using ANOVA followed by a Tukey-Kramer *post-hoc* test. ** $p < 0.01$. Dan, dantrolene. **(b)** Cell viability examined by JC-1 assay. n = 65–81. Error bars indicate s.e.m. Data were analyzed for significance using ANOVA followed by a Tukey-Kramer *post-hoc* test. ** $p < 0.01$, * $p < 0.05$.