Supporting Information

Shimizu *et al.* 10.1073/pnas.0802175105

SI Text

Materials and Methods. ***Antibody.*** We used primary antibodies raised against the following molecules (species immunized); RyR2 (rabbit and guinea pig), VGluT1 (guinea pig, ref. 1; and goat, ref. 2), neurofilament160 (mouse; Sigma N5264), MAP2 (goat; ref. 2). A RyR2 antibody was produced using GST (GST)-fusion protein carrying 4346–4484 aa residues of the mouse RyR2 (GenBank accession no. NM023868) and purified using an affinity column coupled to GST-free RyR2 polypeptides, as reported previously (3). For specificity control, the RyR2 antibody was preabsorbed by overnight incubation with 50 μg/ml of the antigen.

Immunoblot. The microsomal fraction of the brain and the heavy sarcoplasmic reticulum (HSR) fraction of the heart and femoral muscles were prepared for immunoblot analysis. Brains were homogenized using a Potter homogenizer in 9 volumes of a solution containing 0.32 M sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM 2-mercaptoethanol, and 5 mM Tris-HCl (pH 7.4). After centrifugation at $10{,}000 \times g$ for 15 min, the supernatant of brain homogenates was centrifuged at $100{,}000 \times g$ for 1 h to obtain the precipitate (i.e., microsome fraction). The heart and femoral muscles were homogenized using a Polytoron homogenizer in a solution containing 5 mM Tris-maleate (pH7.0), 0.1M NaCl, 2.5 mM EGTA (pH8.0), and 0.1 mM PMSF, and were centrifuged at $4{,}000 \times g$ for 30 min. The supernatant was filtered through two sheets of cheesecloth and centrifuged at $9{,}500 \times g$ for 30 min. The precipitate was suspended in a solution containing 5 mM Tris-maleate (pH7.0), 0.6 M KCl, 2.5 mM EGTA (pH8.0), and 0.1 mM PMSF, and was centrifuged at $10{,}000 \times g$ for 30 min. The precipitate was suspended in a solution containing 5 mM Tris-maleate (pH7.0), 0.1 M KCl, and 0.1 mM PMSF, and was centrifuged at $10{,}000 \times g$ for 30 min to precipitate the crude HSR fraction. The protein concentration was determined by the Lowry's method.

Proteins (20 μg/lane) were separated by 5% SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes (BioTraceNT; PALL). After blocking with 5% skimmed milk for 1 h, membranes were incubated for 2 h with the RyR2 antibody (1 μg/ml). Tris-buffered saline (pH 7.5) containing 0.1% Tween 20 was used as diluent and a washing buffer. Immunoreaction was visualized with the ECL chemiluminescence detection system (Amersham Biosciences).

In situ *hybridization.* Antisense oligonucleotide probes to the mouse RyR2 mRNA were synthesized against the following cDNA regions: 5′-cacagccgagagtataagcaggagaggacatacacacgagacctg-3′ (2506–2550 nucleotide residues) and 5′-tctgtggttgaaggaaaggagctgcctacccgaacctccagcgat-3′ (13573–13617). They were radiolabeled with [^{33}P]dATP using terminal deoxyribonucleotidyl transferase (Invitrogen).

Under deep pentobarbital anesthesia (100 mg/kg of body weight, i.p.), brains were freshly removed from the skull and frozen in powdered dry ice for *in situ* hybridization. Frozen sections were prepared on a cryostat (20 μm in thickness; CM1900; Leica) and mounted on silane-coated glass slides (Muto-Glass). Fresh frozen sections were treated at room temperature with the following incubations: fixation with 4% paraformaldehyde in the PB for 10 min, 2 mg/ml glycine-PBS (PBS, pH 7.2) for 10 min, and acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min. Sections were subjected to prehybridization for 1 h in a buffer containing 50% formamide, 50 mM Tris-HCl (pH 7.5), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA, 0.6 M NaCl, 0.25% SDS, 200 μg/ml tRNA, and 1 mM EDTA. Hybridization was performed at 42°C for 12 h in the prehybridization buffer supplemented with 10,000 cpm/μl of [^{33}P]dATP-labeled probes and 10% dextran sulfate. Slides were washed twice at 55°C for 40 min in 0.1 x SSC containing 0.1% sarcosyl. Sections were exposed to BioMax film (Kodak). The specificity of hybridization was confirmed by identical patterns identified by two non-overlapping probes and by blank signals obtained when hybridization was carried out in the presence of an excess amount of unlabeled probes.

1. Miyazaki T, Fukaya M, Shimizu H, Watanabe M (2003) Subtype switching of vesicular glutamate transporters at parallel fibre-Purkinje cell synapses in developing mouse cerebellum. *Eur J Neurosci* 17:2563–2572.
2. Miura E, *et al.* (2006) Expression and distribution of JNK/SAPK-associated scaffold protein JSAP1 in developing and adult mouse brain. *J Neurochem* 97:1431–1446.
3. Nakamura M, *et al.* (2004) Signaling complex formation of phospholipase C β4 with metabotropic glutamate receptor type 1 α and 1,4,5-trisphosphate receptor at the perisynapse and endoplasmic reticulum in the mouse brain. *Eur J Neurosci* 20:2929–2944.

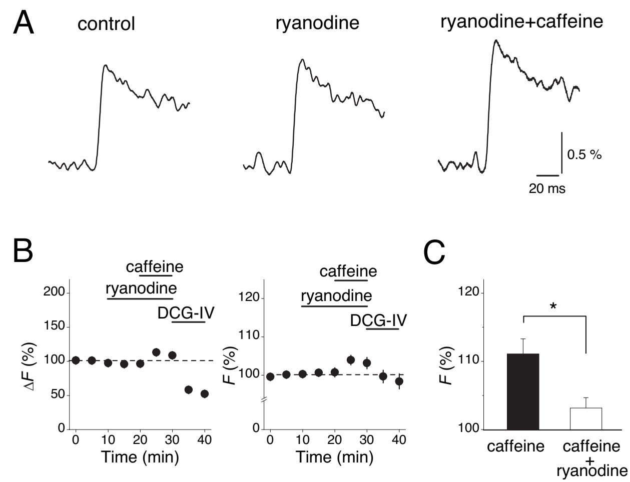

Fig. S1. Suppression of caffeine-induced enhancement of presynaptic Ca^{2+} levels by ryanodine. (*A*) Effects of of caffeine (10 mM) on presynaptic Ca^{2+} signals in the presence of ryanodine (100 μM). (*B*) The time course of the presynaptic Ca^{2+} transient (ΔF, *left*) and baseline fluorescence (*F*, *right*). (*C*) Quantitative data of presynaptic Ca^{2+} levels (*F*) in the experiment in (*B*) are shown as an open bar and are compared with those in Fig. 1*I* shown as a closed bar. The difference is statistically significant ($P < 0.05$, *t* test).

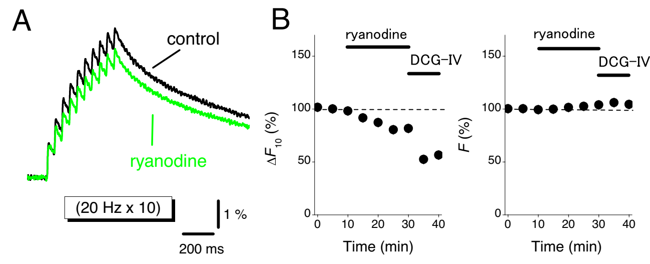

Fig. S2. Use dependent effects of ryanodine on the presynaptic Ca^{2+} transients. (*A*) Effect of 100 μM ryanodine on the presynaptic Ca^{2+} transients elicited by the stimulus train (20 Hz 10 times). (*B*) The time course of the presynaptic Ca^{2+} transient to the 10th stimuli (ΔF_{10}, left) and baseline fluorescence (*F*, right).

Fig. S3. Activity dependent presynaptic Ca^{2+} release is independent from α_7 nicotinic acetylcholine receptors. (*A*) Effect of 10 μM methyllycaconitine (MLA), an α_7 nicotinic acetylcholine receptor-selective antagonist, on the train-induced presynaptic Ca^{2+} transients (20 Hz 10 times). (*B*) The time course of the presynaptic Ca^{2+} transient to the 10th stimuli (ΔF_{10}). (*C*) Summary of the data obtained in the experiments shown in *B* (closed bars) and Fig. 3*A* (open bar).

Fig. S4. Production and specificity of the type 2 ryanodine receptor (RyR2) antibody and immunohistochemistry. (*A*) Alignment of the antigen sequence for RyR2 antibody production (underline) with the sequence of the type 1 ryanodine receptor (RyR1). The antigen sequence has low sequence similarity to the RyR1 (the skeletal muscle type) and is missing in the type 3 ryanodine receptor (RyR3, the brain type). Amino acid residues identical between the RyR1 and RyR2 are indicated by asterisks. (*B*) Immunoblot. An affinity-purified rabbit antibody (anti-RyR2) recognized a single protein band (>500 kDa) in protein extracts prepared from the brain (Br) and cardiac muscle (CM), but not from the skeletal muscle (SM). (*C*-*G*) Immunofluorescence for the RyR2 in the cardiac muscle (*C* and *D*), smooth muscle (*E*), and brain (*F* and *G*). The nucleus was counterstained with DAPI in panels *C*-*E*. (*H*) *In situ* hybridization for the RyR2 mRNA in the adult mouse brain, control experiments with use of the RyR2 antibody preabsorbed with the antigen; Cx, cerebral cortex; Cb, cerebellum; cc, corpus callosum; CPu, caudate putamen; Mb, midbrain; MO, medulla oblongata; OB, olfactory bulb; Th, thalamus. Scale bars: *C*-*E*, 1 μm; *F*-*H*, 1 mm.

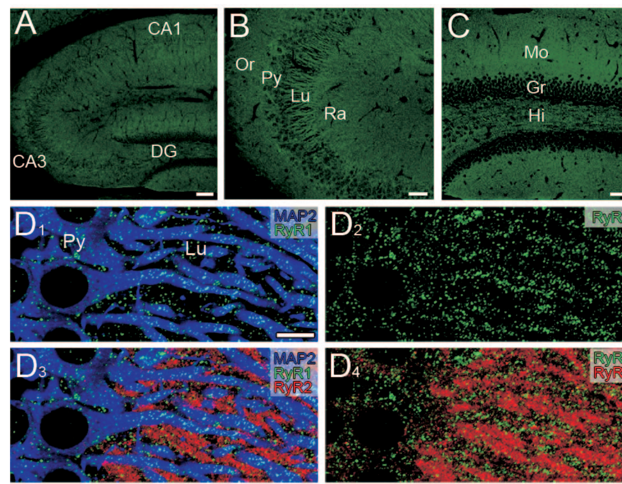

Fig. S5. Predominant postsynaptic distribution of RyR1 in the hippocampus. (*A-C*) RyR1 (green) is expressed at a relatively low level and homogeneously distributed in the adult hippocampus, as compared with the distribution of RyR2 shown in Fig. 5. In the stratum lucidum (Lu) of the CA3 region, signals for RyR1 were arranged in a palisade-like fashion, with their long axis perpendicular to the pyramidal cell layer. (*D*) Triple immunofluorescence for RyR1(green), RyR2 (red), and MAP2 (blue). Punctuate labeling for RyR1 is densely distributed along dendritic shafts and perikarya of CA3 pyramidal cells, whereas it is sparse in the neuropil outside dendritic shafts (i.e., mossy fiber terminal zone). Consequently, postsynaptic RyR1 and axonal RyR2 display almost reciprocal distribution in the stratum lucidum of the CA3 region. See Fig. S5 in Kakizawa *et al.* (32) for the production and specificity of rabbit RyR1 antibody. Scale bars, *A*, 100 μm; *B* and *C*, 50 μm; *D*, 10 μm.