Supporting Information

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SI Methods

Synaptosomal Plasma Membrane Preparation. Synaptosomal plasma membranes (SM) were purified as previously described (1), with slight modifications. Adult rat brains were homogenized in Hepes-buffered sucrose (0.32 M sucrose, 4 mM Hepes/NaOH [pH 7.4]) supplemented with protease inhibitors (1 μg/ml of pepstatin, chymostatin, leupeptin, aprotinin, and 0.4 mM phenylmethylsulfonylfluoride) using a glass homogenizer. The homogenate was centrifuged at 16,000 $\times$ *g* to obtain P2 (crude synaptosomal fraction). The lysed P2 was resuspended in Hepes-buffered sucrose and layered onto a discontinuous sucrose gradient composed of 0.8, 1.0, and 1.2 M sucrose layers and centrifuged for 2 h at 65,000 $\times$ *g*. The SM fraction was recovered from the 1–1.2 M sucrose interface and pelleted by centrifugation. To obtain 1T fraction, the SM pellet was treated for 15 min with 0.5% Triton X-100 in Hepes-EDTA solution (50 mM Hepes, 2 mM EDTA, and protease inhibitors) at 4 °C and pelleted by centrifugation for 20 min at 200,000 $\times$ *g*. To obtain 2T fraction, the fraction 1T was treated a second time with 0.5% Triton X-100 in Hepes-EDTA solution.

Discontinuous Sucrose Density Gradient Centrifugation. Purified synaptosomes were prepared as previously described (2) by pouring over washed P2 fraction into a sucrose density gradient composed of 0.85, 1, and 1.2 M sucrose and centrifuging for 2 h in 150,000 $\times$ *g*. The purified synaptosomal fraction was recovered from the 1–1.2 M sucrose interface, pelleted by centrifugation, and resuspended in fresh Hepes-buffered sucrose. The synaptosomal fraction was subsequently lysed with 1% Triton X-100 in TNE buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, and protease inhibitors) and rotated at 4 °C for 1 h. The suspension was adjusted to 40% sucrose, overlayed with 30% and 5% sucrose, and then centrifuged for 16 h at 180,000 $\times$ *g*. The gradient was collected in 12 equal fractions from the top to the bottom, and the last included the pellet. Each fraction was analyzed by SDS-PAGE followed by Western blotting.

Preparation of Membrane and Cytosol Fractions for Activity Experiments. Adult rat brains were homogenized in 5 volumes of solution A containing 10 mM KPi (pH 7.2), 50 mM KCl, 15 μM pyridoxal 5′-phosphate (PLP), 2 mM EDTA, 2 mM DTT, and protease inhibitors using a glass homogenizer. The homogenate was centrifuged at 1,200 $\times$ *g* for 15 min to remove nuclei and cell debris. The supernatant was centrifuged at 200,000 $\times$ *g* for 2 h to obtain cytosolic (C) and membrane (M) fractions. The fraction C was dialyzed for 6 h against solution A to remove endogenous cytosolic amino acids. To remove any loosely bound SR from the membrane, the fraction M was washed with buffer A supplemented with 0.5% Triton X-100 and 200 mM NaCl. Fraction M was subsequently resuspended a second time with solution A (lacking detergent), washed by centrifugation, and stored along with the cytosolic preparates at −70 °C until use.

Determination of SR Activity in Vitro. Membrane and cytosol extracts from rat brain or SH-SY5Y neuroblastoma cells transfected with SR were diluted in reaction medium containing 20 mM Tris-HCl (pH 8.8), 10 mM L-serine devoid of contaminant D-serine (3), 4 mM $MgCl_2$, 0.5 mM ATPγS, and either 2 mM D-alanine or 0.5 mM sodium benzoate to prevent degradation of D-serine by D-amino acid oxidase enzyme activity. The reaction was carried out at 37 °C for 2–4 h under constant agitation and terminated by a 5-min boiling. Blanks were made by adding 2 mM malonate (a SR inhibitor) (4) or by boiling the samples for 5 min before the addition of L-serine. D-serine was detected by HPLC using D-serine deaminase enzyme to confirm the identity of D-serine peak as described previously (5). D-serine levels were normalized to content of SR in each fraction, which was determined by Western blot and densitometry of the chemiluminescent signal (ImageQuant RT-ECL; GE Healthcare Life Sciences).

Primary Neuronal Cultures. Neuronal cultures were prepared as previously described (6) and plated at a density of 2.3 $\times$ 10^5cells/cm^2. On DIV1, the medium was changed to fresh neurobasal/B27 (NB/B27 medium) with 5% glia conditioned medium (GCM). Thereafter, half the medium was changed every 3 days with fresh NB/B27/GCM. To obtain GCM, astrocytes cultures were prepared from cortices of P1-P2 rats as previously described (6). When the confluence reached 80%, the cells were washed, and NB/B27 medium was added for 2 days. The conditioned medium was replaced with fresh NB/B27 every 2 days for 3–4 times. For immunocytochemistry, neuronal primary cultures were plated on polyD-lysine-treated cover glasses at a density of 6 $\times$ 10^4cells/cm^2 and maintained in NB/B27 medium supplemented with 20% GCM.

Antibodies. Protein expression levels were monitored in Western blots, and immunocytochemical experiments using affinity-purified polyclonal anti-SR (Ab2) (6), mouse anti-synaptophysin, clone SVP-38 (Sigma), mouse anti-flotillin, clone 18 (BD Biosciences), mouse anti PSD-95, clone 7E3-IB8 (Sigma), rabbit anti-phospho-ERK1/2 (Sigma), mouse anti-KDEL (10C3; Santa Cruz Biotechnology), mouse anti-β-tubulin, clone SAP,4G5 (Sigma), mouse anti-GM130, clone 35, (BD Biosciences), mouse anti-HA (Covance), mouse anti-transferrin receptor, clone H68.4 (Zymed), and mouse anti Thy 1.1, clone MRC OX-7 (Serotec). Western blots were developed with secondary antibodies consisting of anti-rabbit or anti-mouse conjugated to peroxidase (Jacksons Laboratory). Immunocytochemical experiments were revealed with anti-rabbit Cy3 and anti-mouse Cy2 (Jacksons Laboratory).

Drugs. NMDA, ifenprodil, Ro-25–6981, forskolin, kainate and A23187 were obtained from Sigma. MK-801, t-ACPD, PPPA, bicucculin, and AP5 were purchased from Tocris.

Mass Spectrometry. For MS analysis, HA-SR transfected SH-SY5Y cells were lysed by sonication with 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 50 mM KCl, protease inhibitors, 2 mM pyrophosphate, 1 mM NaF, and 1 mM orthovanadate. Triton X-100 (0.5%) was then added to the samples and rotated for 10 min. After removing cell debris by a 10-min centrifugation at 1,400 $\times$ *g*, the suspension was centrifuged at 200,000 $\times$ *g* for 1 h to obtain cytosolic and membrane fractions. The membrane fraction was washed once by recentrifugation in the same buffer, solubilized with 20 mM Tris-HCl (pH 7.4), 1% SDS, protease inhibitors, and 1 mM EDTA, and then sonicated for 2 min. The solubilized membranes were diluted with 0.9 ml of solution containing 20 mM Tris-HCl (pH 7.4), 2% Triton X-100, 0.5% deoxycholate, and 1 mM EDTA, and cleared by centrifugation at 16,000 $\times$ *g* for 30 min. Immunoprecipitation of HA-SR from the cytosolic and solubilized membrane fractions was carried out with anti-HA affinity matrix. The SR bands were excised from the gel, cut into roughly 1-mm^3 cubes, and washed twice each

with water and 50% acetonitrile (7). The washed gel pieces were shrunk in ACN, rehydrated with 10 mM DTT in 100 mM NH_4HCO_3, and incubated for 45 min at 56 °C. The cysteines were carbamidomethylated for 30 min at room temperature in the dark using 10 mg/ml iodoacetamide in 100 mM NH_4HCO_3. Subsequently, gel pieces were washed twice each with water and 50% ACN, shrunk in ACN, and rehydrated with 5 μg/ml trypsin (modified, sequencing grade; Promega) in 50 mM NH_4HCO_3 at 4 °C. SR was digested overnight at 37 °C. The tryptic peptides were extracted from the gel with 5% formic acid followed by 5% formic acid in 50% ACN, dried down, and desalted using C18 STAGE tips with 5% formic acid and 5% formic acid in 70% ACN as buffer A and B, respectively. LC-MS analysis was performed on an UltiMate 3000 LC system (Dionex) coupled to an ESI-Q-TOF mass spectrometer (Q-Tof micro; Waters). The peptides were loaded onto a custom-made precolumn (75 μm ID, 1 cm length, packed with ReproSil-Pur C18-AQ, 3 μm, from Dr. Maisch GmbH) with a flow rate of 3 μl/min and separated on a custom-made analytical column (50 μm ID, 8 cm length, ReproSil-Pur C18-AQ, 3 μm) with a flow rate of 0.1 μl/min. Peptides were eluted using a stepped linear gradient (0%–10% B in 5 min, 10%–50% B in 30 min, and 50%–100% B in 5 min) using 0.1% formic acid (solvent A) and 0.1% formic acid in 95% ACN (solvent B). Fused-silica PicoTip emitters (SilicaTip, 360/20 μm OD/ID tubing, 10 μM tip ID, coating 1P–4P; New Objective) were used for nESI. The mass spectrometer was operated in an automated data-dependent acquisition (DDA) mode where each MS scan (m/z 350–1,500, 1-s scan time) was followed by 3 MS/MS scans (m/z 50–1,500, 1-s scan time) of the most intense peptide ions (z = 2–4). The interscan delay was 0.1 s. Argon was used as collision gas, and the collision energy applied was dependent on the mass and charge state of the precursor ion. Source parameters were as follows: capillary voltage, 1905 V; sample cone voltage, 32 V; source temperature, 100 °C; and cone gas flow, 60 l/h (N2).

Lentivirus Transduction. HA-tagged SR and HA-SR T227A were subcloned into pTK-208 as lentivirus produced as described previously (8). At DIV 7, primary neuronal cultures were transduced with 20–40 MOI (multiplicity of infection), and the expression of HA-SR was monitored by both immunocytochemistry and Western blot analysis. For membrane-binding experiments, we used cultures that exhibited about 30% infection efficiency on DIV12.

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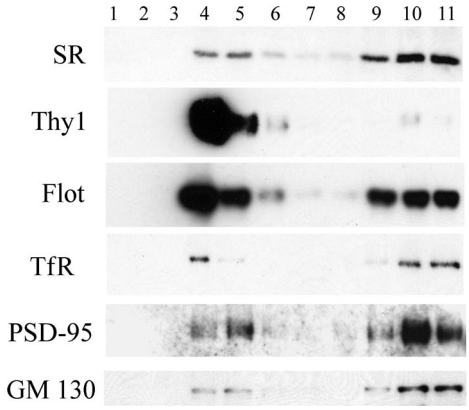

Fig. S1. Distribution of SR on a discontinuous sucrose density gradient. Synaptosomes were lysed with buffer containing 1% Triton X-100 and subjected to discontinuous sucrose density gradient as described in *SI Methods*. Fraction 1 has the lowest density. The fractions were analyzed by Western blot with antibodies against SR, Thy 1, flotillin (Flot), transferrin receptor (TfR), PSD-95, and GM 130 (see *SI Methods*). Lipid rafts are associated with the upper, low-density fractions of the gradient (fractions 4 and 5) enriched with Thy1 and flotillin proteins. SR is enriched in high-density fractions (fractions 9, 10, and 11), composed of a mixture of different types of membranes, containing transferrin receptors, PSD-95, and Golgi matrix protein of 130 kDa (GM130).

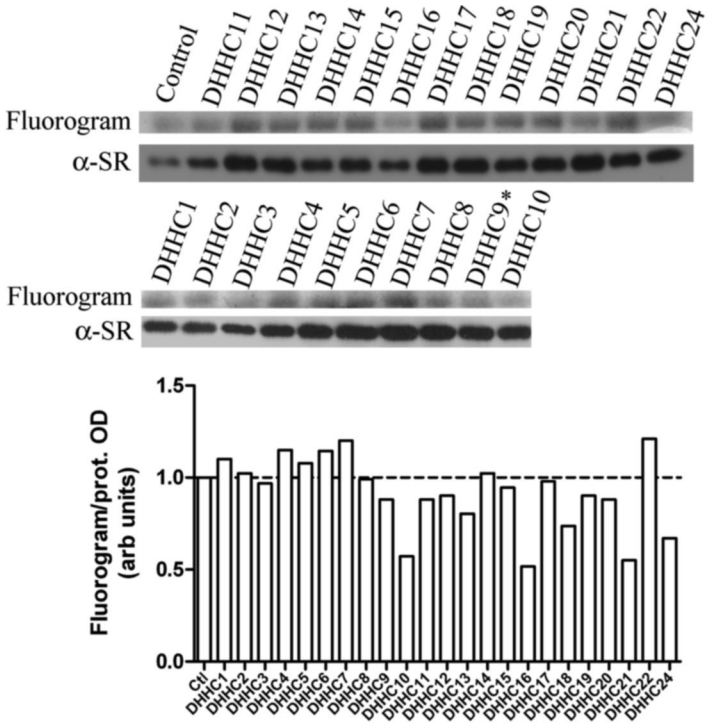

Fig. S2. Lack of SR palmitoylation by DHHC enzymes. HEK 293 cells were cotransfected with 0.4 μg HA-SR and 0.4 μg of each DHHC enzyme. Thirty-six hours after transfection, the cells were labeled with serum-free DMEM containing 1 mCi/ml [^{3}H]palmitic acid in 10 mg/ml BSA (fatty acid free). The cells were washed and lysed, and SR was immunoprecipitated with anti-HA affinity gel. The palmitoylation level was monitored by fluorography (*Upper*) of the SDS-PAGE. Lower panels depict the amount of imunoprecipitated SR by analyzing 1/10th of the immunoprecipitate by Western blotting. *Cotransfected with GCP16 protein, which is required for Ras palmitoylation (Swarthout JT, et al. (2005) DHHC9 and GCP16 constitute a human protein fatty acyltransferase with specificity for H- and N-Ras. *J Biol Chem* 280(35):31141–31148).

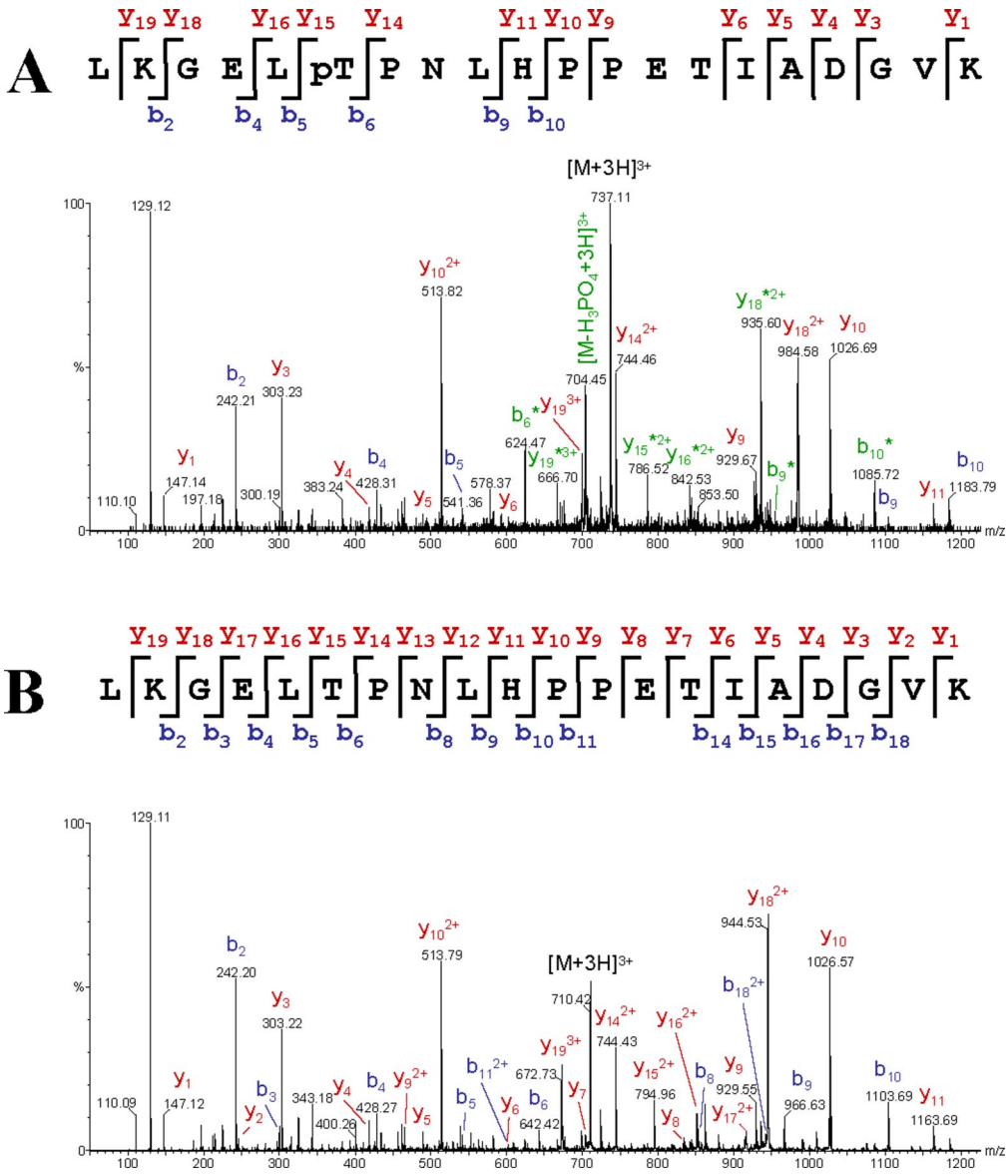

Fig. S3. Membrane-bound SR is phosphorylated at Thr-227. (*A*) ESI-Q-TOF-MS/MS spectrum of the monophosphorylated peptide 222–241 from membrane-bound HA-SR. The fragmentation pattern clearly identifies Thr-227 as the phosphorylation site. (*B*) The ESI-Q-TOF-MS/MS spectrum of the nonphosphorylated peptide 222–241 from cytosolic HA-SR is shown as a comparison. The nonphosphorylated form of this peptide was found in both samples, whereas the peptide showing phosphorylation at Thr-227 was only detected in the membrane fraction. Fragment ions labeled with an asterisk have lost one molecule of phosphoric acid (H_3PO_4).

Table S1. Human *S*-acetyltransferase enzymes employed in this study

Human DHHC[a]	Alternative name	GenBank access
DHHC-1	-	NM_013304
DHHC-2	REAM	BC050272
DHHC-3	GODZ	NM_016598
DHHC-4	-	NM_018106
DHHC-5	-	NM_015457
DHHC-6	-	BC007213
DHHC-7	-	NM_017740
DHHC-8	-	NM_013373
DHHC-9	-	BC012826
DHHC-10	-	NM_024786
DHHC-11	NIDD	NM_173570
DHHC-12	-	NM_032799
DHHC-13	-	NM_207340
DHHC-14	-	NM_024630
DHHC-15	-	BC103980
DHHC-16	Aph2	NM_032327
DHHC-17	HIP14/AKRL1	NM_015336
DHHC-18	-	NM_032283
DHHC-19	-	NM_144637
DHHC-20	-	NM_153251
DHHC-21	-	NM_178566
DHHC-22	HIP14/AKRL2	AB024495
DHHC-24	-	BC117676

[a]The enzymes used in the present study were previously characterized by Fukata *et al.* (1) and were cloned and expressed with FLAG and myc epitope tags in HEK 293 cells as previously described (2).

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