Supporting Information

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SI Materials and Methods

Sample Preparation of SILAM Mouse-Derived Synaptosomes and Cytosol Fractions for MS Analysis. Synaptosomes pellets and cytosol fractions from EE- and control environment-exposed mice brains were further processed using the filter-assisted sample preparation (FASP) method as described earlier (78). Briefly, synaptosome pellets were resuspended in lysis buffer (4% SDS, 0.1 M Tris, pH 7.6, and 100 mM DTT) and heated for 10 min at 95 °C, kept on ice followed by sonication (Bransons) for 30 s for three cycles and heated for another 5 min at 95 °C to ensure complete solubility. Protein lysate was centrifuged at 17,000 × *g* for 10 min and the clear lysates were subjected to the FASP method. SDS and DTT were removed by exchanging the buffer with 9 M urea and 20 mM trimethylamine bicarbonate (TEAB) using 30-kDa cutoff filters (EMD Millipore) and centrifuged at 4,000 × *g* for 10 min. This procedure was repeated two more times. Protein quantification was carried out using bicinchoninic acid assay (BCA). Four hundred micrograms of protein was taken from each sample for MSanalysis. Protein was alkylated using 20 mM iodoacetamide (IAA) and incubated in the dark for 20 min followed by buffer exchange with 9 M urea and 20 mM TEAB two times and with 20 mM TEAB for additional two rounds. The retentate was collected from the filters for the digestion using Lys-C. Twenty micrograms of Lys-C was added to each sample and incubated at 37 °C overnight. The digested peptides were cleaned using Sep-Pak C_{18} cartridges and the eluted peptides were lyophilized. The peptides were fractionated by basic pH reversed-phase LC (bRPLC) on an Agilent 1100 offline HPLC system which consists of a binary pump, variable wavelength detector (VWD), and a fraction collector. The VWD was operated at 280 nm. The pooled peptide digests were dissolved in solvent A (10 mM TEAB) for bRPLC and fractionated by applying a linear gradient of 5–60% solvent B [10 mM TEAB in 90% acetonitrile (ACN)] at a flow rate of 0.5 mL/min on an Xbridge bRPLC column (Xbridge, C_{18}, 5 μm 250 × 4.6 mm; Waters) over 60 min. The peptides were fractionated into 96 fractions followed by concatenation into 12 fractions.

Sample Preparation of SILAC Primary Neuronal Cells for MS Analysis. Rat primary neuronal cells were cultured as described above. The protein lysate was prepared by adding lysis buffer (2% SDS in 50 mM TEAB, pH 8.0) followed by heating at 95 °C for 10 min and then subjected to sonication (Bransons) for 30 s in each cycle. The protein lysate was cleared by centrifuging at 17,000 × *g* for 10 min. The protein amount was quantified by BCA. Sixty micrograms of protein was taken for MS analysis. The protein lysate was reduced by adding 5 mM DTT and incubated at 56 °C for 45 min. Samples were allowed to reach room temperature and alkylated by adding 20 mM IAA and incubating in the dark for 20 min. Samples were subjected to acetone precipitation by adding four volumes ice-cold acetone, vortexed for 30 s, and kept at −20 °C overnight. Samples were centrifuged at 17,000 × *g* for 15 min at 4 °C. The acetone was removed and 80% acetone was added, vortexed for 30 s, and centrifuged at 17,000 × *g* for 15 min. Acetone was removed and the protein was air-dried. Further, protein pellet was reconstituted in 6 M urea and 50 mM TEAB. Trypsin digestion was carried out by adding 1:20 of enzyme-to-substrate ratio and incubated at 37 °C overnight. Trypsin was inactivated by adding 1% TFA to the final concentration and centrifuged at 17,000 × *g* at room temperature for 10 min. The prepared peptides were fractionated using StageTip-based SCX fractionation as described by Kulak et al. (79). Briefly, a SCX StageTip was prepared by using 20-mm syringe plunger from SCX disk (2251; 3M Empore). Three layers were packed in 200 μl pipette tip. The SCX stage tip was activated with ACN and washed with 0.2% TFA. After sample loading, the peptides were fractionated by eluting with six different elution buffers: 50 mM ammonium formate (AF)/20% (vol/vol) ACN/0.5% (vol/vol) formic acid (FA), 75 mM AF/20% (vol/vol) ACN/0.5% (vol/vol) FA, 125 mM AF/20% (vol/vol) ACN/0.5% (vol/vol) FA, 200 mM AF/20% (vol/vol) ACN/0.5% (vol/vol) FA, 300 mM AF/20% (vol/vol) ACN/0.5% (vol/vol) FA, and 5% (vol/vol) ammonium hydroxide/80% (vol/vol) ACN. The eluted fractions were dried in a SpeedVac and stored in a −80 °C deep freezer until further analysis.

LC-MS/MS Analysis. Each peptide fraction from pulse and pulse-chase experiment synaptosomes was analyzed on an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) coupled to Easy nano LC II LC system. The peptides dissolved in solvent A (0.1% FA) were loaded on a trap column (5 μm, 300 Å, 75 μm ID, magic C_{18}; Michrom Biosciences) and resolved on a 20-cm analytical column (2 μm, 100 Å, 75 μm ID, magic C_{18}; Michrom Biosciences) packed in-house by applying a linear gradient of 8–32% solvent B (0.1% FA in 90% ACN) over 90 min at 250 nL/min flow rate. The total run time was 120 min for each fraction. The Orbitrap Elite mass spectrometer was operated in a data-dependent mode. Both survey scan and product ion scans were measured using a high-resolution Orbitrap mass analyzer. The full scans in the range of *m/z* 350–1,800 were measured using an Orbitrap mass analyzer at a mass resolution of 120,000 at *m/z* 400. The top 15 most-intense precursor ions were selected for MS2 fragmentation by enabling monoisotopic precursor selection (MIPS) option and fragmented using beam type higher-energy collisional dissociation (HCD) at 32% normalized collision energy with the isolation window of 1.8 *m/z*. The MS2 scans were acquired in an Orbitrap mass analyzer at a mass resolution of 30,000 at *m/z* 400. The ion filling times and AGC targets for MS1 were set at 100 ms and 1 million, respectively. The ion filling times and AGC target for MS2 were set at 200 ms and 50,000, respectively. Dynamic exclusion was set to 45 s. Internal calibration was carried out using lock mass option (*m/z* 445.1200025) (80) from ambient air. For the cytosol fractions and SILAC-labeled neuronal culture samples, each bRPLC fraction was analyzed on an Orbitrap Fusion Tribrid or Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) interfaced with Easy nLC 1000 LC system (Thermo Fisher Scientific) and Easy nLC 1200 LC systems (Thermo Fisher Scientific), respectively. The peptides dissolved in solvent A (0.1% FA) were loaded on a trap column (2 μm, 100 Å, 100 μm ID. C_{18}, 2 cm, ES 801; Thermo Fisher Scientific) and resolved on a 50-cm analytical column (2 μm, 100 Å, 75 μm ID C_{18}, 50 cm, ES 803; Thermo Fisher Scientific) using an Easy-Spray ion source by applying a linear gradient of 8–32% solvent B (0.1% FA in 95% ACN) for 90 min at flow rate of 250 nL/min. The total run time was 120 min. The Orbitrap Fusion Tribrid and Orbitrap Fusion Lumos Tribrid mass spectrometer were operated in a data-dependent mode. The full scan and product scans were acquired at a high resolution using an Orbitrap mass analyzer. Both survey scan and product ion scans were measured using a high-resolution Orbitrap mass analyzer. The full scans in the range of *m/z* 350–1,800 were measured using an Orbitrap mass analyzer at a mass resolution of 120,000 at *m/z* 200. The data were acquired in top speed for 3 s. The precursor ions were

selected for MS2 fragmentation by enabling the MIPS option and fragmented using beam type HCD at 32% normalized collision energy with the isolation window of 1.6 *m/z*. The MS2 scans were acquired in an Orbitrap mass analyzer at a mass resolution of 30,000 at *m/z* 200. The ion filling times and AGC targets for MS1 were set at 60 ms and 1 million, respectively. The ion filling times and AGC target for MS2 were set at 100 ms and 50,000, respectively. Dynamic exclusion was set to 45 s. Internal calibration was carried out using lock mass option (*m/z* 445.1200025) (80) from ambient air.

Quantification and Statistical Analysis. Statistical significance was calculated using an unpaired Student's *t* test. All statistical details are indicated in the figure legends.

Data Analysis. MaxQuant 1.5.3.8 software suite was used for identification and quantitation (81, 82). The tandem MS data were searched using Andromeda search algorithm against a RefSeq protein database (version 78 for mouse, version 79 for rat) supplemented with frequently observed contaminants. The search parameters used were as follows: (*i*) Lys-C and trypsin as proteolytic enzymes for mouse and neuronal cell cultures, respectively; (*ii*) two missed cleavages; (*iii*) 20 ppm and 4.5 ppm of peptide mass error tolerances for first search and second search, respectively; (*iv*) fragment mass error tolerance of 20 ppm; (*v*) carbamidomethylation of cysteine (+57.02146 Da) as fixed modification and oxidation of methionine (+15.99492 Da) as variable modifications; and (*vi*) minimum of 6 aa per a peptides. Peptides and proteins were filtered at 1% false discovery rate. The match between runs and second peptides options were enabled. Protein inference from identified peptides were conducted in MaxQuant 1.5.3.8 software. Briefly, all proteins that could not be distinguished based on the identified peptides were collapsed into a protein group. When all peptides of protein A were a subset of the peptides of protein B, these proteins were merged into one protein group. On the contrary, when protein A and protein B had their own unique peptides as well as common peptides, these two proteins were reported separately as two protein groups. Quantification of SILAC pairs was performed with standard settings of MaxQuant 1.5.3.8 software using a minimum ratio count of 2. Both shared and unique peptides were used for the quantification. For the calculation of protein ratio, the median value of all peptide ratios (light to heavy) for the protein of interest was taken. The correlation plots were generated in Perseus 1.5.2.6 software (83). The chromatograms and MS/MS spectra were extracted from the raw files using Xcalibur 4.1 software. Tables of identified and quantified peptides are provided in Dataset S1 for the SILAM experiment and Dataset S2 for the SILAC experiment. We analyzed the gene ontology of biological processes, molecular functions, and cell component as well as pathway analysis by PANTHER 11.1 (containing 78,442 subfamilies and 1,064,054 genes annotated) (84, 85).

Protein Turnover Calculation. The protein turnover was calculated as previously described (86). In brief, relative isotope abundance at a designated time point t (RIA_t), was calculated by dividing the abundance of heavy isotope (A_H) by the sum of the abundance of both light and heavy isotopes (A_L and A_H, respectively):

$$RIA_t = \frac{A_H}{(A_H + A_L)}.$$

The data obtained from SILAM and SILAC fit to the following exponential equation relating the RIA at specific time point, t, to RIA at $t = 0$ (RIA_0) and $t = \infty$ (RIA_∞, $t_{max} = 49$ d):

$$RIA_t = RIA_\infty + (RIA_0 - RIA_\infty) \times e^{(-k_{loss} \times t)}.$$

RIA_∞ is relative isotope abundance at infinite time after starting chase experiment. RIA_0 is relative isotope abundance at the moment of starting chase experiment. k_{loss} was then calculated as follows:

$$k_{loss} = -\ln\left(\frac{RIA_t - RIA_\infty}{RIA_0 - RIA_\infty}\right) \div t.$$

RIA_∞ was set to zero because the majority of heavy isotope would have been lost due to continuous protein turnover or cell death. The fitted equation is simplified as follows:

$$k_{loss} = -\ln\frac{RIA_t}{RIA_0} \div t.$$

k_{loss} value was converted to half-life ($t_{1/2}$):

$$t_{1/2} = -\ln\frac{RIA_{1/2}}{RIA_0} \div k_{loss}.$$

$RIA_{1/2}$ was half of the RIA_0 ($RIA_{1/2} = 1/2 \times RIA_0$) and half-life was calculated by simple division as follows:

$$t_{1/2} = \ln(2) \div k_{loss}.$$

Data and Software Availability. All MS data and search results have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD007156 and project name Identification of Long Lived Synaptic Proteins by Comprehensive Proteomic Analysis of Synaptosome Protein Turnover (87).

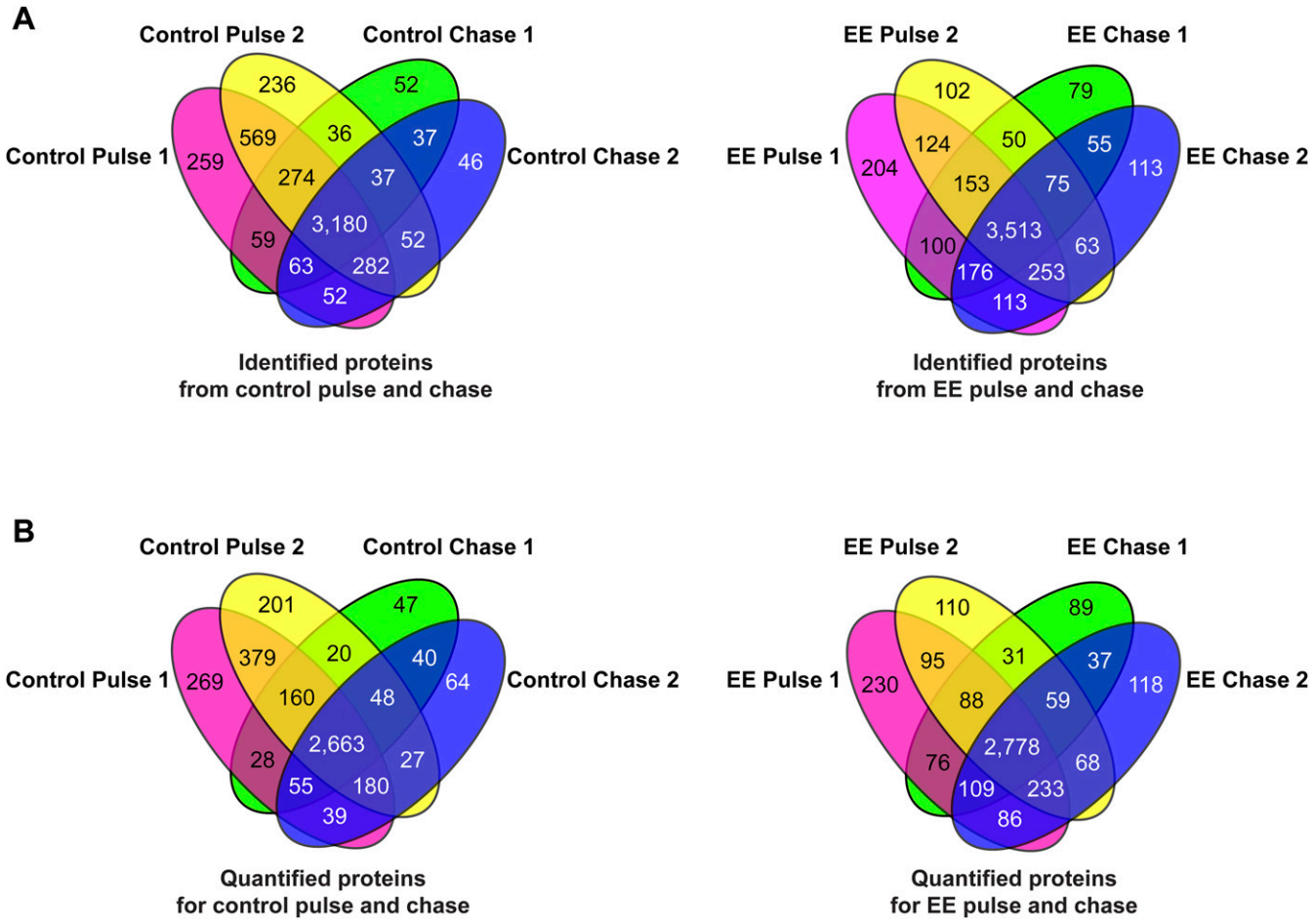

Fig. S1. Profiles of identified and quantified proteins from SILAM. Related to Figs. 1 and 2, and Table 1. (*A*) Venn diagrams show the number of identified proteins from control or EE pulse/chase SILAM mice synaptosome. (*B*) Venn diagram shows the number of quantified proteins from control and EE pulse/chase SILAM mice synaptosome.

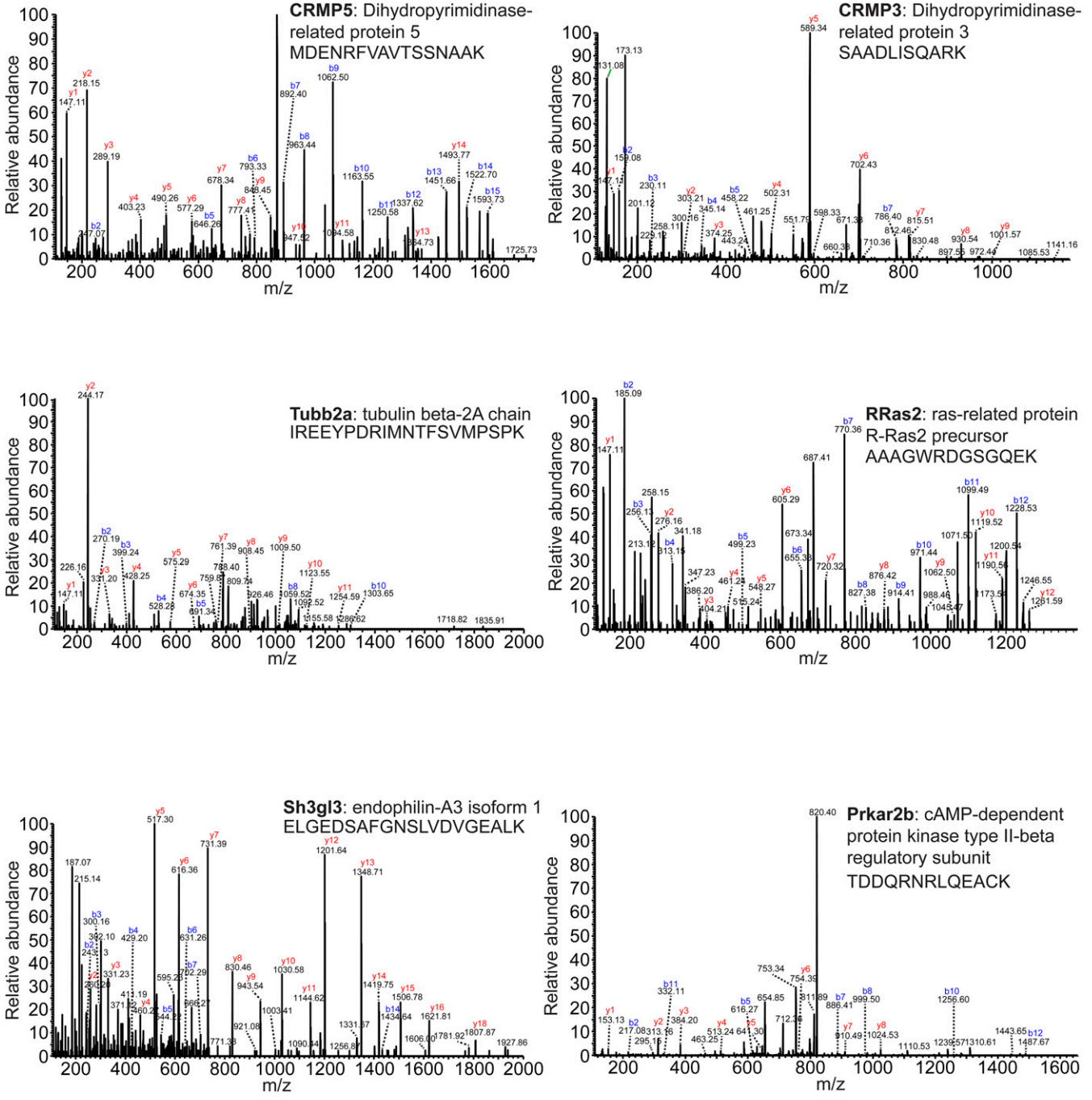

Fig. S2. Example MS2 spectra of identified synaptosome LLPs. Related to Fig. 2*C*. MS2 spectra of representative LLP peptides for Crmp5, Crmp3, Tubb2a, RRas2, Prkar2b, and Sh3gl3 (MS1 chromatogram shown in Fig. 2*C*).

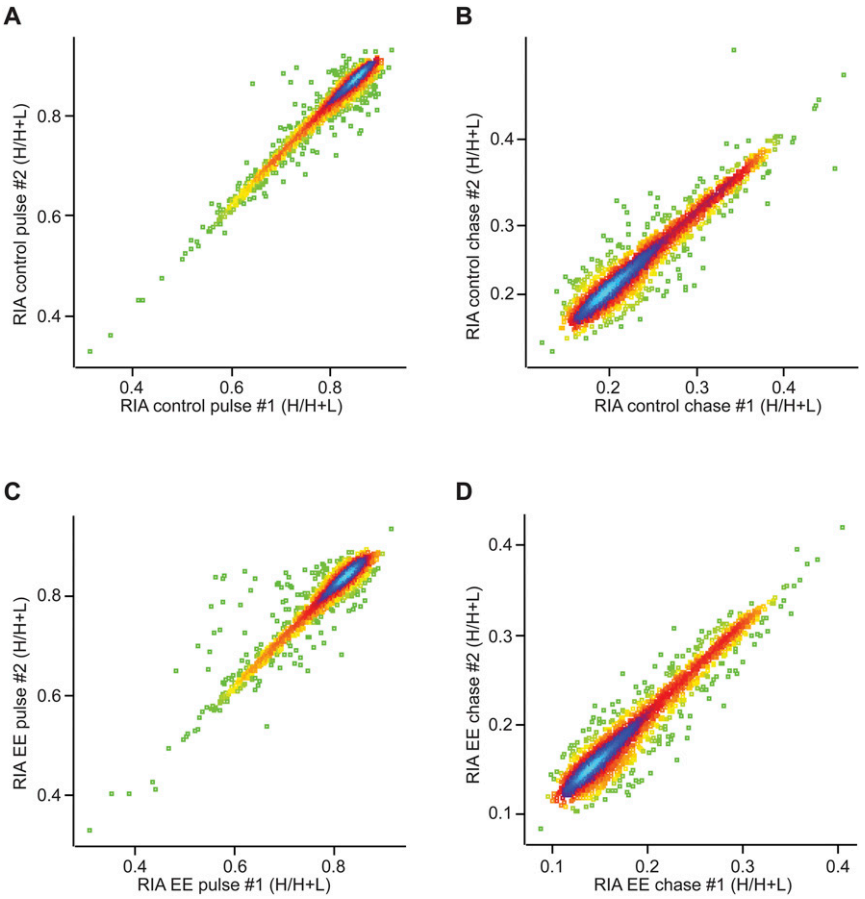

Fig. S3. Correlation analysis of individual SILAM mice. Related to Figs. 2 and 4. Scatter plots show RIA of quantified proteins from all SILAM mice [(*A*) control pulse nos. 1 and 2; (*B*) control chase nos. 1 and 2; (*C*) EE pulse nos. 1 and 2; (*D*) EE chase nos. 1 and 2]. Plots indicate that data obtained from the two replicates of control or EE conditions were highly correlated.

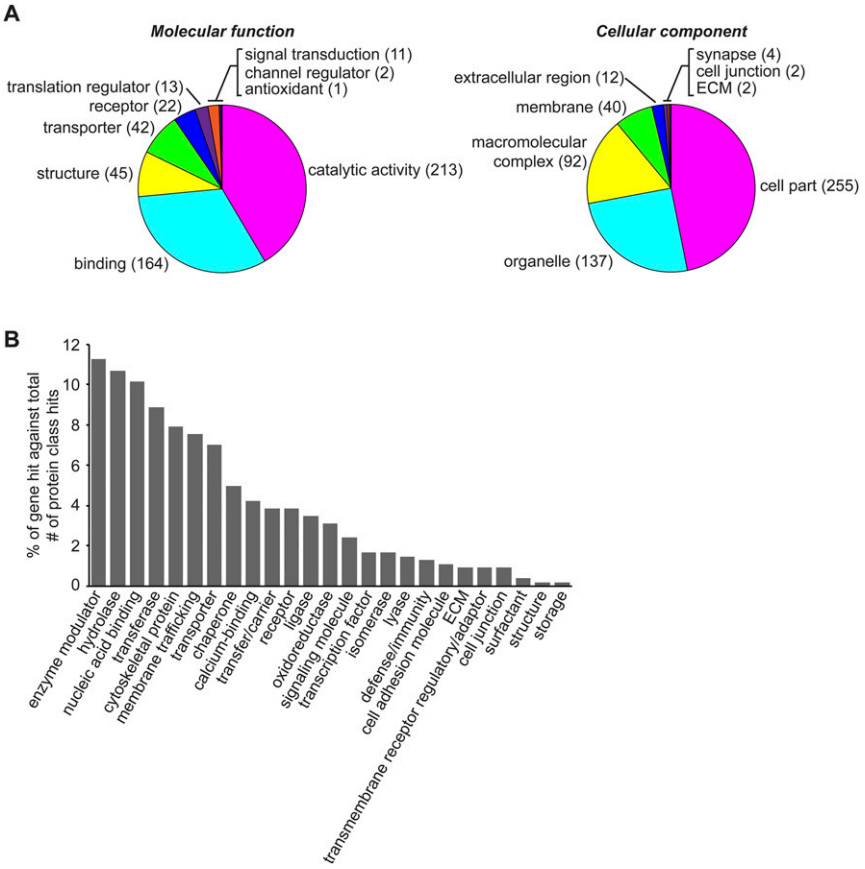

Fig. S4. Molecular functions, cellular components, and protein classes of proteins with higher turnover ratio. Related to Figs. 2*D* and 4. Pie charts indicate (*A*) molecular functions and cellular components or (*B*) protein classes of other proteins with higher turnover ratio (average ratio of RIA of control and EE >5) which were identified from the same SILAM experiment.

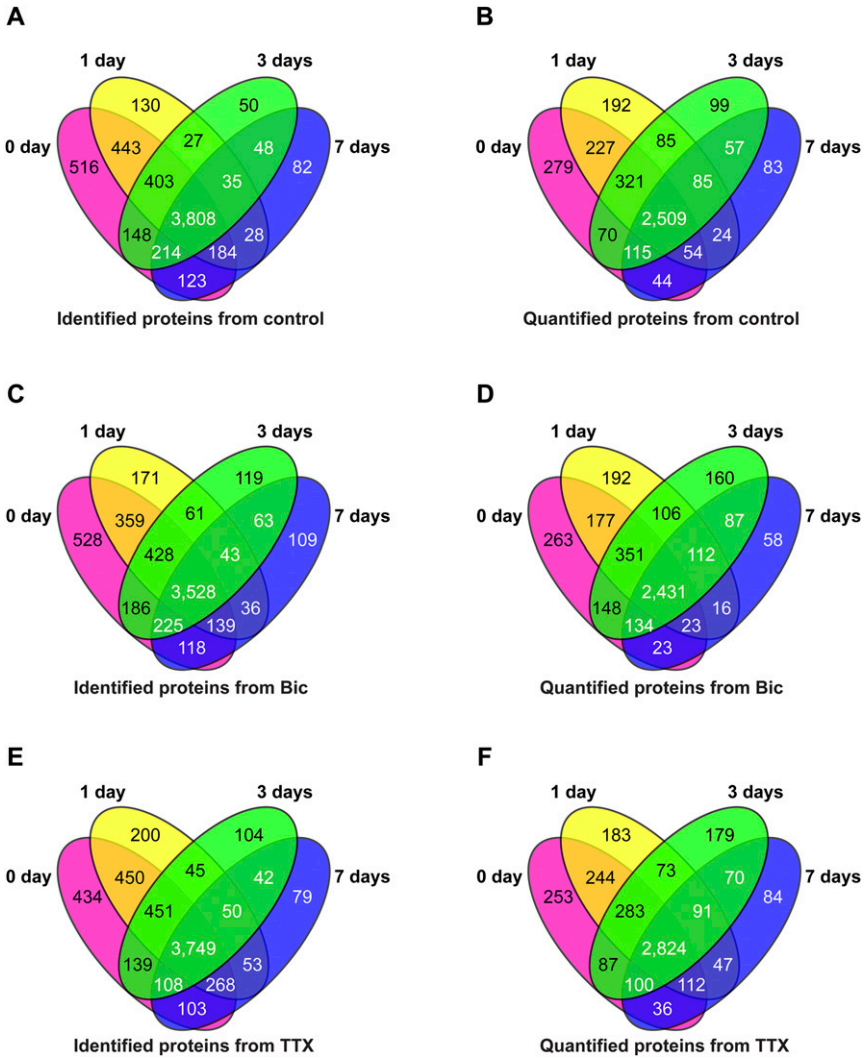

Fig. S5. Profiles of identified and quantified proteins from SILAC. Related to Fig. 5 and Table 2. Venn diagrams show the number of identified proteins from the total lysates of (*A*) control, (*B*) Bic-, or (*C*) TTX-stimulated cortical neurons. Venn diagrams show the number of quantified proteins from the total lysates of (*D*) control, (*E*) Bic-, or (*F*) TTX-stimulated cortical neurons.

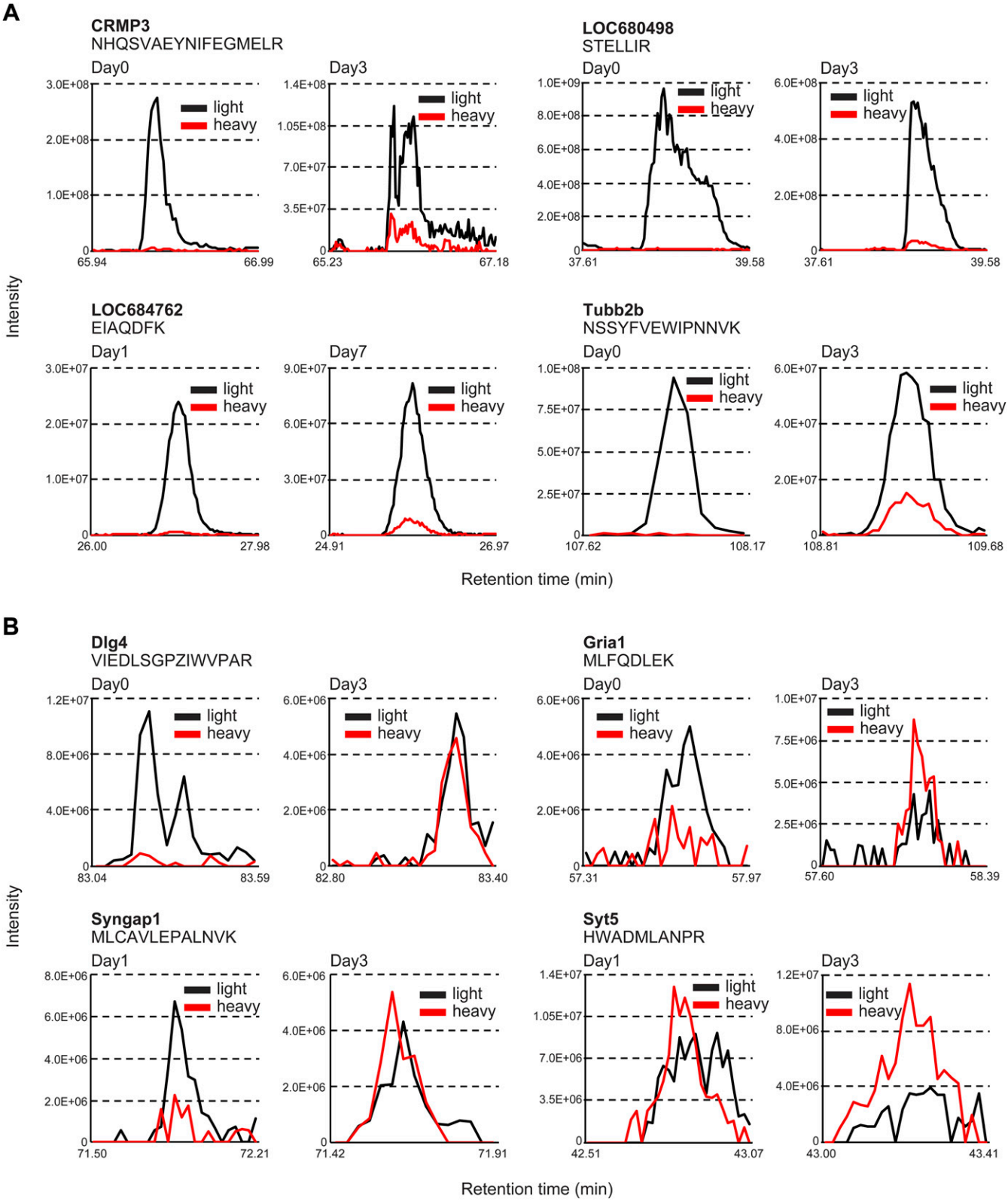

Fig. S6. Characterization of LLPs from SILAC using cortical neurons. Related to Fig. 5 and Table 2. MS1 extracted-ion chromatogram of representative peptides of LLPs from the total neuronal lysates after SILAC. Intensities of isotopes from eluted peptides were aligned according to their retention time and plotted for representative peptides for (*A*) Crmp3, LOC680498, LOC684762, and Tubb2b representing LLPs and (*B*) Dlg4, Gria1, Syngap1, and Syt5 representing rapidly turning-over proteins. Light isotope signal is plotted in black and heavy signal in red.

Dataset S1. List of proteins identified and quantified from mouse brain synaptosomal and cytosolic fractions and kidney after SILAM

Dataset S1

Gene symbols and RIA values of proteins identified and quantified from all eight mice are listed. Proteins from different fractions (synaptosomal and cytosolic fractions) and different tissues (kidney) are listed in separate worksheets.

Dataset S2. List of proteins identified and quantified from cultured rat cortical neurons after SILAC

Dataset S2

Gene symbols, RIA values, calculated average half-lives of proteins identified and quantified from cultured rat cortical neurons are listed.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)