Supporting Information

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SI Materials and Methods

Constructs. Full-length human p35 (pCMV-P35; plasmid 1347; Addgene) was a gift from the laboratory of Li-Huei Tsai, Massachusetts Institute of Technology, Cambridge, MA. Sig-1R-EYFP and Sig-1R-V5 constructs were previously described (1, 2).

Antibodies. The antibodies used in this study are as follows: BiP (Stressgen Bioreagents), cdk5 [sc-173 (Santa Cruz); no. 12134 and no. 2506 (Cell Signaling)], cytochrome c (556432, BD Pharmingen), CP1 (no. 2556; Cell Signaling), CP2 (no. 2539; Cell Signaling), and calpastatin (no. 4146; Cell Signaling). Calpain reg (sc-30065), ERK (sc-271270 and sc-94) were from Santa Cruz; GluR 2/3 (07-598; Upstate), NF-H (SMI-32P), and pNF-H (SMI-31R) were from Covance; NF-200 (ab8135, Abcam), p35/25 (no. 2680), and PP2A C (no. 2259) were from Cell Signaling; SNAP-25 (S9684) and α-tubulin (T5168) were from Sigma-Aldrich; tau (A0024) was from DAKO; and tetramethylrhodamine/TRITC (TAMRA; A6397) were from Life Technology. PHF-1 was a gift from Peter Davies, Albert Einstein College of Medicine of Yeshiva University, New York.

Assessment of Synaptosomal Mitochondria by FACS. Mitochondrial mass was measured in the crude synaptosomal fraction. To prepare the crude synaptosomal fraction (P-2), 5×10^6 cells (for each condition) were suspended and homogenized in ice-cold HS buffer (0.32 M sucrose in 4 mM Hepes, pH 7.4, with protease inhibitor mixture). The homogenates were centrifuged at $1,000 \times g$ for 10 min to remove nuclei and cell debris. The supernatants were further centrifuged at $10,000 \times g$ for 20 min to obtain the crude synaptosomal pellet (P2). The P2 pellets were washed twice with PBS solution and once with Krebs–Ringer phosphate buffer (118 mM NaCl, 5 mM KCl, 4 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM KH_2PO_4, 16 mM sodium phosphate buffer, pH 7.4, and 10 mM glucose), respectively. To label synaptosomal mitochondria with mitochondrial dyes, the P2 pellets were suspended with 500 μL NAO, Rh 123, or MT CMXRos staining solution and incubated for 15 min at 37 °C. Cytometric analysis of mitochondria was performed by using Beckman Coulter flow cytometry, and fluorescence was collected through a 530 ± 30 nm filter. Sample flow rate is kept constant at ∼30,000 events per second; 100,000–1,500,000 events were collected from each sample for analysis. The fluorescence was analyzed by using Quanta MPL software.

Calpain Stimulated p35 Cleavage. To compare the rates of calpain-induced p35 cleavage, p35 cleavage experiments were conducted as described elsewhere (3). Briefly, fresh brain tissues collected from 6-mo-old C57 (WT) and Sig-1R–KO mice were weighed and homogenized in 10 times their volume of Hepes buffer (20 mM Hepes, pH 7.4, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA) at 4 °C by using a tissue glass Dounce homogenizer. The homogenate was centrifuged at $10,000 \times g$ for 15 min, and the resulting supernatant was used as the crude extract. Crude extract was quantified, and 100 μg crude extract was stimulated with 1 mM $CaCl_2$ at 37 °C for various time points to induce cleavage of p35 to p25. The proteolytic reaction was stopped by adding EGTA (final 10 mM) followed by boiling the extract in SDS sample buffer.

P35 Degradation Study. CHO cells were transfected with siSig-1R or siCon in combination with p35. Alternatively, CHO cells were transfected with EYFP or Sig-1R-EYFP in combination with p35. After 48 h of transfection, cells were then treated with 0.1 mg/mL protein synthesis inhibitor CHX for 0–120 min and subsequently harvested. Cell lysates were processed for Western blotting. P35 degradation study was also conducted in primary cortical neurons and hippocampal brain slices harvested from the WT and Sig-1R–KO mice, respectively. Organotypic hippocampal slices were prepared from 1–3-d-old mouse pups. The hippocampi were placed on a tissue chopper and cut at intervals of 300–400 μm.

Preparation of MA-BSA. Preparation of MA-BSA is adapted from Seahorse Bioscience. Ultra Fatty Acid Free BSA was dissolved in 100 mL of 150 mM NaCl and kept in a 37 °C water bath with constant stirring followed by filtration. In a separate container, sodium myristate (Sigma-Aldrich) was dissolved in 44 mL 150 mM NaCl and warmed to 70 °C with constant stirring. Fifty milliliters of the filtered BSA was diluted with 50 mL of 150 mM NaCl solution to make 0.17 mM BSA stock; this was used as vehicle control. The remaining 50 mL filtered BSA was transferred to a warm glass beaker, followed by addition of 40 mL of the 70 °C myristate solution to the BSA solution while stirring at 37 °C. The solution was stirred at 37 °C for 1 h and the water bath temperature was kept between 35 °C and 40 °C. At the end of conjugation, the final volume was brought up to 100 mL with 150 mM NaCl and adjusted to pH 7.4. The stock conjugate solution consists of 1 mM sodium myristate/0.17 mM BSA (6:1 molar ratio of myristate to BSA). Aliquots were stored in glass vials at −20 °C.

Click-iT on-Resin Labeling of Myristoylated p35. Cortical neurons were transduced with siCon or Sig-1R and treated with 25 μM Click-iT myristic acid azide (C10268; Invitrogen) for 4–6 h. Cells were washed and lysed in RIPA lysis buffer containing 1% Triton-X-100 and 1% protease inhibitors. P35 was immunoprecipitated from 500 μg cell lysates by using 5 μg of Protein A/G-crosslinked p35 resin. Following an overnight incubation, the IP resin was washed four times in Tris-buffered saline and then resuspended in 30 μL of 50 mM Tris, pH 8, and labeled with biotin-alkyne using the Click-iT Biotin Protein Analysis Detection Kit (C33372; Invitrogen) for 1 h at 4 °C in a total reaction volume of 100 μL. The IP resin was washed once in TBS, and bound proteins were eluted into 40 μL of 2× LDS buffer, which was then incubated at 90 °C for 5 min. Twenty microliters of the IP eluates were separated on SDS/PAGE, and the myristoylated p35 was detected by anti-TAMRA antibody.

[^{3}H]Myristic Acid Binding to p35 Measured by the p35 Antibody Pull-Down Assay. Myristoylation of p35 was measured in part similarly to previous publications (4). siCon or siSig-1R mouse cortical neurons were cultured in the presence of 0.1 mCi/mL [^{3}H]myristic acid and 2% (wt/vol) fat-free BSA for 5 h at 37 °C. Neurons were washed three times with PBS solution and lysed in 50 mM Tris·HCl, pH 7.5, containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors. P35 was immunoprecipitated to detect the myristoylation levels in siCon and siSig-1R neurons. The pull-down IP beads were subjected to scintillation counting (LS6500; Beckman Coulter) to determine the [^{3}H]-MA p35 activities.

Sig-1R-BiP Association Assay to Characterize the Agonist Property of Myristate. CHO cells stably expressing Sig-1R-V5 were grown in 10-cm dishes and treated with BSA vehicle control, 1 μM MA-BSA, or 3 μM (+)-PTZ in culture medium at 37 °C for 1 h. CHO cells were harvested and suspended in 50 mM Hepes (pH 7.4) followed by cross-linking with 100 μg/mL of dithiobis succinimidyl propionate (Thermo Fisher Scientific) for 30 min at 4 °C.

Reaction was stopped by adding Tris·HCl (pH 8.8, final 50 mM). Fifteen minutes after incubation on ice, cells were lysed with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.3% sodium deoxycholate, 0.1% SDS, protease inhibitor mixture; Sigma-Aldrich). After centrifugation at 12,000 × *g*, the supernatant was incubated overnight at 4 °C with monoclonal anti-V5 antibodies (1:2,000). The cell lysate was incubated with protein A/G PLUS agarose (Santa Cruz) for 90 min. After washing with RIPA buffer, immunoprecipitants were boiled in 30 μL of 2× sample buffer (130 mM Tris·HCl, pH 6.8, 4.2% SDS, and 20% glycerol) and applied to Western blotting. Immunoprecipitated Sig-1R-V5 and coimmunoprecipitated BiP were detected by immunoblotting using antibodies against V5 and BiP (1:1,000), respectively. Intensities of BiP were normalized to those from Sig-1R-V5 for quantification of the activity of Sig-1R ligands.

[^{3}H](+)-PTZ Competition Binding Assay. CHO cells stably expressing Sig-1Rs were seeded into poly-D-lysine–coated 24-well plates. Cells were cultured to confluence and washed with cold PBS solution containing 0.1 mM calcium chloride and 1 mM magnesium chloride (PBSCM) before incubation with BSA or various concentrations of myristate-BSA (1×10^{-4} to 1×10^{-9} M). A total of 10 nM [^{3}H](+)-PTZ was diluted in cold PBSCM and added to compete with myristate-BSA at 4 °C for approximately 2–3 h. A total of 10 μM haloperidol was included in each experiment to subtract nonspecific radio ligand binding. Cells were then washed twice with cold PBSCM and lysed with lysis buffer (1% SDS, 0.1 N NaOH) and mixed with 0.5 mL of scintillation mixture. Bound radioactivity was determined by liquid scintillation spectrometry by using the Beckman LS 6500 Liquid Scintillation Counter (Beckman Coulter). Data were analyzed with Prism 5 (GraphPad Software) for nonlinear curve fitting.

Cell Surface Immunoprecipitation of Sig-1R. NG108-15 cells cotransfected with p35 plus EYFP or Sig-1R-EYPF were cross-linked first by following the Thermo Scientific protocol before lysis. All procedures were performed on ice. Cells were washed twice with cold PBS solution and treated with 0.25 mM dithiobis succinimidyl propionate cross-linker (Thermo Scientific) to allow for chemical cross-linking of intracellular proteins. After 30 min of incubation, the cross-linking reaction was quenched with Tris solution at a final concentration of 25 mM. The fixed cells were harvested by using ice-cold PBS solution containing 0.1% BSA and rotated at 4 °C overnight with rabbit anti-EGFP/EYFP/expanded CFP (Living Colors Full-Length A.v. Polyclonal Antibody, 1:200). After overnight incubation, cells were pelleted by centrifugation at 100 × *g* for 5 min to remove unbound YFP antibodies and then washed five times with PBS solution with 0.1% BSA. The fixed cells were then lysed in 0.3 mL of IP buffer (50 mM Tris·HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, and protease inhibitors) for 30 min. Three hundred micrograms of the lysates were incubated with 30 μL of prewashed protein-A/G Agarose (Santa Cruz) for 90 min at 4 °C. The beads were washed three times with IP buffer and then eluted with SDS sample buffer and heated at 95 °C for 5 min. Proteins in the mixtures were immediately separated by using SDS/PAGE and immunoblotted with anti–Sig-1R, anti-BiP, and anti-p35 antibodies to examine for their potential interactions, if any, on the plasma membrane.

Animals. Oprs1 mutant ($^{+/-}$) OprsGt(IRESBetageo)33Lex litters on a C57BL/6J×129s/SvEv mixed background were purchased from the Mutant Mouse Regional Resource Center at the University of California, Davis. The sigma-1 receptor ($^{+/-}$) males were backcrossed for 10 generations to female on C57BL/6J to ensure that animals had a homogenous background. The resulting mice were genotyped to select Sig-1R WT and KO mice. For primary neuronal cultures, embryonic day 18–19 fetuses were used. WT or Sig-1R–KO mice for brain tissue experiments were usually 6 mo of age. All mice were maintained in constant environmental conditions with a 12-h light/dark cycle. Procedures were optimized to minimize suffering and to reduce number of animals used. Care of all rodents used in this study was in accordance with the guidelines of the National Institutes of Health and the National Institute on Drug Abuse Intramural Research Program Animal Care and Use Program, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

1. Hayashi T, Su T-P (2007) Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival. *Cell* 131(3):596–610.
2. Hayashi T, Hayashi E, Fujimoto M, Sprong H, Su T-P (2012) The lifetime of UDP-galactose:ceramide galactosyltransferase is controlled by a distinct endoplasmic reticulum-associated degradation (ERAD) regulated by sigma-1 receptor chaperones. *J Biol Chem* 287(51):43156–43169.
3. Kusakawa G, et al. (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. *J Biol Chem* 275(22):17166–17172.
4. Asada A, et al. (2008) Myristoylation of p39 and p35 is a determinant of cytoplasmic or nuclear localization of active cyclin-dependent kinase 5 complexes. *J Neurochem* 106(3):1325–1336.

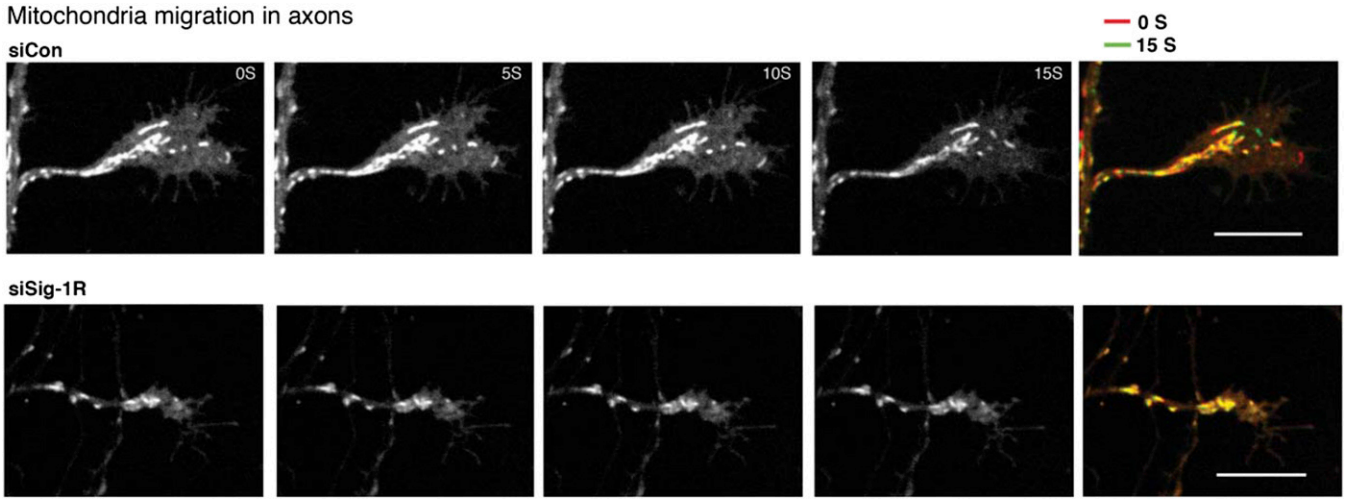

Fig. S1. Axonal mitochondrial dynamic was slower in the Sig-1R–knockdown neurons. Live neurons were labeled with MitoTracker Red and axonal mitochondria movements were monitored and captured every 5 s. Images captured at 0 s (red) and 15 s (green) were merged to identify mitochondrial mobility. Mitochondria shown in yellow indicate no movements over the 15-s period. siCon neurons (*Upper*) showed active mitochondrial movements whereas siSig-1R neurons (*Lower*) exhibited less movement. (Scale bar: 10 μm.)

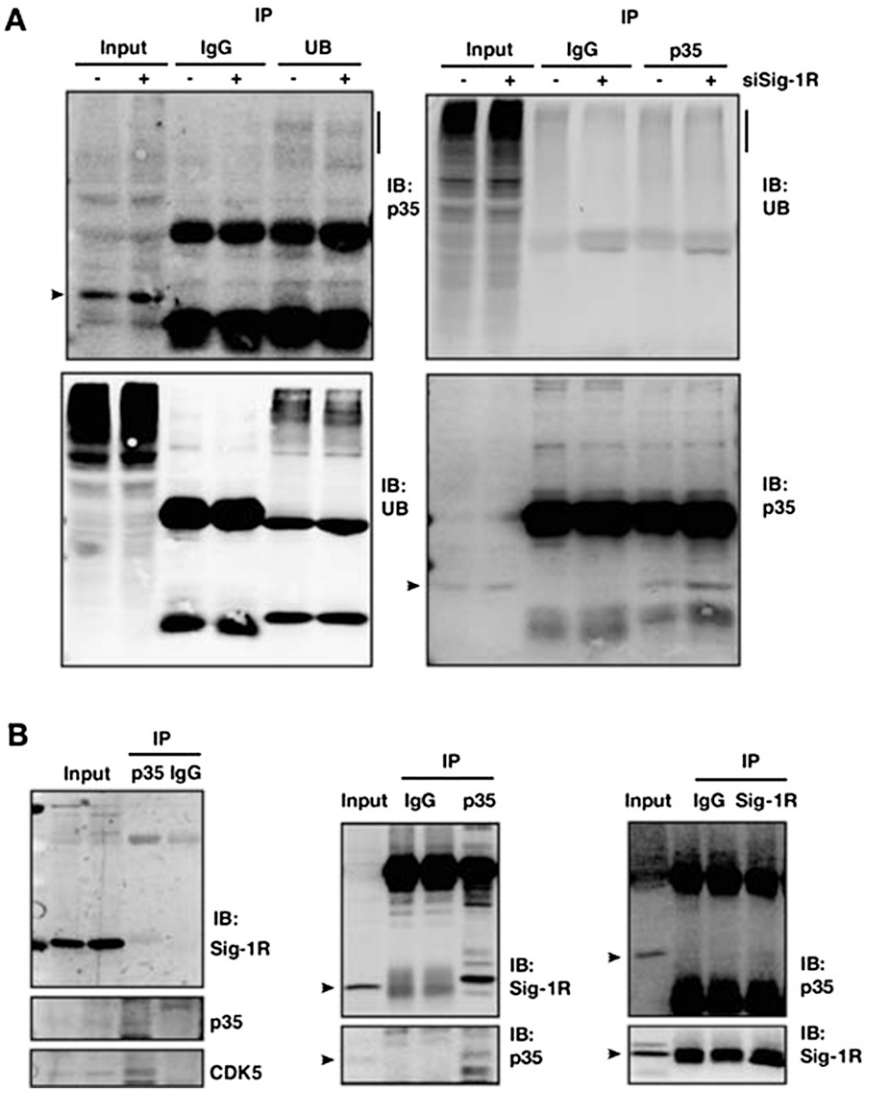

Fig. S2. Sig-1R regulates p35 ubiquitination. (*A*). Coimmunoprecipitation (IP) of ubiquitin (UB) and p35 in neurons expressing siCon or siSig-1R. Cells were treated with proteasome inhibitor for 2 h before harvesting. Although siSig-1R neurons show more total ubiquitinated proteins, ubiquitinated p35 is lower in the siSig-1R neurons. (*B*). CoIP studies examining the interactions between Sig-1R and p35 in neurons. Sig-1R does not physically interact with p35.

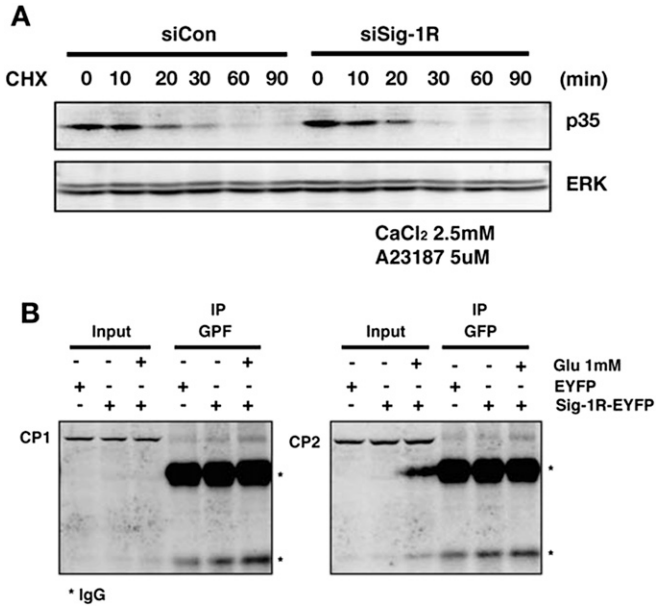

Fig. S3. Sig-1R does not regulate p35 degradation via the calpain activation pathway. (*A*) Calpain activation abolished slower p35 degradations in the Sig-1R–knockdown neurons. (*B*) Sig-1R does not coimmunoprecipitate with calpain subtypes CP1 and CP2. CHO cells overexpressing Sig-1R EYFP were used to study coimmunoprecipitation (IP) of Sig-1R and calpain subtypes 1 and 2, and cells expressing EYFP alone were used as negative controls.

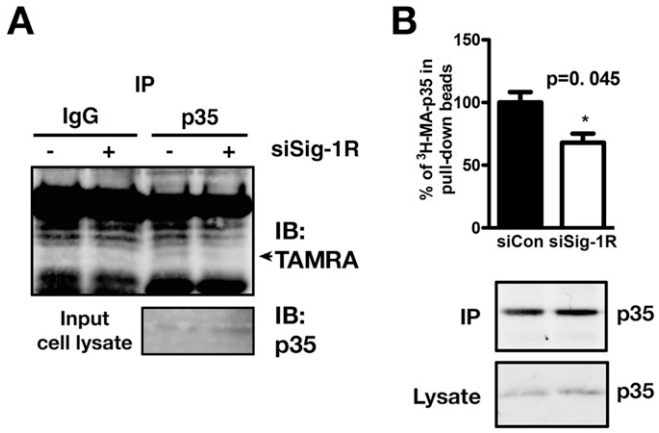

Fig. S4. Myristoylated p35 is reduced in Sig-1R–knockdown neurons. (*A*) Myristoylated p35 level was measured by using Click chemistry (*SI Materials and Methods*). Neuronal lysates labeled with myristic acid azide were subjected to immunoprecipitation (IP). The immunoprecipitated p35 was then subjected to the Click assay. The myristoylated p35 was detected by anti-TAMRA antibody. Sig-1R–knockdown neurons, although they showed higher levels of total p35, expressed less myristoylated p35 compared with control neurons. (*B*) Mouse cortical neurons were cultured in the presence of 0.1 mCi/mL [^{3}H]myristic acid for 5 h. Although siSig-1R neurons expressed more p35 (both in lysates and immunoprecipitated proteins), [^{3}H]-MA p35 activity was less in the siSig-1R neurons, indicating that myristoylation of p35 was hindered by knocking down of Sig-1R. Average disintegration per minute (dpm) is 1,669 in siCon neurons and 1,014.5 in siSig-1R neurons ($n = 3$ independent experiments; error bar indicates SEM; $^*P < 0.05$, *t* test).

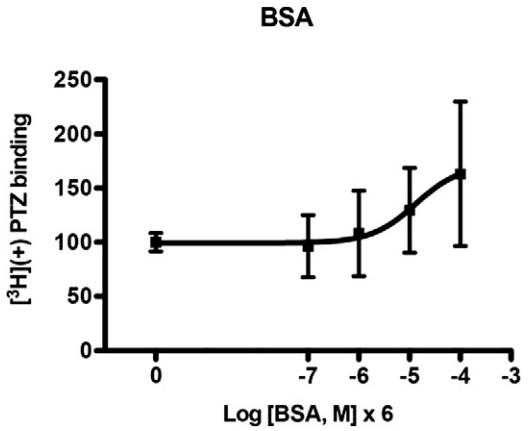

Fig. S5. Effects of BSA on [^{3}H](+)-PTZ binding. BSA was used as a control for MA-BSA. BSA alone at 0.17 E -5 M increased the occupancy of [^{3}H](+)-PTZ in Sig-1R stably expressed CHO cells (n = 3; error bar represents SEM).

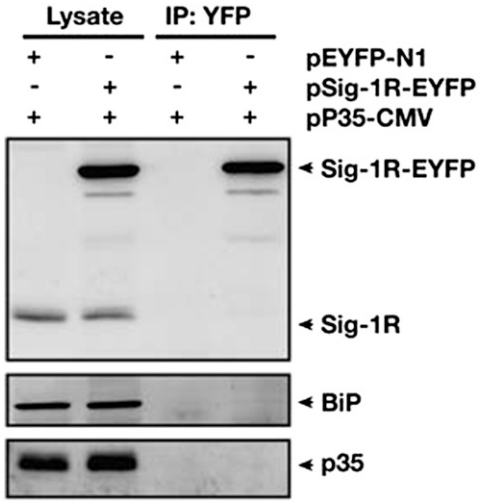

Fig. S6. Sig-1R does not interact with p35 in the plasma membrane. NG108-15 cells were cotransfected with p35 and pEYFP-N1 or pSig-1R-EYFP. Surface-expressing Sig-1R-EYFP coimmunoprecipitated (IP) with BiP but not p35.

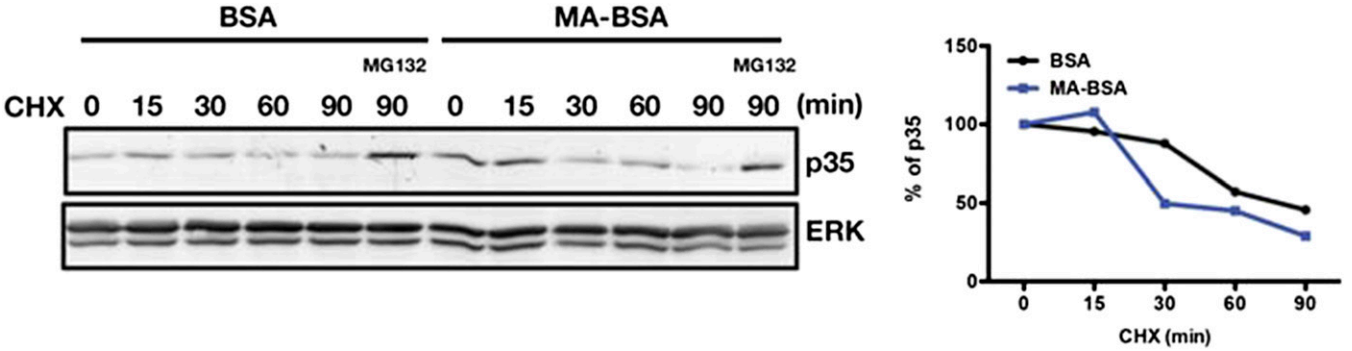

Fig. S7. MA-BSA accelerates p35 degradations. BSA or MA-BSA was treated 24 h before CHX treatments. Cells treated with MA-BSA exhibit a faster p35 degradation rate (n = 3 independent experiments).