Supporting Information

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SI Materials and Methods

Reagents and Plasmids. Cdk5 substrate (PKTPKKAKKL) and Histone H1 were purchased from Anaspec and Upstate Biotechnology, respectively. Purified recombinant protein Cdk5/p35 was purchased from Cell Signaling Technology. $A\beta_{25–25}$, $A\beta_{35–25}$ (reverse sequence control peptide), and $A\beta_{1–42}$ were purchased from Anaspec. $A\beta_{1–42}$ peptide was oligomerized as previously described (1). Glutathione–Sepharose beads were from Amersham Biosciences. Cdk5 polyclonal antibody (C-8) and p35(C-19) were from Santa Cruz Biotechnology; anti-ATM pS1981 was from Rockland Immunochemicals; anti–phospho-Histone H1 was from Millipore; and anti–microtubule-associated protein (MAP)2 was from Sigma. HA-Cdk5, HA-DN-Cdk5, and Myc-P35 plasmids were from Addgene. GST-Cdk5 and His-p35 plasmids were kindly provided by David Park (University of Ottawa, Ottawa, ON, Canada).

Cell Culture and Transfection. HEK293 and SH-SY5Y cells were cultured in DMEM supplemented with 10% heat-inactivated FBS. HEK/NOS1 cells were maintained in the same medium containing geneticin. Cells were transfected with lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. Primary cerebrocortical neurons were cultured from day 16 rat embryos and transfected as we have described previously (1, 2).

Immunoblot Analysis and Immunoprecipitation. Twenty micrograms of protein was electrophoresed on 4–12% denaturing gels (Nupage; Invitrogen), transferred to polyvinylidene difluoride membranes (Immoblin-P; Millipore), and probed with various primary antibodies and HRP-labeled secondary antibodies. Western blots were visualized by X-ray film exposure or with a LI-COR Odyssey infrared imaging system. For immunoprecipitation, 100 μg of cell lysate was incubated with a complex of protein-A/G–agarose beads and primary antibody or antibody-conjugated beads for 3–5 h at 4 °C. Beads were then washed with lysis buffer three times and subjected to immunoblot or kinase assays.

Site-Directed Mutagenesis. Mutations in Cdk5 were made using a commercial kit (Quick Change; Stratagene). Pairs of complementary primers of 30–40 bases were designed with the desired mutations placed in the middle of the sequence. Parental Cdk5 cDNA inserted in pCMV-HA was amplified by using pfu DNA polymerase with these primers for 20 cycles in a DNA thermal cycler. After digestion of the parental DNA with DpnI, the mutants were transferred into XL-Blue *Escherichia coli*. Mutations were confirmed by DNA sequencing.

Biotin-Switch Assay. Analysis of SNO-Cdk5 by the biotin-switch assay was performed as described (1, 2). In brief, cells were lysed with 1% Triton X-100 in HEN buffer (250 mM Hepes, 1 mM EDTA, and 0.1 mM Neocuproine). Free thiols were blocked with 25 mM methyl-methanethiosulfonate (MMTS). Cell extracts were precipitated with acetone and resuspended in HEN buffer with 1% SDS. S-nitrosothiols were selectively reduced by 10 mM ascorbate to reform the thiol group and subsequently biotinylated with 4 mM biotin-HPDP (Pierce Biotechnology). Biotinylated proteins were pulled down with streptavidin–agarose beads (Thermo Scientific) and analyzed by immunoblotting. Controls were performed in the absence of ascorbate or MMTS.

Cdk5 Kinase Luciferase Assay. Cdk5 protein was precipitated from whole-cell lysates (400 μg) and incubated with HA-conjugated beads or a combination of protein-A/G–Sepharose and primary antibody in modified lysis buffer [50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mg/mL leupeptin, and 10 mg/mL aprotinin] for 3 h at 4 °C and then pelleted by centrifugation and washed three times using modified lysis buffer. For the Cdk5 kinase luciferase assay, the precipitate was incubated for 30 min at 30 °C in kinase reaction buffer [25 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 100 mM ATP] containing 0.1 mM histone H1 peptide (PKTPKKAKKL) in a final volume of 50 mL. After the kinase reaction was complete, the remaining amount of ATP was determined by conducting a Kinase-Glo Plus Luminescent Kinase Assay according to the manufacturer's instructions (Promega). Samples were added to an equivalent amount (1:1) of Kinase-Glo Plus reagent, and the mixtures were transferred to a white-well plate. Luminescence was read on a WALLAC VICTOR-2 plate reader. For detecting phosphorylated Histone H1, the same precipitates were incubated with 10 mg Histone H1 protein in kinase reaction buffer for 30 min at 30 °C and then detected by anti–phospho-Histone H1 antibody. The amount of ATP thus measured is inversely correlated to Cdk5 kinase activity. Calpeptin was included in the culture medium to block calpain-mediated activation of Cdk5.

Quantification of Dendritic Spine Density. Primary mixed neuronal/glia cerebrocortical cultures were transfected at 14–16 d in vitro with enhanced green fluorescent protein (EGFP) and Cdk5. Two days after transfection, neurons were exposed to Aβ peptide or NMDA. L-arginine (1 mM) was included in the culture medium as a substrate for NO production by NOS1. Cultures were fixed in 4% paraformaldehyde for 10 min followed by permeabilization with 0.2% Triton X-100 in PBS. Anti-MAP2 antibody was used for immunocytochemistry to specifically label neuronal cells. Images of EGFP-expressing cells were acquired by deconvolution microscopy equipped with SlideBook 4.2 software (Intelligent Imaging Innovations). For EGFP-expressing and MAP2-positive neurons, three distinct fields of secondary or tertiary dendrites were randomly selected and dendritic spine protuberances counted in a masked fashion. Neuronal apoptosis was monitored in a masked fashion by quantification of Hoechst-stained nuclei that were small and brightly labeled compared with normal nuclear morphology. Surviving neurons were scored by MAP2 labeling and normal morphology.

Human and Animal Brain Samples. Postmortem human brain samples (frontal cortex) were obtained from patients diagnosed with Alzheimer's disease or from nondemented, age-matched control subjects. Characteristics of these patients are shown in Table S1. All cases were characterized by clinical examination and neuropsychological testing during life and by detailed histopathological analysis postmortem. Human brain samples were analyzed with institutional permission under state of California and National Institutes of Health guidelines. Informed consent was obtained according to procedures approved by Institutional Review Boards at University of California, San Diego and the Sanford-Burnham Medical Research Institute. Mouse brains were obtained from transgenic Tg2576 mice that express the Swedish mutation of APP ($APP_{K670N,M671L}$) at a high level under control of the hamster prion protein promoter (3) and from age-matched control, wild-type animals.

Transnitrosylation Assay. For the transnitrosylation assays, we followed our published procedure (4). Recombinant Cdk5 (0.1 μM)

was prepared from bacteria and incubated for 1 h at room temperature (RT) in the dark in the presence of 100 μM SNOC. The resulting S-nitrosylated protein was then used as an NO donor by incubating with recombinant Drp1 (0.5 μM) for 30 min at RT in the dark to test for potential transnitrosylation. Resulting samples were subjected to the NO–biotin-switch assay to monitor transnitrosylation from Cdk5 to Drp1. The relative redox potential and change in Gibbs free energy for the transnitrosylation reaction were calculated as previously described (4). In brief, SH-SY5Y cells were exposed to 50 μM SNOC for 20 min, and cell lysates were subjected to the biotin-switch assay. Band intensities in Fig. S9 were analyzed with a LI-COR Odyssey infrared imaging system to estimate the relative levels of S-nitrosylated protein and total protein (i.e., both the reduced and the S-nitrosylated forms, obtained by performing the biotin-switch assay in the absence of MMTS). We then acquired an estimate of the reduced form of the protein by subtracting the amount of oxidized (S-nitrosylated) protein from the total protein level. Next, these values were used in Eq. **S1**, which is a variant of the Nernst equation, to determine the difference in the standard redox potentials between Drp1 and Cdk5. Note that this calculation assumes that the transnitrosylation reaction had reached equilibrium when the samples were obtained and that there is a one-electron transfer between these molecules:

$$E^{0'}_{\mathrm{Cdk5}} - E^{0'}_{\mathrm{Drp1}} = -\frac{RT}{zF} \cdot \ln\left(\frac{[\mathrm{Cdk5_{SNO}}][\mathrm{Drp1_{red}}]}{[\mathrm{Cdk5_{red}}][\mathrm{Drp1_{SNO}}]}\right). \quad \textbf{[S1]}$$

We then used Eqs. **S2** and **S3** to calculate the change in Gibbs free energy associated with the transnitrosylation reaction and obtained a negative value for the transfer of NO from CDK5 to Drp1, indicating that the reaction would occur in intact cells.

$$\Delta G = -zF\Delta E \quad \textbf{[S2]}$$

$$\Delta G^{0'} = -RT \cdot \ln\left(\frac{[\mathrm{Cdk5_{red}}][\mathrm{Drp1_{SNO}}]}{[\mathrm{Cdk5_{SNO}}][\mathrm{Drp1_{red}}]}\right). \quad \textbf{[S3]}$$

1. Cho DH, et al. (2009) S-nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury. *Science* 324:102–105.
2. Uehara T, et al. (2006) S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. *Nature* 441:513–517.
3. Hsiao K, et al. (1996) Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. *Science* 274:99–102.
4. Nakamura T, et al. (2010) Transnitrosylation of XIAP regulates caspase-dependent neuronal cell death. *Mol Cell* 39:184–195.

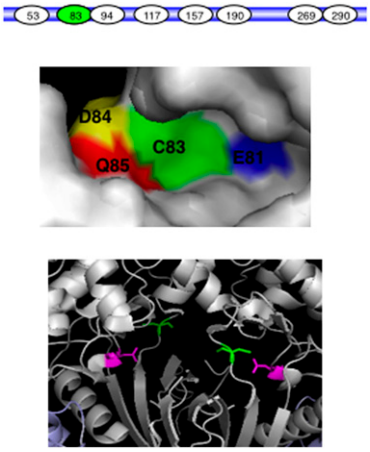

Fig. S1. Schematic representation of Cdk5 cysteine residues (*Top*), model of atomic structure of Cdk5/p25 complex showing putative acid/base S-nitrosylation motif surrounding residue C83 (*Middle*), and ribbon structure of Cdk5/p25 complex showing the spatial position of C83 (green) versus D145 (purple) (*Bottom*). Images were created and rendered by the software program PyMol.

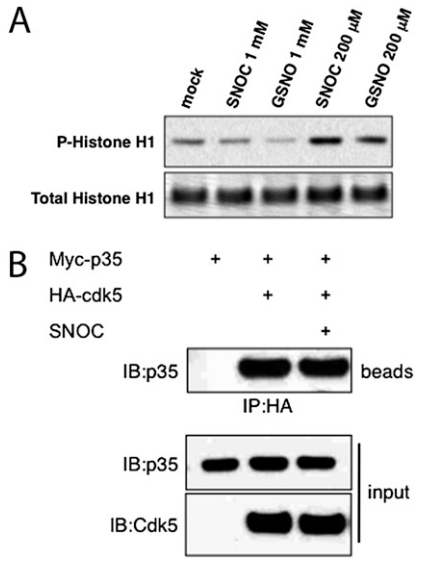

Fig. S2. Effect of NO on Cdk5 and p35. (*A*) Effects of NO on Cdk5 kinase activity: opposite effects of high- and low-dose GSNO or SNOC on Cdk5 kinase activity. HEK/NOS1 cells transfected with wt-Cdk5/p35 were exposed to the indicated concentration of GSNO or SNOC. After 0.5 h, the cells were lysed and Cdk5 kinase activity was quantified by Histone-H1 phosphorylation. (*B*) SNOC exposure does not affect the interaction between p35 and Cdk5 or p35 protein levels. HEK293 cells transfected as indicated were exposed to SNOC (50 μM, 0.5 h) or control solution and then lysed and immunoprecipitated with HA antibody. Total lysate and precipitate were detected by the indicated antibodies.

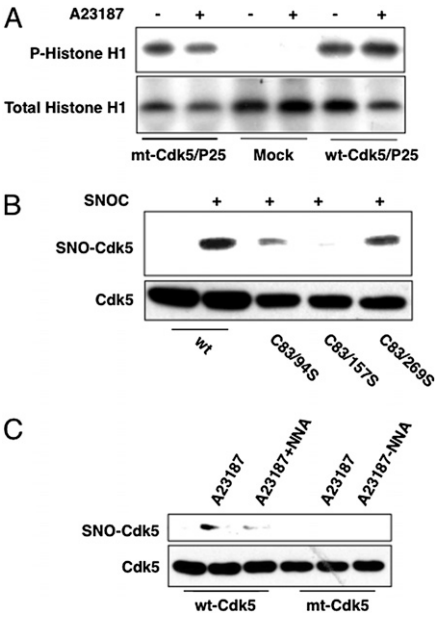

Fig. S3. Effect of endogenous NO on Cdk5 kinase activity and S-nitrosylation of Cdk5. (*A*) HEK/NOS1 cells transfected with wild-type (wt)-Cdk5/p25 or mutant (mt)-Cdk5/p25 were exposed to 5 μM A23187 for 4 h to facilitate Ca^{2+} entry and hence NOS1 activation. The cells were then lysed and Cdk5 kinase activity was quantified by Histone-H1 phosphorylation. Control cells were exposed to vehicle only (DMSO). (*B*) S-Nitrosylation of wild-type and mutant Cdk5. HEK293 cells were transfected with wt or various cysteine-mutated Cdk5s and exposed to 50 μM SNOC. After 0.5 h, cell lysates were subjected to biotin-switch and immunoblot analyses. *Upper*, SNO-Cdk5; *Lower*, total Cdk5. (*C*) Cdk5(C83S/C157S) mutation prevents endogenous NO-induced S-nitrosylation. HEK/NOS1 cells were transfected with wt-Cdk5 or mt-Cdk5 (C83S/C157S) and treated with A23187 in the presence or absence of NNA. *Upper*, SNO-Cdk5; *Lower*, total Cdk5.

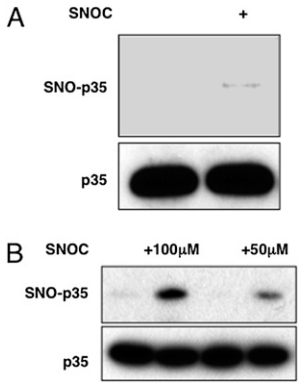

Fig. S4. S-Nitrosylation of p35. (*A*) HEK293 cells were transfected with p35 and then exposed to 50 μM SNOC. After 0.5 h, cell lysates were subjected to biotin-switch and immunoblot analyses. *Upper*, SNO-p35; *Lower*, total p35. (*B*) S-Nitrosylation of p35 in vitro. Recombinant p35 was exposed to 50 μM or 100 μM SNOC and after 0.5 h subjected to biotin-switch and immunoblot analyses. *Upper*, SNO-p35; *Lower*, total p35.

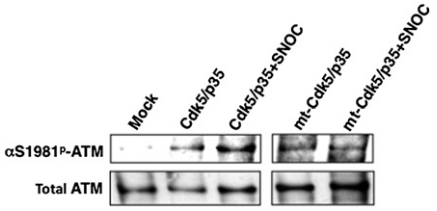

Fig. S5. Phosphorylated ataxia telangiectasia mutated (ATM) as an indicator of Cdk5 kinase activity. HEK293 cells transfected with wt-Cdk5/p35 or mt-Cdk5/p35 were exposed to 200 μM SNOC or old SNOC (as a control). After 30 min, cells were lysed and immunoprecipitated with anti-ATM antibody. The immunoprecipitates were probed with anti-ATM (*Lower*) or ATM phospho-1981–specific antibody (*Upper*).

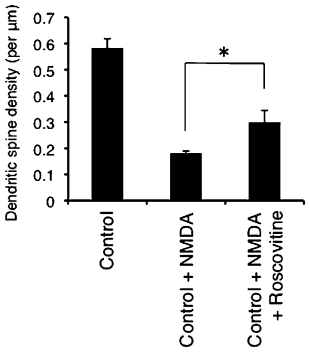

Fig. S6. NMDA-induced dendritic spine loss is partially abrogated by the Cdk5 inhibitor Roscovitine. Cortical neurons were transfected with pEGFP and exposed to 50 μM NMDA in the presence or absence of Roscovitine (10 μM). The next day, the cells were fixed and stained with anti-MAP2 antibody to identify neurons. Spines were visualized by the presence of EGFP in dendritic protuberances and quantified per micrometer of dendrite length. Values are mean + SEM ($n \geq 6$; *t* test with Bonferroni correction, $^*P < 0.05$).

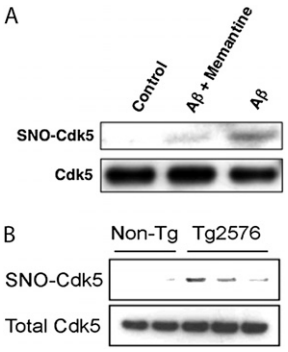

Fig. S7. S-Nitrosylation of Cdk5 in Alzheimer's disease models. (*A*) Detection of SNO-Cdk5 in primary cortical neurons after exposure to Aβ. (*Upper*) SNO-Cdk5. Cortical neurons were exposed to 10 μM $A\beta_{25-35}$ in the presence or absence of 10 μM memantine. After 5 h, cell lysates were prepared and subjected to the biotin-switch assay. (*Lower*) Total Cdk5. Control cells were incubated in the inactive, reverse sequence peptide, $A\beta_{35-25}$. (*B*) Increased SNO-Cdk5 levels in a mouse model of Alzheimer's disease (AD). Brain tissues from Tg2576 AD and control mice were subjected to the biotin-switch assay to detect SNO-Cdk5 in vivo. *Upper*, SNO-Cdk5; *Lower*, total Cdk5.

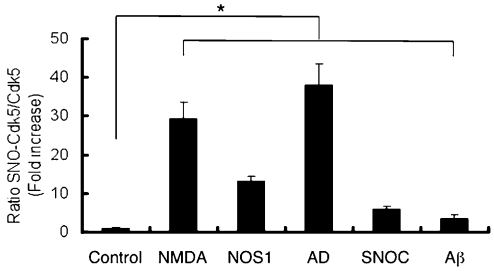

Fig. S8. Ratio of SNO-Cdk5 to total Cdk5 under various conditions. Biotin-switch assays and immunoblot analyses were quantified by densitometry, and the relative ratio of SNO-Cdk5 to total Cdk5 was calculated for the following conditions: in control human brains, in cerebrocortical neurons after NMDA exposure in vitro, in HEK/NOS1 cells after NOS1 activation by A23187 treatment, in human AD brains, in cerebrocortical neurons after SNOC exposure in vitro, and in cerebrocortical neurons after Aβ exposure in vitro. Values are mean ± SEM ($n \geq 3$ for each group; *t* test with Bonferroni correction, $^*P < 0.05$).

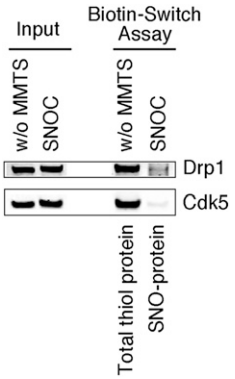

Fig. S9. Biotin-switch assay for calculating standard redox potentials and Gibbs free energy. SH-SY5Y cells were exposed to 50 μM SNOC and cell lysates subjected to the biotin-switch assay. MMTS was used to block free thiols during the assay for S-nitrosylated protein. "Total thiol protein" represents the relative amount of total protein obtained by the biotin-switch assay performed in the absence of MMTS. "SNO-protein" represents the relative amount of S-nitrosylated protein obtained by the biotin-switch assay.

Table S1. List of human brain subjects in this study

Subjects	Diagnosis	Brain region	PMI (h)	Age at time of death	Sex
Control 1	Myocardial infarction	Frontal cortex	2	71	F
Control 2	Myocardial infarction	Frontal cortex	8	63	M
Control 3	Myocardial infarction	Frontal cortex	NA	83	M
Control 4	NA	Frontal cortex	8	74	F
Control 5	Bronchial pneumonia	Frontal cortex	6	91	F
Control 6	NA	Frontal cortex	24	69	F
Control 7	Myocardial infarction	Frontal cortex	9	102	M
Patient 1	AD	Frontal cortex	4	77	F
Patient 2	AD	Frontal cortex	11	78	F
Patient 3	AD	Frontal cortex	NA	76	F
Patient 4	AD	Frontal cortex	6	80	M
Patient 5	AD	Frontal cortex	NA	65	F
Patient 6	AD	Frontal cortex	19	68	F
Patient 7	AD	Frontal cortex	29	75	F
Patient 8	AD	Frontal cortex	NA	80	M
Patient 9	AD	Frontal cortex	12	77	F
Patient 10	AD	Frontal cortex	10	41	M
Patient 11	AD	Frontal cortex	NA	NA	M
Patient 12	AD	Frontal cortex	3	77	F
Patient 13	AD	Frontal cortex	NA	84	M

NA, not analyzed; PMI, postmortem interval.