Supporting Information

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SI Methods

Immunocytochemistry. Cultured hippocampal neurons were fixed and permeabilized sequentially with 4% paraformaldehyde containing 4% sucrose, and 0.1% Triton X-100 in PBS solution. Neurons were then blocked with 5% goat serum in PBS for 1 h and incubated with primary antibodies (overnight, 4°C) and with secondary antibodies (1 h at room temperature). For endogenous surface labeling of AMPA receptors, live hippocampal neurons were incubated with a rabbit anti-GluR1 N-terminal antibody (1, 2) for 20 min at 10°C. After washing, neurons were fixed as described. For NMDA receptor–only synapses, cells were fixed after AMPA receptor labeling and permeabilized with methanol followed by 0.15% Triton X-100 in PBS solution. Neurons were then incubated with mouse anti-NR1 antibody (Affinity Bioreagents, 1:800) overnight at 4°C before incubation with Alexa 568- and Alexa 647–conjugated secondary antibodies. Confocal images were obtained on a Nikon TE300 inverted microscope using a Yokogawa spinning disk confocal scan head (Solamere Technology Group). Images were analyzed using Metamorph image analysis software. For NMDA receptor–only synapse measurements, the percentage of NMDA receptor–only synapses was calculated using the following formula: $100 \times (1 -$ [number of NMDAR puncta containing AMPAR puncta/number of total NMDAR puncta]).

Parkin Mutants and shRNA Constructs. Parkin shRNA sequences 253 and 635: 5′-GGAACAACAGAGTATCGTTC ACATAGTA-3′ and 5′-CCAAACCGGATGAGTGGAGAGTGCCAATC-3′, respectively, were generated against the rat *PARK2* gene. The control scrambled shRNA sequence was obtained from GenScript (5′-CCATTCTGAATCGGTAAGCGACCAATCGCTTACCGATTCAGAATGG-3′).

Electrophysiology. Neurons were held at −60 mV and mEPSCs and mIPSCs were recorded over 5 min using a MultiClamp 700A amplifier (Axon Instruments) controlled with a Pentium PC running MultiClamp Commander and pClamp (Axon Instruments). The extracellular solution contained (in mM): 150 NaCl, 5 KCl, 10 Hepes, 1 $MgCl_2$, 30 D-glucose, 2 $CaCl_2$, 0.001 TTX, and 0.03 bicuculline (330 mOsm/l, pH 7.4). Recording pipettes with resistances between 3 and 5 MΩ were filled with a solution containing (in mM): 30 $CsSO_4$, 70 K_2SO_4, 25 Hepes, 25 N-methyl-D-glucamine, 0.1 $CaCl_2$, 1 EGTA, 2 Na_2ATP, and 0.1 leupeptin (300 mOsm/l, pH 7.2). In experiments recording mIPSCs, the internal solution contained (in mM) 150 KCl, 3 $MgCl_2$, 15 Hepes, 0.1 $CaCl_2$, 1 EGTA, 2 Na_2ATP, and 0.1 leupeptin (300 mOsm/l, pH 7.2). Data were analyzed using MiniAnalysis software (Synaptosoft). Detection criteria for mEPSCs included amplitudes greater than 5 pA and rise times from the onset to the peak of less than 5 msec. In the analysis of NMDA-to-AMPA current ratios, recordings were obtained in the extracellular solution described earlier but increasing Ca^{2+} to 4 mM and omitting Mg^{2+}. The NMDA component was defined as the current 25 msec after the peak. AMPA-mediated mEPSCs obtained in the presence of D-AP5 were completely absent by 20 msec after the peak.

Immunoblot Analysis. COS-7 cells were transfected with GFP-CDCrel-1 alone, GFP-CDCrel-1 and rat WT parkin (GenBank accession no. NM_020093), or GFP-CDCrel1 with parkin-wt and parkin mutants (R42P, R275W, A240R, T415N; Fig. S2) or mRFP, mRFP plus parkin shRNA, parkin-WT, parkin-WT plus parkin shRNA, parkin*, or shRNA plus parkin* (Fig. S6*A*). High-density rat cortical neuron cultures were infected with GFP, GFP–parkin-WT, or GFP–parkin shRNA lentiviral constructs (Fig. S6*B*). Forty-eight hours after transfection, or 72 h after lentiviral infection, 15 μg of whole cell lysate from each group was subjected to SDS/PAGE and immunoblotting with anti–CDCrel-1 or anti-parkin antibodies. Blots were then probed with anti-rabbit HRP secondary antibodies (Amersham), and bands were detected with an ECL-Plus Western blotting detection system (Amersham). Blots were visualized and quantified on an LAS-3000 imaging system (Fuji).

FM Dye Loading. Hippocampal neurons were transfected with GFP, GFP–parkin-WT, or GFP–parkin-R420P on DIV10, 3 to 4 days before FM dye labeling. For puncta number and intensity measurements, neurons were first washed with extracellular solution containing 119 mM NaCl, 2.5 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM Hepes, 30 mM glucose (pH = 7.4), 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 50 μM APV, and 1 μM TTX. The recycling pool of vesicles in presynaptic terminals was then labeled by evoking presynaptic release with 50 mM K^+ solution (78.5 mM NaCl, 50 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM Hepes, 30 mM glucose (pH 7.4), 10 μM CNQX, 50 μM APV, and 1 μM TTX.) in the presence of 10 μM fixable FM dye, AM4–64, for 60 sec. After labeling, neurons were washed with extracellular solution for 10 min and then fixed in 4% paraformaldehyde solution. To measure FM4–64 destaining kinetics, neurons were first washed with extracellular solution containing 119 mM NaCl, 2.5 mM KCl, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM Hepes, 30 mM glucose (pH 7.4), 10 μM CNQX, and 50 μM APV. Presynaptic terminals were then labeled by evoking presynaptic activity with strong electrical stimulation (60 sec stimulation at 10 Hz) in extracellular solution containing 15 μM FM4–64. After washing, FM4–64 destaining was monitored during the application of 2,250 stimuli at 15 Hz (150 sec). Images were acquired for 270 sec in 20-sec intervals (2 frames before destaining, 8 frames during destaining, and 3 more frames after destaining). For each FM4–64 puncta, the integrated intensity of the final three time points was averaged (end value) and this value was subtracted from each previous time point. After subtraction, the value of every point was normalized to the first point to represent the destaining kinetics over time. All experimental statistics were generated using two-sided *t* tests unless otherwise noted.

1. Ehlers MD (2000) Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron* 28:511–525.

2. O'Brien RJ, *et al.* (1998) Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron* 21:1067–1078.

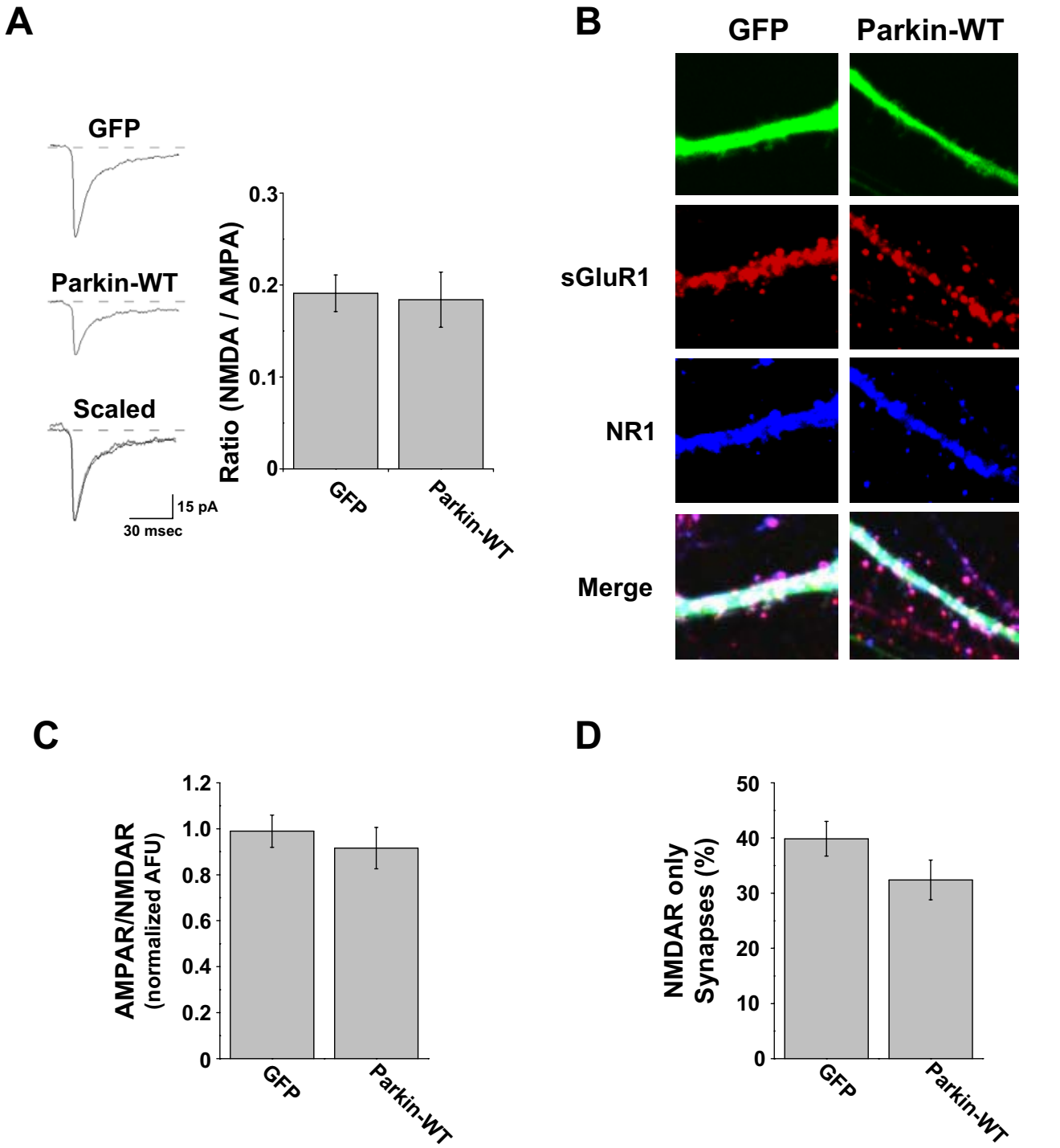

Fig. S1. Parkin does not alter the relative abundance of AMPA receptors and NMDA receptors at glutamatergic synapses. (*A*) Averaged mEPSCs obtained from control neurons (*Top*) and neurons expressing parkin (*Middle*) in Mg^{2+}-free solution. (*Bottom*) Current overlay scaled to the peak current amplitude. The bar graph indicates the ratio of NMDA- and AMPA receptor-mediated currents (NMDA/AMPA). Data represent means ± SEM. (*B*) Hippocampal neurons (DIV14) expressing GFP or GFP–parkin-WT were immuno-labeled for surface GluR1 and total NR1. (*C*) Normalized surface GluR1–to–total NR1 (AMPAR/NMDAR) ratios at synapses of hippocampal neurons expressing GFP or GFP–parkin-WT. (*D*) Quantification of NMDA receptor (NMDAR)–only synapses on hippocampal neurons expressing GFP or GFP–parkin-WT. Data represent mean ± SEM.

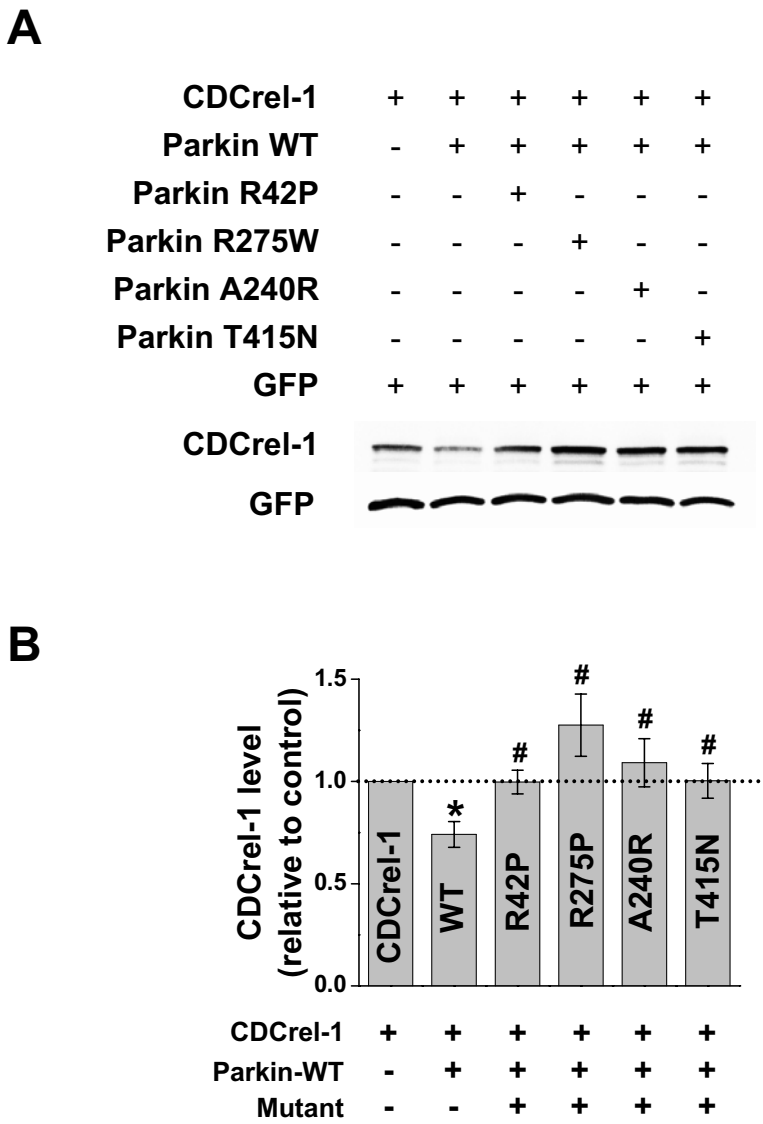

Fig. S2. Co-expression of disease-linked parkin mutants inhibits parkin-mediated degradation of CDCrel-1/septin 5. (*A*) COS-7 cells were transfected with the parkin substrate CDCrel-1 alone, CDCrel-1 plus parkin-WT, or CDCrel-1 plus parkin-WT and parkin mutant as indicated. Twenty-four hours after transfection, 15 μg of cell lysate from each condition was subject to SDS/PAGE and immunoblotting. All cells were co-transfected with pEGFP-N1 expression vector as an expression control. Note that expression of parkin caused a decrease in CDCrel-1 that was prevented by co-expression of disease-linked mutants. (*B*) Quantitative analysis of the immunoblot data from *A*. Data represent mean ± SEM. $^{**}P < 0.05$ relative to CDCrel-1 alone, $^{\#}P < 0.05$ relative to CDCrel-1 plus parkin-WT, $n = 5$, ANOVA.

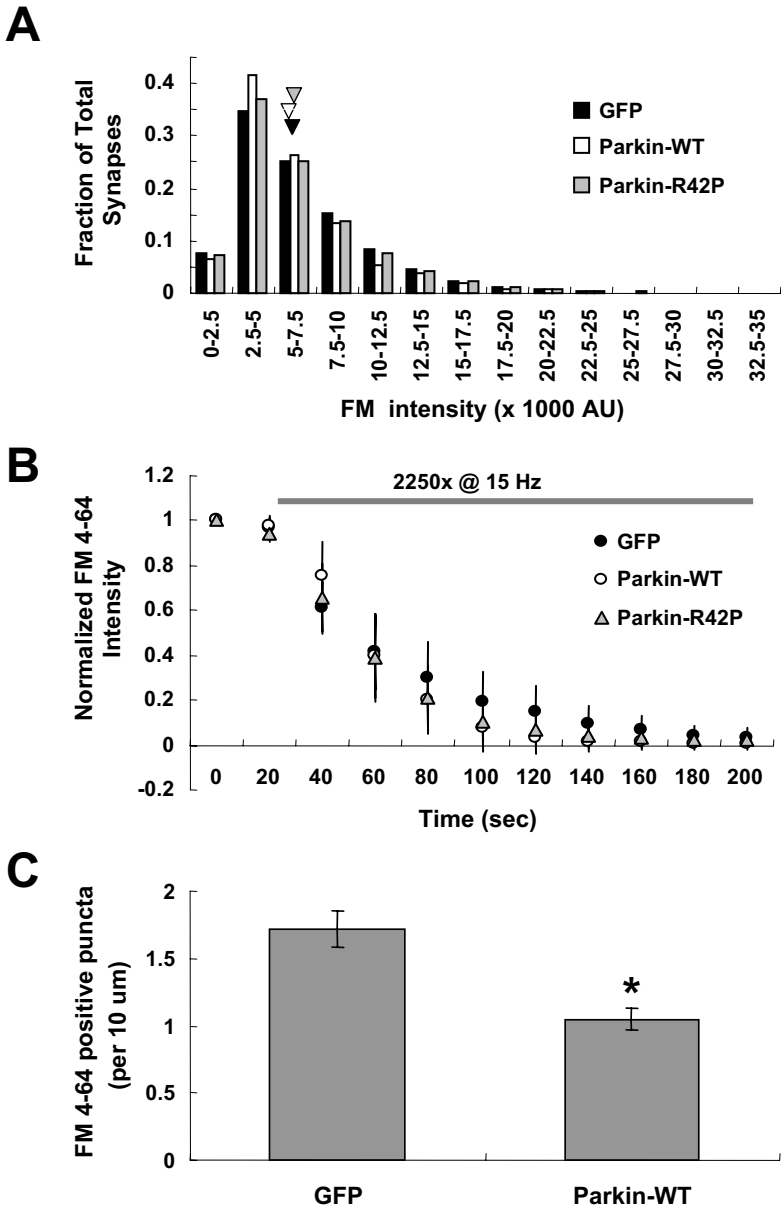

Fig. S3. Postsynaptic expression of parkin decreases the number of functional presynaptic terminals without affecting presynaptic vesicle recycling. (*A*) Parkin does not affect the recycling pool of synaptic vesicles. The distribution of FM4–64 fluorescence intensity values at individual boutons following high K^+ loading (see *Methods* for details). Data were collected from neurons expressing GFP, GFP–parkin-WT or GFP–parkin-R42P (number of puncta/number of neurons, 4,268/16, 2,500/16, and 3,559/16, respectively). The mean intensity of each group is indicated by inverted triangles. (*B*) Postsynaptic parkin does not affect FM4–64 destaining kinetics. Loss of FM4–64 fluorescence over individual puncta during a 15-Hz train of action potentials. Data represent mean ± SD for 102 (GFP), 139 (GFP–parkin-WT), and 127 (GFP–parkin-R420P) puncta (seven neurons for each group). Puncta were maximally destained following 2,250 stimuli at 15 Hz. (*C*) Parkin reduces the number of functional synapses. Presynaptic terminals contacting hippocampal neurons expressing GFP or GFP–parkin-WT for 4 days were loaded by incubation in FM4–64 during high K^+ stimulation (see *Methods* for details). Data represent mean ± SEM of FM positive puncta per 10 μm dendrite ($*P < 0.001$).

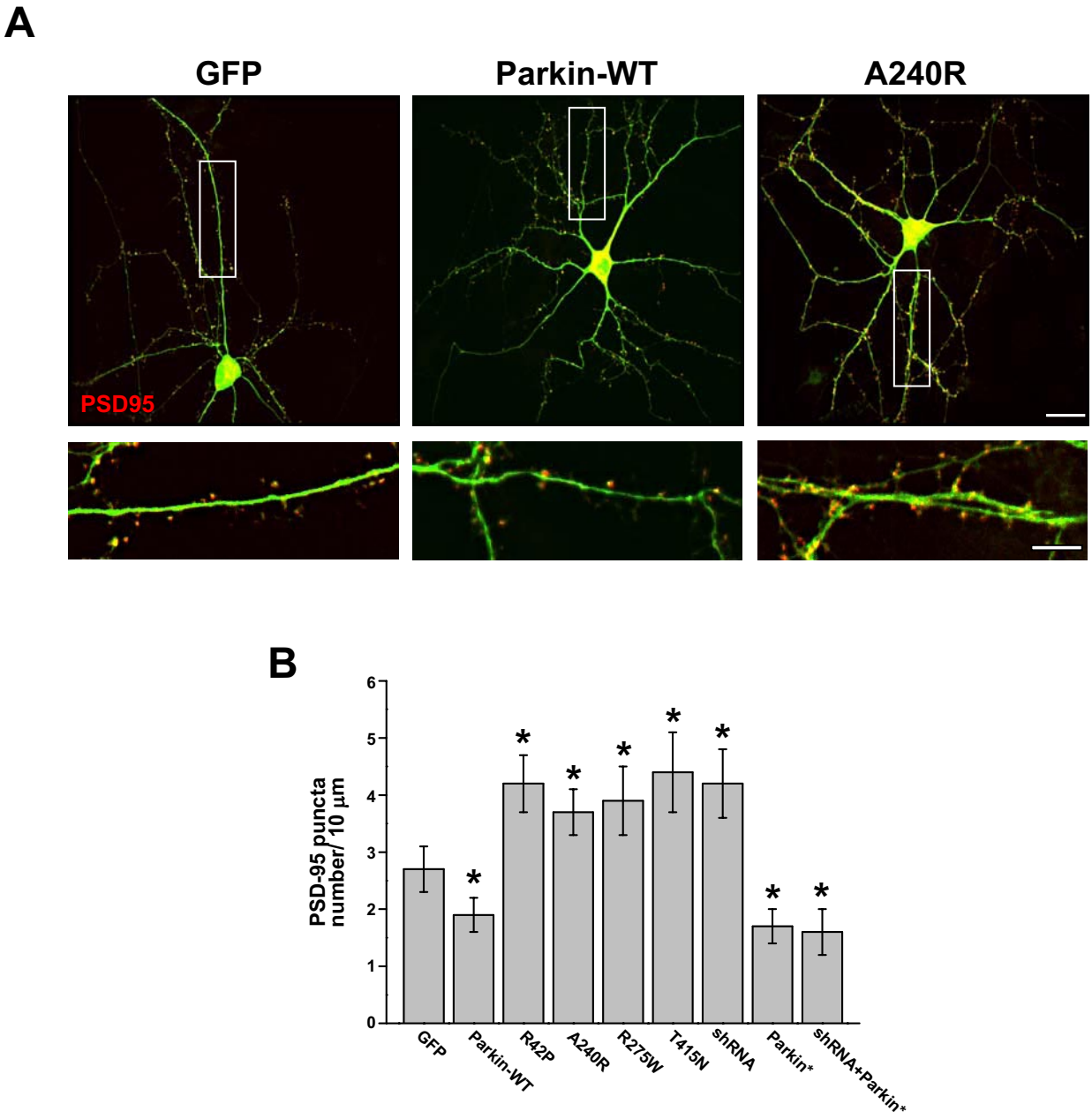

Fig. S4. Parkin causes a loss of glutamatergic synapses. (*A*) Hippocampal neurons (DIV14) expressing GFP, GFP–parkin-WT, or PD-linked parkin mutant (GFP–parkin-A240R; green) for 3 days were fixed and stained for PSD-95 (red). Boxed regions (*Upper*) are also magnified (*Lower*). (Scale bar, 10 μm, *Upper*; 5 μm, *Lower*.) (*B*) Quantitative analysis of the number of PSD-95 puncta on dendrites from neurons expressing GFP, parkin-WT, parkin shRNA, parkin*, parkin shRNA plus parkin*, or the indicated parkin mutants R42P, A240R, R275W, or T415N. Data represent means ± SEM of PSD-95 puncta per 10 μm dendrite, $n = 12$, $^*P < 0.001$ relative to GFP control by *t* test.

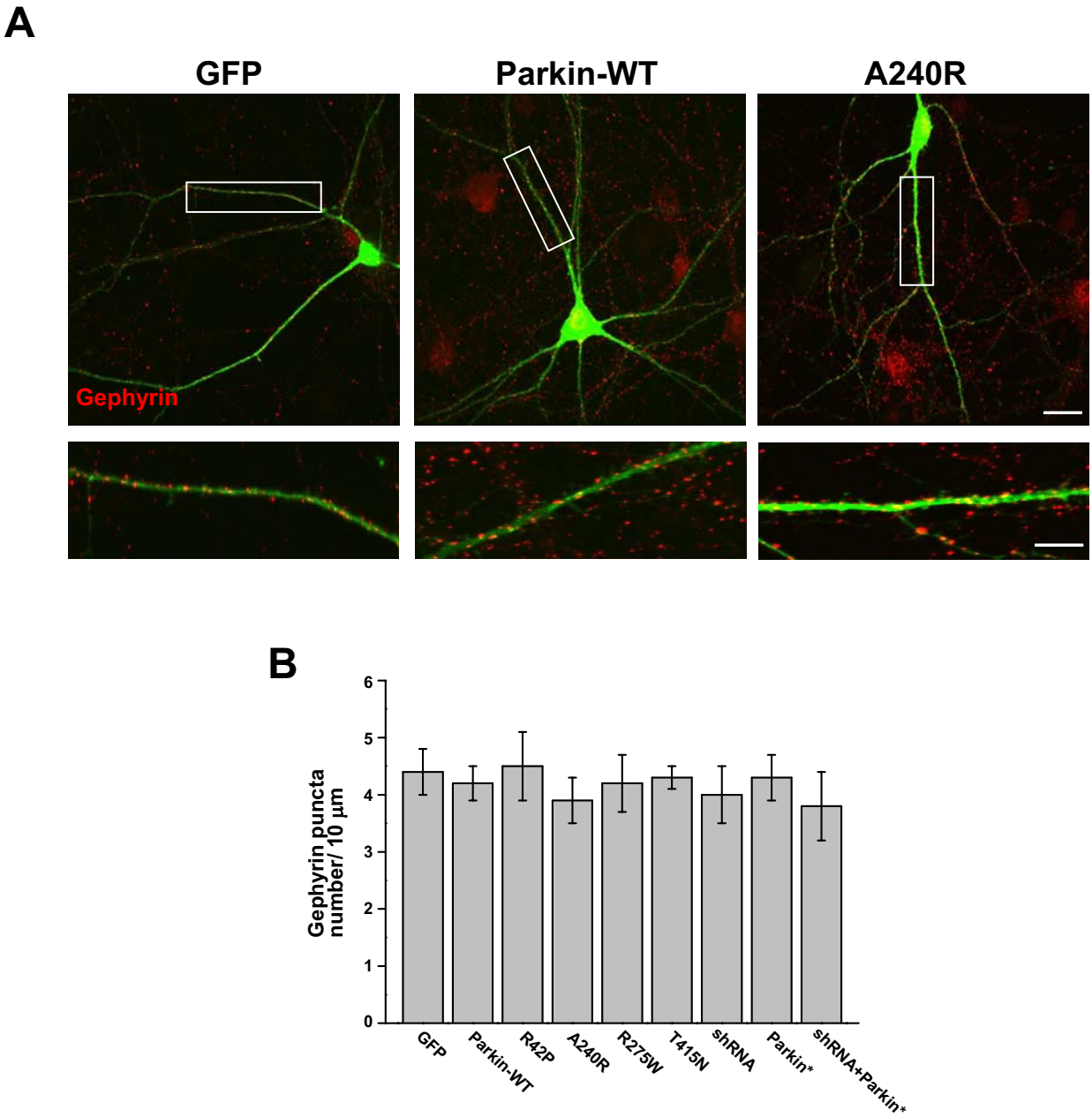

Fig. S5. Parkin has no effect on GABAergic synapse number. (*A*) GABAergic synapses on hippocampal neurons (DIV14, derived from P0 rats) were visualized by immunocytochemical staining for the inhibitory postsynaptic scaffold protein gephyrin (red) expressing GFP, GFP–parkin-WT, or the PD-linked parkin mutants GFP–parkin-A240R (green). (Scale bar, 10 μm, *Upper*; 5 μm, *Lower*.) (*B*) Quantification of gephyrin puncta number along dendrites of neurons expressing GFP, parkin-WT, parkin shRNA, parkin*, parkin shRNA + parkin*, or the indicated parkin mutants R42P, A240R, R275W, or T415N; $n = 14$ each. Data represent mean ± SEM; $P > 0.05$.

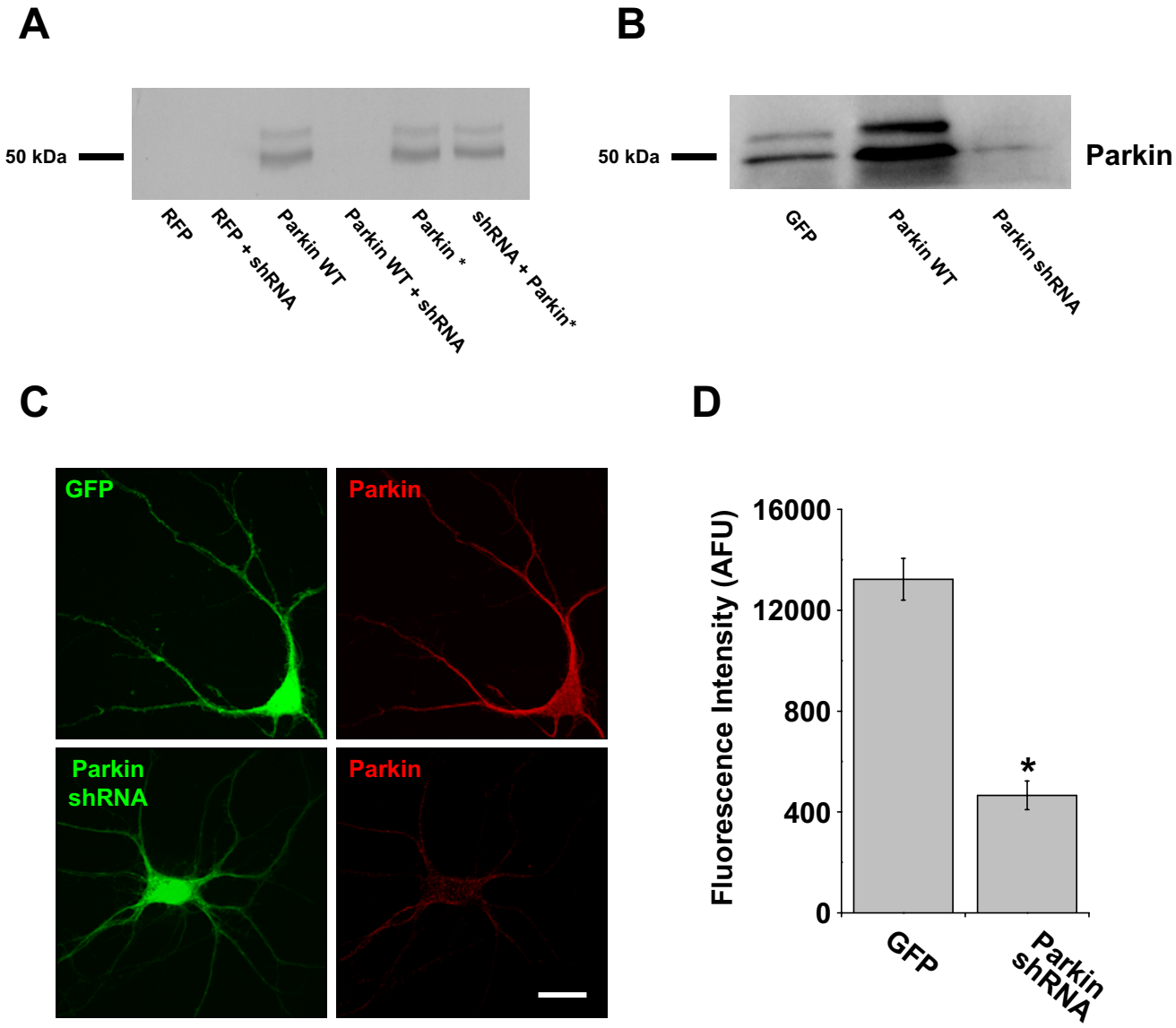

Fig. S6. Knockdown of endogenous parkin by RNAi. (*A*) Parkin shRNA decreases expression of transfected parkin-WT in COS-7 cells. Expression of a parkin mutant bearing silent third codon site mutations to produce mismatches with the shRNA sequence (parkin*) was unaffected by parkin-shRNA. (*B*) Lysates from rat cortical neuron cultures infected with GFP, parkin-WT, or parkin-shRNA lentivirus were subjected to immunoblot analysis with anti-parkin antibody. Neurons infected with parkin-WT lentivirus had a two- to threefold increase in parkin expression whereas neurons infected with parkin shRNA constructs had a >70% decrease in parkin expression compared with GFP-infected control cultures. Note that the infection efficiency was ≈75%, suggesting that knockdown efficiency in individual neurons was at least 70%. (*C*) Hippocampal neurons (DIV14–17) expressing GFP or a bicistronic GFP::parkin-shRNA vector for 5 days were fixed and stained with an anti-parkin antibody. Neurons expressing parkin-shRNA showed a significant decrease in endogenous parkin staining (*Right*) relative to GFP transfected neurons (*Left*). (Scale bar, 10 μm.) (*D*) Quantification of parkin levels in GFP or parkin shRNA–GFP transfected neurons. Shown are mean ± SEM of parkin fluorescence intensity (GFP, 13,227 ± 830 arbitrary fluorescence units, $n = 18$; shRNA, 4,660 ± 569 arbitrary fluorescence units, $n = 21$). *$P < 0.01$ relative to GFP control.

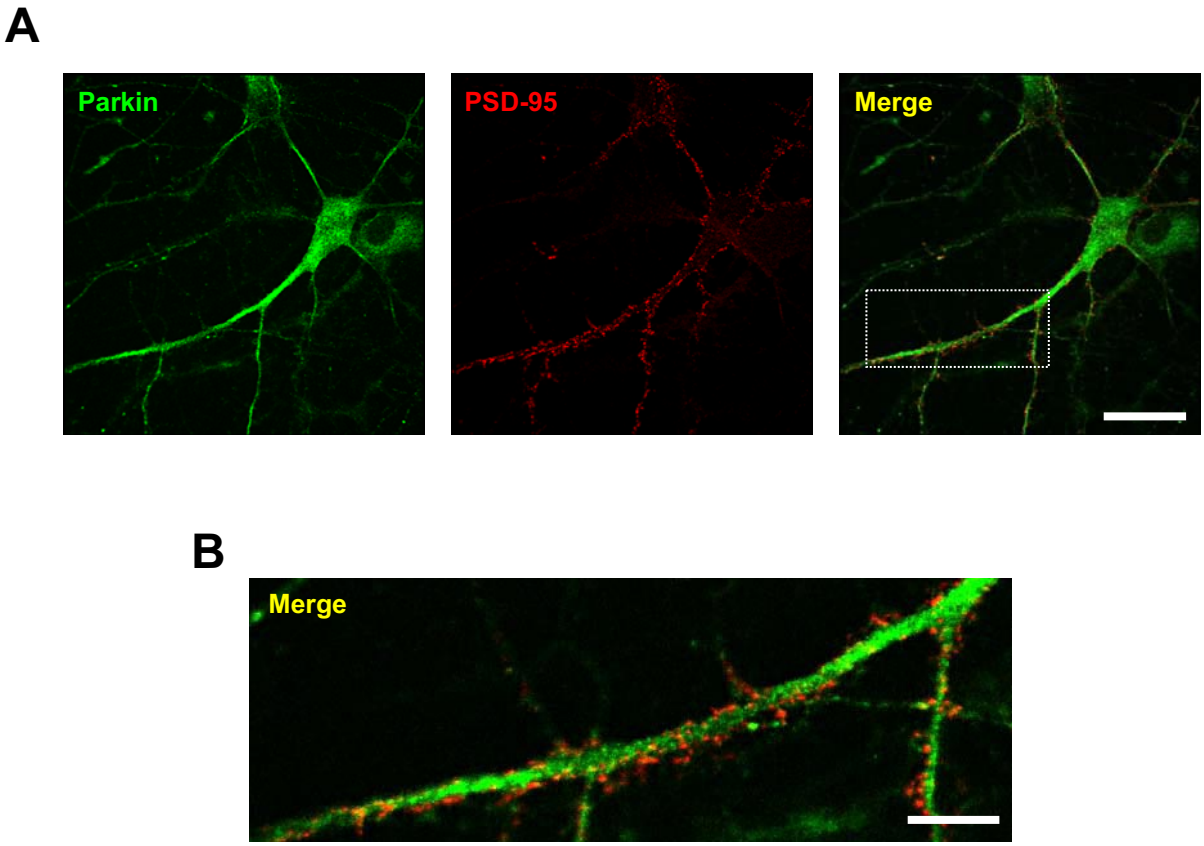

Fig. S7. Parkin is diffusely localized in neurons. Immunocytochemical staining of DIV21 hippocampal neurons for endogenous parkin (green) indicating a diffuse distribution of parkin throughout the cell. Double labeling for the postsynaptic scaffold protein PSD-95 to mark excitatory synapses (red) indicates that parkin is not enriched at glutamatergic synapses at the light microscopy level (merge). Dashed white box in *A* is enlarged in *B*. (Scale bars, 20 μm, *Upper*; 5 μm, *Lower*.)