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# Supplementary Information for

## PICK1 inhibits the E3 ubiquitin ligase activity of Parkin and reduces its neuronal protective effect

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Supplementary materials and methods
Figs. S1 to S11
References for SI reference citations



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## SI Materials and methods

### Mice

PICK1 KO mice have been previously described (1). Parkin KO mice were obtained from the Jackson Laboratory (stock number: 006582). The mice were backcrossed to the C57BL/6J strain (Jackson Laboratory, stock number: 000664). The mice were housed on a 12 h light (7 a.m.)/12 h dark (7 p.m.) cycle with access to water and irradiated rodent chow (Lab Diet 5053) *ad libitum*. Mice were housed three to five mice per cage, and only male mice were used for the experiments. All animal procedures were approved by the Animal Ethics Committee of the Hong Kong University of Science and Technology.

### Antibodies

The guinea pig PICK1 antibody (antigen: the amino acid residues 317-416 of mouse PICK1) used for immunostaining, the rabbit PICK1 antibody (antigen: the amino acid residues 317-416 of mouse PICK1) used for immunoblotting, the rabbit PICK1 antibody (antigen: the amino acid residues 1-29 of mouse PICK1) used for immunoprecipitation, and the GFP antibody (antigen: full length GFP) have been previously described (2). The following primary antibodies were also used in this study: mouse PICK1 antibody (75-040, NeuroMab, Davis, CA), mouse Parkin

antibody (4211S, Cell Signaling Technology, Danvers, MA), mouse HA antibody (H9658, Sigma-Aldrich, St. Louis, MO), mouse actin antibody (A5441, Sigma-Aldrich), mouse PARIS antibody (SAB4200530, Sigma-Aldrich), rabbit myc antibody (sc-789, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit synphilin-1 antibody (sc-98296, Santa Cruz Biotechnology), rabbit p38 antibody (sc-535, Santa Cruz Biotechnology), rabbit α-synuclein antibody (sc-7011-R, Santa Cruz Biotechnology), rabbit cyclin E antibody (sc-481, Santa Cruz Biotechnology), mouse ubiquitin antibody (sc-8017, Santa Cruz Biotechnology), rabbit synaptotagmin XI antibody (sc-135411, Santa Cruz Biotechnology), rabbit CDCrel-1 antibody (11631-1-AP, Proteintech, Chicago, IL), mouse GAPDH antibody (Ab8245, Abcam, Hong Kong), mouse GluA2 (MAB397, Millipore, Hong Kong), rabbit tyrosine hydroxylase antibody (AB152, Millipore), and mouse myc antibody (9E 10, Developmental Studies Hybridoma Bank, Iowa City, IA).

**cDNA Cloning**

The human PICK1 cDNA construct was cloned into the corresponding vectors in frame using the restriction enzyme *SalI/NotI* (New England Biolabs, Ipswich, MA). The construct pDsRed-N1-mito was generated by cloning the mitochondrial targeting sequence into pDsRed-N1 using the restriction enzyme *BamHI/NotI* (New England

Biolabs). Human Parkin, synphilin-1, and α-synuclein have been previously described (3). All constructs were verified using sequencing.

**Cell Culture and Transfection**

Human embryonic kidney 293T (HEK293T) cells (CRL-3216, ATCC, Manassas, VA) and SH-SY5Y cells (CRL-2266, ATCC) were maintained in a humidified incubator with 95% air and 5% $CO_2$. These cell lines were free of mycoplasma contamination. HEK293T cells and SH-SY5Y cells were grown in MEM (Gibco, Grand Island, NY) or DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, GE Healthcare, Logan, UT), 1 mM sodium pyruvate (Gibco), and antibiotics (100 IU penicillin and 100 μg $ml^{-1}$ streptomycin, Gibco). Cells were passaged every 2-3 days when they reached 90-100% confluency. The transfection of HEK293T cells was performed using calcium phosphate co-precipitation. The transfection of SH-SY5Y cells was performed using Lipofectamine according to the manufacturer's instruction (Thermo Fisher Scientific, Waltham, MA). Primary hippocampal neurons were cultured from the brains of Sprague-Dawley embryonic rats. Briefly, the hippocampi were dissected from the embryos and digested using 0.25% trypsin (Gibco) for approximately 15 min at 37°C. Following the termination of the trypsin reaction with horse serum (Gibco), the hippocampal neurons were dissociated using titration. The hippocampal neurons were

plated on coverslips coated with poly-L-ornithine (Sigma-Aldrich). The primary culture was maintained in neuronal culture medium (neurobasal medium supplemented with B27, L-glutamine, and antibiotics; all purchased from Gibco) with half-feed changes every 4 days. The hippocampal neurons were transfected with the indicated constructs using the calcium phosphate co-precipitation method at DIV 7-9 and assayed after 2-3 days.

**Immunocytochemistry**

HEK293T cells and primary cultured hippocampal neurons were fixed using a fixative solution that contained 4% paraformaldehyde (PFA) and 4% sucrose in PBS for 20 min at room temperature (RT). The cells were washed once with PBS at RT to remove the remaining fixative. The cells were subsequently permeabilized using 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 10 min. The cells were washed twice with PBS at RT to remove the remaining detergent. After 1 h of blocking with 10% normal donkey serum (NDS) in PBS at RT, the cells were incubated with primary antibodies in 3% NDS for 1 h at RT or overnight at 4°C. The cells were subsequently washed with PBS three times and incubated with fluorescent-dye-conjugated secondary antibodies (Jackson ImmunoResearch) for 1 h at RT. After washing with PBS (3 x 10 min), the coverslips were mounted and air-dried overnight at RT or 1-3 h at 37°C for imaging.

**Immunohistochemistry**

The brains were first fixed by cardiac perfusion through the left ventricle with the fixative solution (4% PFA and 4% sucrose in PBS), followed by an additional immersion-fixation for 4 h at 4°C. For fluorescent staining, cryoprotection was performed via incubation of the brains in a gradient of sucrose (10% sucrose, 20% sucrose, and 30% sucrose in PBS) at 4°C until the samples dropped to the bottom. Floating 40 μm-thick cryosections in TBS were used for immunostaining. After 1 h of blocking with 10% NDS at RT and primary antibody incubation overnight at 4°C, the slices were incubated with fluorescent-dye-conjugated secondary antibodies for 1 h at RT. DAPI was subsequently used to label the nuclei for 15 min at RT.

**In Vitro Binding**

In brief, 2μg GST or GST fusion proteins were bound to glutathione-Sepharose beads (Life technologies) in binding buffer (50 mM Tris-HCl at pH 7.5, 140 mM NaCl, 0.1% Triton X-100 with protease inhibitors) at 4℃ for 1 h. The beads coupled with GST fusion protein were added to 2μg His-Parkin (Millipore), and incubated for 3 h at 4℃. The beads were washed three times with TBS; bound proteins were processed for SDS-PAGE and Coomassie Blue staining.

**In Vitro Ubiquitination Assay**

In brief, 2μg of purified GST or GST fusion proteins were incubated at 37°C in 50 μl of reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM DTT, and 2 mM ATP) containing 100 ng of E1 (Boston Biochem), 200 ng of E2 (UbcH7, Boston Biochem), 1 μg of His-tagged Parkin (Millipore), and 10 μg ubiquitin (Boston Biochem). After incubation for 3 h, the reaction was stopped by addition of loading buffer. Reaction products were analyzed by western blotting with ubiquitin antibody to detect the autoubiquitination of Parkin. Loading controls were analyzed by Ponceau S staining.

**Co-Immunoprecipitation In Vitro**

Transfected HEK293T cells were harvested and solubilized in 2% Triton X-100 in PBS supplemented with PMSF and aprotinin for 1 h. Protein A beads (GE Healthcare, Uppsala, Sweden) were incubated with antibodies for 1 h. Following centrifugation at a maximum speed (16,873 x *g*) for 20 min, the supernatant was collected and incubated with protein A beads for 2-4 h. The beads were subsequently centrifuged and then washed with 2% Triton X-100 buffer once, 2% Triton X-100 buffer supplemented with

500 mM NaCl twice, and PBS twice. The proteins were eluted with SDS sample buffer and boiled at 95°C for 5 min.

**Co-Immunoprecipitation In Vivo**

The brains from WT and PICK1 KO mice were homogenized using homogenization buffer (10 mM Tris-HCl and 320 mM sucrose supplemented with protease inhibitor cocktail tablets (Roche, Mannheim, Germany), pH 7.4). The homogenates were centrifuged at 700 x *g* for 10 min at 4°C, and the supernatant was retained. Next, 2% Triton X-100 was added to the supernatant to solubilize the proteins for 2 h at 4°C. The solution was centrifuged at a maximum speed (16,873 x *g*) for 20 min. The supernatant was retained, and the protein concentration was determined using the Bradford protein assay. The final concentration was adjusted to 1 mg/ml. Protein A beads were incubated with PICK1 antibodies for 1 h. Then, 0.5 ml of each brain sample was extracted, and immunoprecipitation was performed with protein A beads. The beads were subsequently centrifuged and washed with 2% Triton X-100 in TBS once, 2% Triton X-100 in TBS supplemented with 500 mM NaCl twice, and TBS three times. The proteins were eluted with SDS sample buffer and boiled at 95°C for 5 min. PICK1 KO mice were used as control to test the interaction between PICK1 and Parkin.

**Preparation of Tissue Samples for Western Blotting**

Different mouse tissues were dissected using scissors and fine sharp forceps. Brain micro-dissection was performed to obtain various regions, such as the VM and cortex, under a dissecting microscope. The tissues were homogenized with homogenization buffer (10 mM Tris-HCl and 320 mM sucrose supplemented with protease inhibitor cocktail tablets (Roche), pH 7.4) to obtain the total protein. The samples were centrifuged at 700 x *g* for 10 min at 4 °C, and the supernatant was retained. The protein concentration was determined using the Bradford protein assay (Pierce Protein Biology, Rockford, IL). The samples were mixed with SDS sample buffer and boiled at 95 °C for 5 min. Equal amounts of protein were separated via SDS-PAGE and analyzed using western blotting.

**Quantification of Parkin Aggresomes**

Transfected HEK293T cells were fixed and immunostained. Aggregates larger than 2.6 μm in diameter were designated Parkin aggresomes. The percentage of Parkin aggresomes was quantified as the ratio of the number of cells that contained one or more Parkin aggresomes to the number of total transfected cells.

**Quantification of the Percentage of Axons or Cell Bodies and Dendrites with Parkin Translocation to Mitochondria and the Percentage of Mitochondrial Clearance in Axons**

Transfected hippocampal neurons were treated with DMSO or 13.5 μM CCCP for the indicated times. The percentage of axons with Parkin translocation to mitochondria was calculated as the ratio of the number of axons with Parkin translocation to mitochondria to the number of total transfected axons. The percentage of cell bodies and dendrites with Parkin translocation to mitochondria was calculated as the ratio of the number of cells with Parkin translocation to mitochondria in the cell bodies and dendrites to the number of total transfected cells. The percentage of mitochondrial clearance was calculated as the ratio of the number of axons that exhibited diffuse DsRed signals to the number of total transfected axons.

**Cell Death Assay**

SH-SY5Y cells were transfected by Lipofectamine 2000. Twenty-four hours after transfection, the cells were treated with 10 μM MG132 for 18 h. Hoechst (Molecular Probes, Eugene, OR) and propidium iodide (Molecular Probes) were stained to quantify the percentage of cell death. Transfected hippocampal neurons were treated with DMSO or 25 μM kainate for 2 h. Ventral midbrain neurons were infected with

AAV-DJ expressing the respective proteins and treated with DMSO or 100 μM kainate for 10 h. DAPI was used to label the nuclei. Image J (NIH) was used to analyze the data, and the area of the nuclei determined the percentage of cell death.

**MPTP Administration**

Young (2-month-old) and old (12-month-old) male WT, PICK1 KO, Parkin KO, and PICK1/Parkin DKO mice were administered MPTP or saline. The mice were subcutaneously administered a single low dose of MPTP HCl (25 mg per kg of body weight, calculated as a conjugated salt; Sigma-Aldrich) daily for five consecutive days. The control young and old male mice received an equivalent volume of 0.9% saline. The mice were sacrificed 90 min, 5 or 21 days after the last injection, and the brains were dissected for further analysis.

**Quantification of TH- and Nissl-Positive Neurons.**

The brains were dissected from the mice administered saline or MPTP and were fixed with 4% PFA and 4% sucrose in PBS overnight at 4°C. Cryoprotection was performed via incubation of the brains in different concentrations of sucrose solutions (10% sucrose, 20% sucrose, and 30% sucrose in PBS) at 4°C. Every fourth floating 40 μm-thick cryosection that spanned the SNpc was collected for immunohistochemistry.

The brain sections were first treated with hydrogen peroxide for 10 min at RT to quench the endogenous hydrogen peroxide activity. The sections were subsequently washed with TBS (3 x 5 min). Then, the sections were permeabilized and blocked with 10% NDS plus 0.3% Triton X-100 in TBS for 1 h at RT in a humidified atmosphere. After blocking, the sections were incubated with primary antibodies in 3% NDS plus 0.3% Triton X-100 in TBS overnight at 4°C. The sections were subsequently washed with TBS three times and then incubated with biotinylated rabbit secondary antibody for 1 h. Next, the sections were washed with TBS three times and incubated with VECTASTAIN Elite ABC reagent (Vector Labs, Burlingame, CA) for 30 min. After washing with TBS three times, the sections were stained with 3, 3'-diaminobenzidine (DAB, Sigma-Aldrich) for several minutes; the reaction was stopped by washing in water twice. Finally, the sections were dehydrated using ethanol and xylene and subsequently mounted on slides.

To confirm there is an actual neuron loss in SNpc, sections adjacent to the ones for TH staining were stained with Cresyl violet (Nissl staining). We performed TH staining prior Nissl staining on these sections. Unbiased counts of the total numbers of the TH- and Nissl-positive neurons within the SNpc of one hemisphere were performed using the optical fractionator workflow under Nikon Ni-U upright microscope equipped with MBF Stereo Investigator software. Regions of interest

(ROI) were outlined at low magnification (4X objective) and sampled at high magnification (100X oil objective). The TH labeled whole cell body inside the counting frame without touching the avoidance lines were counted as TH-positive neuron. A neuronal nucleus which was clearly visualized and entirely inside the counting frame without touching the avoidance lines was counted as Nissl-positive neuron. Sampling criteria were empirically chosen and tested based on reported literature parameters. The following parameters were used in our study: sampling frame, 80 μm x 80 μm; grid area, 200 μm x 200 μm; guard region, 1.5 μm; sampling depth, 10 μm. Systematic random sampling was implemented by software once the designated region of interest was covered. Coefficient of error values for TH neurons and nuclei were both under 0.1.

**Quantification of TH Optical Density in Striatum**

TH staining is striatum is similar to the TH staining in SNpc. All images were obtained with the Nikon Eclipse T*i* inverted microscope with SPOT advanced software at the same magnification to allow the visualization of the semi-striatum in a single field. To quantify the TH fibers, the optical density (OD) was measured using ImageJ software. The optical density of the corpus callosum was used as background and was subtracted from every measurement.

**MAO-B activity assay**

Measurement of striatal MAO-B activity was performed using a MAO activity kit (OxiSelect™ Monoamine Oxidase Assay Kit, XPX-5006) according to the manufacturer's protocol. Briefly, crude mitochondria were isolated from mouse striatum, and used as the enzyme source. To increase the specificity of MAO-B, samples were incubated with MAO-A inhibitor prior to the assay. Benzylamine (100 mM) was used as the substrate. Mixture were incubated at room temperature and protected from light. The absorbance was recorded at 570 nm at 45 min (T1) and 60min (T2). The generated $H_2O_2$ during the reaction time ($\Delta T$ = T2- T1) were measured according to the standard curve. Sample MAO-B Activity = $\Delta H_2O_2$ / ($\Delta T$ x protein amount) = pmol/min/mg. Data were normalized to WT control.

**Measurement of DA and MPP+ levels**

1. Reagent

Dopamine hydrochloride (H60255), 1-Methyl-4-phenylpyridinium iodide (MPP+ iodide) (D048), and Dopamine internal standard (IS) Dopamine-$D_4$ hydrochloride solution (D-072) were purchased from Sigma-Aldrich Chemical Company. Formic Acid (21318.297) was purchased from VMR Chemicals.

2. Preparation of tissue samples

After 90 min (for MPP+ measurement) or 5 days (for dopamine measurement) of last dose of MPTP administration, striatal tissue samples were dissected and rapidly frozen and kept in -80 °C for storage. For tissue lysate preparation, striatal tissue was homogenized in lysis buffer (0.5M formic acid with 0.1 ng/μl DA IS) with the concentration of 10 mL/g tissue, then sonicated with Branson Sonifier 250 with 20% duty cycle and 2 output control for 15 times. The lysates were centrifuged at 13,500 x *g* for 10 min at 4 °C. The supernatant was separated and centrifuged one more time, and then put on ice for LC-MS dopamine and MPP+ analysis, or store at -20 °C until analysis. Lysis buffer was used as the blank control.

3. Preparation of standard calibration curve

The calibrating curve solution of DA (10, 20, 50, 100, 200, 1000, 5000 pg/μl) and MPP+ (1, 2, 5, 10, 20, 100, 500 pg/μl) were prepared in 0.5M formic acid and measured. The isotope labeled DA (DA-$D_4$) was used as IS in the calibrators. The linear equation of the line resulting from calibration plots for DA and MPP+ was used to determine sample concentration. The peak areas of standards and samples were measured using QQQ Quantitative Analysis software (MassHunter Workstation, Agilent Technologies).

4. LC-MS analysis of DA and MPP+

Buffer A was composed of 0.1% formic acid plus 3% acetonitrile in $H_2O$ (v/v). Buffer B was consisted of 0.1% formic acid in acetonitrile (v/v). A linear gradient elution profile from 99% A to 90% B over 6 min was run at a flow rate of 0.2 ml/min, with a 2 min re-equilibration between injections. The present study was performed by a high efficiency reversed-phase HPLC separation and an ESI-MS/MS equipped with a Zorbax Eclipse Plus C18 column (2.1 x 100 mm, 3.5 μm, Agilent Technologies). Five microliters of each sample supernatant were injected into LC-MS, the MS/MS spectrum was acquired by the product ion scan mode, and then product ions were selected by SRM scan mode. DA fragment ions used m/z 91.0, 137.0, DA IS fragment ions used m/z 95.0, 141.0, and MPP+ fragment ions used m/z 116.0, 155.0 for qualitative and quantitative analysis.

**Confocal Microscopy**

Confocal images of fixed cells and brain slices were obtained under an inverted microscope (LSM510 or LSM710 Meta; Carl Zeiss, Inc.) with a 63x/1.4 NA oil DIC Plan Apo objective. The images were acquired using LSM or ZEN 2009 software. Adobe Photoshop was used to process the images.

**Statistical Analyses**

Statistical analyses were performed using GraphPad Prism Software. The experiments were not randomized; however, the data collection and analysis were performed blindly to the experimental conditions. No data were excluded from the analyses. Data are represented as mean ± SEM. Statistical methods were not used to predetermine the sample sizes. However, our sample sizes are similar to those generally employed in the field (3-6). The distribution of data was assumed to be normal, but it had not been formally tested. For the comparisons between two groups, unpaired two-tailed Student's t tests were used to analyze the data. For the comparisons across more than two groups, the data were analyzed using one-way analyses of variance (ANOVAs) and adjusted with the Bonferroni's correction. For data with more than one independent variable, two-way ANOVAs with Bonferroni's post hoc analysis were used. A p value $< 0.05$ was considered statistically significant and is indicated by asterisks as follows: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

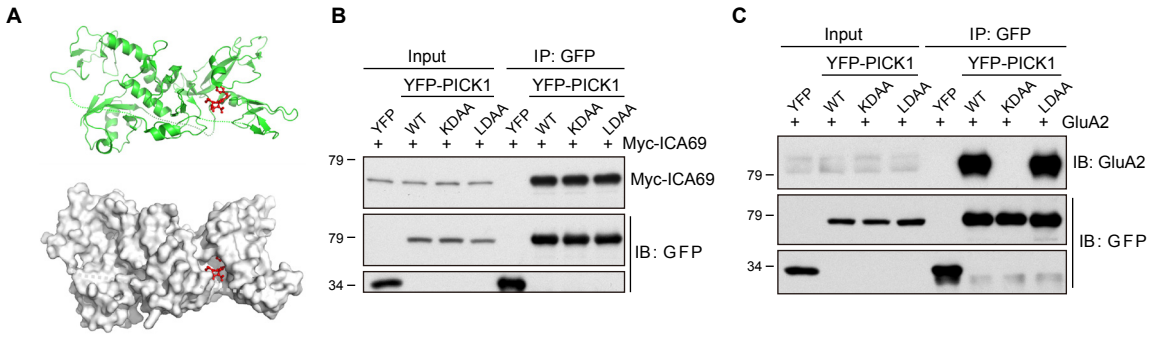



**Fig. S1.** PICK1 LDAA binds to ICA69 and GluA2 normally. (A) Crystal structure of Parkin (PDB code: 4K95) shows that the C-terminal last three residues (FDV) are mostly buried inside the folding core. The upper panel shows ribbon diagram of Parkin and the lower panel shows surface map of Parkin. The last three residues are presented in ball-and-stick model and highlighted in red. (B and C) Co-immunoprecipitation of ICA69 (B) and GluA2 (C) with PICK1 and PICK1 mutants in whole cell lysates from HEK293T cells expressing the respective constructs.

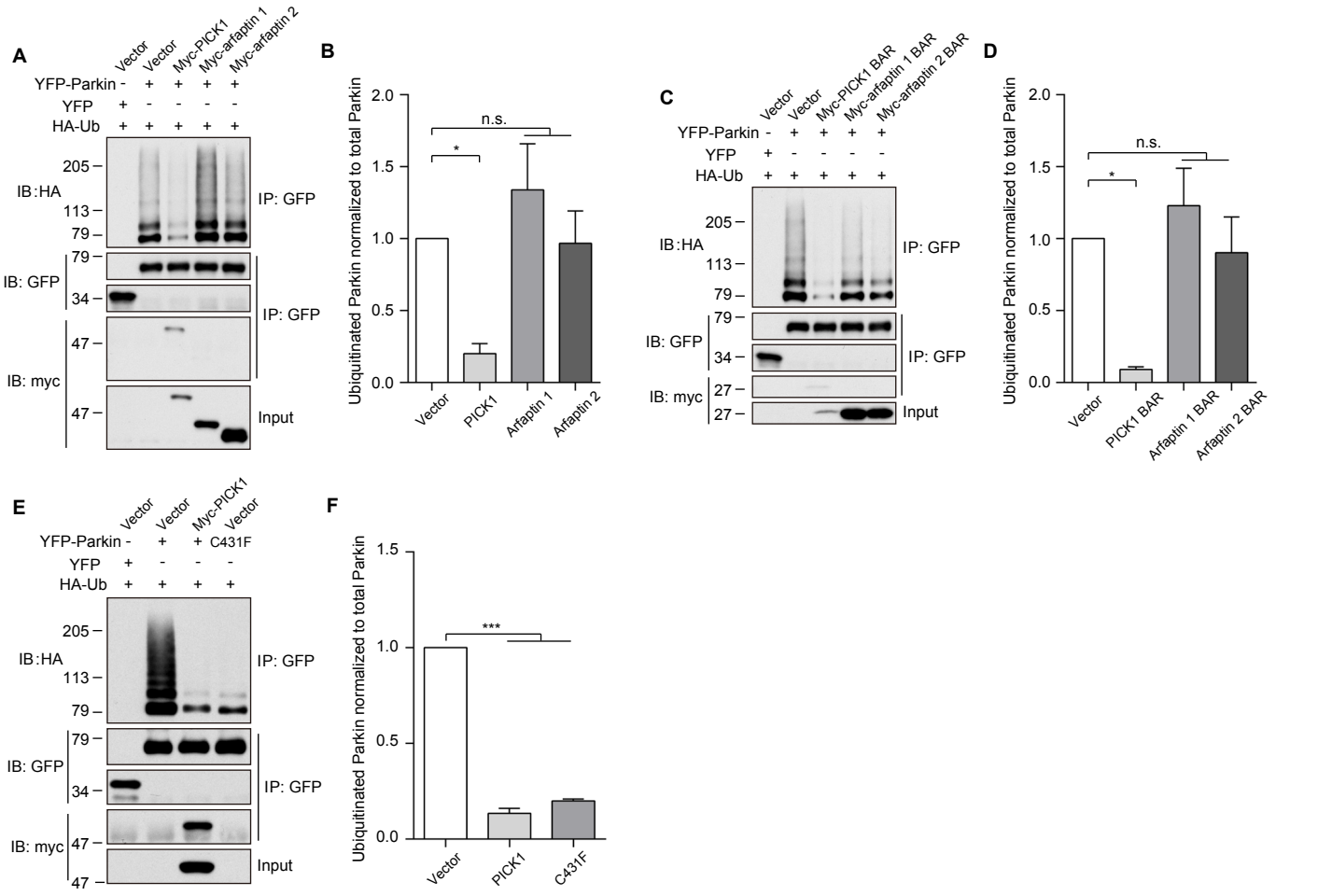



**Fig. S2.** Arfaptin 1 and arfaptin 2 do not affect Parkin E3 ligase activity. (A and C) Western blot analysis of effects of (A) PICK1, arfaptin 1, and arfaptin 2 and (C) their BAR domains on autoubiquitination of Parkin in whole cell lysates from HEK293T cells expressing the indicated constructs. GFP antibodies were used to immunoprecipitate YFP-Parkin. HA antibodies were used to detect polyubiquitinated Parkin. (B) Quantification of ubiquitinated Parkin normalized to total Parkin in (A) (n = 4). (D) Quantification of ubiquitinated Parkin normalized to total Parkin in (C) (n = 4). (E) Western blot analysis of ubiquitination of Parkin C431F and effect of PICK1 on ubiquitination of Parkin. (F) Quantification of ubiquitinated Parkin normalized to total Parkin in (E) (n = 4). Error bars indicate mean ± SEM. One-way ANOVA with post hoc Bonferroni test. *$p < 0.05$; ***$p < 0.001$; n.s., not significant.

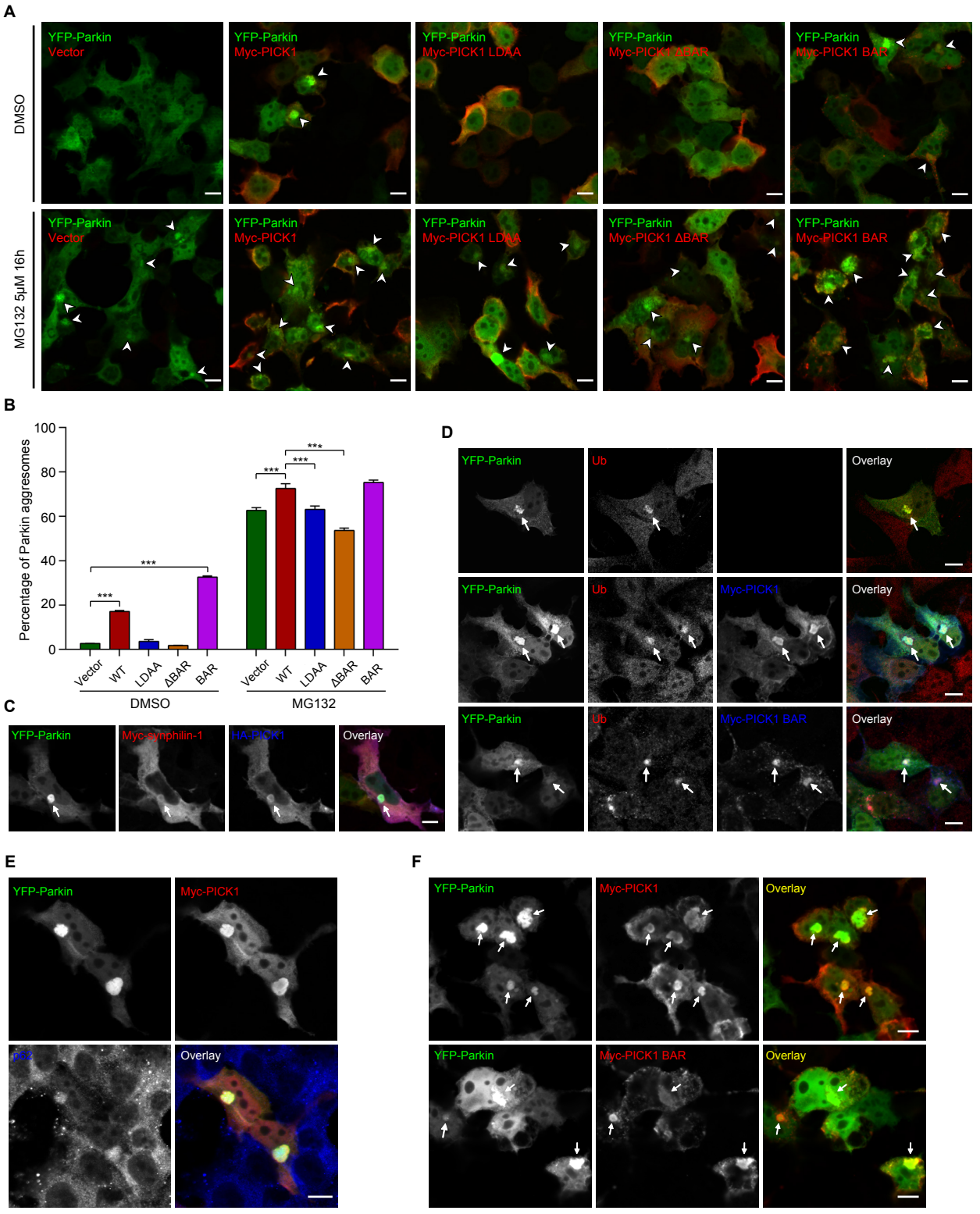
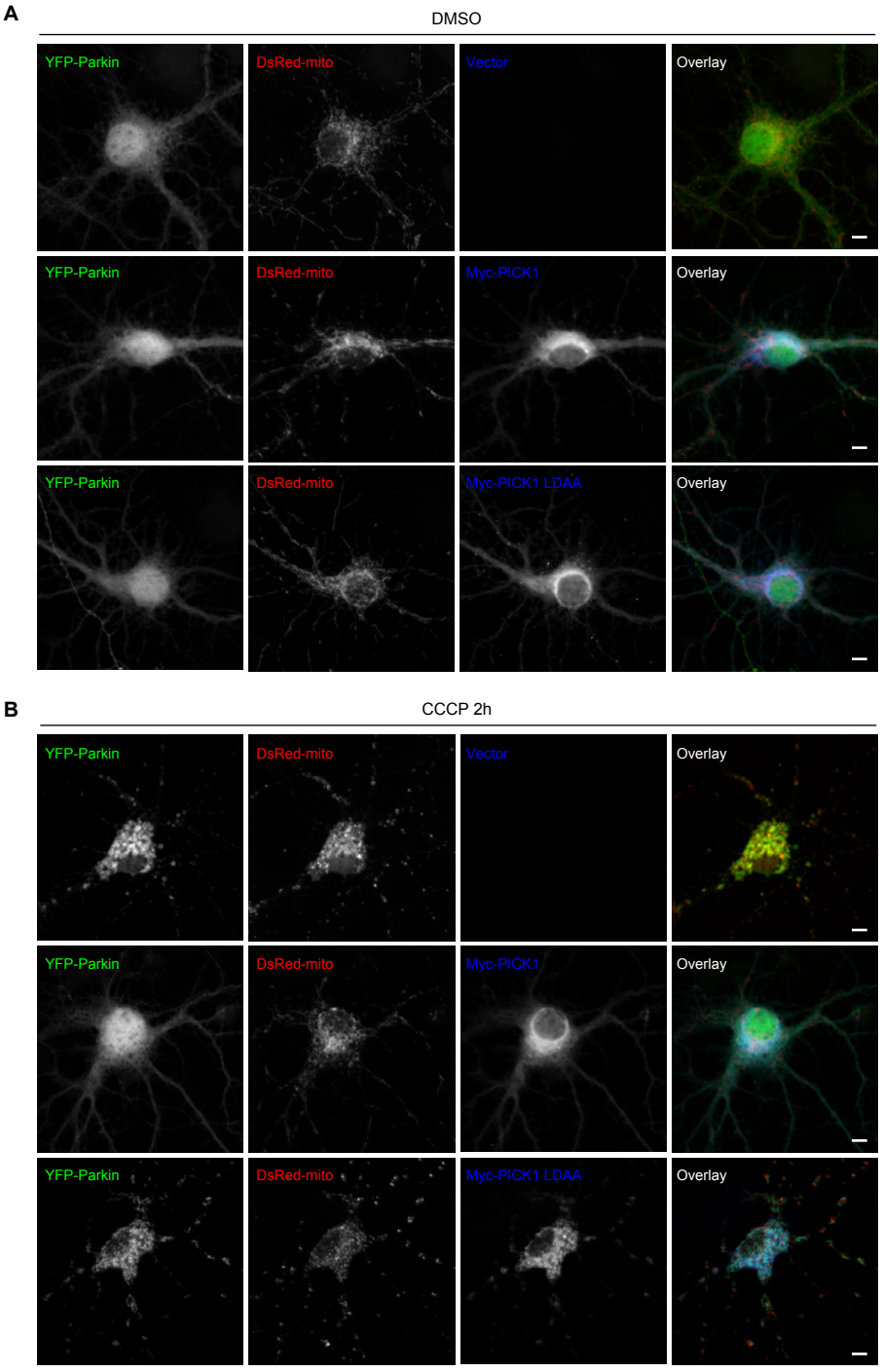



**Fig. S3.** PICK1 promotes Parkin aggresome formation. (A) Immunofluorescence staining for Parkin with PICK1 and PICK1 mutants in HEK293T cells expressing the indicated constructs. HEK293T cells were treated with DMSO or 5 μM MG132 for 16 h. Scale bars, 10 μm. (B) Quantification of the percentage of Parkin aggresomes in (A) (n = 3). Error bars indicate mean ± SEM. Two-way ANOVA with post hoc Bonferroni test. ***p < 0.001. (C) Immunofluorescence staining for Parkin, synphilin-1, and PICK1 in HEK293T cells expressing the indicated constructs. Scale bar, 10 μm. (D) Immunofluorescence staining for Parkin, ubiquitin, PICK1, and PICK1 BAR in HEK293T cells expressing the indicated constructs. Endogenous ubiquitin (Ub, red) was labeled using ubiquitin antibodies. Scale bars, 10 μm. (E) Immunofluorescence staining for Parkin, PICK1, and endogenous p62. Scale bars, 10 μm. (F) Immunofluorescence staining for Parkin with PICK1 and PICK1 BAR in HEK293T cells expressing the indicated constructs. Scale bars, 10 μm. The arrowheads and arrows indicate the Parkin aggresomes.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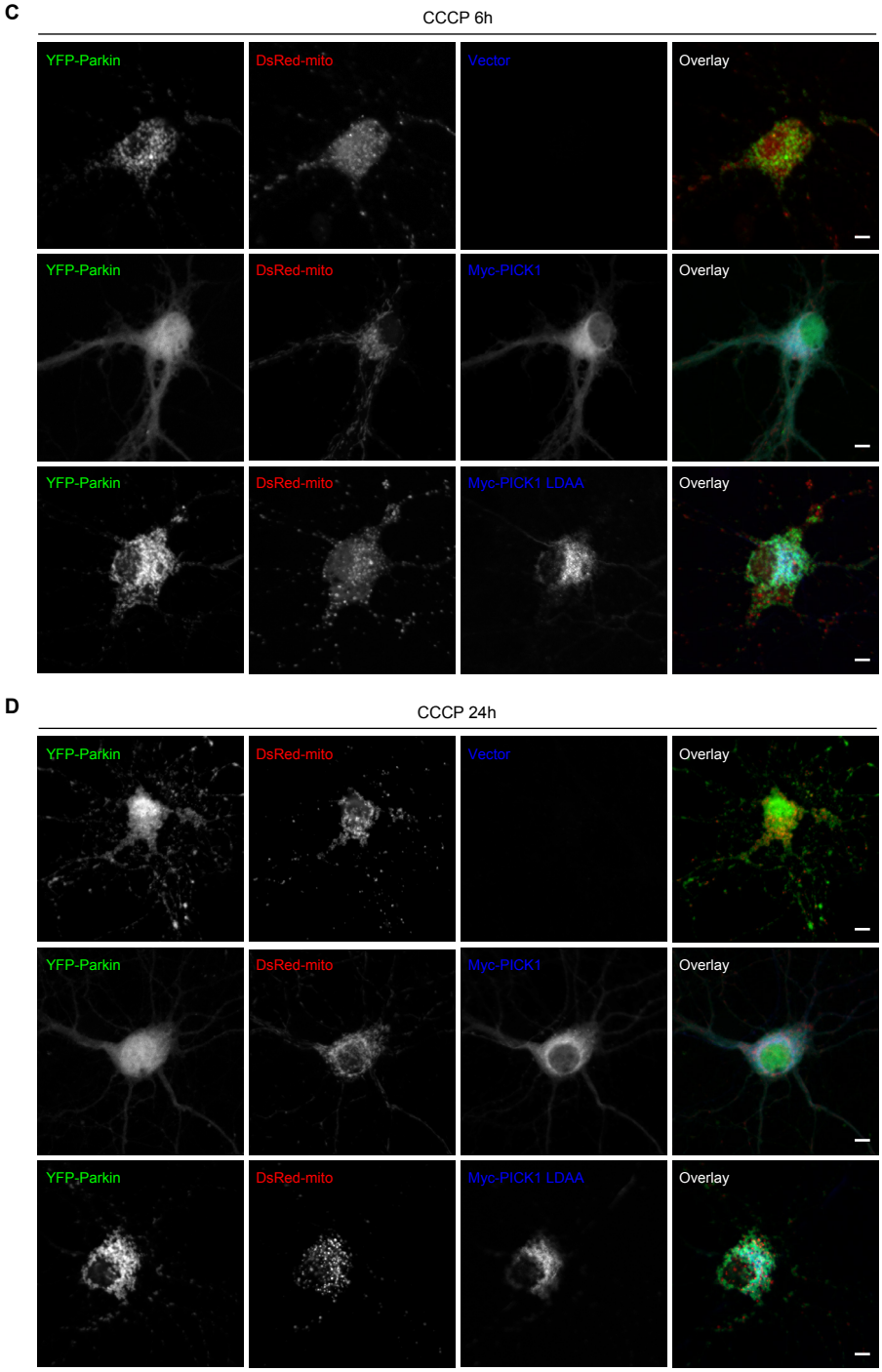
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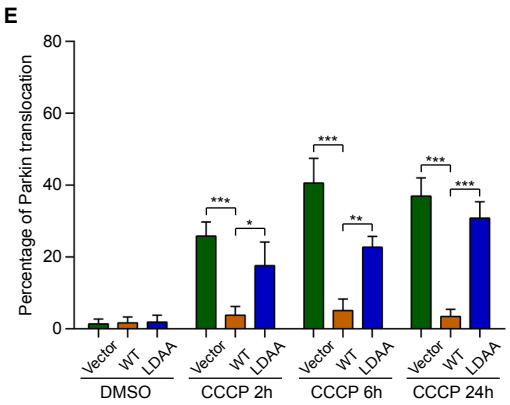


**Fig. S4.** PICK1 inhibits the translocation of Parkin to damaged mitochondria in cell bodies and dendrites in cultured hippocampal neurons. (A-D) Cultured hippocampal neurons were transiently transfected with YFP-Parkin (green) and DsRed-mito (red) in combination with vector control (blue) or myc-PICK1 (blue) or myc-PICK1 LDAA (blue). The neurons were treated with DMSO or 13.5 μM CCCP for indicated times. Scale bars, 10 μm. (E) Quantification of Parkin translocation in cell bodies and dendrites (n = 4). Error bars indicate mean ± SEM. Two-way ANOVA with post hoc Bonferroni test. *p < 0.05; **p < 0.01; ***p < 0.001.

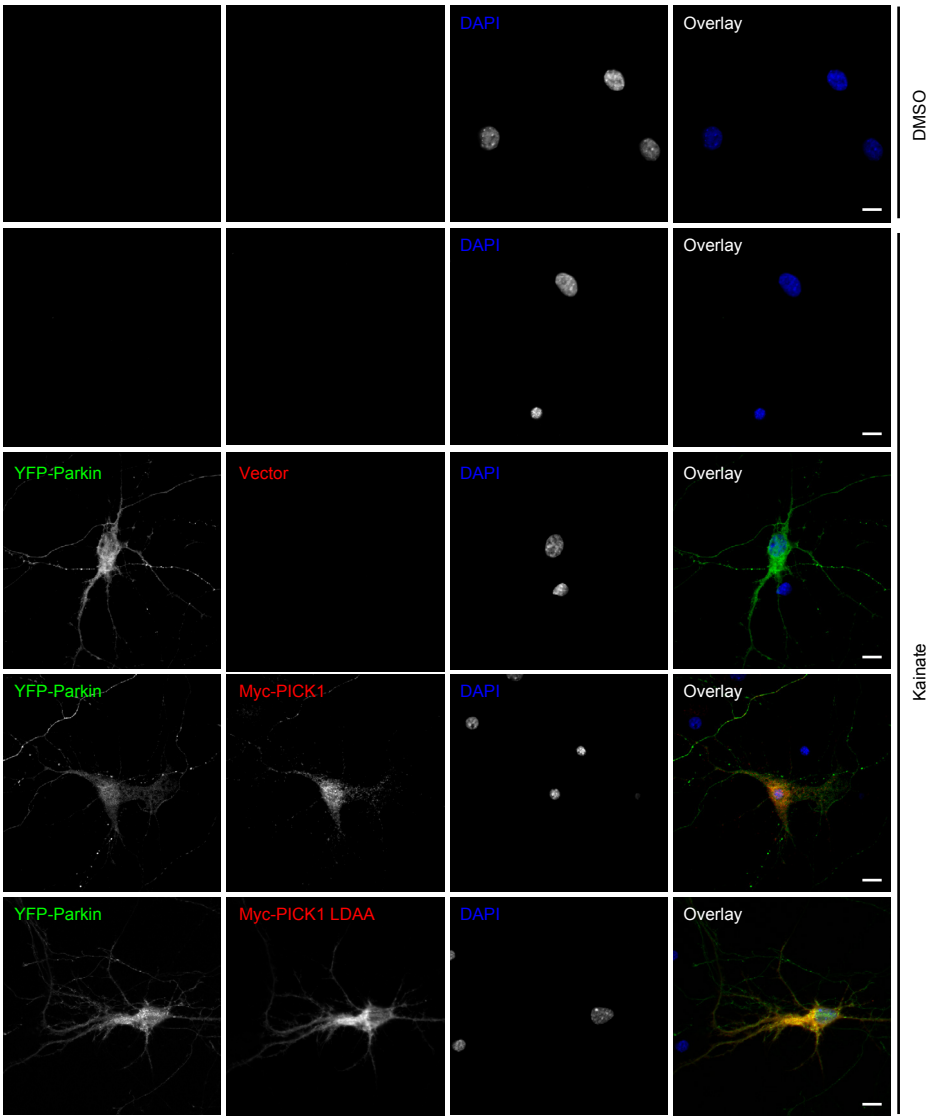


**Fig. S5.** PICK1 inhibits Parkin-mediated cell survival in neurons. Immunofluorescence staining of cultured hippocampal neurons expressing the indicated constructs. The neurons were treated with DMSO or 25 μM Kainate. The nuclei were labeled with DAPI. Scale bars, 10 μm.

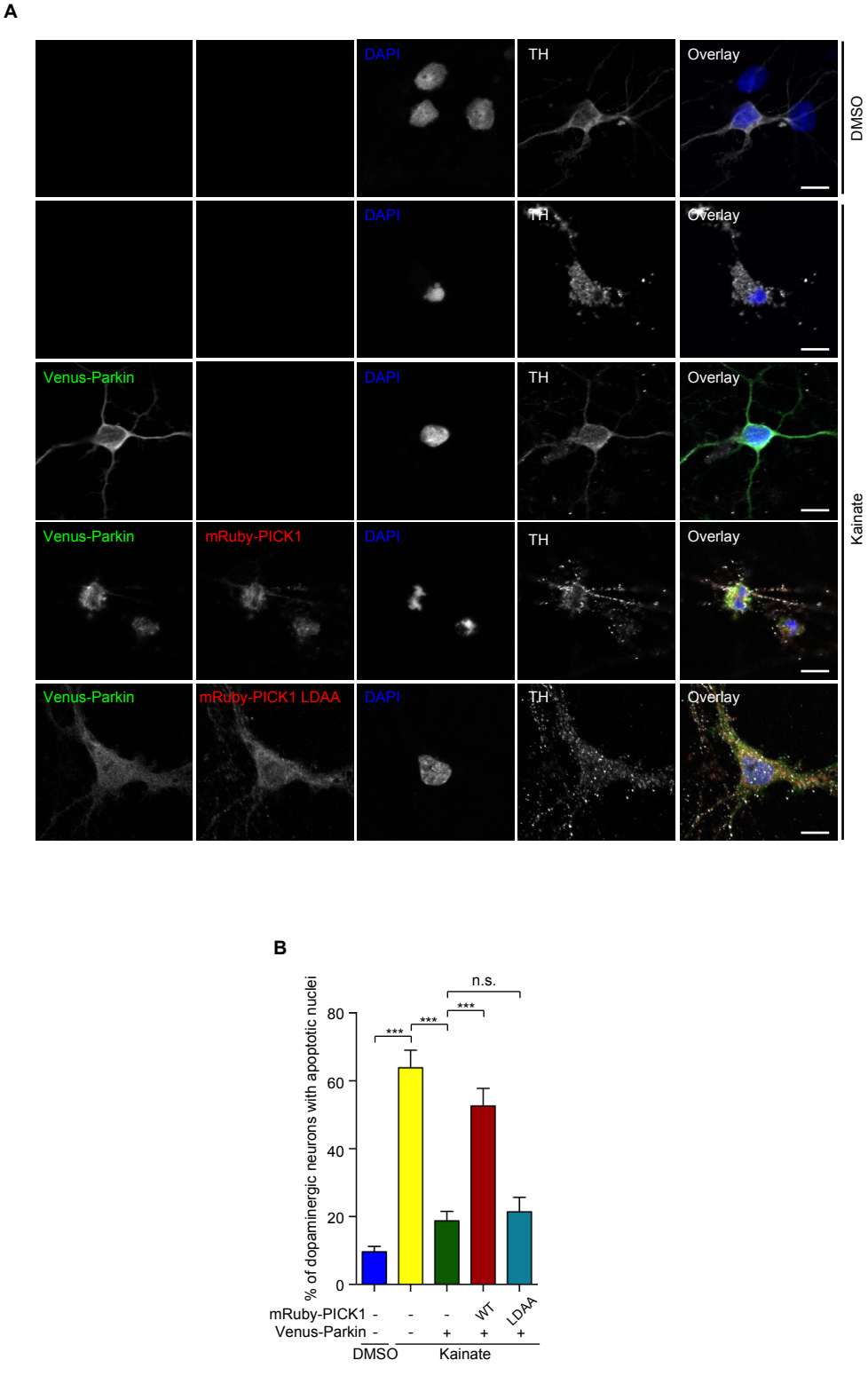


**Fig. S6.** PICK1 inhibits Parkin-mediated cell survival in dopaminergic neurons. (A) Immunofluorescence staining of cultured ventral midbrain neurons expressing the indicated constructs. The neurons were treated with DMSO or 100 μM kainate for 10 h. Scale bars, 10 μm. (B) Quantification of the percentage of TH-positive dopaminergic neurons with apoptotic nuclei in (A) (n = 4). Error bars indicate mean ± SEM. One-way ANOVA with post hoc Bonferroni test. ***$p < 0.001$; n.s., not significant.

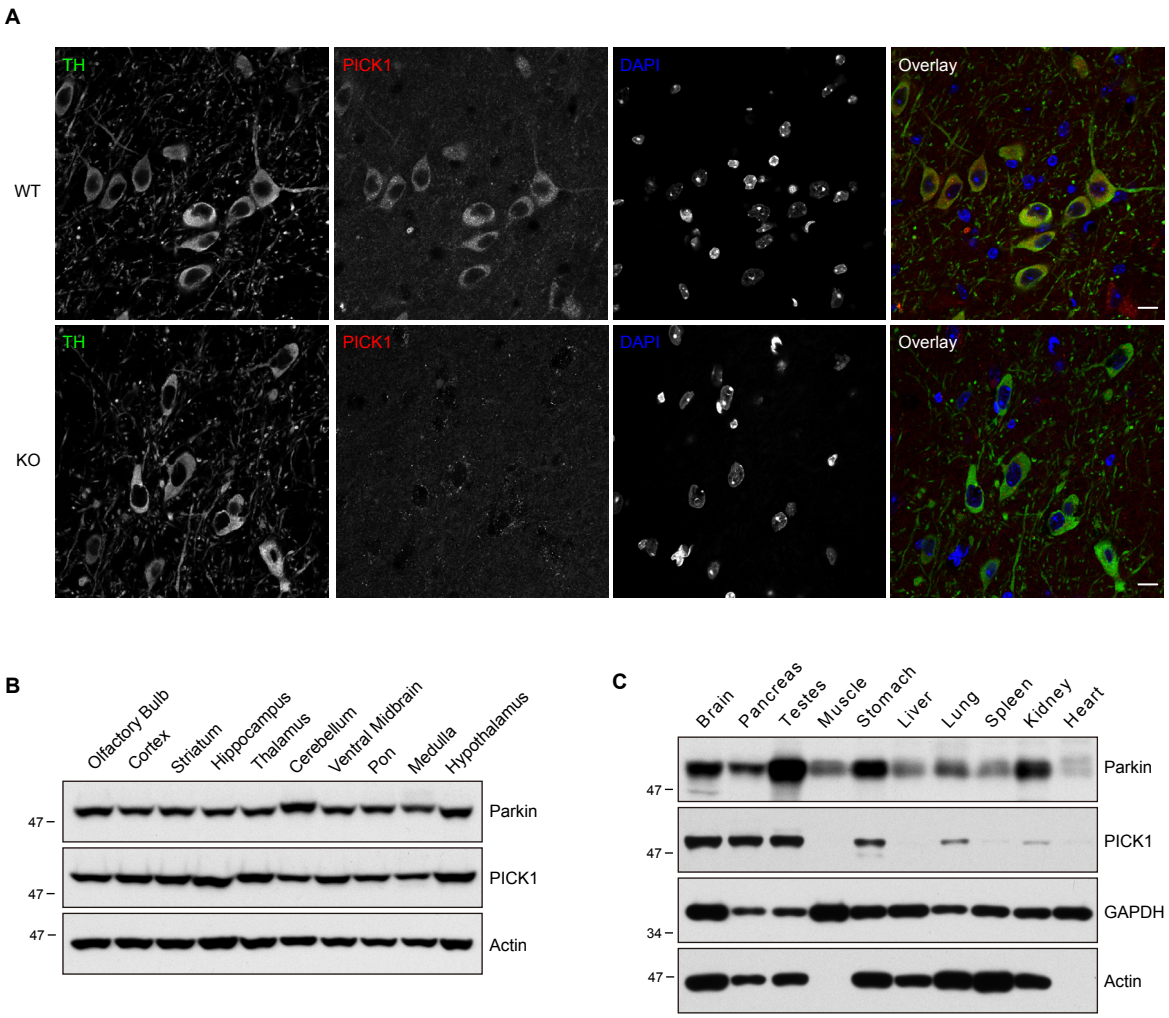


**Fig. S7.** PICK1 is present in dopaminergic neurons. (A) Immunofluorescence staining for TH and PICK1 in SNpc from WT and PICK1 KO mice. Guinea pig PICK1 antibodies were used to label PICK1 (red) and rabbit TH antibodies were used to label TH (green). DAPI (blue) was used to label nuclei. Scale bars, 10 μm. (B) Immunoblot of indicated proteins to examine their expression profile in different parts of brain regions. (C) Immunoblot of indicated proteins to show expression profiles of PICK1 and Parkin in different tissues.

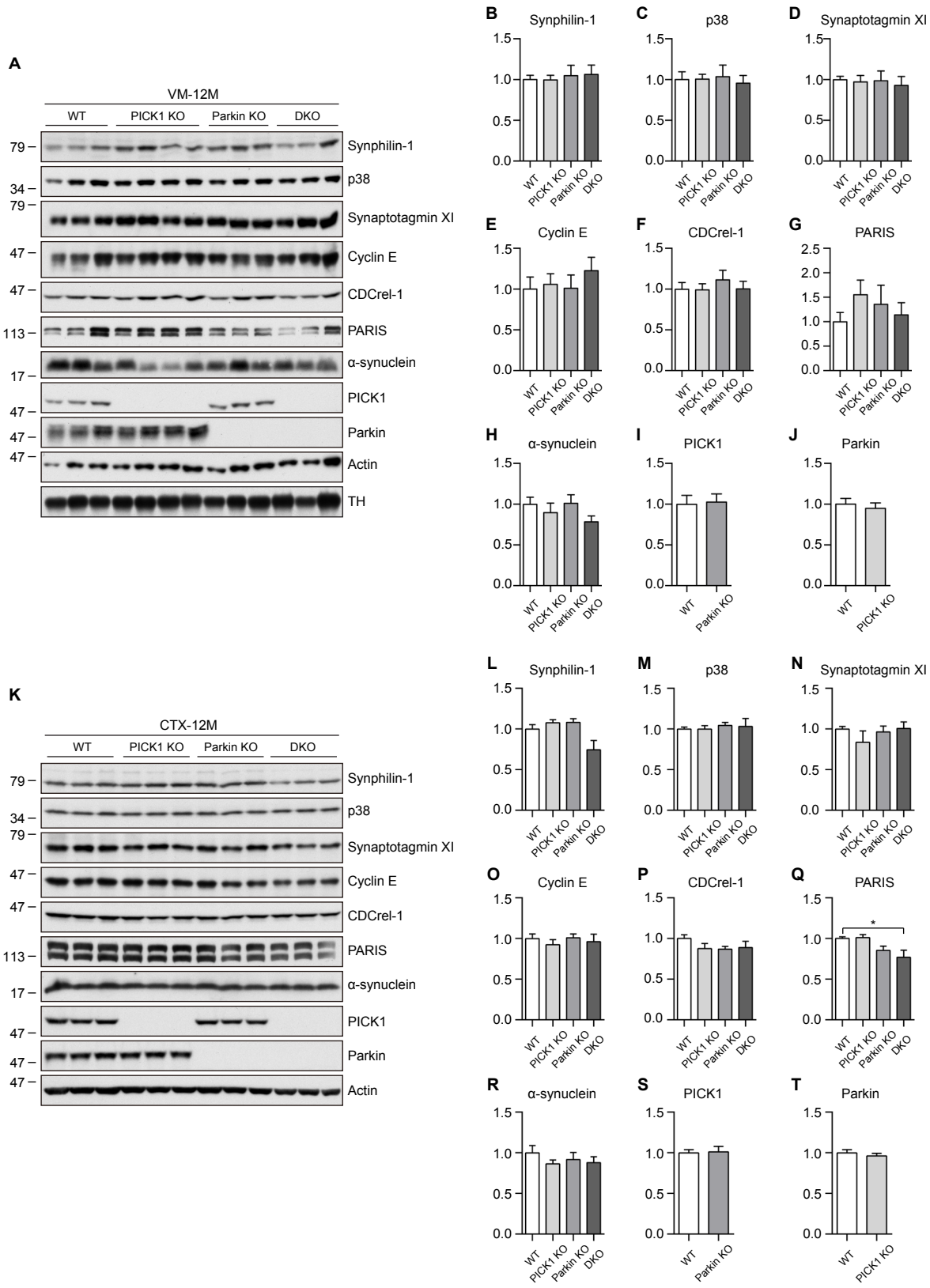


**Fig. S8.** Protein expressions of Parkin substrates in the VM and CTX of old mice. (A-T) Immunoblot (A and K) and densitometric analysis of immunoblots (B-J and L-T) for the indicated proteins to determine their expression in the ventral midbrain (VM) (B-J, WT, n = 8; PICK1 KO, n = 11; Parkin KO, n = 10; DKO, n = 10) and cortex (CTX) (L-T, n = 9 per genotype) of 12-month old WT, PICK1 KO, Parkin KO, and PICK1/Parkin DKO mice. VM samples were normalized to TH and CTX samples were normalized to actin. Error bars indicate mean ± SEM. One-way ANOVA with post hoc Bonferroni test. *$p < 0.05$.

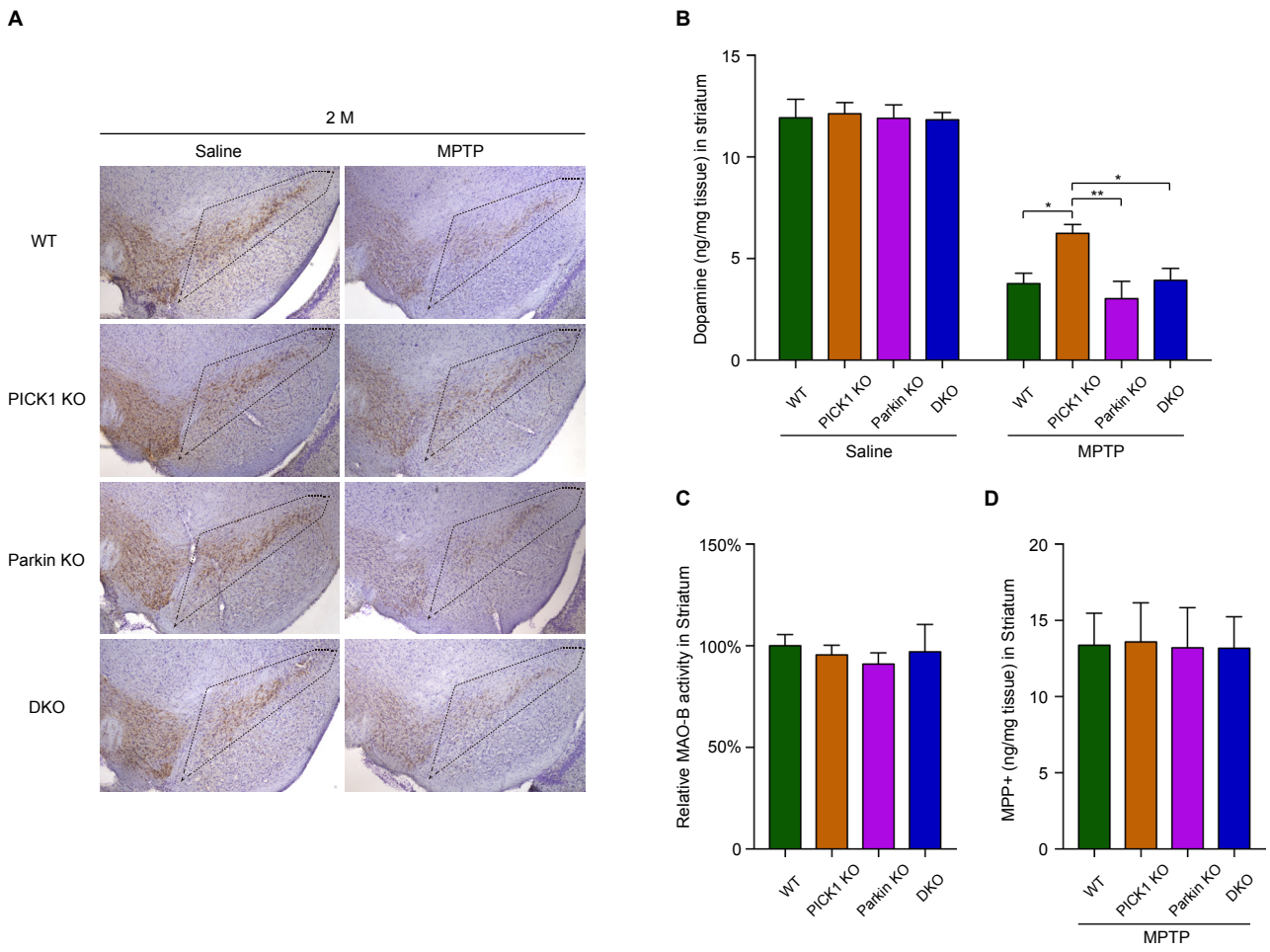


**Fig. S9.** MPTP toxicokinetics in young mice. (A) Representative images for Nissl staining of young mice (TH staining was performed prior to Nissl staining). (B) Dopamine concentration in striatum of saline or MPTP administered young mice. Saline: WT, n = 6; PICK1 KO, n = 6; Parkin KO, n = 6; DKO, n = 5. MPTP: WT, n = 5; PICK1 KO, n = 8; Parkin KO, n = 6; DKO, n = 8. (C) MAO-B activity in striatum of young mice. WT, n = 5; PICK1 KO, n = 5; Parkin KO, n = 6; DKO, n = 6. (D) MPP+ concentration in striatum of MPTP administered young mice. WT, n = 7; PICK1 KO, n = 7; Parkin KO, n = 8; DKO, n = 8. Error bars indicate mean ± SEM. Two-way ANOVA with post hoc Bonferroni test for (B). One-way ANOVA with post hoc Bonferroni test for (C) and (D). *$p < 0.05$; **$p < 0.01$.

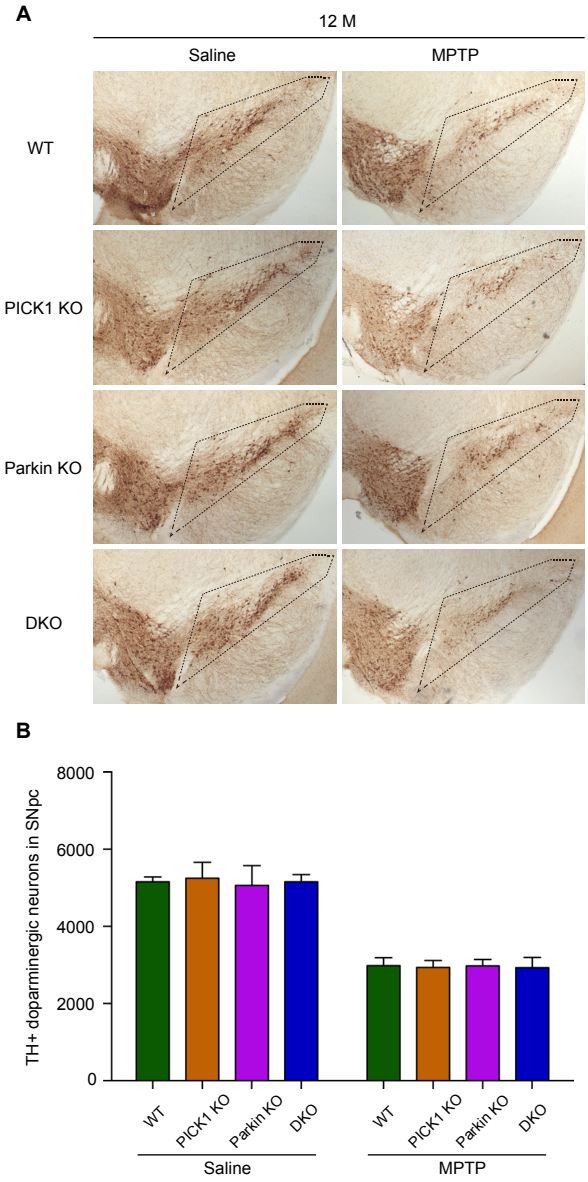


**Fig. S10.** Old PICK1 KO mice are not resistant to MPTP-induced toxicity. (A) Immunohistochemical analysis of dopaminergic neurons from old (12-month-old) mice administered saline or MPTP. The boxed regions indicate the location of the SNpc. (B) Quantification of TH-positive dopaminergic neurons in the SNpc. Old mice administered saline: WT, n = 5; PICK1 KO, n = 5; Parkin KO, n = 5; DKO, n = 8. Old mice administered MPTP: WT, n = 6; PICK1 KO, n = 8; Parkin KO, n = 7; DKO, n = 7. Error bars indicate mean ± SEM.

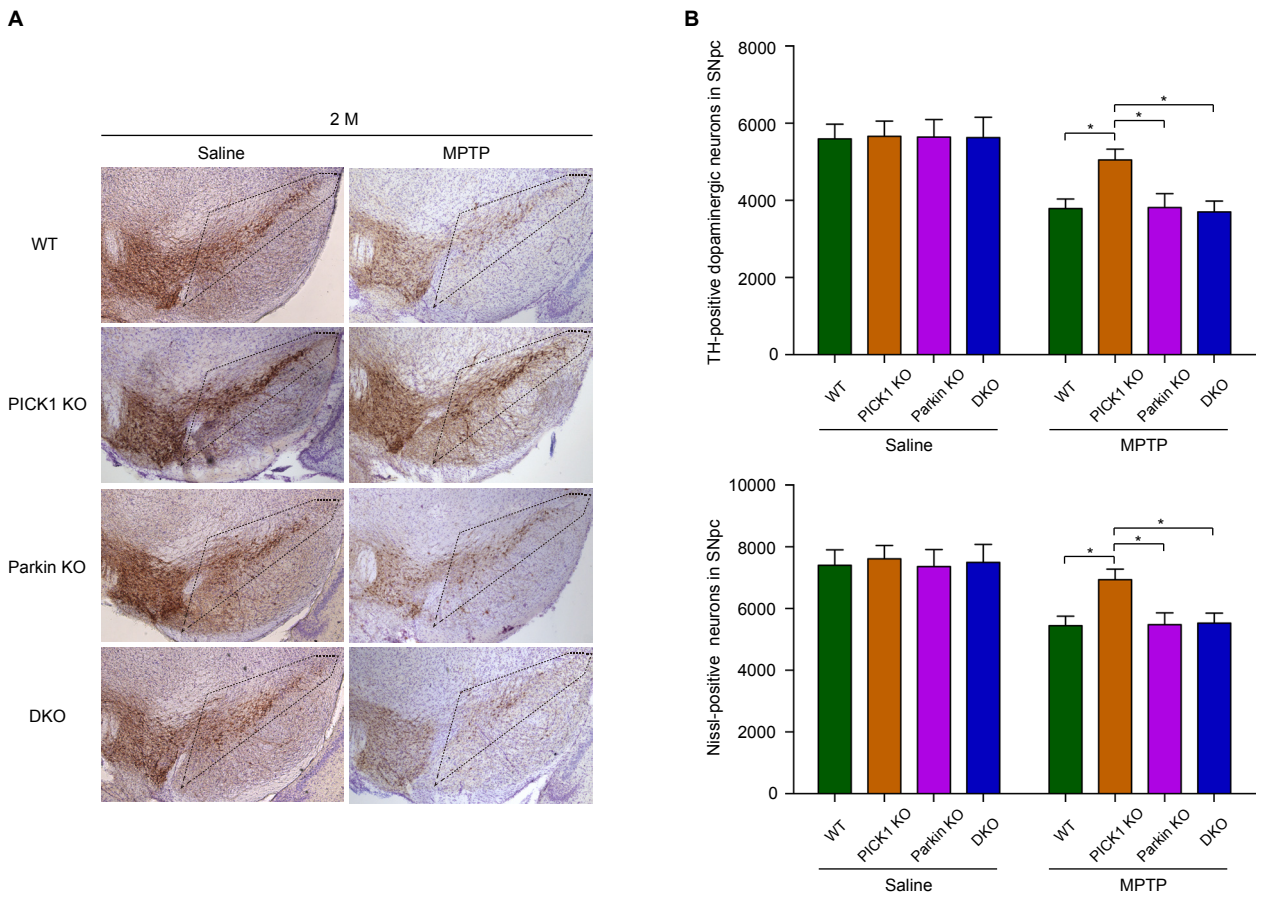


**Fig. S11.** TH and Nissl staining in young mice 21 days post-MPTP injection. (A) Representative images for TH and Nissl staining of young mice. (B) Quantification of TH- and Nissl-positive neurons in the SNpc. Saline: WT, n = 6; PICK1 KO, n = 6; Parkin KO, n = 6; DKO, n = 6. MPTP: WT, n = 8; PICK1 KO, n = 8; Parkin KO, n = 7; DKO, n = 8. Error bars indicate mean ± SEM. Two-way ANOVA with post hoc Bonferroni test. *p < 0.05.

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