Pael Receptor Induces Death of Dopaminergic Neurons in the Substantia Nigra via Endoplasmic Reticulum Stress and Dopamine Toxicity, which is Enhanced under Condition of Parkin Inactivation.

SUPPLEMENTARY FIGURE LEGENDS

**Figure S1. Generation of Parkin<sup>−/−</sup> mice.** (A) Schematic representation of the murine Parkin gene and design of the targeting vector. The region of the Parkin gene that includes proximal exon 3 is shown. Exon 3 (black box) was replaced with a neo cassette (PGK-neo) with two lox P sites (arrowheads). Locations of probes for Southern blot analysis and the sites of related restriction enzymes are indicated. DT-ApA indicates the location of the diphtheria toxin gene with a poly(A) sequence for negative selection. The expected sizes of fragments for the wild-type allele (W) or mutated allele (M) are indicated for digestion of genomic DNA with Hind III or Spe I/Sal I. (B) Southern blotting of representative tail DNA samples. DNA was digested with Hind III and hybridized with the 5’-probe from the Parkin gene. The 8.0-kb band is the wild-type (+) Parkin allele, and the 7.0-kb band represents the mutated allele (-), indicating that recombination has occurred. (C) RT-PCR for Parkin transcripts with the indicated primer pairs (A/B and C/D) in representative brain samples. ‘+’ and ‘-’ indicate the WT and the mutated allele in (b), respectively. PCR products with wild-type mouse Parkin cDNA as template were used as positive controls (PC). A schematic depiction of wild-type and mutant Parkin (∆Ex3) transcripts shows primer binding sites and the number of each exon (lower). M, DNA marker. RT-PCR analysis for Parkin transcripts confirmed the absence of normal transcripts in homozygous mutant mice. (D) RT-PCR products using primers A/B from (c) were cloned into a TA cloning vector, and then sequenced. Sequencing of RT-PCR products confirmed complete deletion of exon 3 and the presence of a frame-shift downstream of exon 2 in mutant mice. (E) Western blotting of whole brain samples with anti-Parkin antibody (Cell Signaling Technologies, Inc.) demonstrated lack of Parkin antigen.

**Figure S2. SNpc neuron-specific cell death is induced by Pael-R.** (A) Schematic presentation of major neurons connecting to the SNpc (1). The abbreviations used in this panels are as follows; GAD; glutamic acid decarboxylase, a marker protein for GABA neurons. (B) Adenoviral vectors including LoxEGFP (5x10<sup>8</sup> p.f.u.), S2NPNCre (10<sup>9</sup> p.f.u.), and LoxPael-R (10<sup>9</sup> p.f.u.) were injected unilaterally into the striatum of Parkin<sup>−/−</sup> mice (right panel), as described in Figure 3. As a control, the LoxPael-R vector was replaced with LoxLacZ (10<sup>9</sup> p.f.u.) and injected on the contralateral
Expression of EGFP protein in the ipsilateral (left small panel) and contralateral striatum (right small panel) 10 days after infection is shown, along with a Nissl-stained slice (middle large panel). (C) Brain images corresponding to the lower panels of Figure 3F are shown. Animals were then perfusion fixed 5 days after injection, and midbrain sections were stained using antibody to activated caspase-3. Images corresponding the motor cortex (M1 and M2, +0.62 mm from the Bregma; left panel), striatum (+0.62 mm from the Bregma, middle panel), and SNpc (-3.52 mm from the Bregma; right panel) on the ipsilateral side were overlapped with EGFP (green) and activated caspase-3 (red) signal. The activated caspase-3 signal (red) is apparent in SNpc, suggesting that neuronal death by Pael-R is specific for dopaminergic neurons in the SNpc. Scale bars, 200 μm.

Figure S3. Decrease of TH signal is dependent on Pael-R expression.

(A) Adenoviral vectors (2 μl), including LoxEGFP (5x10^8 p.f.u.), S2NPNCre (10^9 p.f.u.) and LoxPael-R (10^9 p.f.u.) were injected unilaterally into the striatum of Parkin^+/+ mice (left panels) or Parkin^-/- mice (right panels), as described in Figure 3. As a control, where indicated, LoxPael-R was replaced by LoxLacZ (10^9 p.f.u.), and S2NPNCre + LoxLacZ was injected on the contralateral side. Brains of animals were then perfusion-fixed 10 days later, and midbrain sections were stained using anti-TH antibody. Images at -3.52 mm from the Bregma were obtained to visualize activated caspase-3 (red) and the EGFP signal (green). In each panel, areas indicated by arrowheads are magnified in insets shown in the lower corner. Note that TH signals (red) disappear in the EGFP-positive cells of the SNpc, especially with Pael-R expression (upper row of panels, arrowheads). Scale bar: 200 μm. All images shown in this figure are representative of six repeated experiments. (B) Low magnification images of Figure S3A (derived from Parkin^-/- mice, right two panels) were subjected to image intensity analysis using NIH image software. Relative intensity (0-256 grades) of EGFP (green) and TH (red) signals were measured and plotted in the right panels. The regions of interest (ROIs) are indicated by open stripes (M1-L1 and M2-L2). Intensity analysis demonstrated a marked increase in EGFP signal and a much lower TH signal, consequent to injection of the vector causing overexpresion of Pael-R (left panel). In contrast, there was considerable overlap of both signals when the control (LacZ) was injected (right panel). This suggests that SNpc neurons overexpressing Pael-R have reduced expression of TH antigen. (C) The percentage of TH-negative cells in EGFP-positive neurons was calculated in the ipsilateral (closed bars) or contralateral side (open bars) of either parkin knockout (lower panel) or wild-type littermates (upper panel) at the indicated time points after injection of adenoviral vectors. **denotes p<0.05 in each mouse genotype at day 5 (n=6, and the mean ± S.D. is shown).
Figure S4. Elevation of ORP150 level in Orp150 TG mice. Midbrain sections of either Orp150 TG mice or wild type littermates were subjected to immunohistochemical analysis using anti-TH (green) and anti-ORP150 antibody (red). A Nissl stained image is shown for orientation (A). The open box in panel A is magnified and shown in panel B, where the merged image of TH and ORP150 is shown. The open box in panel B was further magnified in panels C-E. Same analysis was performed in wild type littermates (panels F-J). Marker bars: 200 μm. SNpc; substantia nigra pars compacta, SNpr; substantia nigra pars reticulata.

Figure S5. Dopamine levels in Parkin−/− and Orp150+/− mice after AMPT treatment. AMPT-HCl (150 mg/kg, Sigma) was intra-peritoneally administrated twice per day for up to 12 days. At the indicated times, mice were sacrificed and DA content in the striatum was measured by HPLC-ED (2). n=6, mean ± S.D. is shown. ** denotes p<0.01 by multiple comparison analysis compared to day 0 (before AMPT treatment).

References


**Figure S2.** Kitao et al.

(A) Schematic diagram of the neural circuitry involving the Motor Cortex, Striatum, Thalamus, Brain Stem, and other brain regions. Key neurotransmitters and cellular markers are indicated: GABA (GAD(+)), Dopamine, Substance P, TH(+), and GAD(+).

(B) Images showing gene expression patterns in different regions. From left to right: S2NPNCre+LoxPael-R, Parkin^/-, and S2NPNCre+LoxLacZ.

(C) Close-up views of gene expression in Motor Cortex, Striatum, and SNpc (Substantia Nigra pars compacta). The images display EGFP/αCasp3/Merge staining patterns.

Interpeduncular nucleus, Locus coeruleus, Raphe nucleus (Pons) are also highlighted.
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