On line supporting information

## Materials and Methods

### Immunostaining for activated caspase-3 and α-synuclein

The cells were fixed with 4% paraformaldehyde in PBS at 4 °C for 10 min at indicated times after E46K α-syn transfection. The fixed cells were blocked with 3% horse serum plus 3% goat serum in 0.2% Triton X-100 PBS solution for 40 min at room temperature, then immunostained with anti-α-syn antibody (1:500, BD Pharmingen) and activated caspase-3 antibody (1:250, Promega) for 2 h at room temperature . After washing 3 times with PBS, the cells were incubated with second antibody Alexa Fluor 488 goat anti-mouse IgG secondary antibody (1:1000) or Texas Red goat anti-rabbit IgG secondary antibody (1:200) for 40 min at room temperature, cells were washed 3 times after induction with secondary antibody, then processed with 1:1000 Hoechst for 10 min, washed once with PBS, and the cells were covered with anti-fading agents (Sigma) in cover glass. Four chambers of E46K transfected N2A cells were used for each treatment, 10 random fields were counted at 40 × magnification for each chamber. Cells were imaged with a Zeiss fluorescence microscope (Axiovert 200M) and analyzed by using AxioVision (Carl Zeiss, Germany). Cells with visible aggregates were calculated as percentage of E46K α-syn-transfected cells. Toxicity was measured as the percentage of E46K α-syn-transfected cells staining positively for activated caspase-3.

### Lactate dehydrogenase (LDH) Assay

Cytotoxicity Detection Kit (Roche) was used for measurement of lactate dehydrogenase (LDH) released into the medium by dead cells. 25 μl of culture medium was transferred to 96-well plates for LDH assay. The LDH working solution was made fresh according to manufacture's

instruction. 25 µl of LDH working solution were added to the 25 µl culture medium and incubated for 30 min at room temperature. Absorbance was read at 490 nm with a microplate reader (Beckman Coulter).

**Calcein fluorescent cell viability assay**

Calcein AM is a non-fluorescent, hydrophobic compound that easily permeates intact, live cells. The hydrolysis of Calcein AM by intracellular esterases produces Calcein, a hydrophilic, strongly fluorescent compound that is well-retained in the cell cytoplasm. Cells were grown in black-walled plates and cell viability was quantified using a fluorescence plate reader with excitation at 490 nm and emission at 520 nm.

**LIVE/DEAD assay using Cellomics Array Scan VTI HCS reader**

Cells were incubated with LIVE dye calcein AM (2 µM) which only labels live cells, and DEAD dye ethidium homodimer-2 (4 µM) which only labels dead cells, and nuclear dye Hoechst 33342 (5 µM) at 37ºC for 30 min. Cells were imaged using Cellomics ArrayScan VTI HCS reader (Thermo Fisher Scientific Inc) at three different fluorescence channels. The automatic counting analysis of live cell population and dead cell population was done using the Bioapplications image analysis system (Cellomics, Thermo Fisher Scientific Inc).

**Measurement of proteasome activity**

Proteasome activity was measured at indicated times in PC12 cells expressing E46K after induction of differentiation by NGF. The chymotrypsin proteasome activities were determined by measuring the rate of Suc-Leu-Leu-Val-Tyr-MCA cleavage (Bachem, Torrance, CA, USA) as described previously (Keller et al. 2000). Following experimental treatment, cultures were

placed on ice and homogenized in proteolysis buffer (10 mM Tris-HCl (pH 7.8), 0.5 mM dithiothreitol, 5 mM ATP, and 5 mM $MgCl_2$). Protein determinations were made on the resulting lysates, and 2.5 µl of the fluorogenic substrate (5 mM stock) was incubated in each sample (250 µg/250 µl aliquot). The samples were incubated for 60 min at 37°C. Cleavage products were measured using a CytoFluor Multi-well Plate Reader, Series 400 (Perseptive Biosystems). Background fluorescence was determined by incubating lysates with the proteasome inhibitor lactacystin (50 µM) for 30 min before the addition of proteasome substrate.

**Mitochondrial membrane potential**

Loss of mitochondrial membrane potential was detected with the JC-1 MitoPT$^{TM}$ detection kit (B-bridge International *Inc*.) according to the manufacturer's protocol. Briefly, cells were incubated with MitoPT dye (JC-1) at 37°C for 15 min in a $CO_2$ incubator. Cells from each experimental group were dispensed in 100 µl of assay buffer in a black flat-bottom 96-well plate. The red JC-1 fluorescence was measured using a CytoFluor Multi-well Plate Reader, Series 400 (Perseptive Biosystems) with 485 nm excitation and 590 nm emission filters. By comparing the average of red fluorescent signal in induced versus noninduced control samples, the loss of mitochondrial membrane potential could be monitored.

**Western blot analysis**

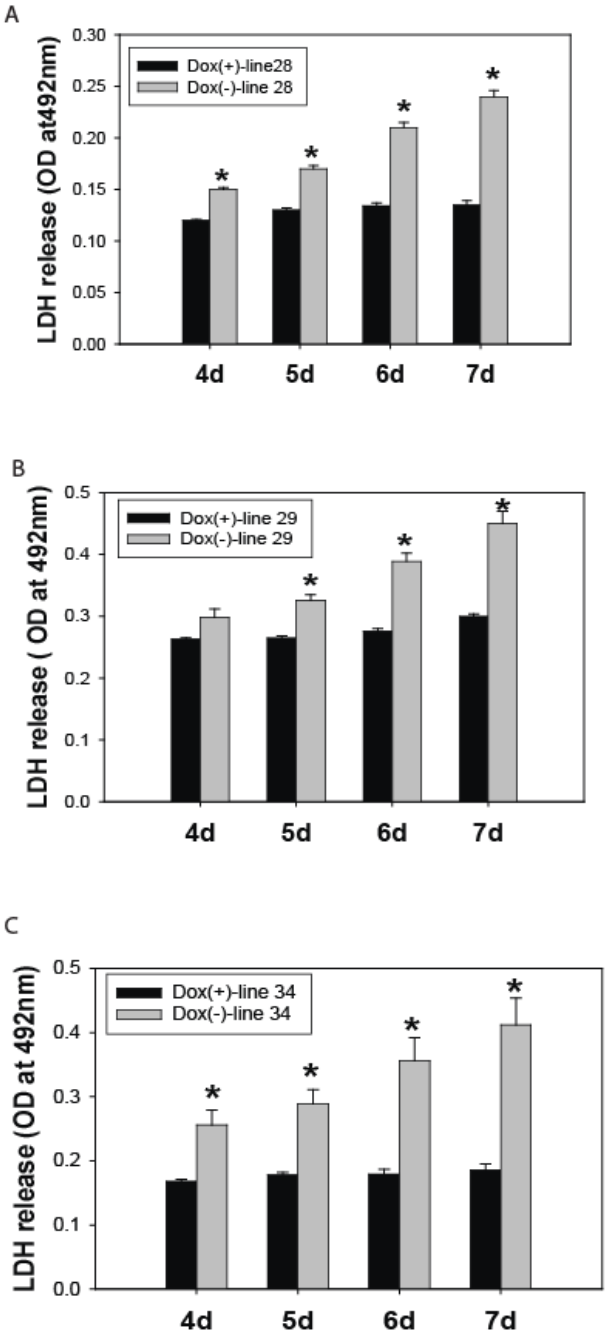
After the generation of cell lines transfected with E46K α-syn, SDS-soluble lysates were assessed by Western blotting for the level of expression of α-synuclein. A total of 20 µg was loaded and resolved by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes, immunoblotted with mouse anti- α-syn (1:500; BD Pharmingen) antibody, and the blots were

visualized by ECL Western blotting detection reagent (Amersham Pharmacia Biotech). Various levels of expression were achieved in different cell lines. The cell lines 28, 29 and 34 showed higher expression levels of E46K among more than 30 lines that we generated (Fig 1).

**Purification of GST- E46K α-syn fusion protein**

Human E46K α -syn fused to the C terminus of GST was expressed in the BL21 (DE3) strain of *Escherichia coli*. Cells were harvested 8 hr post induction and frozen at -80°C. Cell pellets were then thawed and resuspended in lysis buffer (phosphate-buffered saline (PBS), pH 7.4, supplemented with 0.05% Tween 20, 1 mM DTT, 2 mM EDTA, 20 mM AEBSF, Complete protease inhibitor cocktail (Roche Applied Science), leupeptin, and pepstatin). The mixture was lysed by French pressure and clarified by centrifugation at 6000 x g for 15 min at 4°C. To purify GST- E46K α-syn fusion protein, the lysate supernatent was applied to a Gluthathione Sepharose HP column. Next, the column was washed with PreScission Protease cleavage buffer (50 mM Tris (pH 8), 150 mM NaCl, 1 mM EDTA, and 1 mM DTT), followed by addition of 5 units of PreScission Protease enzyme (GE) diluted in 10 ml of cleavage buffer. To complete the on-column cleavage reaction, the enzyme was circulated for 12 h prior to collection of the 10 ml column volume. All chromatography steps were carried out at 4°C using a BioRad DuoFlow QuadTec system. Finally, E46K α-syn eluted protein was lyophilized using a Labconco freeze dry system.

Suppl Fig. 1




**Supplemental Fig. 1.** E46K α-syn induction causes progressive cell toxicity in differentiated PC12 cells. *$p$<0.05 compared to the values of corresponding Dox(+) group by Student's *t*-tests.