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# Apoptosis signal-regulating kinase-1 aggravates ROS-mediated striatal degeneration in 3-nitropropionic acid-infused mice

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ABSTRACT

Apoptosis signal-regulating kinase-1 (ASK1), an early signaling element in the cell death pathway, has been suggested to participate in the pathology of neurodegenerative diseases, which may be associated with environmental factors that impact the diseases. Although it is not entirely elucidated, 3-nitropropionic acid (3-NP) provokes mitochondrial dysfunction and selectively forms striatal lesions similar to those found in Huntington's disease. The current study investigated whether ASK1 is involved in striatal pathology following chronic systemic infusion of 3-NP. The results show that ASK1 acts as a primary mediator of there active oxygen species (ROS) cell death signal cascade in the 3-NP-damaged striatal region by disrupting the positive feedback cycle. In 3-NP-infused striatal lesions, ROS increased ASK1. Superoxide dismutase transgenic (SOD-tg) mice reduced ASK1by scavenging ROS, and reduction of ASK1leads to a reduction in cell death. However, ASK1 down-regulation in 3-NP infusion mice also decreased striatal cell death without scavenging ROS. In contrast decreasing cell death by si-ASK1 treatment along with 3-NP in both SOD tg and wild-type mice (wt), cell death rebounded when ASK1 peptide was added to SOD tg mice. The present study suggests that ROS-inducing ASK1 may be an important step in the pathogenesis of 3-NP infused striatal lesions in murine brains.



## 1. Introduction

The compound3-nitropropionic acid (3-NP) produces selective striatal lesions in animal models [1]. Although not entirely elucidated, the mechanisms of neurotoxicity induced by 3-NP have been shown to include the exhaustion of adenosine triphosphate, mitochondrial membrane depolarization, dysregulation of intracellular calcium homeostasis, calpain activation, and the release of pro-apoptotic proteins from mitochondria [2–5]. The neurotoxic mechanism and the reason for the selective vulnerability of the striatum are not yet well understood.

Superoxide dismutase (SOD) functions to protect cells from the effects of superoxide radicals by eliminating reactive oxygen species (ROS).Several reports have shown that SOD overexpression reduced the size of striatal lesions after 3-NP injection [6]. Increased cytosolic Cu/ZnSOD is a compensatory response to the generation of ROS to reduce the toxic effects of the superoxide anion. In addition, the cumulative effects of ROS exposure cause the activation of various harmful cellular pathways [7–9].

Apoptosis signal-regulating kinase-1 (ASK1), an early signaling element in the cell death pathway [10], is activated by ROS [11] and is required for ROS-induced apoptosis [12]. Previous studies have indicated that increased activated ASK1impacts the presentation of neurodegenerative diseases [13,14]. The rationale for targeting ASK1 was based on findings that oxidative stress is causally related to apoptotic neuronal cell death in neurodegenerative lesions commonly associated with abnormal protein fragments and aggregates [9,15]. Although many investigations have shown that ASK1 is essentially involved in neuronal cell death triggered by various external injuries and expanded polyglutamine, the relationship of ASK1 and ROS in the selective striatal cell death by 3-NP chronic and systemic infusion in mice remains is to be elucidated. In addition, ASK1 was suggested to play a role in mediating the cell death signal in striatal cells of aged mice that received 3-NP injections directly into the striatum.

Here, we examined whether increased levels of ASK1 are reciprocal to the amount of ROS and also investigated the relationship between ASK1 expression and striatal degeneration in 3-NP systemic infusion mice. We suggest that overexpressed ASK1 amplifies ROS damage, which can lead to cell death, and is a deleterious primary responder to oxidative stress in the striatum of 3-NP systemically infused mice.

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## 2. Materials and methods

### 2.1. Animal model

Male SOD1-tg mice (C57BL/6-TgN; Jackson Laboratory, USA) and wild type (wt) male littermates were used in this study. All procedures were performed in accordance with the guidelines for the care and use of laboratory animals (Yonsei University), which have been approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The animals were anesthetized with 2.0% isoflurane under 30% oxygen and 70% nitrous oxide using a vaporizer (VMC Anesthesia Machine, MDS Matrix, USA). The rectal temperatures were maintained at 37 ± 0.5 °C. For the sustained chronic administration of 3-NP infusion, 3-NP (Sigma, USA) was dissolved in saline at a concentration of 0.5 mg per μl (pH 7.4). The prepared 3-NP solution or vehicle control was delivered by sustained infusion (180–220 mg/kg/day) using osmotic mini-pump at an infusion rate of 0.5 μl/h for 7 days (Alzet, USA).

### 2.2. ASK1gene silencing with siRNA and administration of ASK1-peptide

The ASK1 gene was silenced using siRNA against ASK1 (sense, GCUCGUAAUUUAUACACUGtt; antisense, CAGUGUAUAAAUUACGAGCtt; concentration 5 μM; Ambion, Austin, TX, USA). After confirmation of efficiency *in vitro*, the transfecting reagent SiPORT*Neo*FX (Ambion, USA) and ASK1-siRNA or nonfunctional mutantRNA (control siRNA; 5′-AAG AGA AAA AGC GAA GAG CCA-3′; Ambion) were combined, mixed gently, and allowed to form siRNA liposomesfor an additional 10 min at room temperature. An alzet micro-osmotic pump (Durect, USA) containing 100 μl of the transfection reagent or ASK1-siRNA was then placed subcutaneously on the backs of the animals, and a brain infusion cannula connected to the pump was positioned in the striatum (A, 0.7 mm; L, 1.2 mm, and D, 3.3 mm) for 7 days during 3-NP infusion. The mice were sacrificed 7 days after surgery, and the brains were processed for experiments.

To restore ASK1, we synthesizedan ASK1 peptide containing active sites (Thr845) from amino acids 836–875. The ASK1 protein was transported with the dried BioPORTER Quiktease (Sigma–Aldrich) reagent to the striatum of SOD tg mice concomitant with 3-NP infusion for 7 days by a micro-osmotic pump containing 100 μl of ASK1 protein (0.1 mg/ml in saline). As a control, control-peptides were infused in the 3-NP-infused SOD-tg mouse striatum. To confirm the delivery, Western blot analysis was performed with antibodies recognizing the Thr845 region.

### 2.3. Immunohistochemistry

To determine the patterns of DARPP32 (Epitomics, USA) and ASK1 (SantaCruz Biotechnology, Cell signaling) expression, we performed immunofluorescent staining. After sacrificing the animals, brains were removed, fixed, and cut into 20 μm thickness coronal sections on a cryostat. The fixed sections were incubated with a blocking solution, as described previously [16], and incubated with the appropriate primary antibodies. After washing, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Jackson ImmunoResearch, USA). For counter-staining, the sections were incubated with propidium iodide (PI, Sigma). After washing, stained tissue samples were mounted using Vectashield mounting medium (Vector Lab, USA) and coverslipped. The sections were then observed under a LSM510 confocal laser scanning microscope (Carl Zeiss, USA).

### 2.4. Western blot analysis

The protein expression levels were analyzed by Western blotting. Tissues were lysed in lysis buffer (20 mM Hepes–KOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 0.1 mM sodium vanadate, 0.1 mM proteinase inhibitor cocktail), the samples were homogenized with a Teflon homogenizer (Wheaton, USA), and then centrifuged at 8,000*g* for 20 min. The lysates were loaded onto SDS–polyacrylamide gels and after migration, the proteins were electro-transferred onto a polyvinylidenedifluoride membrane (PVDF) (Millipore, USA), which was then blocked in 5% skim milk and incubated with one of the following primary antibodies: Cu/ZnSOD, MnSOD, ASK1, pASK1. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Roche Diagnostics), and the bands were visualized with an enhanced chemiluminescence reagent (Amersham Biosciences).

### 2.5. Apoptotic cell-death assay

Apoptosis-related DNA fragmentation assays were quantified using a commercial enzyme immunoassay kit (Chemicon) and cytoplasmic histone-associated DNA fragment kit (Roche Diagnostics). According to the manufacturers' protocols, cytosolic samples were used in each assay. In addition, we performed a TUNEL assay to detect cell death following the manufacturer's protocol (Roche Diagnostics). Tissue were prepared as described above, and immune-fluorescent labeling was performed as previously described [17,18]. The sections were incubated with 50 μl of the TUNEL reaction mixture, and the sections were counterstained with Hoechst (Molecular Probe, USA), mounted, and observed under a confocal laser scanning microscope (Carl Zeiss). The number of TUNEL-positive cells was counted in the striatum in three sections from each mouse at different levels.

### 2.6. ROS detection by staining

ROS were investigated in the brains of 3-NP-infused mice using green-fluorescence (CM-$H_2$DCFDA) *in situ* detection (Molecular Probes). Each mouse was placed in a stereotaxic frame (Stoelting) under general anesthesia, the CM-$H_2$DCFDA (2 ul, 50 ug/50 ul dimethylsulfoxide) was infused for 10 min into the striatum with a Hamilton syringe. The animals were sacrificed and trans-cardially perfused with heparin and formaldehyde 1 h after infusion of CM-$H_2$DCFDA. After preparing a frozen block of brain tissue, ROS was detected to appear as green dots by CM-$H_2$DCFDAwhen examined under confocal laser scanning microscope (Carl Zeiss).The nucleus was stained with PI (Sigma).

### 2.7. ROS detection by flow cytometry analysis

CMXRosamine (MitoTracker-Red™) and $CMH_2$-DCFDA (Molecular Probe) were obtained from Molecular Probes, dissolved in DMSO, and stored at 20 °C in the dark. Both Mito Tracker (50 μM) and CM-$H_2$DCFDA (50 μ M) were administered into the cortex and striatum at the position described above 30 min before sacrifice. As a control, DMSO was injected in the same locations at the same time before sacrifice in some mice. Following this procedure, the mouse brain was removed, dissected out striatum, and minced finely with a scalpel. Minced striatum tissue was placed in separate tubes containing 2 ml digestion solution, and cells were dissociated with Accumax (Millipore) for 1 h at 37 °C with agitation in the dark. The cell suspension was filtered through a 100 μm mesh (BD Biosciences) and diluted with PBS after fixing the single cells with 3.7% formaldehyde in the dark, the fluorescence of the bound dyes within each cell was analyzed using

a FACSCalibur (BD Biosciences; over 100,000 total events; 5,000 events/gate), and the data were analyzed using Cell Quest software (version 3.3; BD Biosciences). Negative controls were prepared by reacting the tissues with PBS, FITC-conjugated IgG, or PE-conjugated IgG (BD Biosciences).

### 2.8. Detection of DNA oxidation in mouse striatum

We evaluated DNA oxidation usinga monoclonal antibody against 8-OHdG according to a previous report [7]. The brain tissue was fixed, and treated with DNase-free RNase(Roche)at 37 °C for 1 h. Next, the sections were denatured in 4 N HCl and neutralized with 50 mM Tris-base. After washing, the samples were incubated with an anti-mouse 8-OHdG antibody and also incubated with secondary antibody (MOM kit, Vector Laboratory, USA). The slides were mounted with Vectashield and coverslipped.

### 2.9. Rotarod test

The motor function of the animals was assessed with a rotarod apparatus (UgoBasile, USA), where the time that the animals were able to remain on the rod was measured at an accelerating speed from 4 to 40 rpm. Each mouse was pre-trained before implanting the osmotic pump filled with 3-NP or saline. After 30 min rest period, and mice were then placed back on the rotarod for five trials of a maximum of 5 min at an accelerating speed.

### 2.10. Statistical analysis

Data are expressed as the mean ± SD. The statistical comparisons among multiple groups were performed using analysis of variance followed by Fisher's *post hoc* protected least-significant difference test, and comparisons between two groups were performed using the unpaired *t* test (StatView, version 5.01; SAS Institute Inc., Cary, NC, USA).

## 3. Results

### 3.1. Systemic infusion of 3-NP led to the formation of selective striatal lesions in wt, but SOD-tg mice

Mice infused with 3-NP were examined for symptoms, and the degenerated brain region was validated with cresyl violet staining. Primarily, we confirmed an obvious decrease in body weight by 7 days after systemic3-NP reservoir implantation. The mouse body weight reduced an average of 70% of the initial body weight. Seven days after 3-NP administration, most mice presented with severe motor dysfunctionin addition to weight loss. Histological evaluation of the 3-NP-infused mice at day 7 demonstrated striatal abnormalities and cell loss (Fig. 1A).

DARPP32 (dopamine- and cyclic adenosine monophosphate-regulated phosphoprotein), an establishedof striatal neuronmarker, showed weak immunoreactivity in 3-NP-infused wt mice; however, the immunoreactivity in SOD-tg mice was significantly more dense (Fig. 1B). The induction of DNA damage in striatal neurons was evaluated by terminal TUNEL staining (Fig. 1C). The average number of TUNEL-positive nuclei was determined using multiple images in the four different mice of each group. Fig. 1C reveals a statistically significant increase in TUNEL-positive nuclei in the 3-NP-infused wt mice, which was scarcely demonstrated by the DARPP32-positive cells.

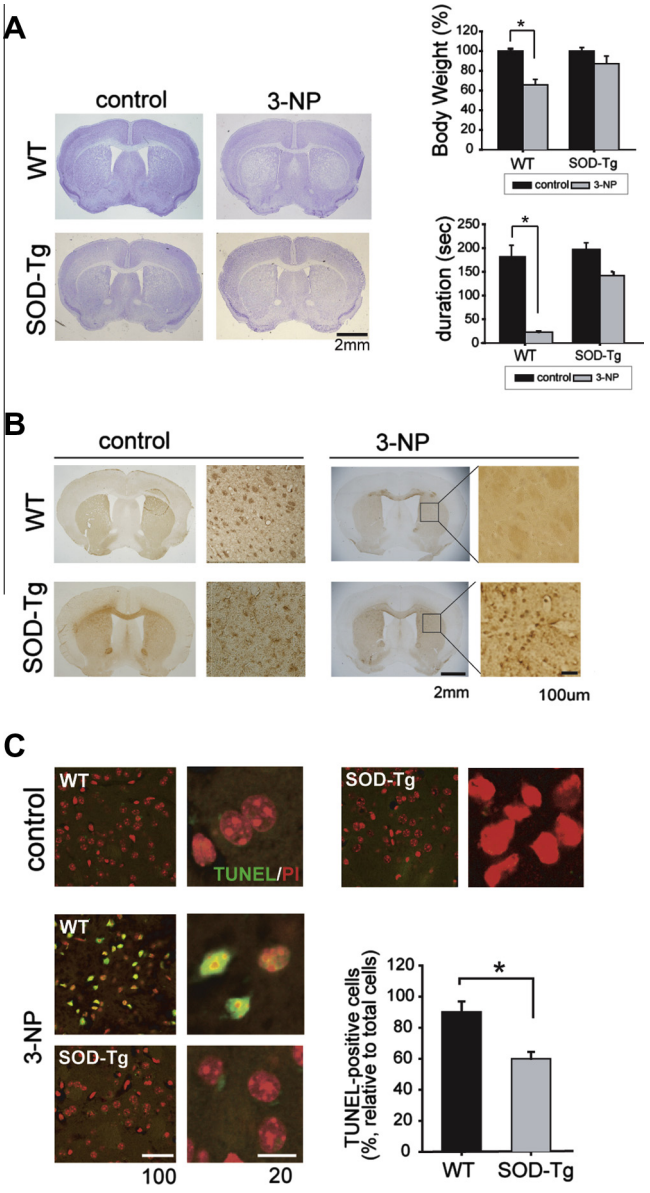


**Fig. 1.** Pathological examination in the striatum after systemic infusion of 3-NP. A, Histological analysis was performed by cresyl violet staining (left). Body weight which measured on day 7 after infusion of 3-NP is expressed as a percentage of the weight before 3-NP infusion (right upper). Motor function is shown the duration sustained on the rotarod (right lower). B, DARPP32-postive cells shown by immunohistochemistry was sparsely detected in the wt mice, while positive cells were more plentiful in the SOD-tg. C, TUNEL-positive cells were more densely detected in the 3-NP-infused wt mice compared to the SOD-tg mice (left). A quantification graph is presented showing relative values of positive cells to counterstained cells in the unit area (right). $^{*}p < 0.05$.

### 3.2. Greater ROS production, oxidative damage and ASK1 levels andactivity were detected in striatal lesions

Using FACS analysis to quantify ROS, the total amount of ROS in the striatum significantly decreased from 99.57% in wt to 28.75% of SOD-tg mice (Fig. 2A). In contrast, there was a slight difference in the ROS quantity in mitochondria that were directly injured after 3-NP infusion between wt and SOD-tg mice (Fig. 2A), as revealed by FACS (wt, 99.99%; SOD-tg, 81.37%).

As an alternative way to compare the changes in the amount of ROS, levels of MnSOD, and Cu/ZnSOD proteins, were determined by Western blot analysis in the lesioned striatum from each brain. MnSOD protein amounts were not different in the damaged

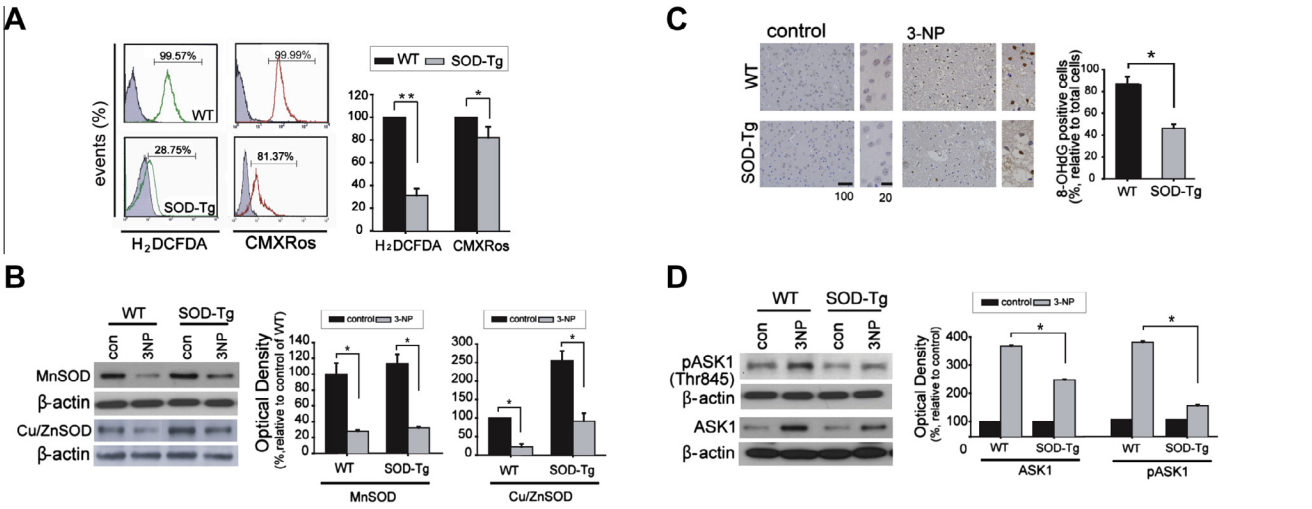


**Fig. 2.** Environmental changes and oxidative damage in the striatum after 3-NP infusion. A, After 3-NP infusion, CM-$H_2$DCFDA-for evaluating total ROS levels, and Mito Tracker-Red for mitochondrial ROS levels were assayed by flow cytometry (left), and the events are presented in a quantified graph (right). B, The MnSOD and Cu/ZnSOD protein levels in the striatum are shown in Western blot analysis (left) and the quantitative values are presented in the graphs (right). C, The 8-OHdG illustrates that the 8-OHdG-positive cells were more densely detected in the wt mice compared to the SOD-tg mice. A quantified graph is presented showing relative values of positive cells to counterstained cells in the unit area. D, The changes of ASK1 and pASK1expression levels were assayed by Western blot (left) and presented the quantified graph (right). $^*p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

striatum between wt and SOD-tg, whereas relatively higher levels of Cu/ZnSOD were detected in SOD-tg than wt (Fig. 2B). Unaltered pattern of MnSOD likely dues the direct attack of the mitochondrial dysfunction reagent, 3-NP. Cytosolic SOD levels, however, were greater in the SOD-tg than in the wt mice because of the restoring effect of SOD overexpression in the SOD tg mice.

Representing DNA oxidation, 8-OHdG-positive cells were scarcely detected in the 3-NP-infused SOD-tg striatum; however, the immune-reactivity was strong in striatum of wt mice (Fig. 2f andC), suggesting that the ROS was produced following mitochondrial dysfunction and subsequent DNA damage (Fig. 2C). Under oxidative conditions, increased ASK1 protein levels, one of the first ROS-responsive molecules, were measured, and also measured increased pASK1 activated by phosphorylationat Thr845. The 3-NP infusion elevated total ASK1 protein amounts the SOD-tg and wt mice however, this increase was meager in the SOD-tg group. Corresponding to total ASK1 amounts, the levels of pASK1 were also significantly lower in SOD-tg mice (Fig. 2D).

### *3.3. ASK1 amounts mediated the striatal cell death without change of ROS level*

ASK1 gene silencing by siRNA sufficiently regulated ASK1 protein down (Fig. 3A). However, ASK1 silencing did not alter the amounts of ROS by 3-NP infusion. Indeed, ROS levels were similar or abundant both in the si-ASK1-treated and the si-control-treated wt mice. ROS detected with CM-$H_2$DCFDA were scant in 3-NP-infused SOD-tg mice, while the CM-$H_2$DCFDA in normal and ASK1-silenced 3NP infusion mice abundantly stained the striatal region with a punctuate shape (Fig. 3B).The results showed that the down-regulation of ASK1 did not affect ROS generation. Although the down-regulation of ASK1 did not affect ROS scavenging, it was sufficient to reduce cell death and improve the motor function (Fig. 3E and F). On the other hand, ASK1-peptide administration triggered cell death without increasing ROS levels.

To confirm whether overexpression of ASK1 plays a role in mediating cell death, synthesized ASK-peptide was infused for 7 days in the SOD-tg mice striatum starting with 3-NP systemic infusion (Fig. 3C). In 3-NP-infused SOD-tg mice, the ROS levels were assessed in the control-peptide and ASK1-peptide treated groups by FACS (Fig. 3D). ASK1-peptide did not alter the amount of ROS generated. A DNA fragmentation assay showed that striatal apoptotic cell death occurred in the 3-NP-infused wt mice and decreased significantly in both SOD-tg mice and siASK1-treated wt mice with 3-NP infusion. In contrast, the DNA fragmentation recurred in the ASK1-peptide-treated SOD tg mice and also aggravate movement ability (Fig. 3E and F).

## 4. Discussion

The results of the present work show an improvement in behavioral impairment by ASK1 down-regulation, despite 3-NP infusion. We propose the hypothesis that ASK1 overexpression by systemic infusion of 3-NP promotes the formation of selective striatal lesions, and this occurs apart from ROS generation. The results of our study showed that increased ASK1andpASK1expression by harmful ROS signals augmented neuronal cell death in the striatum.

Mitochondria are key players in the production of ROS and have been reported to have an important role in 3-NP injury-induced pathology [19]. We evaluated amounts of total ROS produced in mitochondria and cytosol MitoTracker-Red CM$H_2$XRos (MT Red CM-$H_2$XRos) is a molecule that has been used to measure mitochondrial free radicals (MFRs) in various cell cultures. Because this dye has been reported to be sequestered into the nucleus and vesicles [20,21], we administered it to detect the generation of MFRs in 3-NP-infused mice and to measure MFR generation *in situ*. Besides of mitochondrial dysfunction, 3-NP also raises cellular oxidative stress. The alteration of Cu/Zn-SOD is a compensatory mechanism that protects cells from free radical-induced damage, and systemic infusion of 3-NP altered Cu/Zn-SOD enzyme levels in this study. It has been previously reported that 3-NP induced major changes in SOD activity in the striatum of 3-NP-injected rats [22]. Our study displayed that 3-NP caused a reduction in Cu/Zn-SOD, but had no effect on MnSOD protein levels. It can be inferred that the overexpression of Cu/ZnSOD is primarily responsible for cellular anti-oxidant protection in 3-NP-infused SOD-tg mice, possibly by compensating for the overexpression of ROS. In wt mice, however, the endogenous antioxidant system could not enough to eliminate the increased ROS, as Cu/ZnSOD amounts

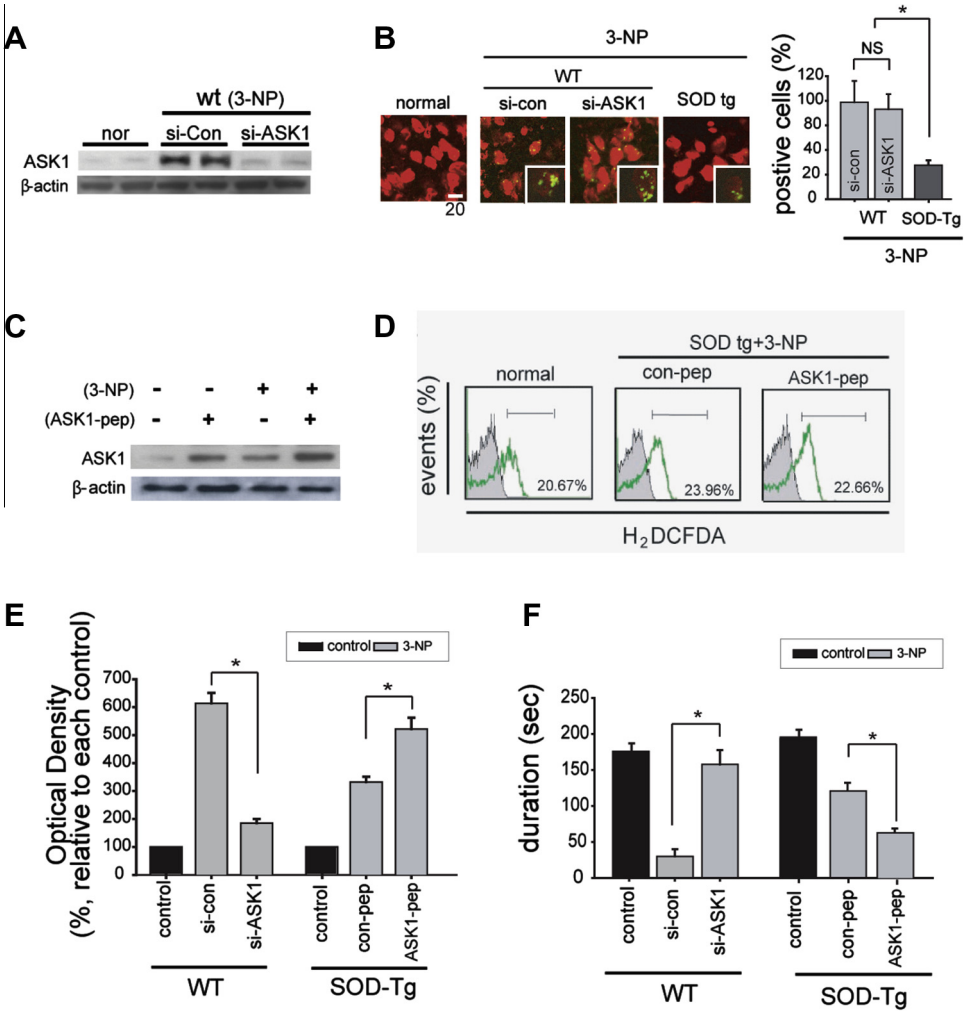


**Fig. 3.** Down regulation and induction of ASK1. A, In the striatum, ASK1 proteins were declined by siRNA-ASK1 (left), and quantified graph (right). B, CM-$H_2$DCFDAin the striatum was detected in abundance in the si-control- or si-ASK1-treated wt mice, but was decreased in the 3-NP-infused SOD-tg mice. $H_2$DCFDA, green; PI, red. C, Western blot analysis shows the expression levels of ASK1 in the striatum of wt or ASK1-peptide-added SOD-tg mice with 3-NP infusion. D, After 3-NP infusion, total ROS levels was evaluated with CM-$H_2$DCFDA in each group (con-pep vs ASK1-pep) by flow cytometry. E, Apoptotic cell death using DNA fragmentation assay was found in each treated mouse striatum. F, Motor function is shown as the duration sustained on the rotarod in each treated group. $^*p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were diminished. The lesions of 3-NP indicate that striatal oxidative damage has occurred, and that this damage is associated with changes in the cellular antioxidant system [23]. The Cu/ZnSOD levels were much greater in the SOD-tg mice compared to the wt mice, likely to overcome the oxidative damage. However, the changes in SOD protein levels during oxidative stress do not determine whether protect or not, although it is certain that overexpressed SOD amounts protect against harmful oxidative environments [8].

Oxidative DNA damage results from direct ROS attacks. There are some types of oxidative DNA damage detective method. Among them, 8-OHdG results in oxidative DNA damage. The results presented here show that the oxidative DNA lesions are markedly augmented in the 3-NP-infused wt mouse brain.

Once ROS are generated, they can damage mitochondria causing additional free-radical generation and loss of antioxidant capacity, leading to a deleterious cycle [24]. Therapeutic prevention of oxidative stress has been proposed to "break the cycle" of cell death [24], and studies have been carried out in the neurodegenerative field attempting to modulate key enzymatic components that regulate oxidative stress [8]. Our study was performed on the assumption that mitochondrial complex II inhibitors generate ROS, and contribute to cell death. Furthermore, because ASK1is known to be involved in ROS-induced cell death, we propose that ASK1plays an instrumental role and even amplifies this process. The ASK1 signaling-mediated cell fate decision appears to depend in part on the extent and duration of ASK1amounts and its activation. A previous report found that a mutated SOD induced ER stress and activated the ASK1-mediated cell death pathway [25]. Also, the deletion of ASK1 mitigated neuronal loss and extended the life span of SOD1-mutated mice [25].

Recent studies with animal models have revealed that oxidative stress is a causative factor in the initiation and progression of Alzheimer's disease (AD) and Parkinson's disease, where antioxidants have the capacity to attenuate the phenotypes associated with these neurodegenerative disorders. Although antioxidants can prevent the oxidative stress-mediated progression of neurodegenerative diseases [26], antioxidant treatment is not sufficient to halt disease progression. We implied that inhibition of ASK1, the major downstream activator, may disrupt the positive feedback Therefore we propose that mitochondrial dysfunction by 3-NP induces an increase in ROS level; ROS-activated ASK1 mediates harmful oxidative signals; and cells eventually undergo apoptosis. The precise mechanisms have not yet been elucidated and have to be investigated.

In conclusion, this study indicates that ROS-induced ASK1 is an important step in the pathogenesis of 3-NP-mediated striatal lesion, and suggests that ASK1 acts as an amplifier of the ROS signal cascade. Taken together, we suggest a combination of ASK1 inhibition and ROS elimination for more effective therapy.

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