PLANT BIOLOGY

Correction for "Impact of clock-associated *Arabidopsis* pseudo-response regulators in metabolic coordination," by Atsushi Fukushima, Miyako Kusano, Norihito Nakamichi, Makoto Kobayashi, Naomi Hayashi, Hitoshi Sakakibara, Takeshi Mizuno, and Kazuki Saito, which appeared in issue 17, April 28, 2009, of *Proc Natl Acad Sci USA* (106:7251–7256; first published April 9, 2009; 10.1073/pnas.0900952106).

The authors note that on page 7255, Fig. 4 appeared incorrectly in part. The regulatory arrow between CCA1/LHY and TOC1 pointed in the opposite direction. This error does not affect the conclusions of the article. The corrected figure and its legend appear below.

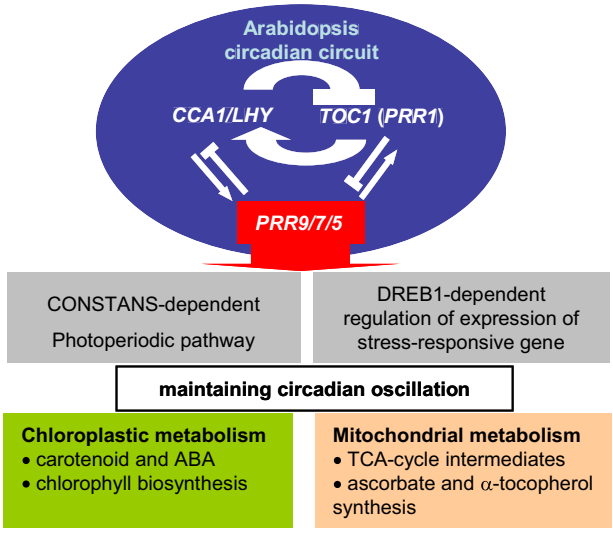

Fig. 4. Proposed output functions of PRR9/7/5. The figure suggests that PRR9/7/5, together with CCA1/LHY and TOC1 (PRR1), generates a normal oscillation rhythm. Furthermore, it shows that PRR9/7/5 is involved in DREB1-dependent regulation of stress-responsive gene expression and photoperiodic control of flowering time. The outputs of PRR9/7/5, such as maintaining central metabolism including that of the TCA cycle and antioxidant vitamins, are also shown. In addition, PRR9/7/5 negatively regulates the biosynthetic pathways associated with chlorophyll, carotenoid-ABA, and α-tocopherol biosynthesis in chloroplasts.

www.pnas.org/cgi/doi/10.1073/pnas.0904226106

NEUROSCIENCE

Correction for "S-nitrosylation of XIAP compromises neuronal survival in Parkinson's disease," by Anthony H. K. Tsang, Yun-IL Lee, Han Seok Ko, Joseph M. Savitt, Olga Pletnikova, Juan C. Troncoso, Valina L. Dawson, Ted M. Dawson, and Kenny K. K. Chung, which appeared in issue 12, March 24, 2009, of *Proc Natl Acad Sci USA* (106:4900–4905; first published March 9, 2009; 10.1073/pnas.0810595106).

The authors note that due to a printer's error, on page 4904, the lower right corner of Fig. 5*A* was cut off. The corrected figure and its legend appear below.

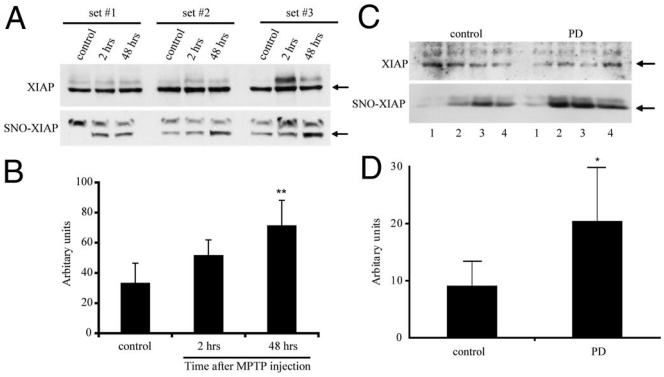

Fig. 5. XIAP is S-nitrosylated in vivo. (*A*) The level of S-nitrosylated XIAP is increased at 2 and 48 h after MPTP injection as observed in 3 independent experiments. (*B*) The amount of S-nitrosylated XIAP was quantified by a densitometer, and a significant increase of XIAP S-nitrosylation was observed in MPTP-treated animals (**, $P < 0.01$). (*C*) A marked increase of S-nitrosylated XIAP in the caudate was observed in PD patients. (*D*) The amount of S-nitrosylated XIAP was quantified by a densitometer, and a significant increase of XIAP S-nitrosylation was observed in PD patients (*, $P < 0.05$). These results were replicated at least 3 times.

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S-nitrosylation of XIAP compromises neuronal survival in Parkinson's disease

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Inhibitors of apoptosis (IAPs) are a family of highly-conserved proteins that regulate cell survival through binding to caspases, the final executioners of apoptosis. X-linked IAP (XIAP) is the most widely expressed IAP and plays an important function in regulating cell survival. XIAP contains 3 baculoviral IAP repeats (BIRs) followed by a RING finger domain at the C terminal. The BIR domains of XIAP possess anticaspase activities, whereas the RING finger domain enables XIAP to function as an E3 ubiquitin ligase in the ubiquitin and proteasomal system. Our previous study showed that parkin, a protein that is important for the survival of dopaminergic neurons in Parkinson's disease (PD), is S-nitrosylated both in vitro and in vivo in PD patients. S-nitrosylation of parkin compromises its ubiquitin E3 ligase activity and its protective function, which suggests that nitrosative stress is an important factor in regulating neuronal survival during the pathogenesis of PD. In this study we show that XIAP is S-nitrosylated in vitro and in vivo in an animal model of PD and in PD patients. Nitric oxide modifies mainly cysteine residues within the BIR domains. In contrast to parkin, S-nitrosylation of XIAP does not affect its E3 ligase activity, but instead directly compromises its anti-caspase-3 and antiapoptotic function. Our results confirm that nitrosative stress contributes to PD pathogenesis through the impairment of prosurvival proteins such as parkin and XIAP through different mechanisms, indicating that abnormal S-nitrosylation plays an important role in the process of neurodegeneration.

nitric oxide | apoptosis | neurodegeneration | inhibitors of apoptosis

A family of proteins that promotes cell survival through their characteristic antiapoptotic property includes X-linked inhibitor of apoptosis (XIAP). This group of proteins contain a variable number of baculoviral IAP repeat (BIR) motifs, which are marked by a sequence of ≈70 amino acids with a specific signature of histidine and cysteine residues within the structure in coordination with a zinc ion (1, 2). In XIAP, there are 3 BIRs followed by a RING finger domain at the C-terminal (3). The RING finger domain is marked by multiple histidine and cysteine residues that coordinate with 2 zinc ions to form the functional structure (4). XIAP is known to mediate a number of important physiological functions in the cell that depend on the BIR and RING finger domains (2). For example, XIAP binds to caspases via the BIRs and inhibits their activation during the execution phase of apoptosis (1). In addition, XIAP can function as an E3 ubiquitin ligase through its RING finger domain to target a number of substrates for ubiquitination (3).

XIAP is a potential target for antitumor therapy because of its up-regulation and promotion of the survival of cancer cells. However, modulation of XIAP's antiapoptotic activity has been implicated not only in cancer biology, but also in neurodegenerative disorders. For instance, overexpression of XIAP is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease (PD) (5, 6). Our previous study suggests that S-nitrosylation of parkin by NO compromises the survival of neurons in the pathogenesis of PD (7). We have also found that there is a high level of nitrosative stress in the brain tissues of PD patients (7). We suspect that S-nitrosylation of proteins that are critical for neuronal survival can also contribute to the pathogenesis of PD. Because the BIR and RING finger domains of XIAP contain multiple cysteine residues and are potential targets for S-nitrosylation, we decided to determine whether XIAP could be S-nitrosylated and whether this modification could compromise its antiapoptotic function.

Results

BIR Domains of XIAP Are S-Nitrosylated by NO. To determine whether XIAP could be S-nitrosylated in vitro, HEK293 cells expressing myc-XIAP were treated with *S*-nitrosoglutathione (GSNO). These samples were then subjected to the biotin switch assay (Fig. 1*A*). HEK293 cells expressing myc-XIAP treated with GSNO were readily S-nitrosylated, but S-nitrosylation was not observed in samples treated with glutathione (GSH) (Fig. 1*A*). The S-nitrosylation of XIAP was specific as under the same conditions, α-synuclein, which contains no cysteines, was not S-nitrosylated as demonstrated (7) (Fig. 1*B*). To confirm that S-nitrosylation not only occurred through the treatment with an exogenous NO donor, we performed a similar experiment with the use of N2A cells, which have been shown to possess endogenous neuronal NO synthase (nNOS) activity (8). After the biotin switch assay on N2A cells transfected with XIAP, we found that XIAP was S-nitrosylated under basal conditions (Fig. 1*C*). S-nitrosylation of XIAP was abolished by treatment with nitro-L-arginine (N-Arg), a nNOS inhibitor, and ascorbate, which reverses the S-nitrosylation modification. These results suggest that S-nitrosylation of XIAP depended on the NO produced by the nNOS in the N2A cells (Fig. 1*C*).

To map the potential S-nitrosylation sites of XIAP, we constructed a series of XIAP truncation mutants and subjected them to the biotin switch assay (Fig. 1 *D–F*). We found that the sites for S-nitrosylation were concentrated in the BIR domains (Fig. 1 *D–F*), but not in the RING finger domain (Fig. 1*D*). All 3 BIR domains are individually S-nitrosylated (Fig. 1 *D–F*). To further confirm that XIAP could be S-nitrosylated, we switched to the 2,3-diaminonaphthalene (DAN) fluorometric assay on recombinant GST-tagged truncated and full-length XIAP. The fluorometic assay showed that both truncated BIR1–3 and full-length XIAP were readily S-nitrosylated at a comparable level, suggesting that S-nitrosylation of XIAP was concentrated primarily in the BIR domains (Fig. 1*G*). To identify the sites for S-nitrosylation in XIAP, we incubated recom-

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The authors declare no conflict of interest.

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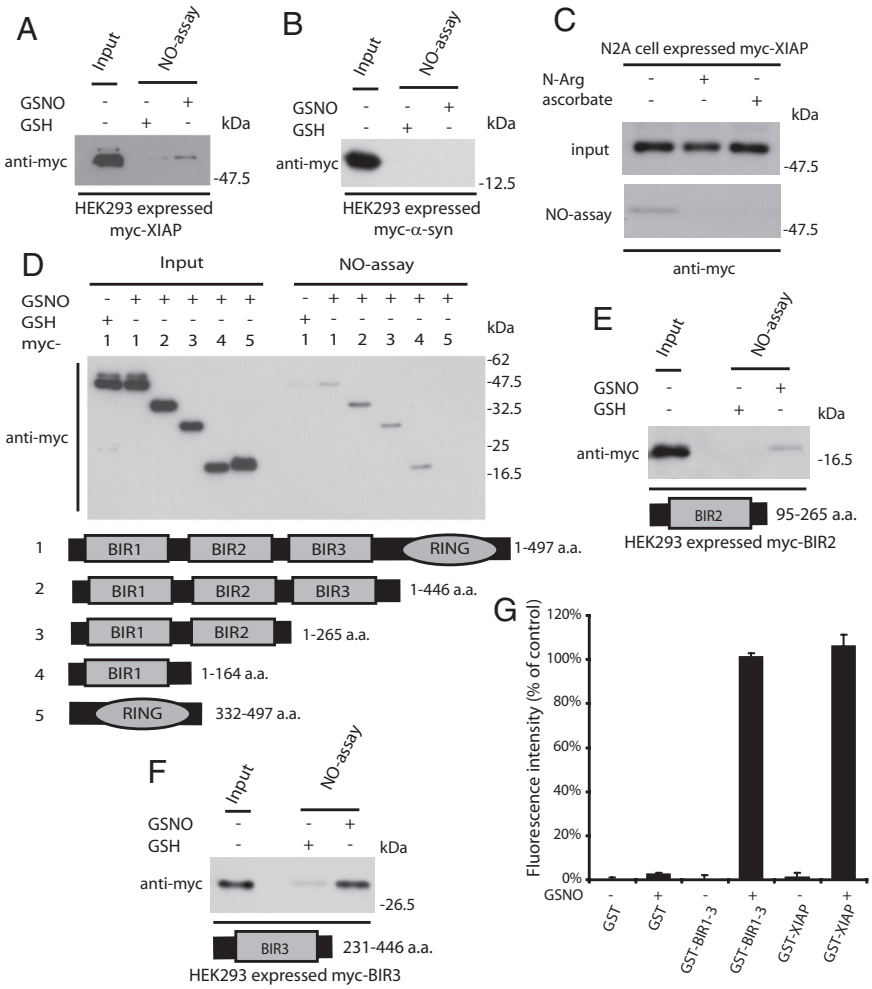

Fig. 1. XIAP can be S-nitrosylated in vitro and ex vivo. (*A*) XIAP was selectively S-nitrosylated when exposed to 250 μM GSNO at 37 °C for 15 min. (*B*) α-Synuclein that contains no cysteine residue was not S-nitrosylated under the same condition, demonstrating the specificity of the assay. (*C*) XIAP was S-nitrosylated ex vivo by endogenous NO in N2A cells. Cell lysate of N2A cells expressing myc-XIAP was directly brought to biotin switch assay. S-nitrosylation of XIAP in N2A cells could be prevented by incubating the cells with 3 mM ascrobate and 1 mM N-Arg. (*D–F*) Domain mapping reveals BIR domains are the major targeting domains for S-nitrosylation. (*D*) The myc-tagged truncated fragments of XIAP encoding amino acids 1–446 (BIR1–3), 1–265 (BIR1–2), 1–164 (BIR1), and 232–497 (RING) was generated and analyzed for their capability of S-nitrosylation by biotin switch assay. (*E* and *F*) Two additional truncated fragments encoding amino acid 95–265 (BIR2) (*E*) and 231–446 (BIR3) (*F*) of XIAP could also be S-nitrosylated. (*G*) S-nitrosylation of recombinant GST-XIAP and GST-BIR1–3 could be detected by the fluorometric method. These results were replicated at least 3 times.

binant GST-tagged XIAP BIR1–3 with GSNO and then performed the biotin switch reaction to label S-nitrosylated cysteine residues with biotin. We then used mass spectrometry to identify peptides that were biotinylated at the cysteine residues. Consistent with the biochemical data, we identified cysteine residues within the BIR domains that were modified by NO mainly within each of the BIR 1–3 domains of XIAP (Table S1).

XIAP S-Nitrosylation Does Not Affect Its E3 Ligase Activity. Through its E3 ligase activity, XIAP targets a number of substrates for ubiquitination (3). XIAP also targets itself for autoubiquitination, which can be used as an indicator for its E3 ligase activity (3). To determine whether NO could affect XIAP's E3 ligase activity, HEK293 cells transfected with myc-XIAP and HA-tagged ubiquitin (Ub) were treated with the NO donors GSNO and NOC18. Both treatments of GSNO and NOC18 had no effect on XIAP autoubiquitination, which indicated that the E3 ligase activity was not affected by XIAP S-nitroyslation (Fig. 2 *A* and *B*).

XIAP is known to form dimers, and this dimerization is important for its physiological function and E3 ligase activity (2, 9, 10). To determine whether NO could disrupt the dimerization of XIAP, HEK293 cells transfected with myc-XIAP and HA-XIAP were treated with GSNO and NOC18 and then followed by anti-myc immunoprecipitation (IP). Treatment of GSNO and NOC18 had no effect on XIAP dimerization (Fig. 2 *C* and *D*).

XIAP S-Nitrosylation Impairs Its Ability to Inhibit Caspase-3 Activity. A number of physiological functions of XIAP are associated with its BIR domains. For instance, the BIR2 domain of XIAP binds to caspase-3 and inhibits its caspase activity. We suspected that XIAP S-nitrosylation could affect its caspase-3 inhibition activity because one of the modified cysteines we identified by MS resided within the BIR2 domain and is close to the conserved residues of the IAP-binding motif (IBM) interacting groove (1). To test this hypothesis, we incubated recombinant GST-tagged XIAP and His-tagged caspase-3 together and monitored caspase-3 activity by measuring the fluorescence intensity generated by the cleavage of the caspase-3 fluorogenic substrate Ac-DEVD-AFC (Fig. 3). Incubation of XIAP with caspase-3 selectively inhibited its caspase activity (Fig. 3 *A* and *B*). In contrast, treatment of XIAP with GSNO before incubation with caspase-3 resulted in loss of XIAP's anticaspase-3 activity (Fig. 3 *A* and *B*). This loss of XIAP's anticaspase-3 activity by NO could be restored by the treatment of DTT, which suggests that the NO modification on XIAP was reversible (Fig. 3 *A* and *B*).

Caspase-3 has a number of cellular substrates and one of them is poly(ADP-ribose) polymerase 1 (PARP-1). To confirm that XIAP S-nitrosylation could affect its caspase-3 inhibition activity, we decided to test whether S-nitrosylation of XIAP could block its inhibition on caspase-3 cleavage of PARP-1. We set up an in vitro caspase-3 activity assay by combining GST-XIAP, caspase-3, and HEK293 cell lysate, and then monitored the PARP-1 cleavage by using an antibody specific for the caspase-3 cleaved PARP-1 fragment. Incubation of caspase-3 with HEK293 lysate resulted in the cleavage of PARP-1 (Fig. 3*C*). In contrast, coincubation of XIAP and caspase-3 reduced the amount of caspase-3-generated PARP-1 cleaved fragment (Fig. 3*C*). However, treatment of XIAP with GSNO before the incubation with caspase-3 abolished this

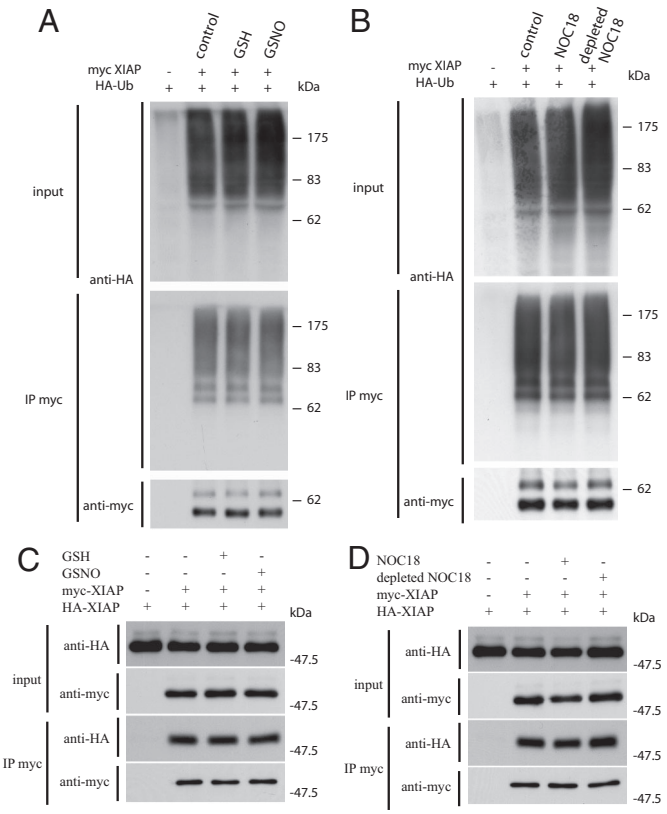

Fig. 2. NO does not affect E3 ligase activity and dimerization of XIAP. (*A* and *B*) NO has no effect on XIAP E3 ligase activity. HEK293 cells transfected with myc-XIAP and HA-Ub were incubated with either 100 μM GSNO for 6 h or 100 μM NOC-18 for 24 h. The E3 ligase activity of XIAP was assessed by IP with anti-myc antibody and analyzed by Western blot. (*C* and *D*) NO has no effect on XIAP dimerization. HEK293 cells transfected with myc-XIAP and HA-XIAP was treated with 100 μM GSNO for 6 h or 100 μM NOC18 for 24 h. Dimerization of XIAP was assessed by IP by anti-myc antibody and analyzed by Western blot. These results were replicated at least 3 times.

anticaspase-3 activity, confirming that XIAP S-nitrosylation could affect its caspase-3 inhibition activity (Fig. 3*C*). Again, this inhibition of XIAP anticaspase-3 activity was restored by the treatment of DTT, which suggests that the NO modification of XIAP was reversible (Fig. 3*C*). To confirm that this observation was specific for NO modification on XIAP, we used GST protein as control and found that different treatments had no effect on the generation of PARP-1 -cleaved fragment by caspase-3 (Fig. 3*D*).

Different studies suggest that the XIAP's anticaspase-3 activity depends on the direct physical interaction of XIAP with caspase-3 (1). Thus, XIAP S-nitrosylation may lead to loss in XIAP's anticaspase-3 activity by interfering with the direct interaction between XIAP and caspase-3. To determine whether XIAP S-nitrosylation could affect the interaction between XIAP and caspase-3, lysates from HEK293 cells transfected with myc-XIAP were incubated with recombinant caspase-3 followed by anti-myc IP. We found that XIAP specifically coimmunoprecipitated with caspase-3 (Fig. 3*E*). Treatment of cell lysate with GSNO before incubation with caspase-3 abolished this XIAP caspase-3 interaction (Fig. 3*E*). This interaction could be restored by the treatment of DTT, which suggests that the NO modification on XIAP was reversible (Fig. 3*E*). Taken together, these results showed that S-nitrosylation of XIAP impairs its binding with caspase-3 and directly inhibits XIAP's anticaspase-3 activity.

XIAP's Antiapoptotic Function Is Impaired by S-Nitrosylation. XIAP is well known to possess antiapoptotic activity against a variety of cell death paradigms (2, 11, 12). Because we found that S-nitrosylation of XIAP impaired its anticaspase-3 activity, we suspected that S-nitrosylation of XIAP could also impair its antiapoptotic activity in cells exposed to various cell death stimuli. To test this hypothesis, HEK293 cells transfected with and without XIAP were treated with 50 ng/mL of TNF-α and 0.1 μg/mL of actinomycin D for 24 h to induce apoptosis. Consistent with previous studies, treatment of HEK293 cells with TNF-α induced cell death, but expression of XIAP significantly attenuated the cell death induced by TNF-α (13) (Fig. 4*A*). This protection was abolished by the treatment of NOC18, but not with the NO-depleted NOC18 (Fig. 4*A*).

Because we suspected that nitrosative stress could compromise the protective effects of XIAP and possibly contribute to the development of PD, we tested whether NO could impair XIAP's antiapoptotic function by using PD cell-based models. Rotenone inhibits mitochondrial complex I and exposure to rotenone like herbicides may lead to degeneration of dopaminergic neurons (14). Similarly, the high propensity of dopamine to oxidize among catecholamines may account for why dopaminergic neurons are more susceptible to degeneration in PD (15, 16). To test whether S-nitrosylation of XIAP could compromise its ability to protect neurons against rotenone- and dopamine-induced toxicity, we transfected cells with XIAP and then treated cells with rotenone and dopamine. Treatment of cells with rotenone (50 μM) and dopamine (2 mM) induced a significant increase of cell death (Fig. 4 *B* and *C*). In contrast, cells transfected with XIAP were resistant to rotenone or dopamine challenge (Fig. 4 *B* and *C*). However, the protection offered by XIAP was abolished by pretreatment of cells with the NO donor, NOC18 (Fig. 4 *B* and *C*).

Proteasomal dysfunction and protein aggregation-induced toxicity have been considered as other major contributors in the pathogenesis of PD (17). Consistent with this hypothesis, neurons exposed to proteasomal inhibitors or proteins prone to aggregation are more vulnerable to cell death (18–20). To test whether S-nitrosylation of XIAP could comprise its ability to protect neurons against proteasomal dysfunction and protein aggregation, we treated cells with proteasomal inhibitor, MG132 (3 μM), or expressed an aggregation-prone GFPu protein to induce cell death (21). Inhibiting proteasomal function or exposure of cells to aggregation-prone GFPu protein induced a significant increase in cell death (Fig. 4 *D* and *E*). This cell death was prevented by expression of XIAP (Fig. 4 *D* and *E*). However, this protection afforded by XIAP was abolished by pretreatment of cells with the NO donor NOC18 (Fig. 4 *D* and *E*). Taken together, these results suggest that S-nitrosylation of XIAP can compromise the survival of dopaminergic neurons in the process of neurodegeneration in cellular models of PD.

XIAP S-Nitrosylation Is Increased in an Animal Model of PD and in PD Patients. Our previous study showed that S-nitrosylated proteins are significantly increased in animal model of PD and PD patients (7). In this study, our results suggest that S-nitrosylation of XIAP could compromise its protective function in cells. Thus, we hypothesized that during the pathogenesis of PD, XIAP S-nitrosylation is elevated, which could possibly compromise the survival of neurons in the process of neurodegeneration. To test this hypothesis, we first used the well-established MPTP animal model of PD to determine whether S-nitrosylation of XIAP is increased in this model. Administration of MPTP in animals selectively induces the degeneration of the nigrostriatal dopaminergic neurons as observed in PD (22). To determine whether XIAP S-nitrosylation was increased in nigrostriatal system in mice after MPTP treatment, mice were injected with MPTP as described (7, 23). After MPTP treatment, mice were killed at 2- and 48-h time points, their brains were harvested, and XIAP S-nitrosylation in the striatum was determined by the biotin switch assay. We selected these 2 time points because from our previous study we found that 2 and 48 h after MPTP treatment

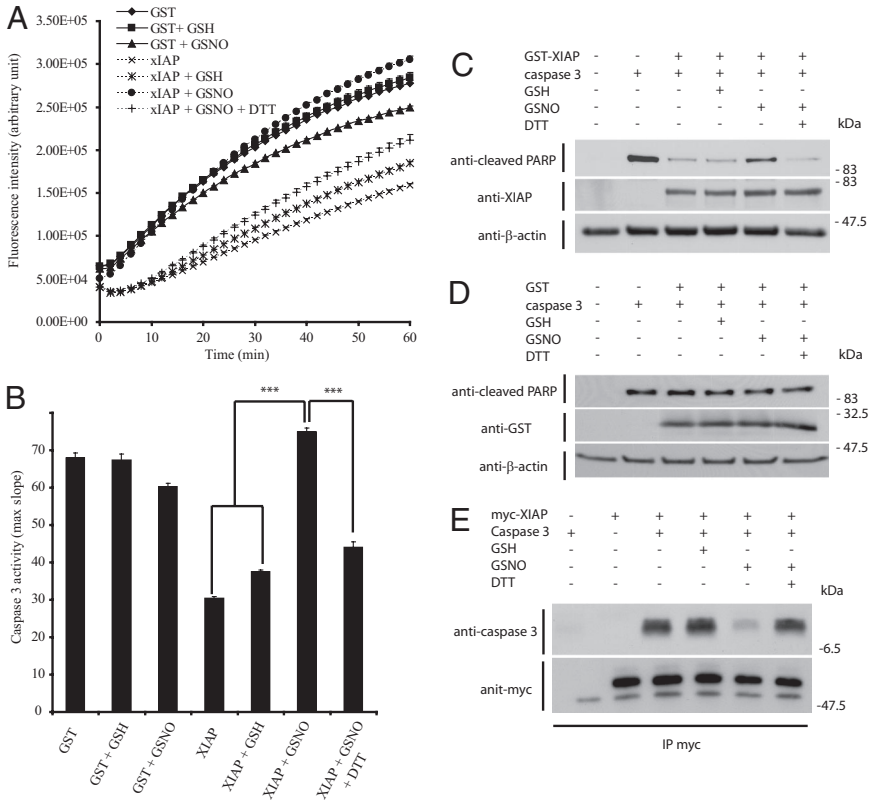

Fig. 3. S-nitrosylation of XIAP regulates its inhibitory effect on active caspase-3. (*A*) NO abolishes XIAP inhibitory effect on caspase-3 activity. Recombinant GST or GST-XIAP (0.2 μM) was pretreated with GSNO (500 μM). Recombinant active caspase-3 (20 nM) was then incubated with pretreated or mock-treated GST-XIAP or GST at room temperature for 10 min. Activity of recombinant caspase-3 (20 nM) was monitored by the fluorescence generated by the cleavage of the fluorogenic caspase-3 substrate Ac-DEVD-AFC (50 μM). (*B*) Quantification of the caspase-3 activity in the presence of pretreated or mocked treated recombinant proteins. The rate of caspase-3 activity incubated with recombinant proteins with or without pretreatment was determined from the maximum slope as in *A* (***, $P < 0.001$). (*C*) NO attenuates the inhibitory effect of XIAP on caspase-3-dependent PARP cleavage. Recombinant GST-XIAP pretreated with 500 μM GSNO was incubated with 0.5 μg of active recombinant caspase-3 in equal molar ratio at room temperature for 10 min. HEK293 cell lysate (10 μg) was then added and the samples were further incubated at 37 °C for 30 min. Cleavage of PARP was analyzed by Western blot by anticleaved PARP antibody. (*D*) Experiment as in *C* was repeated with the use of GST instead of GST-XIAP. No effect on caspase-3-induced PARP cleavage was observed. (*E*) NO reduces the binding of XIAP to active caspase-3. HEK293 cell lysate overexpressing myc-XIAP was treated with GSNO (500 μM) and then incubated with 7.5 μg of active recombinant caspase-3. Binding was assessed by co-IP with anti-myc antibody and analyzed by Western blot with anti-active caspase-3 antibody. These results were replicated at least 3 times.

marked the highest levels of parkin S-nitrosylation in the brain (7). After MPTP treatment, XIAP S-nitrosylation was markedly increased in the striatum (Fig. 5*A*). This increase in XIAP S-nitrosylation was particularly significant 48 h after MPTP treatment (Fig. 5 *A* and *B*).

We next wanted to address whether increased XIAP S-nitrosylation could also be observed in PD patients. To determine whether XIAP S-nitrosylation was increased in PD patients, we performed the biotin switch assay on postmortem brain tissue from caudate from normal control and PD patients. We found that the protein levels of XIAP were similar in normal control and PD patients (Fig. 5*C*). However, we observed a significant increase in S-nitrosylated XIAP in PD patients (Fig. 5 *C* and *D*). Taken together, these results suggest that XIAP is S-nitrosylated in vivo and is selectively increased in the MPTP animal model of PD and in the postmortem brain tissues of PD patients.

Discussion

XIAP is an antiapoptotic protein that is known to be crucial for cell survival. However, overexpression of XIAP is commonly observed in tumors and is implicated in the development of different types of cancer (11, 24). In this study we show that XIAP can be S-nitrosylated at the BIR domains. XIAP S-nitrosylation compromised its antiapoptotic function by inhibiting its anticaspase-3 activity. We further found that a significant increase of S-nitrosylated XIAP can be observed in the MPTP animal model of PD and in PD patients. These results support our previous finding that nitrosative stress is an important contributor in the pathogenesis of PD.

We first reported S-nitrosylation of parkin can compromise its protective functions (7). The mechanism by which S-nitrosylation of parkin impairs its protective function appears to occur through inhibition of its E3 ubiquitin ligase activity (7, 25). This idea contrasts with the mechanism of impairment of XIAP antiapoptotic function by S-nitrosylation. XIAP antiapoptotic function is inhibited by S-nitrosylation through preventing XIAP's binding to caspase-3. Several recent studies have also reported that other neuroprotective proteins such as peroxiredoxin and protein-disulphide isomerase are modified by S-nitrosylation, and this modification compromises their normal protective functions (26, 27). Thus, the repertoire by which S-nitrosylation can compromise cellular survival is diverse.

Apart from impairing neuroprotection proteins, S-nitrosylation is also involved in mediating cell death through GAPDH (28, 29). Recent studies showed that S-nitrosylation of GAPDH can mediate the translocation of GAPDH–Siah1 protein complex to the nucleus and initiates apoptosis (29, 30). Our findings that S-nitrosylation of XIAP can affect its antiapoptotic function further suggest that nitrosative stress can affect the survival of neurons through targeting a number of pathways. Because we found that XIAP S-nitrosylation was increased in the MPTP animal model of PD and in PD patients, these results suggest that neurons would be more vulnerable to cell death in face of unfavorable conditions such as proteasomal dysfunction and protein aggregation-induced toxicity. Our findings also suggest that fully understanding how nitrosative stress can contribute to PD will help develop new therapeutic approaches for this disease.

Materials and Methods

Chemicals and Cell Culture. All chemicals were purchased from Sigma–Aldrich unless otherwise stated. HEK293T and N2a cells were maintained in DMEM (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco) at 37 °C with 5% CO_2. Transfection was performed with Lipofectamine and Plus Reagent (Invitrogen) according to the manufacturer's instruction.

Generation of Plasmids. XIAP and procaspase-3 were cloned from the SuperScript human brain cDNA library (Invitrogen). Full-length XIAP was cloned into pRK5-myc and HA vectors for cell culture study and into pGEX4T-2 vector for recombinant protein production. The truncated fragments of XIAP encoding amino acids 1–446 (BIR1–3), 1–265 (BIR1–2), 1–164 (BIR1), 95–265 (BIR2), 231–446 (BIR3), and 332–497 (RING) were generated by PCR with the full-length XIAP as template and cloned into pRK5-myc vector. The XIAP fragment BIR1–3 was also cloned into

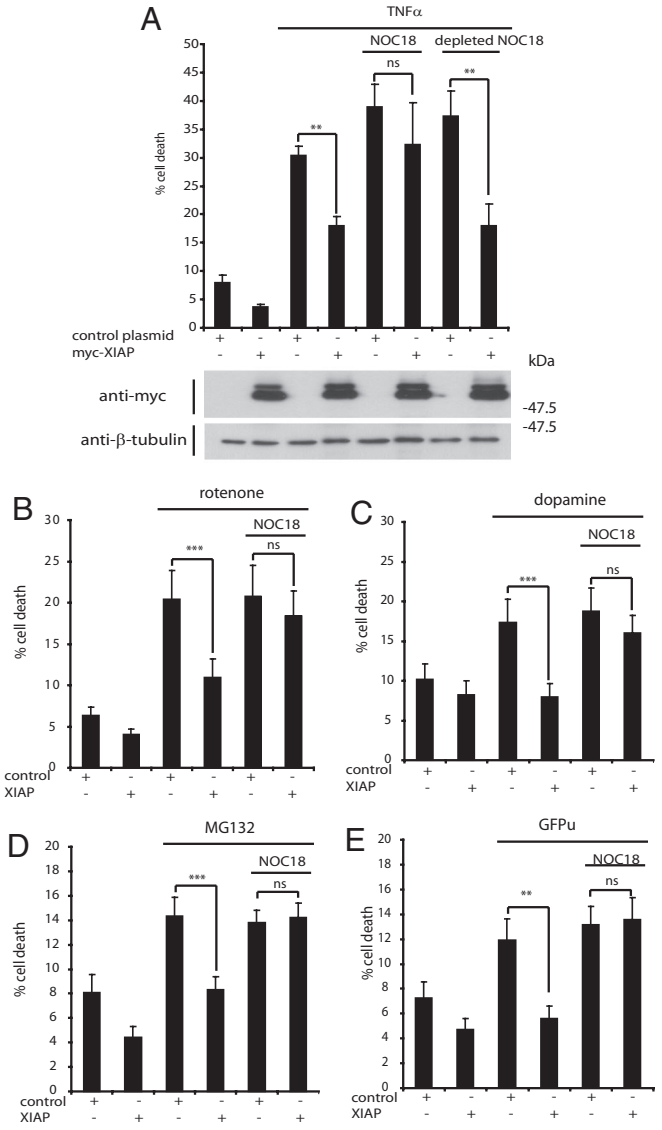

Fig. 4. S-nitrosylation of XIAP impairs its cytoprotective effects against various cell death stimuli. (*A*) NO inhibits XIAP's antiapoptotic function against TNF-α-induced cell death. HEK293 cells were transfected with 0.25 μg of myc-XIAP. Thirty hours after transfection, cells were preexposed with either 100 μM NOC-18 or depleted NOC-18 for 6 h and then treated with 50 ng/mL TNF-α and 0.1 μg/mL actinomycin D for an additional 24 h. Cell death was assayed by trypan blue exclusion method (**, $P < 0.01$; ns = nonsignificant). Representative protein levels of myc-XIAP after treatment as indicated are shown. (*B–E*) NO inhibits XIAP's antiapoptotic function against various cell death stimuli. HEK293 was transfected and preexposed to NOC-18 as described in *A*. After that, cells were challenged with various cell death stimuli (50 μM rotenone for 16 h, 2 mM dopamine for 24 h, and 3 μM MG132 for 24 h), and cell death was analyzed by trypan blue exclusion assay. In GFPu experiment (*E*), 0.125 μg of myc-XIAP was cotransfected with either 0.125 μg of GFPu or control plasmid. 30 h after transfection, cells were exposed to 100 μM NOC-18 and cell death was assessed 60 h after transfection (**, $P < 0.01$; ***, $P < 0.001$; ns = nonsignificant). These results were replicated at least 3 times.

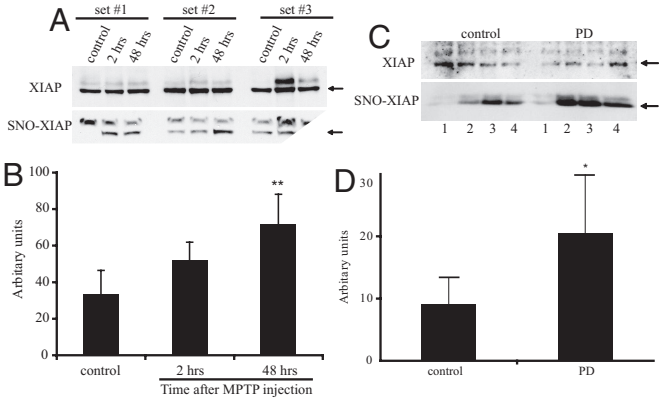

Fig. 5. XIAP is S-nitrosylated in vivo. (*A*) The level of S-nitrosylated XIAP is increased at 2 and 48 h after MPTP injection as observed in 3 independent experiments. (*B*) The amount of S-nitrosylated XIAP was quantified by a densitometer, and a significant increase of XIAP S-nitrosylation was observed in MPTP-treated animals (**, $P < 0.01$). (*C*) A marked increase of S-nitrosylated XIAP in the caudate was observed in PD patients. (*D*) The amount of S-nitrosylated XIAP was quantified by a densitometer, and a significant increase of XIAP S-nitrosylation was observed in PD patients (*, $P < 0.05$). These results were replicated at least 3 times.

pGEX4T.2 vector for recombinant protein production. Procaspase-3 was cloned into pET28C vector for expression of recombinant proteins. The cDNAs of α-synuclein and ubiquitin were generated as described (7). Sequence integrity of all constructs was verified by sequencing.

Overexpression and Purification of Recombinant Proteins. Recombinant GST, GST-XIAP, and GST-BIR1–3 were expressed in Rosetta (DE3) pLys *Escherichia coli* (Novagen). Overexpression of bacterial culture in linear growing phase (0.6 OD) were induced by 0.2 mM IPTG at 18 °C overnight, and the recombinant proteins were then purified by GSH-Sepharose (GE Healthcare). His-tagged recombinant active caspase-3 was produced according to Stennicke and Salvesen (31) and was purified by Ni-NTA Sepharose (GE Healthcare). Concentrations of the recombinants protein were quantified by SDS/PAGE with the use of BSA as standard.

Preparation of *S*-GSNO. *S*-GSNO was prepared according to Cook et al. (32). GSNO was prepared freshly at the day of each experiment.

In Vitro S-Nitrosylation Assay. The biotin switch assay was performed according to Jaffrey and Snyder (33) with some modifications. Nitrosylated cell lysates or recombinant proteins in HENT buffer (250 mM Hepes, 1 mM EDTA, 0.1 mM Neocupoine, 1% Triton X-100) were incubated with 10 mM methyl methanethiosulfonate (MMTS) (Thermo Scientific) at 50 °C for 20 min and then excess MMTS was removed by passing through the G25 Sephadex spin column 3 times. The samples were then incubated with 5 mM ascrobate and 0.4 mM biotin-HPDP (Thermo Scientific) for 1 h at room temperature with rotation. Unreacted biotin-HPDP was then removed by G25 Sephadex spin column and biotinylated samples were then incubated with 50 μL of Neutravidin-agarose (Thermo Scientific) for 1 h. Pellets were then washed 5 times with neutralization buffer [20 mM Hepes (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100] with 0.6 M NaCl and eluted by SDS sample buffer and subjected to Western blot analysis.

Fluorometric Detection of S-Nitrosylated XIAP. Fluorometric assay was performed according to Cook et al. (32). In brief, GSNO- or GSH-treated GST-tagged recombinant proteins were immunoprecipitated with polyclonal anti-GST antibody. The pellet was then washed 5 times with TBST (1% Triton X-100 in TBS) buffer. After washing, 100-μL assay buffer containing 100 μM DAN and 100 μM $HgCl_2$ in TBS was added to pellets and incubated for 2 h at room temperature in darkness. The fluorescence generated by the formation of fluorometric product 2,3-napththyltrazole was then measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

XIAP Dimerization and Autoubiquitination. For dimerization assay, HEK293T cells were transfected with myc-XIAP and HA-XIAP. After 24 h, cells were treated with GSNO (100 μM) or NOC-18 (100 μM) and then harvested at the selected time points with IP buffer (1% Triton X-100, 10% glycerol, 1 mM aprotinin, 1 mM leupeptin, 1 mM benzamidine, 10 mM PMSF in TBS) at 4 °C for 1 h and cleared by centrifugation. The cell lysates were subjected to anti-myc IP by incubating with 0.5 μg of anti-myc antibody (Roche) together with 50 μL of protein A agarose (GE Healthcare) for 2 h at 4 °C with rotation. The immuno-complexes were washed 5 times with IP buffer and eluted by SDS sample buffer and subjected to Western blot analysis. For ubiquitination assay, 24 h after transfection, HEK293T cells overexpressed with myc-XIAP and HA-ubiquitin were treated with GSNO (100 μM) or NOC-18 (100 μM) as indicated. After treatment, cells were lysed by IP buffer and followed by anti-myc IP protocol as described.

XIAP Caspase-3 Interaction. HEK293T lysates with or without expressing myc-XIAP were treated with GSH (500 μM), GSNO (500 μM), or DTT (1 mM) for 15 min at 37 °C as indicated. The lysates were then passing through G25 Sephadex spin column once and recombinant active caspase-3 (7.5 μg) was added to the lysates and co-IP protocol was then carried out as described. Caspase-3 was detected by anticleaved caspase-3 antibody (Sigma).

Caspase-3 Activity Assay. In caspase-3 activity assay, recombinant GST-XIAP and GST were first treated with 5 mM DTT for 20 min at room temperature, and DTT was then removed by a G25 Sephadex desalting column (GE Healthcare). After passing through the column, 0.2 μM recombinant proteins were treated with 500 μM GSH or GSNO for 15 min at 37 °C followed by passing the samples again through the Sephadex desalting column to remove GSH and GSNO. The samples were then incubated with 20 nM active caspase-3 for 10 min at room temperature with rotation. After incubation, 50 μM DEVD-AFC (Sigma) was added as substrate. Caspase-3 activity was monitored by the fluorescence generated at an excitation wavelength of 355 nm and an emission wavelength of 460 nm at 30 °C.

PARP Cleavage. Recombinant GST-XIAP and GST were first treated with DTT, GSH, and GSNO as in the caspase activity assay. Treated proteins were then incubated with 0.5 μg of active caspase-3 (1:1 molar ratio) for 10 min at room temperature with rotation. After 10 min, 10 μg of 293T cell lysate was added to the mixture and incubated at 37 °C for 30 min. The reaction was stopped by adding SDS sample buffer, and PARP cleavage was analyzed by Western blot with the anticleaved PARP antibody (BD Bioscience).

Cell Death Analysis. HEK293T cells were transfected with 0.25 μg of myc-XIAP or control vector. Thirty hours after transfection, cells were pretreated with 100 μM NO donor NOC-18 (Calbiochem) for 6 h. Cells were then treated with selected drugs as indicated and conditions as in the following: 50 ng/mL TNF-α and 0.1 μg/mL actinomycin D for 24 h; 50 μM rotenone for 16 h; 2 mM dopamine for 24 h; 3 μM MG132 for 24 h. In GFPu-induced cell death assay, apart from myc-XIAP and control vector, cells were also cotransfected with GFPu or GFP as control. Cell death was analyzed 60 h after transfection by Trypan blue exclusion assay.

Animals and Treatment. All experiments followed the guidelines set by the Institutional Animal Care Committee. Eight-week-old male wild-type C57B/L mice (Charles River Laboratories) were used. Mice received 4 i.p. injections of MPTP-HCl (20 mg/kg of free base equivalent; Sigma) in sterile saline at 2-h intervals. Mice were killed at 2 and 48 h after the last injection. Control mice received saline-only injections. The mouse brains were harvested, and S-nitrosylation of xIAP in the striatum was determined as described (7, 23)

Human Tissue. Human brain tissue was obtained through the brain donation program of the Morris K. Udall Parkinson's Disease Research Center at Johns Hopkins Medical Institutions according to Health Insurance Portability and Accountability Act regulations. This research proposal involves anonymous autopsy material that lacks identifiers of gender, race, or ethnicity. The Johns Hopkins Medical Institutions Joint Committee on Clinical Investigations decided that the studies in this proposal are exempt from human subjects approval because of Federal Register 46.101 exemption 4. Four age-matched control brains, 4 PD and/or DLBD brains, were used for S-nitrosylation of XIAP by in vivo S-nitrosylation assay (Table S2).

In Vivo S-Nitrosylation Assay of XIAP in Human Brain Tissue. The assay was basically the same as the in vitro assay except tissues were homogenized in HENS buffer [250 mM Hepes (pH 7.7), 1 mM EDTA, 0.1 mM neocuproine, and 1% SDS] without the incubation with NO donors. S-nitrosylated protein was detected by using specific XIAP antibody (BD Bioscience).

Statistical Analysis. Data are expressed as mean ± SEM. Significance was determined by ANOVA or Student's *t* test.

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1. Eckelman BP, Salvesen GS, Scott FL (2006) Human inhibitor of apoptosis proteins: Why XIAP is the black sheep of the family. *EMBO Rep* 7:988–994.
2. Srinivasula SM, Ashwell JD (2008) IAPs: What's in a name? *Mol Cell* 30:123–135.
3. Vaux DL, Silke J (2005) IAPs, RINGs, and ubiquitylation. *Nat Rev Mol Cell Biol* 6:287–297.
4. Weissman AM (2001) Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* 2:169–178.
5. Crocker SJ, et al. (2003) Attenuation of MPTP-induced neurotoxicity and behavioural impairment in NSE-XIAP transgenic mice. *Neurobiol Dis* 12:150–161.
6. Eberhardt O, et al. (2000) Protection by synergistic effects of adenovirus-mediated X-chromosome-linked inhibitor of apoptosis and glial cell line-derived neurotrophic factor gene transfer in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. *J Neurosci* 20:9126–9134.
7. Chung KK, et al. (2004) S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science* 304:1328–1331.
8. Lopez-Figueroa MO, et al. (2001) Characterization of basal nitric oxide production in living cells. *Biochim Biophys Acta* 1540:253–264.
9. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD (2000) Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* 288:874–877.
10. Lu M, et al. (2007) XIAP induces NF-κB activation via the BIR1/TAB1 interaction and BIR1 dimerization. *Mol Cell* 26:689–702.
11. Holcik M, Gibson H, Korneluk RG (2001) XIAP: Apoptotic brake and promising therapeutic target. *Apoptosis* 6:253–261.
12. Liston P, Fong WG, Korneluk RG (2003) The inhibitors of apoptosis: There is more to life than Bcl2. *Oncogene* 22:8568–8580.
13. Li L, et al. (2004) A small molecule Smac mimic potentiates TRAIL- and TNF-α-mediated cell death. *Science* 305:1471–1474.
14. Greenamyre JT, Betarbet R, Sherer TB (2003) The rotenone model of Parkinson's disease: Genes, environment, and mitochondria. *Parkinsonism Relat Disord* 9(Suppl 2):S59–S64.
15. Danielson SR, Andersen JK (2008) Oxidative and nitrative protein modifications in Parkinson's disease. *Free Radical Biol Med* 44:1787–1794.
16. Stokes AH, Hastings TG, Vrana KE (1999) Cytotoxic and genotoxic potential of dopamine. *J Neurosci Res* 55:659–665.
17. Dawson TM, Dawson VL (2003) Molecular pathways of neurodegeneration in Parkinson's disease. *Science* 302:819–822.
18. Tanaka Y, et al. (2001) Inducible expression of mutant α-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis. *Hum Mol Genet* 10:919–926.
19. Rideout HJ, Lang-Rollin IC, Savalle M, Stefanis L (2005) Dopaminergic neurons in rat ventral midbrain cultures undergo selective apoptosis and form inclusions, but do not up-regulate iHSP70, following proteasomal inhibition. *J Neurochem* 93:1304–1313.
20. Stefanis L, Larsen KE, Rideout HJ, Sulzer D, Greene LA (2001) Expression of A53T mutant but not wild-type α-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. *J Neurosci* 21:9549–9560.
21. Link CD, et al. (2006) Conversion of green fluorescent protein into a toxic, aggregation-prone protein by C-terminal addition of a short peptide. *J Biol Chem* 281:1808–1816.
22. Dawson T, Mandir A, Lee M (2002) Animal models of PD: Pieces of the same puzzle? *Neuron* 35:219–222.
23. Thomas B, et al. (2007) MPTP and DSP-4 susceptibility of substantia nigra and locus coeruleus catecholaminergic neurons in mice is independent of parkin activity. *Neurobiol Dis* 26:312–322.
24. Schile AJ, Garcia-Fernandez M, Steller H (2008) Regulation of apoptosis by XIAP ubiquitin-ligase activity. *Genes Dev* 22:2256–2266.
25. Yao D, et al. (2004) Nitrosative stress linked to sporadic Parkinson's disease: S-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. *Proc Natl Acad Sci USA* 101:10810–10814.
26. Fang J, Nakamura T, Cho DH, Gu Z, Lipton SA (2007) S-nitrosylation of peroxiredoxin 2 promotes oxidative stress-induced neuronal cell death in Parkinson's disease. *Proc Natl Acad Sci USA* 104:18742–18747.
27. Uehara T, et al. (2006) S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. *Nature* 441:513–517.
28. Hara MR, et al. (2005) S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. *Nat Cell Biol* 7:665–674.
29. Hara MR, et al. (2006) Neuroprotection by pharmacologic blockade of the GAPDH death cascade. *Proc Natl Acad Sci USA* 103:3887–3889.
30. Sen N, et al. (2008) Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis. *Nat Cell Biol* 10:866–873.
31. Stennicke HR, Salvesen GS (1999) Caspases: Preparation and characterization. *Methods* 17:313–319.
32. Cook JA, et al. (1996) Convenient colorimetric and fluorometric assays for S-nitrosothiols. *Anal Biochem* 238:150–158.
33. Jaffrey SR, Snyder SH (2001) The biotin switch method for the detection of S-nitrosylated proteins. *Sci STKE* 2001:PL1.