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OXIDANTS INDUCE ALTERNATIVE SPLICING OF α -SYNUCLEIN: IMPLICATIONS FOR PARKINSON'S DISEASE

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Abstract

α -Synuclein (α -syn) is a presynaptic protein that is widely implicated in the pathophysiology of Parkinson's disease (PD). Emerging evidence indicates a strong correlation between α -syn aggregation and proteasomal dysfunction as one of the major pathways responsible for destruction of the dopamine neurons. Using Parkinsonism mimetics (MPP⁺, rotenone) and related oxidants, we have identified an oxidant-induced alternative splicing of α -syn mRNA, generating a shorter isoform of α -syn with deleted exon-5 (112-syn). This spliced isoform has an altered localization and profoundly inhibits proteasomal function. The generation of 112-syn was suppressed by constitutively active MEK-1 and enhanced by inhibition of the Erk-MAP kinase pathway. Overexpression of 112-syn exacerbated cell death in a human dopaminergic cell line compared to full length protein. Expression of 112-syn and proteasomal dysfunction were also evident in the substantia nigra and to a lesser extent in striatum, but not in the cortex of MPTP treated mice. We conclude that oxidant-induced alternative splicing of α -syn plays a crucial role in the mechanism of dopamine neuron cell death and thus contributes to PD.

Introduction

α -Synuclein (α -syn) is a presynaptic protein that is widely implicated in the pathophysiology of Parkinson's disease (PD), a neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons and formation of Lewy body aggregates [1–4]. Reports indicate that α -synuclein (α -syn), parkin and the ubiquitin-proteasome pathway are responsible for generating protein aggregates as well as Lewy bodies [2,5]. Defects in mitochondrial complex-1 activity, dopamine deficiency, proteasomal dysfunction and

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markers of oxidative stress are all detected in patients suffering from PD [6–8]. Specific mitochondrial toxins or complex-1 inhibitors such as 1-methyl-4-phenylpyridinium (MPP⁺), a metabolic product of 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP), and rotenone induce symptoms similar to those of PD and increase the expression and aggregation of α -syn [9–11].

α -Syn has multiple effects on cellular function. α -Syn is an inhibitor of phospholipase D₂ [12,13]; binds to dopamine transporters altering dopamine homeostasis (14); acts as a molecular chaperone [15,16]; and interacts with various other proteins altering their cellular function [17]. The gene structure of α -syn reveals the presence of 6 exons, ranging in size from 42 to 1110 bp [18]. The translation start codon (ATG) is encoded in exon 2 and the stop codon (TAA) is encoded by exon 6 [18]. Functional studies indicate that an acidic C-terminus region of α -syn is required for its normal physiological function; deletion of the C-terminus makes the protein more vulnerable to aggregation [19,20]. Pathways that could lead to the generation of shorter isoforms of α -syn in cellular systems or *in vivo* models include C-terminal truncation or alternative splicing of pre-mRNA. The C-terminus truncated forms/shorter isoforms of α -syn have been recently detected in cells and in post mortem samples of PD subjects [21]. Although the existence of alternatively spliced transcript of α -syn (112-Syn) was discovered earlier [22,23], no evidence exists, to our knowledge, regarding the induced expression of 112-syn and the factors regulating the induction of alternative-splicing of α -syn and its existence in the preclinical mouse model of PD. In the present study, we provide evidence that Parkinsonism mimetics such as MPP⁺, rotenone and 6-hydroxy dopamine (6-OHDA) can induce the alternative splicing of α -syn, leading to the deletion of exon-5. Deletion of this exon results in the generation of a 112 aa protein (112-syn) instead of the 140 aa wild-type counterpart (WT α -syn). The physiological significance of this finding and its relevance to PD pathophysiology are discussed.

Experimental Procedures

Materials

1-Methyl-4-phenyl tetrahydropteridine (MPP⁺ iodide), 1-Methyl-4-phenyl-1,2,3,6-tetrahydropteridine (MPTP), rotenone, 6-hydroxydopamine (6-OHDA) and other chemicals for buffer preparations were purchased from Sigma. *N,N'*-Bis(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid (HBED) was obtained as a gift from Dr. Cherakuri Muralikrishna (National Institutes of Health). Adenovirus containing full-length GPx1 was a generous gift from Dr. Larry Oberley (University of Iowa). Adenovirus expressing constitutively active and dominant negative forms of MEK-1 were obtained from the adenoviral core facility, Medical College of Wisconsin (Milwaukee, WI). MAP kinase inhibitor U0126 (tyrophostin) was obtained from Calbiochem.

Cell cultures

Human SH-SY5Y dopaminergic cells were maintained as described earlier [11]. Twelve hours before the start of treatment, medium was replaced with Dulbecco's modified Eagle's medium containing 2% FBS. The above conditions were applied to all of the experiments performed in this study. Primary cultures of Mesencephalic Neurons (MN) from timed

pregnant Sprague Dawley rats (from embryonic day 14 (E14) fetuses was prepared according to the previous protocol [24]. Using sterile techniques, ventral mesencephalic tissue was dissected from rat embryos and maintained on ice in calcium free Hank's balanced salt solution (HBSS). Cell suspensions of embryonic mesencephalic tissue was digested in HBSS containing trypsin (0.2 mg/ml) and 40 mg/ml DNase in a water bath at 37°C for 20 min. Treatment was terminated by the addition of HBSS solution containing 80 mg/ml trypsin inhibitor and the cells were collected by gentle centrifugation (750 rpm for 2 min). The tissue was triturated gently to disperse the cells into solution and cells were collected by centrifugation (2000 rpm for 5 min). The obtained cell pellet was re-suspended in Neurobasal Medium (NBM; GibcoBRL Life Technologies) supplemented with B27 components, L-glutamine (500µm) penicillin (100 units/ml) streptomycin (100 units/ml) KCL (25.4mm) and Fetal Bovine Serum (10%) for 24 h. Cells were seeded in 6-well culture plates (corning) coated with poly D lysine (20µl/ml) at a density of at a density of 1.1×10^5 cells/cm² and were maintained in a humidified CO₂ incubator. On day two, the medium was aspirated and replaced with serum free Neurobasal Medium (NBM; GibcoBRL Life Technologies) supplemented with B27 components, L-glutamine (500 µm) penicillin (100 units/ml) streptomycin (100 units/ml) and KCL (25.4 mm) and 50% of the culture medium was replaced after 4 days *in vitro*. The percentage of tyrosine hydroxylase positive neurons was ~5% in all the experiments. Animals were treated in accordance with guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

RT-PCR Analysis

Following the termination of experiments, the medium was aspirated, and 1 ml of TRIzol reagent (Invitrogen) was added to cells in 6-well plates, and RT-PCR was performed as described previously [11]. In some experiments, tissues from substantia nigra, striatum and cerebral cortex from mouse brain were directly homogenized in Trizol reagent and followed the above mentioned methodology.

Immunoblotting

Following treatments, cells were washed with DPBS and resuspended in 100 µl of radioimmune precipitation buffer (RIPA) (20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 100 mM NaCl, 100 mM sodium fluoride) containing protease inhibitor cocktail (Roche). Phosphatase inhibitor cocktail (Bio-Rad) was also included while examining phospho Erk levels. In some experiments, cell pellets were freeze-thawed five times and the resulting suspension was centrifuged at 16,000 g × 30 min in order to isolate cytosol. The obtained pellet was washed with DPBS, collected by centrifugation and resuspended in 100 µl of RIPA buffer and was referred as membrane / particulate fraction. Protein concentrations were determined by the Lowry method (Bio-Rad) and proteins (40 µg) were resolved on a 15% SDS-PAGE for α-syn and 10% for Erk and blotted onto nitrocellulose membranes. Blots were developed as described previously [11]. α-Syn was detected using antibodies raised against the N-Terminus (Cell Signaling, USA) or C-terminus epitope of α-syn (Zymed Laboratories, USA). Total and phospho-Erk antibodies were purchased from Cell Signaling Inc. β-Actin antibody was purchased from Chemicon Inc.

Cloning of WT-syn and 112-syn

SH-SY5Y cells were treated with 2 mM MPP⁺ for 24 h and the total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Full-length wild-type as well as 112-syn mRNA were amplified using high fidelity PCR supermix (Hi FI PCR supermix, Invitrogen Inc) using the forward (5'-**AAGCTTAGGAATTC** ATTAGCCATGGATGTATTC-3') and reverse (5'-**CTCGAGAGATATTTTC** TTAG GCTTCAGGTTCGTAGT-3') primers containing *Hind III* and *Xho I* restriction sites (shown in bold and underlined) as described previously [11].

Measurement of 26 S Proteasomal Activity

Proteasome function was measured using the substrates SucLLVY-AMC (chymotrypsin-like) and Z-Leu-Leu-Lys-AMC (trypsin-like) as described earlier [25].

MTT Assay

Cellular toxicity was measured by the addition of one-tenth volume of 0.5% MTT dye to cells following transfection with either WT-syn or 112-syn as described previously [26].

Tyrosine hydroxylase immunohistochemistry

Following the termination of incubation, cells were washed once with DPBS and fixed (3% paraformaldehyde, 0.02% glutaraldehyde in PBS) for 15 min at room temperature. Cells were then permeabilized by dipping cells for 10 seconds in 100% methanol (−20°C) and then incubated for 10 min × 3 each with 0.5 mg/ml NaBH₄ in PBS, pH 8.0 to reduce aldehyde groups followed by rinsing with PBS three times (5 min each). Cells were permeabilized with 0.01% Triton X-100 in PBS for 30 sec and again washed with PBS three-times (5 min each). Cells were blocked in 1% BSA, PBS pH 7.5 for 30 min to block unspecific binding of the antibodies and incubated with TH primary antibody (Cell Signaling Inc) in 1% BSA, 0.1% saponin, PBS pH 7.5 for 24h. Cells were washed with PBS pH 7.5, three-times (10 min each) and incubated with FITC labeled secondary antibody (Chemicon) in 1% BSA, 0.1% saponin, PBS pH 7.5, 120 min at room temperature. Finally, cells were washed three-times (10 min each) with PBS and immediately observed under a fluorescence microscope (Nikon) equipped with FITC filter settings.

MPTP mouse model

Male C57BL/6 mice (8 weeks old, weighing ~ 22–25 gm) were procured from the Center for Cellular and Molecular Biology, India. Animals were housed according to standard animal care protocols, fed *ad libitum* and kept on a 12h light / dark cycle. Five animals in each group (Control, MPTP 7-D and MPTP-14D) were used for the study. Acute mouse model of PD was created by injecting MPTP (20 mg / kg body wt in 0.2 ml PBS, intraperitoneal) four times (injections at 2h intervals for a total of four injections per animal), whereas, control animals were injected on the same schedule with equal volumes of PBS. At the end of experimentation, animals were sacrificed by decapitation, brains were removed immediately and the substantia nigra, striatum and cerebral cortex were dissected on a chilled surface and kept frozen until use. All the experimental protocols used were

approved by Institutional Animal Ethical Committee (IAEC) and were in accordance with the NIH guide for the care and use of laboratory animals.

Statistics

Unless otherwise stated, the experimental values for all the data are presented as mean $\pm$ SD. Sigma plot software was used to analyze statistical significance employing student's t-test between control and experimental groups. Values were considered significant at $p < 0.001$ (***) and $p < 0.05$ (*) from three separate experiments and each time performed in triplicates.

Results

Parkinsonism mimetics induce alternative splicing of α -syn mRNA

The addition of MPP^+ (2 mM) to SH-SY5Y cells induced a time-dependent increase in the alternative splicing of α -syn mRNA (Fig. 1A). Splicing of α -syn mRNA was evident by 16 h, that gradually predominated by 24 h (Fig. 1A). Sequence analysis of the spliced transcript demonstrated a deleted exon-5 (corresponding to 84 bases which encodes 28 aa) resulting in the generation of a 112 aa protein (referred as 112-syn) instead of a 140 aa WT-syn. The Western blots demonstrating α -syn protein levels showed a similar trend (Fig. 1B). MPP^+ induced the generation of smaller isoforms of α -syn in a time dependent manner which were detectable with an antibody that was raised against the N-terminus epitope of α -syn (Cell Signaling) but not against the C-terminus epitope (Zymed) and this substantiates our finding that the smaller isoform has a missing C-terminus region (Fig. 1B). When cells treated with MPP^+ (2 mM) were fractionated into the cytosol and particulate fractions, we noticed that WT-syn was more enriched in the cytosolic fraction, whereas 112-syn was more abundant in the particulate fraction of the cells (Fig. 1C).

Treatment of cells with different concentrations of MPP^+ , rotenone or 6-OHDA for 24 h also induced the expression of 112-syn in a dose dependent manner (Fig. 2A). Pretreatment of cells with the lipophilic iron chelator HBED (2 μ M), adenoviral overexpression of glutathione peroxidase (GPx1) or with glutathione ester (2 mM) inhibited the expression of 112-syn mRNA, indicating that the alternative splicing of α -syn was activated by oxidative stress (Fig. 2B). Treatment of cells with H_2O_2 (200 μ M for 6 h) induced the expression of 112-syn, albeit to a lesser extent than the mitochondrial toxins (Fig. 2A). These results suggest that α -syn protein expression in response to oxidants can be regulated at a post-transcriptional level through alternative splicing.

Role of Erk-MAP kinase on the alternative splicing of α -syn

Treatment of SH-SY5Y cells with MPP^+ (2 mM) for 24 h significantly inhibited Erk 1/2 phosphorylation (Fig. 3). Phospho-Erk 1/2 levels were further diminished when cells were pretreated with a specific Erk-MAP kinase inhibitor (U0126, 40 μ M) followed by MPP^+ (2 mM). These events were reversed when MPP^+ was incubated with cells over-expressing constitutively active form of MEK-1, which is an Erk 1/2 kinase (Fig. 3B). Consistent with this finding, MPP^+ -induced 112-syn mRNA as well as protein expression were exacerbated in cells with reduced phospho-ERK 1/2 levels (Fig. 3B). However, cells demonstrating increased phospho-Erk 1/2 levels have reduced expression of 112-syn following MPP^+

treatment (Fig. 3B and C). Inhibition of phospho-Erk 1/2 (using U0126 or dominant-negative MEK-1) restored the alternative splicing of α -syn (Fig. 3C). These results demonstrate that the phosphorylation status of Erk-MAP kinase plays a crucial role in regulating the spliced isoforms of α -syn.

Overexpression of 112-syn in dopaminergic cells: Effect on proteasomal function

In order to examine the functional consequence of the alternative splicing of α -syn, we investigated the cellular effects of 112-syn by overexpressing plasmids encoding either the WT-syn or 112-syn in SH-SY5Y cells (Fig 4 A and B). Results indicate that compared to the WT-syn, cells expressing 112-syn exhibited proteasomal dysfunction (Fig. 4A). The trypsin-like activity of the proteasome was inhibited to a greater extent by 112-syn than the chymotrypsin-like activity by (Fig. 4A). Data from cell viability assay revealed that overexpression of 112-syn reduced cell viability to a larger extent than the wild-type counterpart (Fig. 4B), suggesting that 112-syn overexpression is deleterious to the dopaminergic cells.

Expression of 112-syn in rat mesencephalic neurons

Treatment of the primary cultures of rat mesencephalic neurons (MN) with MPP⁺ (10 μ M) for 48 h also induced alternative splicing of rSyn1 (human homolog of rat α -syn) (Fig. 4C), but, to a much smaller extent. The DNA sequence demonstrated the deletion of exon 5 (84 bases which encodes 28 aa) in rSyn1. Incidentally, this is the same exon as the one that was excised in human α -syn gene upon exposure to oxidants. To our knowledge, this is the first demonstration of an additional sub-variant of rSyn1 in addition to the known three splice variants, rSyn1, rSyn2 and rSyn3 [27]. Inhibition of Erk 1/2 phosphorylation by U0126 (30 μ M) exacerbated the generation of rSyn1-112 (Fig. 4C). However, with increasing doses of MPP⁺, we noticed a decrease in the level of the alternative spliced transcript (Fig. 4C). Under similar incubation conditions, there was a substantial loss of tyrosine hydroxylase (TH)-positive neurons in cells treated with increasing dose of MPP⁺ (Fig. 4D). Virtually, no intact TH positive neurons were observed at higher concentrations of MPP⁺ (40 μ M) (Fig. 4D). This result indicates that there is a highly stringent signaling mechanism that regulates the exon-5 of α -syn in response to oxidant treatment.

Expression of 112-syn in the MPTP mouse model of PD

There was a significant decline in the trypsin-like activity of the 20 S proteasome on day 7 and 14 in the substantia nigra and striatum of MPTP treated mice, a parameter that is widely used to assess the affects of Parkinsonian mimics (Fig. 5A and B) [28,29]. Under similar treatment conditions, we also noticed a significant decline in the chymotrypsin-like activity on day 7 in substantia nigra and striatum, however, the activity was not significantly different on day 14 (Fig. 5B). Both the trypsin-like and chymotrypsin-like activities were not altered in the cerebral cortex (unaffected region) during the course of treatment (Fig. 5A and B). Under similar treatment conditions, we have found a gradual and substantial increase in the up-regulation of both wild type α -syn and 112-syn (nearly two-fold) in both substantia nigra and striatum (to a greater extent in substantia nigra than striatum) on day 7 and 14 in the MPTP treated mice; these changes were not evident in control animals (treated with PBS) or in the cortex (unaffected region of brain) of MPTP treated mice (Fig.5C–H).

Moreover, the basal expression levels of 112-syn in the cortex and striatum were much lower or insignificant compared to the substantia nigra (Fig. 5C and H). Based on the present findings, it is evident that induced-expression of 112-syn is demonstrable under both *in vitro* cell culture studies and *in vivo* mouse model of PD, indicating that the alternative-splicing of α -syn could play a crucial role in the pathophysiology of PD.

Discussion

The basic mechanisms underlying of the etiology of PD are still not completely understood. Published data suggest a prominent role for the involvement of oxidative stress in the onset or progression of the disease [2]. A strong correlation between the decreased proteasomal activity and increase in α -syn aggregation has been identified in several PD subjects; these pathophysiological changes lead to the formation of Lewy body aggregates [6]. The significant role of α -syn in the progression of the disease is also supported by the following recent discoveries: the familial mutant forms of α -syn, and its association with the PD subjects [30]; the oxidative modification of α -syn in sporadic cases of PD [31] and its effects on cellular dopamine homeostasis [14]; as well as the resistance of α -syn null-mice towards parkinsonism mimetic-induced toxicity [32]. Although α -syn is presumably more susceptible to aggregation, the exact nature and function of this protein are still not clear. Reports indicate that nitration of tyrosine residues is critical for α -syn fibril formation [15,33]. Phosphorylation of ser 129 was found in association with PD pathophysiology [34]. Recent evidence indicates the existence of C-terminus truncated forms and / or smaller isoforms of α -syn which are associated with PD subjects [21].

The present study identified an oxidant-mediated alternative splicing of α -syn mRNA which generates a shorter mRNA transcript that has deleted exon 5 resulting in the generation of 112 aa synuclein protein (112-syn), instead of a 140 aa wild-type protein (WT-syn) (Fig 1). The domain structure of the α -syn protein reveals three primary regions, the N-terminus first half contains several repeat regions and is involved in membrane interaction, the middle region, which is referred to as a non-amyloid-component region (NAC), and is the region prone to aggregation; and the highly acidic C-terminus region which maintains the protein in a solubilized form and functions as a chaperone (Scheme 1) [4,16]. Consistent with this model, we found that 112-syn, which lacks a major proportion of the C-terminus, was found to be abundantly localized to the particulate / membrane fraction of cells compared to the WT-syn, a predominantly cytosolic protein (Fig. 1).

Reports indicate that parkinsonism mimetics such as MPP⁺ and rotenone increase the expression and aggregation of α -syn [9–11]. Enhanced α -syn levels were shown to inhibit the Erk-MAP kinase pathway and potentiate dopamine neuron cell death [35,36]. Emerging evidence indicates that Erk-MAP kinase also plays a crucial role in the alternative splicing events resulting in the skipping of exon/s [37,38]. In the present study, we noticed a crucial role of Erk-MAP kinase in regulating the alternative splicing of α -syn (Fig 3) that gives rise to shorter isoforms of the protein. These processes are deleterious to the dopaminergic cells, as overexpression of 112-syn exhibited a more pronounced effect on proteasomal dysfunction and enhanced cell death than wild type α -syn (Fig. 4 A and B).

Treatment of the primary cultures of rat mesencephalic neurons (MN) with MPP⁺ also resulted in the generation of α -syn spliced product. Sequence analysis revealed the excision of the same region of the gene (exon 5, encoding 84 bases) even in rSyn1 and the spliced product was referred as rSyn1-112. This process appears to be more pronounced in dopamine neurons, as treatment of MN with increasing doses of MPP⁺ reduced the survival of TH positive neurons as well as the generation of rSyn1-112 (Fig. 4D) indicating that dopamine neurons were the main source for 112-syn expression. We also found a time dependent increase in the expression of both WT and 112-syn in the substantia nigra and to a lesser extent in the striatum (possibly due to the presence of dopaminergic nerve terminals) but not in the cortex of the MPTP model of PD (Figure 5C–H). Under similar treatment conditions there was a significant decline in the trypsin-like and chymotrypsin-like activities of the 20 S proteasome in the substantia nigra and striatum but not in the cortex of MPTP treated mice (Fig. 5A and B); these data are consistent with other studies showing that MPTP selectively targets dopamine neurons and not the other regions of brain [9]. These results demonstrate a direct correlation between 112-syn and proteasomal dysfunction under both *in vivo* and *in vitro* conditions (Fig. 4A).

Recent studies demonstrated the existence of several smaller protein isoforms of α -syn in cellular models as well as in human diseased subjects [21]. The abundance of 112-syn has been demonstrated in human subjects suffering from dementia with Lewy bodies [39]. These results strongly suggest a crucial role for the alternatively-spliced transcripts of α -syn. In light of recent reports indicating the role of pre-mRNA splicing in neurodegenerative diseases, such as, presenilin-2 and Tau in Alzheimer's disease models [40] and peripherin in ALS mouse model [41], we propose that oxidant-induced alternative splicing could be common to many neurodegenerative diseases.

Overall, based on the present findings of oxidant-induced alternative splicing of α -syn, the localization of spliced isoforms to the particulate fraction and their potential inhibitory effect on the proteasomal activity and dopamine neuron cell death, we suggest that regulated alternative splicing of α -syn may play a crucial role in the mechanisms mediating dopamine neuron cell death and possibly in the pathophysiology of PD. Clearly, additional experiments aimed at understanding the comprehensive affects of this alternatively-spliced isoform in mouse models in highly warranted to have a complete understanding of its role as a potential mediator in PD.

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Figure 1. Time dependent induction of 112-syn by MPP⁺

Human SH-SY5Y cells were treated with 2 mM MPP⁺ for indicated time intervals. (A) Following termination of the experiment, total RNA was extracted from the cells and full-length α -syn mRNA levels were analyzed on a 2% agarose gel following RT-PCR. (B) Cells were treated under the same conditions as (A) and immuno-blotting for α -syn was performed from 40 μ g of cell lysates resolved on a 15% SDS-PAGE. The α -syn immuno-reactivity was analyzed using antibodies raised against either the N-terminus or C-terminus epitope of WT α -syn as indicated (B). Cells were treated either with MPP⁺ (2 mM) for 24 h

or over-expressed with WT and 112-syn. (C) Forty eight hours following transfection the cells were collected and freeze-thawed five times and the cytosol and membrane bound proteins were collected as described in “Methods section”. Forty μ g protein from cytosolic and particulate fractions were resolved on a 15 % SDS-PAGE and α -syn immuno-blot was developed with α -syn antibody raised against the N-terminus of the protein and the bands were developed with ECL detection. Data presented is a representative of three separate experiments.

Figure 2. ROS dependent generation of 112-syn by Parkinsonism mimetics

(A) SH-SY5Y cells treated with different concentrations of MPP⁺, rotenone or 6-OHDA for 24 h induced the generation of 112-syn in a dose dependent manner as analyzed by RT-PCR. (B) Cells were pretreated with a lipophilic iron chelator, HBED (2 μM), GSH-ester (2 mM) or adenoviral over-expression of GPx1 followed by MPP⁺ (2 mM) for 24 h. Following the termination of experiment, total RNA was extracted by Trizol reagent and α-syn mRNA was analyzed using the primers that amplify the full-length transcript of α-syn. Data shown is a representative of three separate experiments.

Figure 3. Inhibition of Erk-MAP kinase exacerbates alternative splicing of α -syn mRNA

Cells were treated with MPP⁺ (2 mM) for 24 h in the presence or absence of adenoviral over-expression with either constitutively active MEK-1 (20 Pfu) or in presence of the Erk-MAP kinase inhibitor U0126 (40 μ M). (A) Immuno-blot indicating the total and phosphorylated forms of Erk 1/2 under the treatment conditions. (B) Wild-type and 112-syn levels under the same conditions as (A) as analyzed by RT-PCR. (C) Immuno-blot demonstrating the wild-type and spliced protein isoforms of α -syn under the conditions of

increased or decreased phosphorylation of Erk 1/2. β -Actin was used as a loading control. These experiments were repeated three times and similar results were obtained.

Figure 4. Overexpression of 112-syn induces proteasomal dysfunction and cell-death

Human SH-SY5Y cells were individually transfected with either pcDNA3 alone (control), or pcDNA3 containing either WT or 112-syn and the cells were allowed to recover for 48 h. (A) At the termination of experiment, cells were collected and both Trypsin and chymotrypsin-like activities of the proteasome were measured as described in “Methods Section”. (B) At the termination of incubation 100 μ l of MTT (5 mg/ml) was added to cells and incubated for 1 h and MTT formazon crystals formed by the cells were solubilized as described in “Methods Section” and the absorbance was measured at 570 nm in a

spectrophotometer. Data shown are the mean $\pm$ SD of three separate experiments. * $p < 0.05$; ** $p < 0.01$ compared to control group. (C) Primary cultures of MN were incubated with MPP⁺ (10 to 40 μ M) in the presence or absence of the Erk-MAP kinase inhibitor U0126 (40 μ M) for 48 h. Following the termination of experiment, total RNA was extracted by Trizol reagent and α -syn mRNA was analyzed using the primers that amplify the full-length transcript of human α -syn as described in “Methods Section”. (D) MN were treated as in (C) and following the termination of experiment, cells were fixed and TH immunostaining was performed as described in “Methods Section”. Data shown is a representative of three separate experiments.

Figure 5. Induced expression of 112-syn in the MPTP mouse model of PD

Following MPTP treatment, animals (n=5) were sacrificed by decapitation and the substantia nigra, striatum and cortex were dissected and (A) trypsin-like and (B) chymotrypsin-like activities of the 20 S proteasome from substantia nigra, striatum and cerebral cortex were measured by a fluorimetric assay as described in the “Methods Section”. (C–H) Total RNA was extracted from substantia nigra, striatum and cortex by employing Trizol reagent and the first strand cDNA synthesis was performed using random hexamers. Both, α -syn and 112-syn expression was analyzed by PCR using the primers that

amplify the full-length transcript of human α -syn as described in “Methods Section”. Data shown are the mean $\pm$ SD of three separate experiments. ** $p < 0.01$; for A and B. Data shown is the representative of three separate experiments for C–H.

Scheme 1. Schematic representation of alternative splicing of human α -syn pre-mRNA

* indicates the start codon for translation. Numbers 1–6 indicate the Exons. Dotted lines indicate the splice sites.