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ORIGINAL PAPER

Aggregation of α-synuclein by DOPAL, the monoamine oxidase metabolite of dopamine

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Abstract Parkinson's disease (PD) is a neurodegenerative disease characterized by the selective loss of dopamine (DA) neurons and the presence of α-synuclein (AS) aggregates as Lewy bodies (LBs) in the remaining substantia nigra (SN) neurons. A continuing puzzle in studying PD pathogenesis is that although AS is expressed throughout the brain, LBs and selective dopaminergic cell loss lead to characteristic clinical signs of PD, suggesting that there is a link between AS aggregation and DA metabolism. One potential candidate for this link is the monoamine oxidase (MAO) metabolite of DA, 3,4-dihydroxyphenylacetaldehyde (DOPAL), as neither DA nor DA metabolites other than DOPAL are toxic to SN neurons at physiological concentrations. We tested DOPAL-induced AS aggregation in a cell-free system, in vitro in DA neuron cultures and in vivo with stereotactic injections into the SN of Sprague–Dawley rats by Western blots, fluorescent confocal microscopy and immunohistochemistry. We demonstrate that DOPAL in physiologically relevant concentrations, triggers AS aggregation in the cell-free system, and in cell cultures resulting in the formation of potentially toxic AS oligomers and aggregates. Furthermore, DOPAL injection into the SN of Sprague–Dawley rats resulted in DA neuron loss and the accumulation of high molecular weight oligomers of AS detected by Western blot. Our findings support the hypothesis that DA metabolism via DOPAL can cause both DA neuron loss and AS aggregation observed in PD.

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Abbreviations

PD	Parkinson's disease
DA	Dopamine
DAQ	Dopamine quinone
CA	Catecholamine
AS	α-synuclein
LB	Lewy bodies
MAO	Monamine oxidase
mPTP	Mitochondrial permeability transition pore
DOPAL	3,4-Dihydroxyphenylacetaldehyde
SN	Substantia nigra
PVDF	Polyvinilidene difluoride
TBS	Tris buffered saline
PCR	Polymerase chain reaction
5Y	SHSY-5Y cells
ECL	Enhanced chemiluminescence
TH	Tyrosine hydroxylase
DAB	Diamonobenzidine dichloride
DOPAC	3,4-Dihydroxyphenylacetic acid
HVA	Homovanillic acid
WT	Wild type
IR	Immunoreactivity
DAT	Dopamine transporter
ALDH	Aldehyde dehydrogenase
PAGE	Polyacrylamide gel electrophoresis
VTA	Ventral tegmental area

Introduction

Parkinson's disease (PD) is the most common movement disorder characterized pathologically by selective loss of dopamine (DA)-producing neurons in the substantia nigra (SN) and the accumulation of α-synuclein (AS) aggregates in the form of Lewy bodies (LBs) in the remaining neurons. DA or one of its metabolites has long been suspected to be involved in the death of SN neurons [3]. However, DA itself is not sufficiently toxic at physiological levels to initiate cell death [7, 23]. Interestingly, over 50 years ago, Blaschko postulated that monoamine metabolites of monoamine oxidase (MAO) would be highly reactive and toxic in tissues in which they are formed [3]. 3,4-Dihydroxyphenylacetaldehyde (DOPAL) is the MAO metabolite of DA and under normal conditions is converted to 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase [15]. DOPAL is present in human SN at physiological concentrations of 2–3 μM in neurologically normal human patients at autopsy [23]. Our prior work with in vitro [23] and in vivo [7] models of DA neurons has confirmed the neurotoxicity of DOPAL at physiological concentrations. Additionally, AS aggregation is also implicated in nearly all forms of sporadic PD [44] and in certain genetic forms of PD, AS mutations lead to early onset of PD [35]. Investigators have posited that a byproduct of DA metabolism might be required for AS toxicity in vivo [48]. The high reactivity of DOPAL due to its aldehyde moiety [15] and its neurotoxic potential [30] led us to testing whether DOPAL could trigger AS aggregation.

Materials and methods

Chemicals and reagents

DOPAL was prepared as described earlier [29]. Human-recombinant AS 1-140 was purchased from Calbiochem (EMD Biosciences, Inc., San Diego, CA, USA), while NuPage gels and polyvinylidene difluoride (PVDF) membranes were from Invtirogen (Invitrogen Corporation Carlsbad, CA, USA). All other chemicals were from Sigma Chemical Company (St Louis, MO, USA). The AS antibody (AS 202) was a gift from J. Trojanowski.

Recombinant protein aggregation

DOPAL was dissolved in 1% benzyl alcohol, then diluted to a final concentration of 1.5–1,500 μM as described previously [7]. AS (2 μM) was incubated at 37°C in 20 μl of 100 mM tris–HCl buffer (pH 7.2) with or without DA, DOPAL, DOPAC, or homovanillic acid (HVA) for up to 4 h. The reaction was stopped by heating at 70°C for 3 min in SDS buffer. The entire mixture was transferred to the appropriate gel (vide infra).

SDS-PAGE silver staining and immunoblotting

Ten microgram of protein (estimated by Bio Rad protein reagent) was resolved by electrophoresis on 4–12% bis-tris gels run with MES SDS buffer, and 3–8% tris-acetate gels using tris-acetate running buffer. The silver staining of the gel was done by the conventional method; briefly the gel is fixed in ethanol, followed by reduction using 1 mM DTT and crosslinking with glutaraldehyde. The gel was reacted with 0.1% silver nitrate solution, washed and developed in 3% sodium carbonate by fixing in 2.3 M sodium citrate. For immuno-blotting, the protein was transferred to PVDF blotting membranes with 2 μm pore size, blocked with 5% milk protein in tris buffered saline (TBS) containing 0.1% Tween 20, and incubated for 1 h with a AS 202 monoclonal antibody (1:2,500 dilution). Blots were washed five times with 5% milk protein in TBS and probed with horseradish peroxidase-conjugated antimouse secondary antibody (1:2,500).

Blots were washed five times with TBS containing 0.1% Tween 20 before detection with Super Signal (Pierce).

Thioflavin-S staining of AS aggregates

After a 4-h incubation of 2 μM AS, either with or without 1.5 mM DOPAL, the reaction mixture was stained with triple-filtered thioflavin-S (final concentration 10 mg/ml). A 10-μl aliquot of reaction mixture was observed and photographed with a Nikon Optiphot fluorescence microscope as previously described [19].

Generation of AS model

Wild-type AS cloned in PRK-172 was a gift from M. Goedert. The AS gene was amplified from PRK-172 with following primers which introduced Hind III and Xba 1 site into the amplicon.

5′-ACGTAAGCTTTCTCGAGATGGATGTATTCAT GAAAGGACT-3′ (AS_FP1)

5′-CCGTTCTAGACTCGAGGGATGGAACATCTGT CAGCAG-3′ (AS_RP1)

Digestion of the PCR product with Hind III and Xba 1 was followed by ligation into similarly digested pcDNA3.1 (Invitrogen) to generate wild type α-synuclein construct (AS_WT), as described [39].

Cell culture and immunofluorescence

SHSY-5Y (5Y) cells were grown in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2. Cells were transfected by Lipofectamine 2000 (Invitrogen) as per manufacturer's protocol; briefly, cells were trypsinized and plated at 1 to 3 million cells per well on a six-well plate or one-fifth the above number for four-well chamber slide (Nunc, USA) a day before transfection. On the next day, 5 μg of appropriate plasmid DNA (estimated by O.D.) was diluted in 250 μl of OPTI-MEM (Invitrogen) and mixed with equal volume of OPTI-MEM containing 5 μl Lipofectamine 2000 [39]. The mix was incubated for 30 min at room temperature and added directly to the cells in six-well plate. For four-well chamber slide one-fifth volumes were used and the experiment continued as described above. The cells were allowed to grow in OPTI-MEM for 6 h, after which normal medium containing antibiotics was added.

The cells were grown for 48–72 h before culturing cells in the presence or absence of increasing concentrations of DOPAL for 2 or 8 h. The cells were then fixed for immunofluorescence assays or harvested for immunoblot analysis. One hundred transfected cells containing the aggregates were counted twice in multiple view fields. The cells were initially screened for aggregates at 20× magnification, and aggregates were then confirmed by examinations at higher magnifications of 40× and 60×. The percent of cells containing AS aggregates at the three highest DOPAL concentrations was compared to the control group using Student's *t* test.

Immunofluorescence was done as per standard protocol, AS 202 antibody was used at a dilution of 1:2,000 for immunofluorescence assays. Appropriate secondary antibodies were used at a dilution of 1:2,000 for immunofluorescence experiments and cells observed under a confocal microscope (Zeiss LSM 5 PASCAL system).

Western blot of extracts from 5Y cells

Forty-eight hours after transfection, IMDM medium of 5Y cells were replaced by serum-free reduced medium (OPTI-MEM) and a concentration of DOPAL ranging from 3 μM to 1.0 mM was added to growing cells. Cells were incubated for 2 or 8 h in DOPAL-containing medium following which, they were harvested and lysed by sonication pulses. The lysis buffer was RIPA buffer containing SDS: 150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deooxycholate, 5 mM EDTA plus 100 μM sodium orthovanadate plus 1 tablet of protease cocktail (Boehringer Mannheim, for 10 ml of lysis buffer). The lysed samples were centrifuged at 12,000*g* for 10 min at 4°C and 25 μg of supernatant protein samples were electrophoresed on NuPAGE 3–8% tris-acetate gels with a 500-kDa exclusion (Invitrogen) and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). Immunoblotting was done as per standard protocols detailed above. AS 202 antibody (1:4,000) and *β*-actin (Abcam, UK) antibody (1:1,000) were utilized as primary antibodies. The immune complexes were visualized with a horseradish peroxidase-conjugated secondary antibody (1:6,000) and the use of the enhanced chemiluminescence (ECL) Plus or the ECL Advance kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's protocol.

Intranigral DOPAL injections and tissue preparation

The housing and nutrition of the rats used in this study and all procedures performed on them conformed to standards set forth in the Guide for the Care and Use of Laboratory Animals of the National Research Council (National Academy Press, 1996). The experimental protocols reported herein were reviewed and approved by the Animal Care Committee of the Saint Louis University. The details of the surgical and immunohistochemical methods have been published [7]. Briefly, 2-month-old male Sprague–Dawley rats (300 g; Harlan, Indianapolis, IN, USA) were deeply anesthetized with 4% induction and maintained with 1%

isoflurane. The heads of the rats were fixed in a stereotaxic apparatus (David Kopf, Tujunga, CA, USA). Single injections of DOPAL were made into the SN unilaterally (AP-5.5 mm, ML 2 mm and DV -7.4 mm from bregma) via a glass micropipette (O.D. 30–50 μm) glued to a 1-μl Hamilton syringe. Control injections of similar volumes of the vehicle (1.0% benzyl alcohol in phosphate-buffered saline, pH 7.4) were made on the opposite side. Three rats were injected with 400 nl/1.0 μg DOPAL, while six were injected with 200 nl/0.2 μg DOPAL. Black microspheres (6.0 μm; Molecular Probes, Eugene, OR, USA) were added to mark the injection sites.

Rats for Western blot analysis were killed after 4 h by injecting pentobarbital (100 mg/kg, IP). Their brains were exposed, the midbrain removed stereotaxically, and a punch of the injection site obtained using a 15-gauge needle. The resultant biopsies were frozen immediately on dry ice and stored at −80°C until analysis. Tissue biopsies were homogenized in five volumes and centrifuged at 13,000*g* for 10 min at 4°C. The black microspheres were identified in the pellets and supernatants were analyzed with Western blot as described above using AS 202 and *β*-actin antibodies. For the 4-h 1.0-μg DOPAL and control SN injections, the density of the bands on the blot was determined using the UnScan it program (Silk Scientific, Orem, UT, USA). The results were expressed as units of AS aggregate/unit of actin.

Immunochemistry

Rats for immunohistochemistry were reanesthetized 4, 24 or 48 h after surgery and perfused first with saline followed by fixative (4% paraformaldehyde in 0.1 M phosphate buffer). The brains were removed, postfixed for at least 4 h, sunk in 20% sucrose, and sectioned frozen at 40 μm with a sliding microtome. A 1:4 series of sections was immunostained using antibodies against tyrosine hydroxylase (TH, made in mouse; 1:10–12,000; ImmunoStar, Inc., Hudson, WI, USA) while another series was immunostained with antibodies against AS 202 (monoclonal, made in mouse; 1:20,000). Briefly, the sections were immersed in phosphate buffer (PB; pH 7.3) containing 0.3% Triton X-100, 1% goat serum and primary antibody and kept on a shaker overnight. They were washed 3× the next morning and then immersed for 1 h in PB/Triton serum containing biotinylated secondary antibodies (1:400) against antigens of the species where the primary antibodies were produced. The sections then were incubated in Vectastain ABC Elite solution (1:200; Vector, Burlingame, CA, USA) for 1 h, washed with three rinses of PB, and reacted with diaminobenzidine dihydrochloride (DAB) intensified with nickel ammonium sulfate for 4–10 min. Hydrogen peroxide at a concentration of 0.6% catalyzed the reaction. The sections were then rinsed, mounted on gelatinized slides, air-dried, counterstained with Neutral Red, dehydrated in alcohols, defatted in xylenes, and coverslipped with Permount.

Results

Effect of DOPAL on AS aggregation in a cell free system

Aggregation of AS monomers showed a dose response to concentrations of DOPAL from 15 to 1,000 μM (Fig. 1a) on a silver-stained gel. In contrast to DOPAL, DA, DOPAC or HVA, at a concentration of 1,400 μM, did not trigger AS aggregation as detected on a silver stained gel (Fig. 1b).

Aggregation of AS monomers to higher molecular weight oligomeric forms was visualized by Western blot within 1 min of exposure to DOPAL and continued up to 4 h, generating increasingly high molecular weight complexes (Fig. 2a). In lanes 7,8, there appears to be some self-aggregation of the AS monomer after the 4-h incubation with DOPAL or control. Dose–response experiments at 60 min using bis-tris and tris-acetate gels demonstrated that dimers of AS can form at DOPAL concentrations as low as 1.5 μM (Fig. 2b). Higher-order oligomeric forms of AS were induced by increasing concentrations of DOPAL. At 300 μM and above, DOPAL induced large multimeric AS aggregates (Fig. 2c). In contrast to DOPAL, neither DA nor its other major metabolites (DOPAC, HVA) triggered AS aggregation at concentrations of up to 500 μM using a bis-tris gel (Fig. 2d1). With a tris-acetate gel there neither DA, DOPAC, nor HVA, triggered AS aggregation at concentrations of up to 1,500 μM (Fig. 2d2). With exposures to DOPAL longer than 60 min, very high molecular weight AS aggregates (>500 kDa) did not enter the tris-acetate gel. These aggregates were visualized after thioflavin-S staining with fluorescent microscopy (Fig. 2e, f).

Effect of DOPAL on AS aggregation in SHSY-5Y cells

The effects of DOPAL on aggregation of AS was examined in a human DA cell line, 5Y cells overexpressing wild type AS (AS-WT) (Fig. 3). The addition of DOPAL greatly accelerated AS aggregation. At DOPAL concentrations ≥30 μM, large intracellular aggregates increased from 106 to 133% above baseline AS-WT transfection. The percentage of cells showing large AS aggregates at the three highest DOPAL concentrations is significantly higher than in controls ($P<0.002$; Student's t test). At DOPAL doses greater than 150 μM, increased toxicity was observed with approximately 60% dead cells after 8 h based on the appearance of floating cells compared to those attached to the dish. The remaining cells were rich in large intracellular

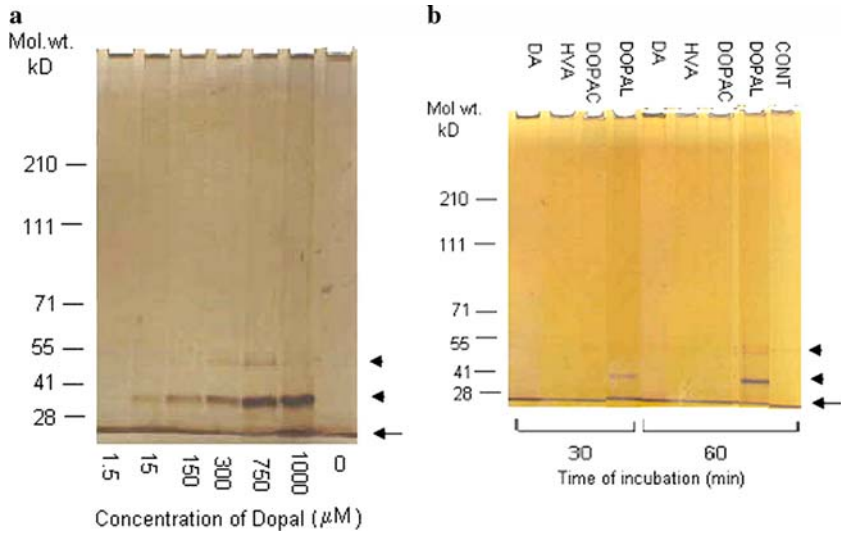

Fig. 1 Aggregation of AS 1-140 in vitro induced by application of DOPAL detected by silver stain. **a** AS (2 μM) was incubated for 60 min with or without increasing concentrations of DOPAL. At each concentration of DOPAL, 1 μg of AS was electrophoresed on tris acetate gel. **b** AS (2 μM) was incubated for 30 or 60 min with or without 1,400 μM DA, HVA, DOPAC, or DOPAL. At each concentration, 1 μg of AS was electrophoresed on tris acetate gel. After PAGE, gels were silver stained as described in "Methods" section. *Arrows* point to bands corresponding to AS monomer while arrowheads indicate AS aggregates

AS aggregates compared to control cells. These aggregates appear both in cell bodies and in axons.

Western blot of the extracts of 5Y cells incubated with increasing DOPAL concentrations for 8 h showed small oligomers up to 55 kDa induced by 3 μM DOPAL (Fig. 4a). The size of the oligomers increased up to 500 kDa after incubation of cells with 30 and 60 μM DOPAL (Fig. 4a). At 60 μM DOPAL most of the AS aggregates (Fig. 3g, j) were too large to enter the gel (Fig. 4a). To check for specificity of DOPAL-AS interaction, a blot was probed with antibodies against β-actin. No low or high MW oligomers were detected (data not shown). At supraphysiological doses of DOPAL (1 mM), 5Y cells were dead within 2 h. Although no intracellular aggregates could be visualized within this brief period, Western blot analysis revealed low and high MW AS oligomers up to 500 kDa (Fig. 4b).

Effect of DOPAL on AS aggregation in vivo

AS monomers were present in biopsies from the SNs injected either with DOPAL or vehicle, but aggregates were at very low levels from biopsies injected with vehicle (Fig. 5a). Western blot of extracts of rat SN injected with 1 μg of DOPAL showed an increase in AS oligomers ranging in size from 50 to 170 kDa at 4 h, but not at 1 h (Fig. 5a). These oligomers accounted for 63% of the total AS on the blot. There was a 2.75-fold increase in AS aggregates after 4 h in DOPAL-injected SN compared to the control injection when corrected for the amount of actin protein on the gel (DOPAL: 0.44 AS aggregate units/actin unit vs. Control: 0.16 AS aggregate units/actin unit). A blot probed for actin showed no evidence of aggregation in the DOPAL-injected SN either at 1 or 4 h (Fig. 5a). Western blot of extracts of rat SN injected with 0.2 μg of DOPAL showed a lesser increase in 50 to 170 kDa oligomers compared to the 1.0-μg DOPAL injection (Fig. 5b). Injections of both 1.0 and 0.2 μg DOPAL into the SN resulted in a loss of TH immunoreactivity (ir) at 24 and 48 h (Fig. 6a–d). Large AS aggregates were not detected with AS immunohistochemistry after DOPAL injections at 4, 24, or 48 h (data not shown).

Discussion

This study demonstrates that application of DOPAL at physiologically relevant concentrations triggers rapid AS aggregation in a cell-free system, in vitro and in vivo. Portions of the in vitro study shown in Fig. 3 were published earlier [15]. In contrast to DOPAL, DA, DOPAC and HVA do not trigger either low or high molecular weight AS aggregates at physiologically relevant, concentrations. These findings are consistent with earlier reports [11, 28, 32, 36] which show that DA quinones (DAQ) and other DA oxidation products, at concentrations of 100 μM, (11) bind to AS and inhibit, rather than trigger, aggregation of AS oligomers from forming fibrils. Our results do not confirm an earlier report [9] that, at concentrations of 100 μM and above, DA triggers AS aggregation. In comparison, we show that DOPAL triggers AS aggregation at concentrations as low as 1.5 μM. The difference between our results

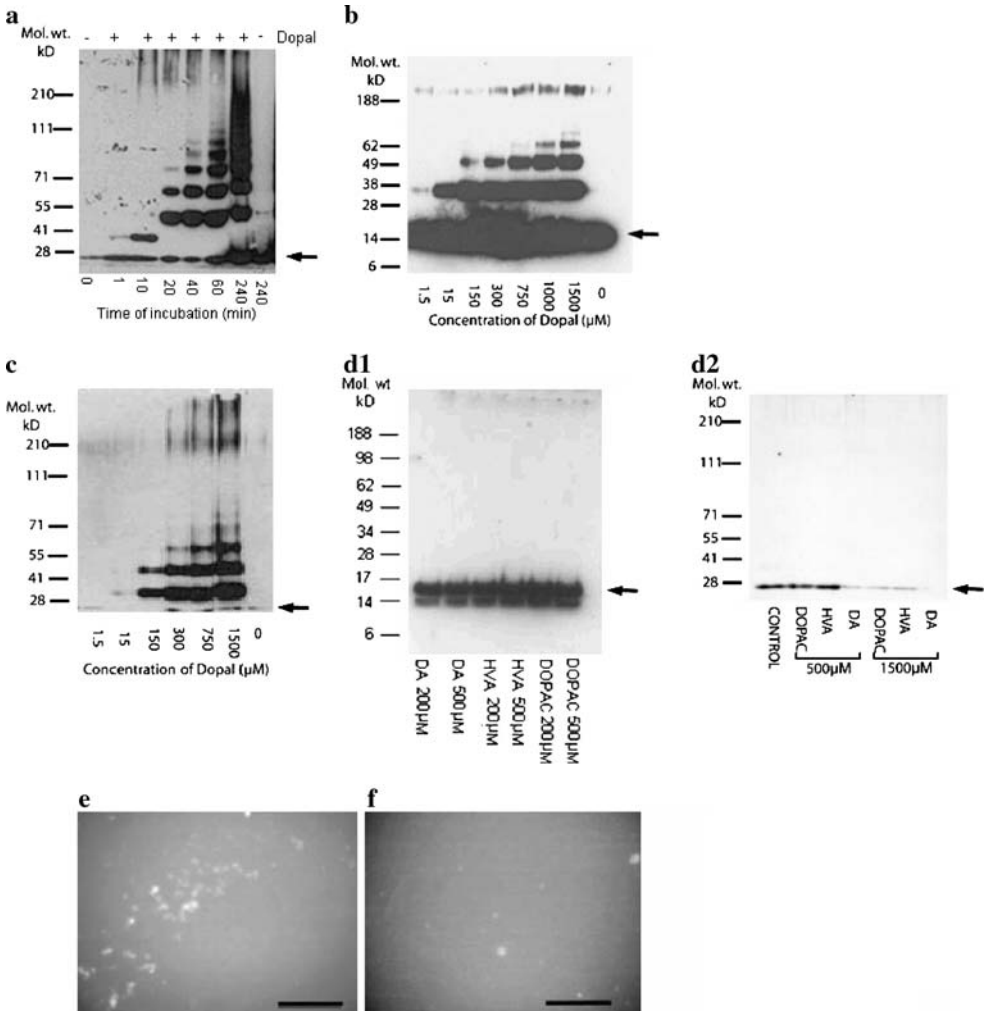

Fig. 2 Aggregation of AS 1-140 in vitro induced by application of DOPAL detected by Western blot using AS 202 antibody. **a** AS (2 μM) was incubated with or without 1.5 mM DOPAL for various times and at each time point, 1 μg of AS was electrophoresed on a tris-acetate gel. **b**, **c** AS (2 μM) was incubated for 60 min with or without increasing concentrations of DOPAL. At each concentration of DOPAL, 1 μg of AS was electrophoresed on bis-tris gel (**b**) or tris-acetate gel (**c**). **d1**, **d2** AS (2 μM) was incubated for 60 min with or without increasing concentrations of DA, DOPAC or HVA. At each concentration, 1 μg of AS was electrophoresed on bis-tris (**d1**) or tris-acetate (**d2**) gels. After PAGE, immunoblotting was performed as described in "Methods". *Arrow* indicates the monomeric species of AS (MW 14.5 kDa). **e**, **f** Fluorescence microscopy of thioflavin-S stained, DOPAL-induced AS aggregates. AS (2 μM) was incubated either with (**e**) or without (**f**) 1.5 mM DOPAL for 4 h. The incubation mixtures were stained with thioflavin-S and viewed under a fluorescence microscope. Scale bars in **e** and **f** equal 100 μM

with DA and those described earlier [9] may be due to a different propensity of different batches of AS to aggregate. An increasing age of a particular AS batch may increase the propensity of the AS to aggregate. In this regard AS self-aggregates with prolonged storage. Further, unlike DOPAL, DA does not trigger AS monomer aggregation to form oligomers in vitro [37]. These results taken together suggest that a combined increase in cytosolic levels of DOPAL and DAQ could lead to large increases in levels of toxic AS oligomers [1]. In addition, our thioflavin-S and in vitro studies indicate that DOPAL can trigger formation of large intracellular AS aggregates, which have a beta-pleated sheet structure [37]. The lack of a DOPAL-induced β-actin aggregation shows the specificity of this effect on AS. The in vivo and in vitro toxicity of DOPAL is consistent with our earlier demonstration of a dose-related DOPAL toxicity to DA SN neurons in vivo [7] and to PC-12 cells in vitro [23]. These results provide a link between the two central hypotheses explaining the selective death of DA neurons in PD, namely the DA [13] and the AS [12] hypotheses for PD pathogenesis.

PD is a common neurodegenerative disorder characterized clinically by rigidity, bradykinesia and postural tremor [40] and pathologically by selective loss of DA neurons in the SN and LBs [44]. The DA hypothesis has suggested that DA or its metabolites contributed to the

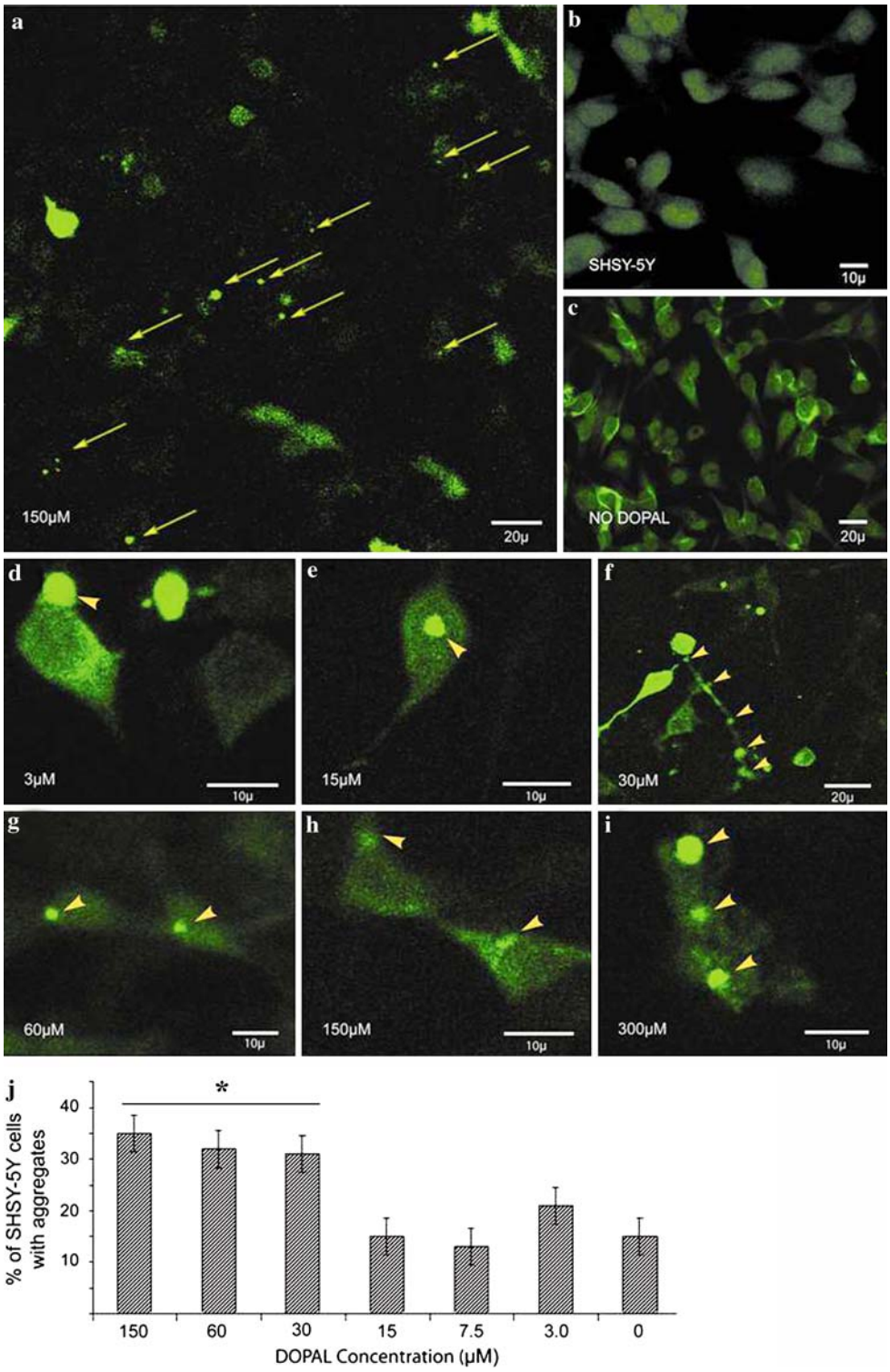

Fig. 3 Effect of DOPAL on AS aggregation in the neuronal SHSY-5Y cell line. Immunofluorescent photomicrographs of transfected 5Y (overexpressing AS-WT protein) cells after immunostaining with AS 202 antibody. **a–c** Confocal images of transfected 5Y cells treated with 150 μM of DOPAL (**a**), or without it (**c**) and an untreated control 5Y cells with endogenous AS expression and lacking AS overexpression (**b**). **d–i** Magnified images of 5Y cells exposed to 3–300 μM DOPAL. Cytoplasmic aggregates in the cell bodies and neuritic processes are highlighted by *arrows*. *Scale bar* is at the bottom right of panels. **j** Plot of AS aggregation propensity with respect to varying concentration of DOPAL. Each *bar* represents two separate counts of 100 cells each in multiple fields. As aggregation increases at DOPAL concentrations of 30 μM or greater compared to controls (*$P < 0.002$; Student's *t* test)

selective vulnerability of DA neurons [13, 15]. However, no direct genetic link between DA and PD has been described [12]. Toxicity to neurons in vivo requires a 400-fold higher concentration of DA than DOPAL [7,14], suggesting that DA itself is not toxic in normal physiological conditions. We postulate instead that DOPAL mediates DA toxicity [7] most likely due to the fact that unlike DA, DOPAC or HVA, DOPAL is highly reactive [3]. DOPAL aldehyde moiety [15] increases its capacity to generate a free radical [30] and to activate the mitochondrial permeability transition pore (mPTP) [23], a trigger for cell death. We now show that DOPAL also triggers AS

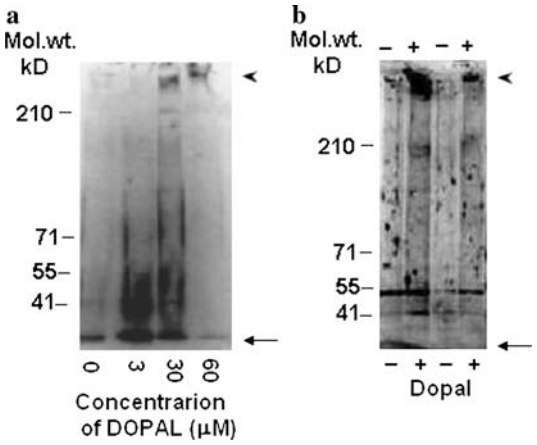

Fig. 4 Western blot of extracts from 5Y cells transfected with AS-WT and treated with DOPAL. The same amount of supernatant protein (25 μg) was added to each lane of the gels. **a** 5Y cells were transfected with AS and incubated either without or with increasing concentrations of DOPAL for 8 h, and probed with AS 202 monoclonal antibodies. **b** In an acute toxicity study, 5Y cells were transfected with AS and incubated either with (lanes 2, 4) or without (lanes 1, 3) 1.0 mM DOPAL for 2 h. The blot was developed with AS 202 antibody. *Arrows* point to bands corresponding to the AS monomer, while *arrowheads* indicate large AS aggregates

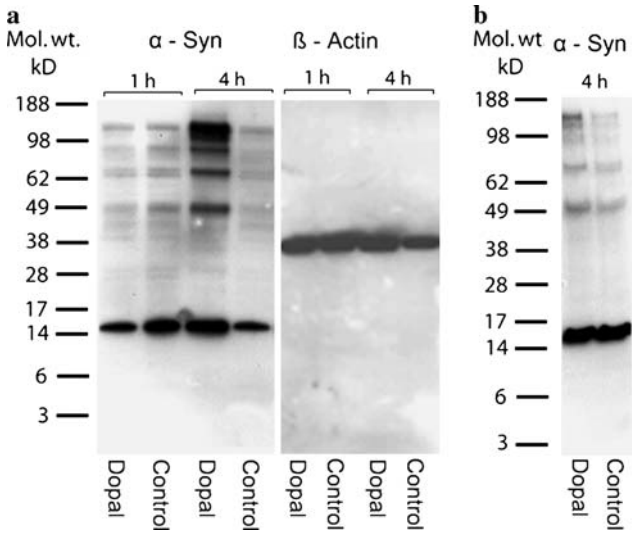

Fig. 5 Western blot of extracts from rat SN injected with DOPAL. **a** SN was injected with either 1.0 μg DOPAL or vehicle control (lanes 2, 4). After either 1 or 4 h, rats were killed, the SN biopsied and, after PAGE, immunoblotted using AS 202 monoclonal antibody as described in "Methods". The blot was stripped and reprobed with *β*-actin antibody. **b** SN was injected with either 0.2 μg DOPAL or vehicle control, and after a 4-h survival, immunoblotting was performed using AS 202 antibody as described above. Compare the DOPAL dose effect after 4 h in (**a**) to that in (**b**)

aggregation, similar to the effect of the highly reactive free hydroxyl radical on AS aggregation [38]. In contrast, DA is not chemically reactive, does not form a free radical and is not toxic at physiologically relevant levels [7, 23, 30]. However DAQ, a DA an oxidation product, is reactive.

Non-DA neurons through the brainstem may also be affected in PD [22]. However these are mainly other catecholamine (CA) neurons [34] which have a MAO metabolite, 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL), with in vitro and in vivo toxicity similar to DOPAL [5, 8]. In contrast to DA SN neurons, none of the other CA neuronal groups was affected in PD control motor activity. It is the loss of DA SN neurons in PD that results in the loss of motor control, the major cause of disability in PD [13].

In contrast to DA, AS mutations have been linked to PD pathogenesis [12]. However, AS is widely distributed in neurons and glia throughout the brain [16], and therefore by itself cannot explain the selective DA neuron loss in the SN in sporadic forms of PD. There are however, several links between AS and DA metabolism [30, 46, 48]. For instance, AS binds to the DA transporter (DAT) and increases DA uptake into the cytosol of cells transfected with DAT and AS [27]. Thus, these cells become more susceptible to DA toxicity [27]. In addition, oligomers of AS permeabilize vesicles [47] and allow DA to leak out [46], which could lead to increased cytosolic DA and its metabolites, including DOPAL. Furthermore, the toxicity of AS oligomers is mediated by a toxic DA metabolite [48]. We showed that DOPAL, but not its other metabolites (DOPAC, DOPET, HVA) is toxic in vitro and in vivo [7, 23, 30].

Thus, we propose that DOPAL not only triggers aggregation of AS to its toxic oligomeric form, but also mediates the toxicity of these oligomers [7, 23, 48]. Other possible sources of DA toxicity include its reactive oxidation products DAQ [11] and dopaminochrome (DC) [36], which inhibit AS fibrillization. However, neither free unbound DAQ nor DC have been directly detected or quantitated in human brain. The presence of DAQ and DC in brain is presumed, as they appear to be one of the multiple-postulated intermediates in the neuromelanin pathway. Therefore, the relevance of DAQ and DC to PD is speculative.

Another common theory implicates a complex I deficit found in SN neurons in PD pathogenesis [43, 45]. The complex I dysfunction may be inherited through maternal mitochondrial DNA [45] or acquired through pesticide exposure [2]. For instance, the pesticide rotenone, which inhibits complex I in all brain cells [2], provides a model of PD with selective loss of DA SN neurons and LB-like inclusions. The loss of DA SN neurons in the rotenone model is not due to a bioenergetic defect with ATP depletion [2], which would affect all brain cells. Rather, rotenone, which produces a partial inhibition of complex I in vivo [2], produces increased DOPAL levels in an in vitro model of DA neurons [26]. This effect of rotenone may be due to the fact

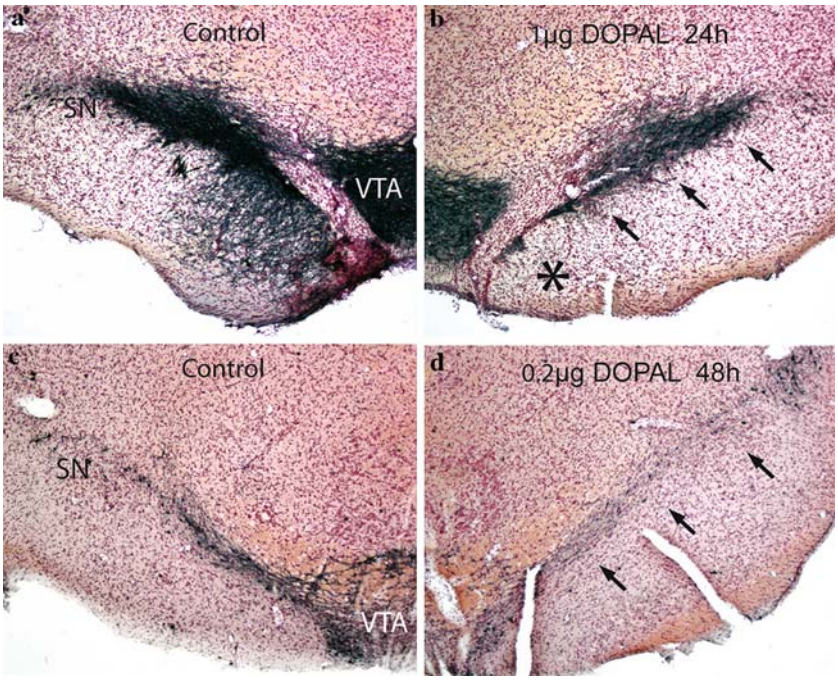

Fig. 6 Photomicrographs of sections illustrating the effect of microinjections of DOPAL or vehicle into rat substantia nigra (*SN*). Compare the substantia nigra in sections taken from the same level immunostained for tyrosine hydroxylase either 24 h (**a**, **b**) or 48 h (**c**, **d**) after an injection of either vehicle (**a**, **c**) or DOPAL (**b**, 1.0/0.4 µl; **d**, 0.2/0.2 µl) and note the diminished immunoreactivity on the side injected with DOPAL (*arrows* and *asterisk*)

that complex I inhibition reduces levels of nicotinamide adenine dinucleotide, a cofactor of aldehyde dehydrogenase (ALDH) required for the metabolism of DOPAL to DOPAC [26]. A study of the enzyme itself showed that ALDH gene expression is decreased in SN of patients with sporadic PD [15, 31].

Our results suggest that AS oligomers, and not the large AS aggregates, may mediate AS toxicity [1]. This hypothesis is supported both by the presence of oligomers in extracts of transfected 5Y cells treated with 1.0 mM DOPAL, and the large intracellular AS aggregates in cells remaining after exposure to 300 µM DOPAL. We also show that both 1.5 µM concentrations of DOPAL in a cell free system and 3 µM DOPAL in 5Y cell cultures rapidly trigger formation of potentially toxic oligomers of AS of the same size as those found in PD SN neurons [48]. Similarly, injection of DOPAL into SN of rats leads to a dose-dependent AS oligomerization and to a loss of TH ir. We did not visualize intracellular aggregates with either high or low doses of DOPAL in vivo. This suggests that rapid injection of DOPAL onto SNneurons results in neuronal death before these large aggregates can form and may reflect the difficulty in reproducing the gradual build up of DOPAL necessary to trigger large aggregates in vivo [2]. These results are consistent with cell culture data supporting the view that AS oligomers, and not the large AS aggregates, trigger neuronal cell death [1].

DOPAL levels of 2–3 µM have been measured in autopsy brains from neurologically normal patients [23]. This concentration of DOPAL is the same concentration that we used in vitro and twice that was required in a cell-free system to trigger AS aggregation. This finding suggests that under normal in vivo conditions, DA neurons may contain factors that protect against low-level DOPAL-induced injury [7]. In PD, however, these protective factors may become depleted [20], and other deficits associated with PD, such as a complex I deficit, may contribute to increased DOPAL levels [2, 43, 45]. AS aggregates could fibrillize into large multimeric β-pleated sheets, forming LBs with prolonged exposure to DOPAL [35].

Concentrations of both DOPAL and AS [19] may contribute to the propensity for aggregation via two possible mechanisms. First, DOPAL could react with H_2O_2 formed by oxidative DA metabolism to produce hydroxyl radicals [30], which induce AS aggregation [37]. Second, we show here that DOPAL directly triggers AS aggregation. DOPAL reactivity could be due to either the aldehyde group or to quinones formed from the ring hydroxyls [6]. However, the aldehyde group distinguishes DOPAL from catechol quinones [11], which do not trigger AS aggregation at low concentrations suggesting that the aldehyde moiety of DOPAL may be essential for its effect on AS aggregation.

Accumulation of AS either due to multiplication of SNCA locus [33], proteasomal defects [42], or mutation in AS gene [24, 41, 49], may also enhance formation of DOPAL-triggered oligomers and aggregates of AS. Oligomeric forms of AS could permeabilize DA storage vesicles [47]. Such pathologic function of AS could increase cytosolic DOPAL levels and as a consequence further increase AS oligomerization and aggregation, with a feed forward reaction culminating in SN cell death and LB formation as evidenced in our in vivo and cell culture experiments.

These findings provide evidence for new targets in drug development for PD [4].

Our experiments, showing DOPAL-induced AS aggregation in vitro and in vivo support the hypothesis that there are common convergent pathogenic mechanisms underlying PD [7, 18]. Taken together, our results are consistent with the view that DOPAL may account for the selective loss of DA neurons mediated by toxic AS oligomers [48], as well as the formation of LBs [44] in remaining SN neurons. Our results also provide a plausible link between DA production and its metabolism, AS and the pathogenesis of PD. Finally, this is one of the first studies to demonstrate that a neurotransmitter metabolite can trigger aggregation of a protein linked to a neurodegenerative disease. Recent reports indicate that synaptic function is altered by toxic protein oligomers before other parts of the neuron are affected in a number of neurodegenerative diseases including PD [10, 17, 21, 25]. Neurotransmitters, their metabolites and AS protein, are in highest concentration at the synapse. Our results provide a mechanism for selective protein oligomerization and loss of synaptic function early in neurodegenerative diseases.

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References

1. Arasate M, Mitra S, Schweitzer ES, Segal MR (2004) Inclusion body formation reduces levels of mutant huntingtin and risk of neuronal death. Nature 431:805–810
2. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT (2000) Chronic systemic pesticide exposure reproduces feature of Parkinson's disease. Nature Neurosci 3:1301–1306
3. Blashko H (1952) Amine oxidase and amine metabolism. Pharmacol Rev 4:415–453
4. Burke WJ (2003) 3,4-Dihydroxyphenylacetaldehyde: a potential target for neuroprotective therapy in Parkinson's disease. Current Drug Targets. CNS Neurol Disord 2:143–148
5. Burke WJ, Li SW, Anwar M, Glickstein SB, Ruggiero DA (2001) Catecholamine-derived aldehyde induces apoptosis in adrenergic neurons in rostral ventral lateral medulla. Brain Res 891:218–227
6. Burke WJ, Li SW, Chung HD, Ruggiero DA, Kristal BS, Johnson EM, Lampe P, Kumar VB, Franko M, Zahm DS (2004) Neurotoxicity of MAO metabolites of catecholamine neurotransmitters: role in neurodegenerative diseases. Neurotoxicology 25:101–115
7. Burke WJ, Li SW, Williams EA, Nonneman R, Zahm DS (2003) Dihydroxyphenylacetaldehyde is the toxic dopamine metabolite in vivo: implications for Parkinson's disease pathogenesis. Brain Res 989:205–213
8. Burke WJ, Schmitt CA, Gillespie KN, Li SW (1996) 3,4-Dihydroxyphenylglycolaldehyde, the MAO-A metabolite of norepinephrine, is selectively toxic to differentiated rat pheochromocytoma cells. Brain Res 772:232–235
9. Cappai R, leck S-L, Tew DJ, Williamson NA, Smith DP, Galatis D, Sharples RA, Curtain CC, Ali FE, Cherney RA, Culvenor JG, Bottomley SP, Masters CL, Barnham KJ, Hill AF (2005) Dopamine promotes α-synuclein aggregation into SDS-resistant soluble oligomers via a distinct folding pathway. FASEB J 19: 1377–1396
10. Coleman P, Federoff H, Kurlan R (2004) A focus on the synapse for neuroprotection in Alzheimer's disease and other dementias. Neurology 63:1155–1162
11. Conway KA, Rochet JC, Bieganski RM, Lansbury PT (2001) Kinetic stabilization of the α-synuclein protofibril by a dopamine α-synuclein adduct. Science 292:1346–1349
12. Dawson TM, Dawson VL (2003) Molecular pathways of degeneration in Parkinson's disease. Science 302:819–821
13. Fahn S (1997) Levo-dopa-induced neurotoxicity. CNS Drugs 8:276–393
14. Filloux F, Townsend JJ (1993) Pre- and postsynaptic neurotoxic effects demonstrated by intrastriatal injection. Exp Neurol 119:79–88
15. Galvin JE (2006) Interaction of alpha-synuclein and dopamine metabolites in pathogenesis of Parkinson's disease: a case for selective vulnerability of substantia nigra. Acta Neuropathol 112:115–126
16. Galvin JE, Lee VM, Trojanowski JD (2001) Synucleinopathies: clinical and pathological implications. Arch Neurol 58:186–190
17. Gong Y, Chang L, Viola KL, Lacor PN, Lambert MP, Finch FE, Krafft GA, Klein WL (2003) Alzheimer's disease-affected brain: presence of oligomeric AB ligands (ADDLS) suggest a molecular basis for reversible memory loss. Proc Natl Acad Sci USA 100:10417–10422
18. Greenamyre JT, Hastings TG (2004) Parkinson's—divergent causes, convergent mechanisms. Science 304:1120–1122
19. Hashimoto M, Hsu LJ, Sisk A, Xia Y, Taked A, Sundmo M, Masliah E (1998) Human recombinant NACP/α-synuclein is aggregated and fibrillated in vitro: relevance for Lewy body disease. Brain Res 99:301–306
20. Hirsch EC, Faucheux B, Damier P, Mouatt-Prigent A, Agid Y (1997) Neuronal vulnerability in Parkinson's disease. J Neural Transm 50:79–88
21. Hornykiewicz O (1996) Dopamine (3-hydroxytyramine) and brain function. Pharmacol Rev 18:925–965
22. Jellinger K (1987) Overview of morphological changes in Parkinson's disease. J Adv Neurol 45:1–18
23. Kristal BS, Conway AD, Brown AM, Jain JC, Ulluci PA, Li SW, Burke WJ (2000) Selective dopaminergic vulnerability: 3,4-dihydroxyphenylacetaldehyde targets mitochondria. Free Radic Bio Med 30:24–931
24. Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT (1998) Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nature Genet 18:106–108
25. Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola K (1998) Diffusible, nonfibrillar ligands derived from AB 1–42 are potent central nervous system neurotoxins. Proc Natl Acad Sci USA 95:6448–6453
26. Lamensdorf I, Eisenhofer G, Harvery-White J, Nechustan A, Kirk K, Kopin IJ (2000) Dihydroxyphenylacetaldehyde potentiates the toxic effects of metabolic stress in PC 12 cells. Brain Res 868:191
27. Lee FJS, Liu F, Pristupa ZB, Niznik HB (2001) Direct binding and functional coupling of α-synuclein to dopamine transporters accelerate dopamine-induced apoptosis. FASEB J 15:916–926

28. Li HT, Lin DH, Luo XY, Zhang F, Ji LN, Du HN, Hi J, Zhou JW, Hu HY (2005) Inhibition of α-synuclein fibrillation by dopamine analogs via reaction with amino groups of α-synuclein: Implication for dopaminergic neurodegeneration. FEBS J 272:3661–3672
29. Li SW, Burke WJ (1998) Synthesis of a biochemically important aldehyde, 3,4-dihydroxyphenylacetaldehyde. Bioorg Chem 26:45–50
30. Li SW, Li HT, Minteer S, Burke WJ (2001) 3,4-Dihydroxyphenylacetaldehyde and hydrogen peroxide generate a hydroxyl radical: possible role in Parkinson's disease pathogenesis. Brain Res 93:1–7
31. Mandel S, Grunblatt E, Riederer P, Amariglio N, Hirsch JJ, Rechari G, Youdim MBH (2007) Gene expression profiling of sporadic Parkinson's disease substantia nigra pars compacta reveals impairment of ubiquitin proteosome subunits, SKP1A, aldehyde dehydrogenase, and chaperone HSC-70. Ann NY Acad Sci 1053:356–375
32. Mazzulli JR, Mishizen AJ, Giasson BJ, Lynch DR, Thomas SA, Nakashima A, Nagatsu T, Ota A, Ischiropoulos H (2006) Cytosolic catechols inhibit α-synuclein aggregation and facilitate the formation of intracellular soluble oligomeric intermediates. J Neurosci 26:10068–10078
33. Miller DW, Hague SM, Clairmon J, Baptista M, Gwinn-Hardy K, Cokson MR, Singleton AB (2004) Alpha-synuclein in blood and brain from familial Parkinson disease with SNCA locus triplication. Neurology 62:1835–1838
34. Nagatsu T, Sawada M (2006) Cellular and molecular mechanisms of Parkinson's disease: neurotoxins, causative genes and inflamatory cytokines. Cell Mol Neurobiol 26:779–800
35. Narhi L (1999) Both familial Parkinson's disease mutations accelerate α-synuclein aggregation. J Biol Chem 274:9843–9846
36. Norris EH, Giasson BI, Hodara R, Xu S, Trojanowski JQ, Ischiropoulos H, Lee VM (2005) Reversible inhibition of α-synuclein fibrillizationation by dopaminochrome-mediated conformational alterations. J Biol Chem 280:21212–21219
37. Osterova-Golts N, Petrucelli L, Hardy J, Lee JM, Farer M, Wolozin B (2000) The A53T α-synuclein mutation increases iron-dependent aggregation and toxicity. J Neurosci 20:6048–6054
38. Paik SR, Shin HJ, Lee JH (2000) Metal catalyzes oxidation of α-synuclein in the presence of copper (II) and hydrogen peroxide. Arch Biochem Biophys 378:269–277
39. Pandey N, Schmidt RE, Galvin JE (2006) Mutations of KTKEGV repeat region of alpha-synuclein promotes aggregation in cultured cells. Expl Neurol 187:1515–1520
40. Parkinson J (1817) An Essay on Shaking Palsy
41. Polymeropoulos MD, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Stenroos ES, Chandraskharappa s, Athanassiadou H, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Dilorio G, Golbe LI, Nussbaum RL (1997) Mutation in α-synuclein gene identified in families with Parkinson's disease. Science 275:2045–2047
42. Sawada H, Khono R, Kihara T, Izumi Y, Sakka N, Ibi M, Nakanishi M, Nakamizo T, Yamakawa K, Shibasaki H (2004) Proteasome mediates dopaminergic neuronal degeneragion and its inhibition causes alpha-synuclein inclusions. J Biol Chem 279:10710–10719
43. Schapira AHV, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD (1990) Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 54:823–827
44. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goerdert M (1997) α-Synuclein in Lewy bodies. Nature 388:839–840
45. Swerdlow RH, Parks JK, Davis JN, Cassarino BS, Trimmer PA, Currie LJ, Dougherty S, Bridges WS, Bennet JP, Wooten GF, Parker DW (2004) Matrilineal inheritance of complex I dysfunction in a multigenerational Parkinson's disease family. Ann Neurol 44:873–881
46. Volles MJ, Lansbury PT (2002) Vesicle permeabilization by protofibrillar α-synuclein: comparison of wild type with Parkinson's disease-linked mutants and insights in the mechanism. Biochemistry 41:4595–4602
47. Volles MJ, Lee SJ, Rochet JC, Schtelerman MD, Ding JT, Kessler JC, Lansbury PT (2001) Vesicle permeabilization by protofibrillar α-synuclein: implications for the pathogenesis and treatment of Parkinson's disease. Biochemistry 40:7812–7819
48. Xu J, Kao SY, Song W, Jin LW, Yankner BA (2002) Dopamine-dependent neurotoxicity of α-synuclein: a mechanism for selective neurodegeneration in Parkinson's disease. Nat Med 8:600–606
49. Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E, Ros RB, Ampuero I, Vidal L, Hoeniaka J, Rodríguez O, Atares B (2004) The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol 55:164–173