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Dopamine and Paraquat Enhance α-Synuclein-Induced Alterations in Membrane Conductance

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Abstract We have previously demonstrated that α-synuclein overexpression increases the membrane conductance of dopaminergic-like cells. Although α-synuclein is thought to play a central role in the pathogenesis of several neurodegenerative diseases including Parkinson's disease, multiple system atrophy, and diffuse Lewy body disease, the mechanism of action is not completely understood. In this study, we sought to determine whether multiple factors act together with α-synuclein to engender cell vulnerability through an augmentation of membrane conductance. In this article, we employed a cell model that mimics dopaminergic neurons coupled with α-synuclein overexpression and oxidative stressors. We demonstrate an enhancement of α-synuclein-induced toxicity in the presence of combined treatment with dopamine and paraquat, two molecules known to incite oxidative stress. In addition, we show that combined dopamine and paraquat treatment increases the expression of heme oxygenase-1, an antioxidant response protein. Finally, we demonstrate for the first time that combined treatment of dopaminergic cells with paraquat and dopamine enhances α-synuclein-induced leak channel properties resulting in increased membrane conductance. Importantly, these increases are most robust when both paraquat and dopamine are present suggesting the need for multiple oxidative insults to augment α-synuclein-induced disruption of membrane integrity.

Keywords Parkinson's disease · Oxidative stress · Pore · Leak channel · Dopamine · Paraquat

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Introduction

Parkinson's disease is the second most common neurodegenerative disorder affecting 1.5 million Americans and 4 million people worldwide (Kempster et al. 2007). Less than 10% of Parkinson's disease cases derive from a direct genetic cause, while the majority of patients present with sporadic/idiopathic disease and lack a clearly defined etiology (Mandel et al. 2005; Klein and Schlossmacher 2007). However, both familial and sporadic Parkinson's disease patients present with similar pathological hallmarks, including a progressive loss of substantia nigra pars compacta (SNpc) dopamine neurons, loss of dopamine terminals in the putamen, increased microglial activation, and the presence of large intracytoplasmic proteinaceous inclusions within the remaining SNpc dopamine neurons called Lewy bodies (Spillantini et al. 1997; Braak et al. 2004; Croisier et al. 2005). The Lewy bodies are replete with α-synuclein, a protein that was initially linked to Parkinson's disease through genetic studies. In fact, both mutations in and overexpression of the gene that encodes for α-synuclein, *SNCA*, lead to familial forms of Parkinson's disease (Polymeropoulos et al. 1997; Spillantini et al. 1997; Krüger et al. 1998; Papadimitriou et al. 1999; Singleton et al. 2003; Zarranz et al. 2004; Paleologou et al. 2010). Furthermore, genome-wide association studies (GWAS) link polymorphisms in *SCNA* with an increased risk of developing Parkinson's disease, supporting a role for α-synuclein in both familial and sporadic/idiopathic forms of this disease and extending the relevance of this protein to a larger cohort of patients (Satake et al. 2009; Simon-Sanchez et al. 2009; Hamza et al. 2010).

The mechanism by which α-synuclein incites pathogenesis is multifarious but commonly proposed to be due to a toxic gain of function. Toxicity has been linked to its

propensity to misfold, and in this article, we explore one potential mechanism for α-synuclein-induced neuronal toxicity in the presence of an oxidative stress environment involving alterations in membrane function leading to increased cellular membrane conductance. The ability of α-synuclein to disrupt membrane integrity and/or form pore-like structures is supported by studies demonstrating the formation of annular α-synuclein in vitro using atomic force and electron microscopy. In addition, we and others have shown increased membrane permeability in α-synuclein-containing synthetic vesicles and cell lines that overexpress this protein (Goldberg and Lansbury 2000; Volles et al. 2001; Ding et al. 2002; Caughey and Lansbury 2003; Pountney et al. 2004; Furukawa et al. 2006; Danzer et al. 2007; Kostka et al. 2008; Tsigelny et al. 2008; Auluck et al. 2010; Feng et al. 2010). Increased membrane permeability is likely to disrupt cellular ionic balance and facilitate the misregulation of intracellular calcium levels, subsequently leading to increased oxidative stress. Furthermore, the autonomous pacemaking of substantia nigra dopamine neurons increases the influx of calcium resulting in increased mitochondrial oxidative stress making these neurons more susceptible to toxins (Surmeier et al. 2010a, b). This finding is relevant to Parkinson's disease since oxidative stress accumulates with age, the leading risk factor for Parkinson's disease (Ames et al. 1993; Bishop et al. 2010). Indeed, the evidence for oxidative stress in the form of oxidatively modified proteins, lipids, and nucleic acids has been observed in post-mortem Parkinson's disease brains (Dexter et al. 1989, 1994; Sian et al. 1994; Alam et al. 1997a, b; Mattson et al. 1999; Lotharius and Brundin 2002; Beal 2003; Jenner 2003; Miller et al. 2009).

A potential source of oxidative free radicals within the nigrostriatal system is extravesicular dopamine, a highly reactive molecule that interacts with and incites α-synuclein misfolding (Graham et al. 1978; Conway et al. 2001; Weingarten and Zhou 2001; Lotharius and Brundin 2002; Cappai et al. 2005; Kim et al. 2005; Maguire-Zeiss et al. 2006; Moussa et al. 2008; Outeiro et al. 2009). However, since α-synuclein and dopamine are normally found within the nigrostriatal system, there is likely another factor that contributes to the pathogenic process in idiopathic Parkinson's disease. In fact, accumulating evidence points to the involvement of multiple insults that cumulatively compromise the nigrostriatal system beyond a limiting threshold resulting in Parkinson's disease (Maguire-Zeiss and Federoff 2003; Maguire-Zeiss et al. 2005; Elbaz et al. 2007; Klein and Schlossmacher 2007; Sulzer 2007; Migliore and Coppedè 2009a, b). For instance, epidemiological evidence establishes pesticide exposure as one risk factor for Parkinson's disease (Barbeau et al. 1985; Hubble et al. 1993; Gorell et al. 1998; Engel et al. 2001; Herishanu et al. 2001; Lai et al. 2002). Specifically, paraquat (1,1′dimethyl-4,4′-bipyridilium dichloride), a herbicide widely utilized to control weed growth, is associated with Parkinson's disease (Smith 1985; Rajput et al. 1987; Liou et al. 1997; Schmuck et al. 2002; Dinis-Oliveira et al. 2006). Paraquat is used experimentally as a redox cycler to increase the formation of reactive oxygen species in the form of superoxide free radicals, and paraquat-treated animals display some of the pathological features of Parkinson's disease such as decreased tyrosine hydroxylase fiber density in the striatum, loss of tyrosine hydroxylase positive neurons in substantia nigra, evidence for α-synuclein aggregates, and increased oxidative stress (Brooks et al. 1999; Manning-Bog et al. 2003; Thiruchelvam et al. 2004; McCormack et al. 2005, 2006; Prasad et al. 2007; Cocheme and Murphy 2008; Fei et al. 2008; Kang et al. 2009; Kang et al. 2010).

We previously reported that α-synuclein caused an increase in membrane conductance reminiscent of leak channels (Feng et al. 2010). In this article, we sought to determine whether Parkinson's disease relevant oxidative stressors would augment α-synuclein-mediated alterations in membrane conductance and subsequently cell death. In this article, we demonstrate for the first time that combined treatment of dopaminergic cells with paraquat and dopamine enhanced α-synuclein-induced leak channel properties resulting in increased membrane conductance and cell death. In addition, these increases are most robust when both paraquat and dopamine are present suggesting the ability of multiple oxidative insults to potentiate α-synuclein's toxic effects.

Methods

Antibodies

The following antibodies were used for western blotting: anti-dopamine transporter (DAT; 1:1000; Novus, Littleton, CO), anti-heme oxygenase-1 (HO-1; 1:1000; Abcam, Cambridge, MA), anti-α-synuclein for immunoblotting (Syn; 1:1000; BD Biosciences, San Jose, CA), anti-α-tubulin (1:1000; Abcam), anti-tyrosine hydroxylase (TH; 1:1000; Chemicon/Millipore, Billerica, Massachusetts), horseradish peroxidase conjugated goat anti-rabbit IgG antibody (1:2000; Chemicon/Millipore), horseradish peroxidase conjugated goat anti-mouse IgG antibody (1:2000; Chemicon/Millipore). The following antibodies were used for immunocytochemistry: anti-α-synuclein antibody for immunocytochemistry (Syn; 1:1000; NeoMarkers, Fremont, CA), anti-vesicular monoamine transporter 2 (VMAT2; 1:200; Chemicon/Millipore), Alexa Fluor 594 conjugated goat anti-mouse secondary antibody (1:2000; Invitrogen, Camarillo, CA), Alexa Fluor 594 conjugated goat anti-rabbit secondary antibody (1:2000; Invitrogen).

Cell Culture

MN9DwtsynIRESgfp (MN9Dsyn) cells were engineered and cultured as previously described (kind gift of Dr. Howard Federoff; Luo et al. 2007; Su et al. 2008; Feng et al. 2010). MN9Dsyn is an immortalized dopaminergic-like cell line that harbors an integrated transgene affording doxycycline-regulated (DOX; 2.0 μg/ml media, Sigma-Aldrich, St. Louis, MO) human wild type α-synuclein (Syn) expression and separately, using an internal ribosome entry site (IRES), green fluorescent protein (GFP) expression. The parental cell line (MN9D) was derived from mouse embryonic mesencephalon fused to a neuroblastoma cell line (kind gift of Dr. A. Heller; Choi et al. 1991b).

MTT (3-[4,5-Dimethylthiazol]-2,5-diphenyltetrazolium) Assay

MN9Dsyn cells (1×10^4 cells/well) were grown in 96-well plates (Nunclon™, Thermo Fisher, Waltham, MA) in the absence or the presence of DOX to induce Syn and GFP expression. Twenty-four hours after induction, cells were treated with vehicle, dopamine (DA; 100 μM), paraquat (PQ; 50 μM), or both DA and PQ for an additional 24 h. In experiments using L-3,4-dihydroxyphenylalanin (L-DOPA) in place of dopamine, cells were treated with vehicle, L-DOPA (100 μM), paraquat (PQ; 50 μM), or both L-DOPA and PQ for 24 h following Syn induction (DA, PQ and L-DOPA were purchased from Sigma-Aldrich). Cell viability was quantified by means of an MTT assay as described by the manufacturer (Roche, Indianapolis, IN). Each experiment was performed with an *N* of 8 and repeated at least three times. Data are expressed as mean percentage of cell death with respect to untreated control cells (−DOX) ± SEM.

Western Blot Analysis

MN9Dsyn cells (5×10^6 cells/10 cm plate; Nunclon™) were grown in the absence or the presence of DOX to induce Syn and GFP expression. Twenty-four hours after induction, cells were treated with dopamine (DA; 100 μM), paraquat (PQ; 50 μM), or both for 24 h. Following experimental treatment, cells were washed with phosphate-buffered saline (PBS) and subsequently lysed on ice in modified RIPA buffer (50 mM Tris HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl) supplemented with protease inhibitor cocktail (Sigma-Aldrich; cat. No. P2714), phenylmethanesulfonylfluoride (PMSF; 1 mM; Sigma-Aldrich), and Halt Phosphatase Inhibitor (Thermo Scientific) using a hand-held-motorized homogenizer. Lysates were centrifuged at 13,300 rpm × 15 min at 4°C and cleared supernatants retained. The supernatants were subjected to polyacrylamide gradient gel electrophoresis under denaturing conditions (4–16% SDS-PAGE) followed by transfer to polyvinylidene difluoride (PVDF) membranes (Perkin Elmer, Waltham, MA) and western blot analyses as described in the figure legends. Immunoreactive complexes were visualized following Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) treatment and exposure to Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ). Membranes were re-probed with a primary antibody against α-tubulin as a loading control. Quantification of protein levels was performed using the EC3 Imaging System (UVP, LLC; Upland, CA). For α-synuclein oligomer density measurement, bands above 16 kDa were included and total optical density of α-synuclein oligomers from each treatment group was quantified and normalized to α-tubulin density.

Immunocytochemistry

For VMAT2 ICC, MN9Dsyn cells (4×10^4 cells/well; 24-well plate; Nunclon™) were grown on polyethyleneimine-coated (PEI) glass coverslips (diameter = 12 mm, Deckglaser, Germany) for 48 h without DOX and subsequently processed for immunocytochemistry. For α-synuclein ICC, MN9Dsyn cells were grown on PEI-coated glass coverslips in the absence/presence of DOX (Syn and GFP induction). Twenty-four hours after induction, cells were treated with dopamine (DA), paraquat (PQ), DA, and PQ, vehicle or untreated for an additional 24 h (see figure legends for specific treatment paradigms). In both cases, cells were subsequently fixed with 4% paraformaldehyde/4% sucrose/PBS for 15 min at room temperature, permeabilized and blocked in TBS/4.5% non-fat dry milk (NFDM)/0.1% Triton-X 100, and incubated with an anti-VMAT2 antibody (VMAT2 ICC; Fig. 1b) or an anti-α-synuclein antibody (α-synuclein ICC; Fig. 2) overnight at 4°C. After washing with TBS/4.5% NFDM/0.05% Triton-X 100, cells were further incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (VMAT2 ICC; Fig. 1b) or Alexa Fluor 594-conjugated goat anti-mouse IgG secondary antibody (α-synuclein ICC; Fig. 2a). After two additional washes in TBS/4.5% NFDM/0.05% Triton-X 100, nuclei were stained with DAPI (100 nM; 4′,6′-diamidino-2-phenylindole; Invitrogen), cells mounted with mowiol (Calbiochem, La Jolla, CA) and imaged. All fluorescent complexes were visualized using a Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY). Images were acquired with a Photometrics Coolsnap-fx (Roper Scientific, Tucson, AZ) camera using the Scanalytic's IPLab software (Fairfax, VA) and further processed with ImageJ (National Institutes of Health, Bethesda, MD). Each experiment was repeated three times.

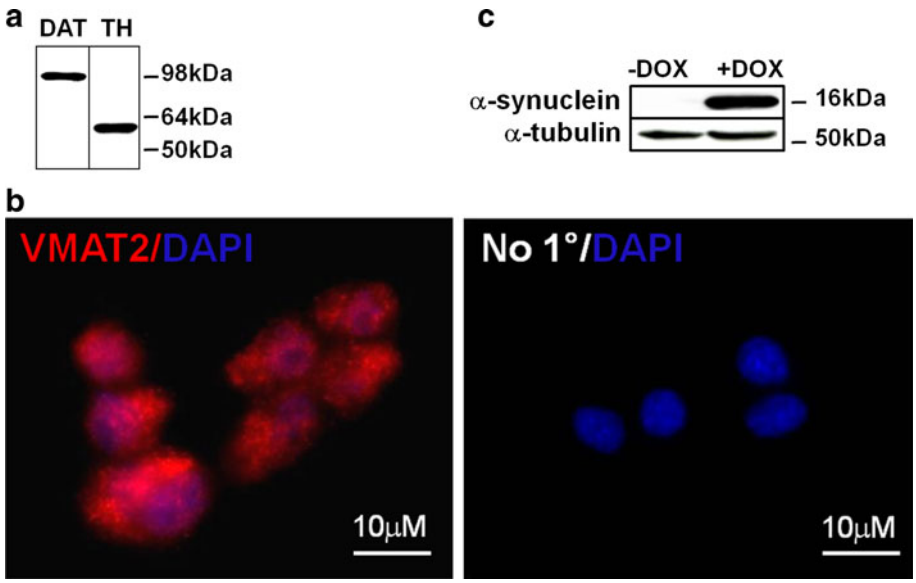


Fig. 1 Expression of α-synuclein and markers of dopaminergic neurons in MN9Dsyn cells. **a** Representative western blots of MN9Dsyn cell lysates (20 μg/lane) demonstrating the presence of dopaminergic neuron markers, tyrosine hydroxylase (TH), and dopamine transporter (DAT). **b** Representative image of MN9Dsyn cells immunostained for vesicular monoamine transporter 2 (VMAT2; *red*) and subsequently stained with DAPI to visualize nuclei (*blue*) (*left panel*). No primary antibody control counterstained with DAPI (*right panel*). **c** Representative western blots of MN9Dsyn lysates (20 μg protein/lane) immunoblotted for α-synuclein and reprobed for α-tubulin as a loading control. Administration of doxycycline (+DOX) induces robust α-synuclein overexpression (+DOX) compared with uninduced MN9Dsyn (−DOX)

Electrophysiological Analysis/Whole Cell Patch Clamp Recordings

MN9Dsyn cells (4×10^4 cells/well; 24-well plate; Nunclon^{TM}) were grown on PEI-coated coverslips in the absence or presence of DOX (induction of Syn and GFP). Twenty-four hours after induction, both induced and uninduced cells were treated with dopamine (DA), paraquat (PQ), DA + PQ, or vehicle for an additional 24 h. The MN9Dsyn-containing coverslips (diameter = 12 mm, Deckglaser, Germany) were placed on the stage of a Nikon TM2000 inverted microscope (Nikon Instruments, Melville, NY), and the cells were continuously perfused with an extracellular solution containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM Hepes, 5 mM glucose, 0.25 mg/l phenol red, 10 μM D-serine, and 23.4 mM sucrose pH 7.4 (all reagents obtained from Sigma-Aldrich). Electrodes were pulled on a vertical pipette puller to a resistance of 4–6 MΩ from borosilicate glass capillaries (Wiretrol II, Drummond, Broomall, PA) and filled with intracellular recording solution containing 145 mM CsCl, 10 mM Hepes, 5 mM MgATP, 0.2 mM NaGTP, and 10 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) pH 7.2. Whole cell voltage clamp recordings were performed at room temperature using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). To measure input resistance, the membrane potential was held at 0 mV and stepped to levels from −45 to 45 mV in 5 mV increments. Currents were filtered at 2 kHz with an 8-pole low pass Bessel filter (Frequency Devices, Haverhill, MA), digitized at 5–10 kHz with Digidata 1322 A data acquisition board and pCLAMP9.2 software (both from Molecular Devices). All data were analyzed with Clampfit 9.2 software (Molecular Devices) and are the average of 9–20 cells/treatment condition from four independent experiments. Statistical analyses for multiple comparisons were performed using a one-way analysis of variance (ANOVA), and a paired *t*-test with Bonferroni adjustment was performed subsequent to the ANOVA.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analyses for multiple comparisons was performed using a one-way analysis of variance (ANOVA) with Tukey post hoc test or paired *t*-test with Bonferroni adjustment for observations of Syn and DA/PQ-induced cell death, changes in HO-1 protein levels, as well as electrophysiological analyses. All statistical analyses were conducted using SPSS18.0 (SPSS Inc., Chicago, IL). Results were considered statistically significant at $P \leq 0.05$.

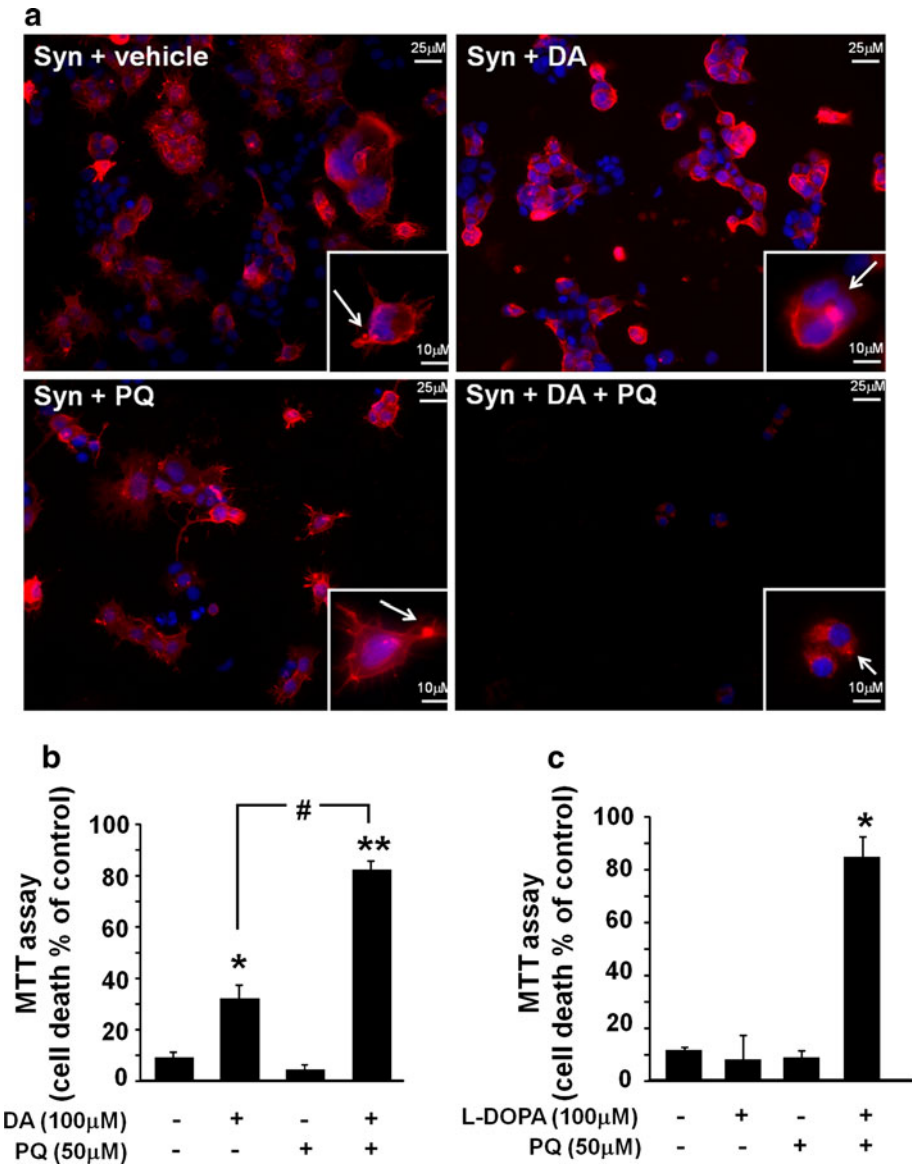


Fig. 2 Treatment with dopamine and paraquat augments α-synuclein-induced cell death. **a** Representative images of MN9Dsyn cells overexpressing α-synuclein (+DOX/Syn) and treated with vehicle (Syn), dopamine (DA), paraquat (PQ), or both following immunocytochemistry for α-synuclein (*red*). Membrane localized, nuclear, and cytosolic α-synuclein as well as aggregates (*white arrows*) are present in the α-synuclein overexpressing cells. DAPI (*blue*) was employed to visualize the nuclei. Scale bar = 25 μm. Syn-specific aggregates are more apparent in the higher magnification inset photomicrograph (*white box*; *arrow*; scale bar = 10 μm). Cell loss and shrunken/punctate nuclei are evident in Syn-overexpressing cells treated with both DA and PQ (Syn + DA + PQ). **b** MTT assay of MN9Dsyn cells treated with DA (100 μM), PQ (50 μM), or both. Cell death was calculated as the percentage of mitochondrial activity reduction following α-synuclein overexpression (+DOX/Syn) as compared with uninduced (−DOX) controls for the same treatment group (DA, PQ, or both). Values are expressed as percent cell death ± SEM ($N = 8$ wells/treatment). Each experiment was repeated at least three times. One-way ANOVA and Tukey HSD post hoc test, *significant difference as compared with untreated controls, $P < 0.05$. Syn overexpression alone resulted in 9% cell death; treatment with DA or PQ resulted in 32.1% [(+DOX) vs. (+DOX + DA) $^{*}P = 6 \times 10^{-5}$] and 4.5% [(+DOX) vs. (+DOX + PQ) $P = 0.79$)] cell death, respectively; treatment with both DA and PQ induced 82.2% cell death [(+DOX) vs. (+DOX + DA + PQ) $^{**}P = 5 \times 10^{-13}$; (+DOX + DA) vs. (+DOX + DA + PQ) $^{\#}P = 5 \times 10^{-13}$). **c** MTT assay of MN9Dsyn cells in the presence and absence of DOX treated with L-DOPA (100 μM), PQ (50 μM), or both. Cell death was calculated as the percentage of mitochondrial activity reduction following Syn overexpression (+DOX/Syn) as compared with uninduced (−DOX) controls for the same treatment group (L-DOPA, PQ, or both). One-way ANOVA and Tukey HSD post hoc test, *significant difference as compared with untreated controls, $P < 0.05$. Treatment with L-DOPA or PQ resulted in 8.2% [(+DOX) vs. (+DOX + L-DOPA) $^{*}P = 0.98$] and 8.8% [(+DOX) vs. (+DOX + PQ) $P = 0.99$)] cell death, respectively; treatment with both L-DOPA and PQ induced 84.8% cell death [(+DOX) vs. (+DOX + L-DOPA + PQ) $^{**}P = 1.16 \times 10^{-5}$). In the absence of Syn overexpression dopamine (100 μM) and paraquat (50 μM) induced 42 and 12.7% cell death respectively (data not shown)

Results

Treatment with Dopamine and Paraquat Augments α-Synuclein-Induced Cell Death

Pathogenesis of sporadic Parkinson's disease likely involves multiple factors including genetic vulnerability and environmental insults (Maguire-Zeiss and Federoff 2003; Maguire-Zeiss et al. 2005; Cicchetti et al. 2009). To investigate how various insults may act in concert to enhance cell vulnerability, we utilized an immortalized dopaminergic cell line that harbors an integrated transgene affording doxycycline (DOX)-regulated human wild type α-synuclein (Syn) expression and using an internal ribosome entry site (IRES) also expresses green fluorescent protein (Choi et al. 1991b; Strathdee et al. 1999; Su et al. 2008; Feng et al. 2010). First, we showed that MN9Dsyn cells express the characteristic dopaminergic neuronal markers, tyrosine hydroxylase (TH), dopamine transporter (DAT) (Fig. 1a), and vesicular monoamine transporter 2 (VMAT2) (Fig. 1b). The dopamine content in the parental cell line (MN9D cells) was previously estimated to be 102.0 ± 2.1 fg/cell (Choi et al. 1991a, 1992). Next, we established that the MN9Dsyn cell line overexpresses α-synuclein following DOX treatment (Fig. 1c).

Using this cell line, we previously demonstrated toxicity induced by α-synuclein overexpression (Feng et al. 2010). To determine the effects of multiple insults on α-synuclein-induced cell vulnerability, we treated MN9Dsyn cells with the oxidative stressors, dopamine (DA; 100 μM) and paraquat (PQ; 50 μM). Using immunocytochemistry, we initially determined whether human α-synuclein and subsequent treatment with oxidative stressors caused accumulation of this protein. Consistent with our previous observation, α-synuclein localized to the cell membrane, nucleus, and cytosol (Fig. 2a; red; Feng et al. 2010). In addition, α-synuclein-positive aggregates (white arrows) were apparent in all treatment groups. There was an obvious decrease in cell number when MN9Dsyn cells were treated with both dopamine and paraquat compared with any other treatment group. We next quantified the effect of these stressors on cell viability by measuring the reduction of 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium (MTT; Fig. 2b). In order to determine α-synuclein-specific effects, we calculated the cell death of DOX-induced cells as a percentage of the uninduced controls for the same treatment group (dopamine, paraquat, or both). Here, we show that α-synuclein overexpression resulted in 9.1% cell death while the addition of dopamine or paraquat treatment resulted in 32.1 and 4.5% cell death, respectively, as compared with uninduced controls (−DOX). Interestingly, combined treatment with dopamine and paraquat enhanced the α-synuclein-induced cell death to 82.2% demonstrating an increase in α-synuclein-induced cell death when the combined oxidative stressors are present (Fig. 2b). To determine whether the dopamine precursor, L-DOPA, could affect similar changes, we included L-DOPA in place of dopamine. In Fig. 2c, we show a significant increase α-synuclein-induced cell death only when all both L-DOPA and PQ are present (84.8%; Fig. 2c).

Dopamine and Paraquat Activate Cellular Anti-Oxidant Response Mechanisms

Since we observed an augmentation of α-synuclein-induced cell death following treatment with dopamine and paraquat, we next sought to examine the cellular response to these oxidative stressors. An important component of the cellular anti-oxidant response mechanism is the expression of Nrf2 (NF-E2-related factor 2)-regulated phase II detoxification enzymes. The Nrf2 translocates to the nucleus and binds to the anti-oxidant response element facilitating transcription of these genes (Calkins et al. 2009; Chen et al. 2009). We chose to interrogate heme oxygenase-1 (HO-1) protein levels because this Nrf-2-regulated gene has been implicated in Parkinson's disease (Hung et al. 2008; Schipper et al. 2009; Song et al. 2009). Following the treatment of MN9Dsyn cells, we observed the most robust increase in HO-1 protein expression following combined treatment with dopamine and paraquat (three-fold increase compared to untreated controls) indicating a cumulative effect of multiple stressors in response to oxidative stress. Neither α-synuclein nor paraquat alone increased HO-1 expression, while dopamine had a small but significant effect (Fig. 3). These data demonstrate that the combination of paraquat and dopamine enhance the oxidative stress microenvironment of these cells.

Oxidative Stress Increases Membrane Permeability in α-Synuclein Overexpressing Cells

Having established an elevated oxidative stress environment with combined treatment of dopamine and paraquat, we next investigated if the increased levels of oxidative stress resulted in changes in membrane permeability related to α-synuclein overexpression. We performed whole-cell patch-clamp recordings of induced (+DOX/Syn) and uninduced (−DOX) MN9Dsyn cells treated with dopamine, paraquat, or dopamine and paraquat. First, we eliminated potential contributions from voltage-gated potassium or sodium channels by means of an intracellular solution containing cesium chloride and a holding potential at 0 mV. Second, to eliminate potential differences due to total cell surface areas, we normalized conductance changes by membrane capacitance. We also normalized the data

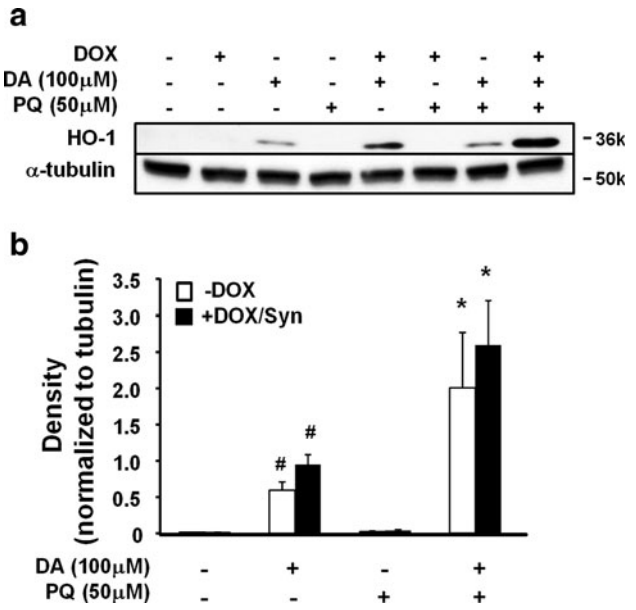


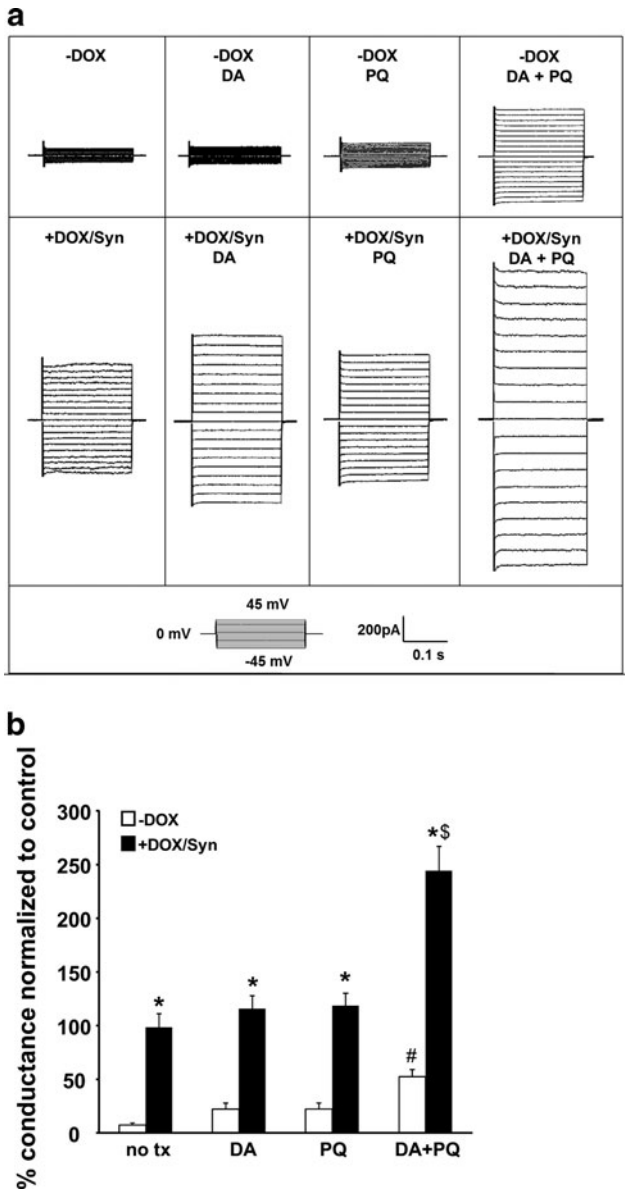
Fig. 3 Dopamine in combination with paraquat increase heme oxygenase-1 expression. **a** Representative western blots illustrating upregulation of heme oxygenase-1 (HO-1) protein levels in MN9Dsyn cells (±DOX) treated with dopamine (DA), and dopamine plus paraquat (PQ). Samples (20 μg protein/lane) were immunoblotted for HO-1. The same blots were subsequently stripped and reprobed for α-tubulin as a loading control. **b** HO-1 immunoprotein complexes were quantified by densitometric analysis of western blots and values normalized to α-tubulin. Values are expressed as the mean band intensity ± SEM from six samples/treatment. One-way ANOVA and Tukey HSD post hoc test, *significant difference as compared with untreated controls, $P < 0.05$. Only cells treated with both DA and PQ demonstrated a significant increase in HO-1 protein levels. (ANOVA, *significance as compared to non-treated control: (+DA + PQ) $P = 0.006$, (+DOX + DA + PQ) $P = 0.00017$.) HO-1 protein levels were increased to a lesser extent in cells treated with DA alone as compared with non-treated control (statistically insignificant by ANOVA with Tukey HSD post hoc test; $^{\#}P < 0.05$ significant by paired *t*-test with Bonferroni adjustment)

to control α-synuclein-overexpressing MN9Dsyn cells to reflect the percent changes in membrane conductance resultant of various treatments. Consistent with our previous results, α-synuclein overexpression (+DOX/Syn) alone increased membrane conductance compared with uninduced cells (−DOX; Fig. 4) (Feng et al. 2010). Individual treatment with either dopamine or paraquat did not increase membrane conductance as compared with untreated control ($P > 0.05$, DA or PQ treated cells versus untreated control) either in the presence or the absence of α-synuclein overexpression. The combined treatment of dopamine and paraquat resulted in elevated membrane permeability in the absence of α-synuclein suggesting that while dopamine or paraquat alone was not sufficient to alter membrane permeability, the combined treatment of both stressors significantly increased membrane conductance indicating compromised membrane integrity ($^{\#}P < 0.05$, significant difference as compared with uninduced cells that were treated with DA, PQ or vehicle). Importantly, the combination of α-synuclein overexpression, dopamine, and paraquat led to a more robust and significant increase in membrane conductance when compared with any stressor alone ($^{\$}P < 0.05$, significant difference as compared with induced cells that were treated with DA, PQ or vehicle). These electrophysiology results are consistent with our data demonstrating increased cell death when MN9Dsyn cells are exposed to this combination of stressors (Fig. 2).

Since our previous study and that of others demonstrated an alteration in membrane conductance due to the presence of membrane-localized α-synuclein, we next asked whether the elevation of α-synuclein-mediated membrane conductivity in MN9Dsyn cells treated with dopamine and paraquat was due to increases in soluble α-synuclein oligomers (Furukawa et al. 2006; Danzer et al. 2007; Tsigelny et al. 2007; Feng et al. 2010). Protein lysates from induced (+DOX/Syn) and uninduced (−DOX) MN9Dsyn cells treated with dopamine and paraquat were prepared in modified RIPA buffer and subjected to polyacrylamide gel electrophoresis under denaturing conditions followed by α-synuclein western blot analysis to analyze soluble oligomers. Monomeric α-synuclein and SDS-stable α-synuclein oligomers (black vertical line) were present following DOX induction (Fig. 5a). We did not observe a significant difference in monomeric or SDS-stable oligomeric α-synuclein density among the induced (+DOX/Syn) MN9Dsyn cells treated with dopamine, paraquat, or both (Fig. 5b). These results in combination with the membrane conductance (Fig. 4) and immunocytochemical (Fig. 2a) data suggest that the combined dopamine and paraquat augmentation in membrane conductivity is not due to increased α-synuclein aggregation.

Discussion

Emerging evidence points to a complex process in the pathophysiology of neurodegenerative disorders involving multiple factors (Maguire-Zeiss and Federoff 2003; Elbaz et al. 2007; Sulzer 2007; Migliore and Coppedè 2009a). It is interesting that advancing age is the predominant risk factor for Parkinson's disease and that oxidative stress increases with age (Beal 2003; Mariani et al. 2005; Bishop et al. 2010; Hindle 2010). Our previous study suggests that one cytotoxic role of α-synuclein results from misfolding of this protein into pore-like structures producing leak channel properties and compromised membrane integrity (Feng et al. 2010). In the current study, we hypothesized that elevated levels of oxidative stress would contribute to cell vulnerability by increasing the α-synuclein-mediated membrane conductivity changes resulting in cell death



◀ **Fig. 4** Oxidative stress increases membrane permeability in α-synuclein overexpressing cells. **a** Representative traces from α-synuclein overexpressing (+DOX/Syn) and uninduced (−DOX) MN9Dsyn cells treated with ±DA ± PQ showing currents elicited by stepping membrane voltage from a holding potential of 0 mV to levels between −45 and 45 mV (*inset*: step voltage protocol). **b** Percent conductance change from α-synuclein overexpressing (+DOX/Syn) and uninduced (−DOX) MN9Dsyn cells treated with ±DA ± PQ. Data were normalized to control α-synuclein-overexpressing MN9Dsyn cells to reflect the percent changes in membrane conductance as a result of various treatments. Values are expressed as percent conductance ± SEM ($N = 9$–20 cells/treatment group from four independent experiments). α-Synuclein overexpression (+DOX/Syn) increased membrane conductance [$^{*}P < 0.05$, one-way ANOVA and paired *t*-test with Bonferroni adjustment, significant difference as compared with uninduced control; (+DOX/Syn) compared with (-DOX)]. Dopamine or paraquat treatment alone did not increase membrane conductance as compared with the untreated control group either in the uninduced (−DOX) or induced (+DOX/Syn) MN9Dsyn cells [statistically insignificant by ANOVA and paired *t*-test with Bonferroni adjustment; (−DOX + DA) and (−DOX + PQ) compared with (−DOX); (+DOX + DA) and (+DOX + PQ) compared with (+DOX)]. Combined treatment of dopamine and paraquat resulted in elevated membrane permeability indicating compromised membrane integrity [$^{\#}P < 0.05$, ANOVA and paired *t*-test with Bonferroni adjustment, (−DOX + DA + PQ) compared with (−DOX)]. Importantly, the combination of α-synuclein overexpression, dopamine and paraquat led to a more robust and significant increase in membrane conductance when compared with any stressor alone [$^{\$}P < 0.05$, ANOVA and paired *t*-test with Bonferroni adjustment, (+DOX + DA + PQ) compared with (+DOX)]

(depicted schematically in Fig. 6). To test this hypothesis, we utilized a dopaminergic cell line with doxycycline-inducible α-synuclein overexpression, MN9Dsyn cells, and assessed the effects of oxidative stress, in the form of extracellular exposure to dopamine and paraquat, on α-synuclein-mediated cell vulnerability. In this article, we report for the first time increased membrane conductivity indicative of compromised membrane integrity, an enhanced antioxidant response, and augmentation of cell death related to α-synuclein overexpression following exposure to both dopamine and paraquat. It is interesting that α-synuclein overexpression in combination with exposure to either stressor alone did not significantly enhance the synuclein-mediated changes in membrane integrity supporting the need for multiple stressors.

The initial genetic clue that α-synuclein was involved in the pathogenic mechanism(s) of Parkinson's disease emerged with the discovery of familial disease caused by point mutations and multiplications of the α-synuclein gene (*SNCA*) (Polymeropoulos et al. 1997; Krüger et al. 1998; Papadimitriou et al. 1999; Singleton et al. 2003; Zarranz et al. 2004; Paleologou et al. 2010). Although these mutations and multiplications account for a limited number of familial Parkinson's disease cases, α-synuclein remains at the center of Parkinson's disease pathogenesis in part because it is localized to the hallmark pathological feature of this disorder, the Lewy body, and recent GWAS studies associate *SNCA* polymorphisms with an increased risk of developing sporadic Parkinson's disease (Spillantini et al. 1997; Satake et al. 2009; Simon-Sanchez et al. 2009; Hamza et al. 2010). α-Synuclein is ubiquitously expressed in the brain and exists under normal conditions in a random coil structure serving various physiological functions including synaptic maintenance and vesicle recycling (Abeliovich et al. 2000; Murphy et al. 2000; Cabin et al. 2002; Steidl et al. 2003; Fortin et al. 2005; Burre et al. 2010; Darios et al. 2010; Nemani et al. 2010). In the presence of molecular crowding, changes in pH and oxidative stress, α-synuclein misfolds into protofibrils and the more densely packed fibrils which are components of Lewy bodies (Fig. 6, step 1a; Shtilerman et al. 2002; Uversky et al. 2002b; Caughey and Lansbury 2003; Fink 2006).

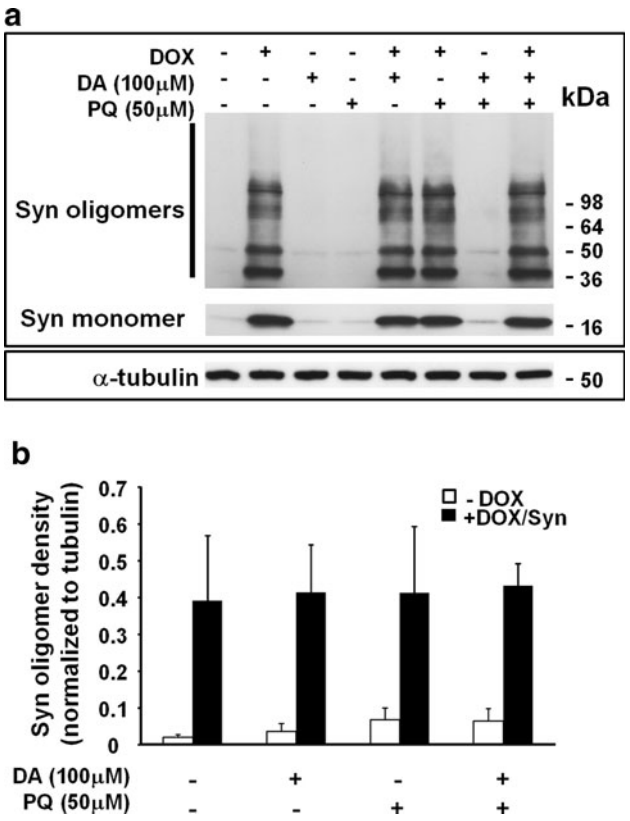


Fig. 5 Western blot analysis of α-synuclein protein levels in treated MN9Dsyn cells. **a** MN9Dsyn cells were treated with (+) and without (−) doxycycline (DOX), dopamine (DA) and paraquat (PQ). Protein lysate samples were subjected to 4–16% SDS-PAGE and immunoblotted for α-synuclein. The same blots were subsequently stripped and reprobed for α-tubulin as a loading control. Representative western blots revealing the presence of monomeric and SDS-resistant α-synuclein (Syn) oligomers (vertical line; $N = 3$/treatment; 20 μg protein/lane). **b** α-Synuclein immunoprotein complexes were quantified by densitometric analysis of western blots and values normalized to α-tubulin. Values are expressed as mean band intensity ± SEM from three samples and analyzed by one-way ANOVA and Tukey HSD post hoc test. There was no statistically significant difference in α-synuclein protein levels among the DOX-treated groups

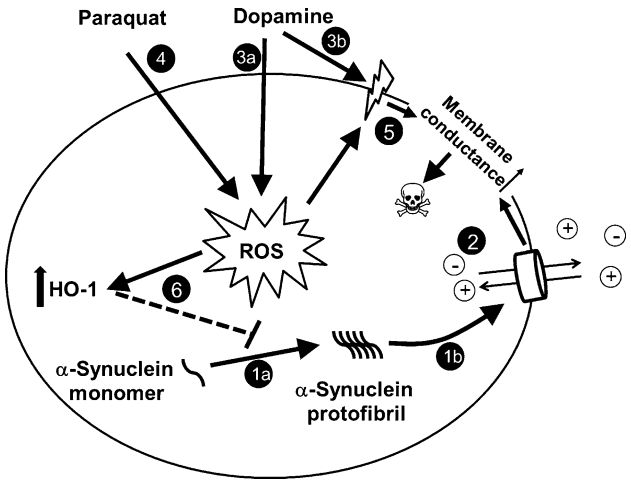


Fig. 6 Hypothesized effect of oxidative stress and α-synuclein on membrane integrity. α-Synuclein induces neuronal toxicity by misfolding into pore-like structures and increasing membrane conductance (1 and 2). Dopamine and paraquat each contribute to elevated levels of oxidative stress by increasing intracellular levels of ROS (3a and 4). Dopamine auto-oxidizes extracellularly leading to free radical production and consequently compromised membrane integrity (3b). Intracellular oxidative stress also increases membrane leakage through oxidation of the lipid membrane (5; lightning bolt). Importantly, combined treatment of neurons with dopamine and paraquat enhances the α-synuclein-induced effects in part by increasing α-synuclein leak channel conductivity leading to a disruption of ionic imbalance, and eventually cell death (1–5). Cells attempt to compensate for the increased oxidative stress through activation of anti-oxidant response mechanisms including upregulation of heme oxygenase-1 (HO-1; 6-*solid line*). HO-1 in turn has been shown to inhibit α-synuclein fibrillization (6-*dotted line*). In our model, this anti-oxidant response is not sufficient to inhibit the combined effects of α-synuclein and oxidative stressors

Protofibrils are generally considered the toxic species, proposed to form annular structures that can function as leak channels (Fig. 6, steps 1b and 2; Duda et al. 2000; Goldberg and Lansbury 2000; Uversky et al. 2001a, b; Lashuel et al. 2002; Caughey and Lansbury 2003; El-Agnaf et al. 2003; Cookson 2005; Uversky 2007; Cookson and van der Brug 2008). Since α-synuclein is ubiquitously expressed throughout the brain, we hypothesize that a micro-environment which promotes α-synuclein-mediated membrane conductance changes may facilitate this protein's toxicity and result in the selective vulnerability associated with this protein (Maroteaux et al. 1988).

The link between the oxidative stress and toxicity induced by α-synuclein is especially relevant in the case of Parkinson's disease which is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta and attendant nigrostriatal projections (Davie, 2008; Hawkes et al. 2009; Lees et al. 2009). An important feature of this population of neurons that has been put forth to explain their selective vulnerability is the presence of the neurotransmitter dopamine and the autonomous pacemaker firing of these neurons, both of which contribute to an increased oxidative environment (Greenamyre and Hastings 2004; Sulzer 2007; Guzman et al. 2010). Dopamine is relatively stable in the low pH vesicular environment where it is normally securely sequestered (Eisenhofer et al. 2004), however, extravesicular dopamine results in rapid oxidation by monoamine oxidase or iron-mediated catalysis producing free radicals and highly reactive quinones which can react with various cellular components including the plasma membrane inciting cell death (Fig. 6, steps 3a and 3b; Maguire-Zeiss et al. 2005; Sulzer 2007; Mosharov et al. 2009). Oxidized dopamine has also been shown to stabilize protofibrillar α-synuclein, which is considered the toxic species, possibly by radical coupling or nucleophilic attack (Conway et al. 1998; LaVoie and

Hastings 1999; Conway et al. 2001; LaVoie et al. 2005; Li et al. 2005; Norris et al. 2005; Bisaglia et al. 2007; Outeiro et al. 2009). Furthermore, both computational modeling and in vitro studies have demonstrated the importance of α-synuclein C-terminal residues including ^{125}YEMPS^{129} in the noncovalent interactions with the aromatic ring in dopamine which result in inhibition of α-synuclein fibrillization leading to stabilization of the protofibrillar form, and these nonspecific hydrophobic interactions are further enhanced by electrostatic interactions with glutamate^{83} in the NAC region of α-synuclein (Mazzulli et al. 2006, 2007; Herrera et al. 2008). The metabolic product of dopamine, DOPAC, at low concentrations also inhibits α-synuclein fibrillization by noncovalent interactions with the N-terminus of α-synuclein (Zhou et al. 2009). Interestingly, one group demonstrated that α-synuclein-induced toxicity requires the presence of dopamine (Xu et al. 2002).

Despite the purported neurotoxic role of dopamine, the initiation of pathogenesis in most Parkinson's disease patients is not likely attributable to dopamine dysregulation but instead a complex event involving multiple factors. For example, exposure to the environmental toxicants including paraquat has long been established as a risk factor for Parkinson's disease (Smith 1985; Hubble et al. 1993; Engel et al. 2001; Lai et al. 2002; McCormack et al. 2002; Liu et al. 2003; Dinis-Oliveira et al. 2006; Migliore and Coppedè 2009a). Paraquat has been shown to enter the CNS via the neutral amino acid transporter, System L, and affect mitochondrial function (McCormack and Di Monte 2003; Cocheme and Murphy 2008). NADPH cytochrome reductases and the mitochondrial complex I (NADH:ubiquinone oxidoreductase) both reduce paraquat into a cation radical PQ^+ which is re-oxidized by cellular diaphorases back to paraquat initiating a toxic chain of redox cycling resulting in the production of superoxide free radicals (Clejan and Cederbaum 1989; Dicker and Cederbaum 1991; Day et al. 1999; Dinis-Oliveira et al. 2006). As a consequence, paraquat has been shown to induce ROS, lipid peroxidation, DNA damage, and cytotoxicity in vitro (Fig. 6, steps 4 and 5; Schmuck et al. 2002; Peng et al. 2004; Dinis-Oliveira et al. 2006; Black et al. 2008; Cocheme and Murphy 2008). Likewise, in vivo, rodents treated with paraquat demonstrate an increase in oxidative stress and substantia nigra dopaminergic neuron vulnerability (Thiruchelvam et al. 2000; McCormack et al. 2002, 2005, 2006; Manning-Bog et al. 2003; Cicchetti et al. 2009; Kang et al. 2010). Other studies have demonstrated the ability of paraquat to increase α-synuclein fibrilization in vitro and aggregation in dopaminergic neurons in vivo (Uversky et al. 2001b, 2002a; Manning-Bog et al. 2002, 2003). Interestingly, in some cases increased α-synuclein aggregation in vivo was accompanied by the absence of nigral degeneration and motor behavioral deficits, while others reported a protective role of α-synuclein overexpression against paraquat toxicity through upregulation of Hsp70 (Manning-Bog et al. 2003; Fernagut et al. 2007; Norris et al. 2007). These discrepancies suggest that the experimental model influences the interaction between the α-synuclein and paraquat. Therefore, the α-synuclein effects on paraquat-induced toxicity may depend on the transgenic mouse model, cell culture model, and/or specific treatment schemes utilized. Because of the multifactorial nature of sporadic PD pathogenesis, a dopaminergic cell line is a useful model that allows us to dissect out components of the complex interactions between genes (α-synuclein) and oxidative insults (dopamine and paraquat). Furthermore, dopamine and paraquat were chosen in our study because of their relevance to oxidative stress in the nigrostriatal pathway.

First we established that our model, MN9Dsyn cells, express the rate-limiting enzyme for catecholamine synthesis, tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter 2, which is consistent with previous studies that demonstrated the ability of the MN9D parental cells to produce, transport, and store dopamine (Choi et al. 1991a; Chen et al. 2005; Zhou et al. 2006; Dong et al. 2008). We also demonstrated an enhancement of α-synuclein-induced toxicity in the presence of both dopamine and paraquat. Similar results were observed when we employed the dopamine precursor, L-DOPA. In this model, we cannot distinguish between the effects of intracellular and extracellular dopamine or L-DOPA. In both cases, we can envision that these extracellularly applied compounds will become oxidatively modified in the media leading to MN9Dsyn membrane disruption. However, the treatment of MN9Dsyn cells with dopamine induced the production of the Nrf2-regulated phase II detoxifying enzyme, heme oxygenase-1 (HO-1) indicating elevated levels of oxidative stress within the cell following exposure to dopamine. Importantly, combined treatment with dopamine and paraquat induced a significant increase in HO-1 expression above the dopamine-mediated increase (Fig. 6, step 6). Consistent with our previous observation, α-synuclein overexpression alone increased the membrane conductance of MN9Dsyn cells compared to non-α-synuclein-overexpressing cells. In this article, we report for the first time that in the presence of enhanced oxidative stress induced by the combined treatment of dopamine and paraquat an augmentation in membrane conductance in α-synuclein-overexpressing MN9Dsyn cells and elevated leak channel conductivity.

In our MN9Dsyn model, α-synuclein overexpression alone engendered the formation of SDS-stable soluble α-synuclein oligomers, but we did not observe a further increase in soluble oligomer levels or cellular aggregates in the presence of oxidative stress despite a robust

augmentation in the membrane conductance. We posit that in our experimental paradigm dopamine, paraquat and α-synuclein have a robust combined endpoint effect, enhanced membrane conductance, but this may occur in the absence of enhanced formation of soluble α-synuclein structures. We envision that extracellular dopamine acts by oxidatively modifying membrane components. It is also probable that a portion of the extracellular dopamine enters the MN9Dsyn cells through the dopamine transporter, and this cytosolic dopamine provides a separate source of free radicals via autooxidation or enzymatic degradation by monoamine oxidase (Fig. 6, step 3; Conway et al. 2001; Cappai et al. 2005; Caudle et al. 2008; Outeiro et al. 2009). Similarly, paraquat enhances the formation of free radicals in the form of superoxides also affecting membrane integrity. We know that paraquat exposure results in an elevated state of oxidative stress and compromised mitochondrial energy production via redox cycling targeting the mitochondrial electron transport chain (Fig. 6, steps 4 and 5; Heller et al. 1984; Day et al. 1999; Macianskiene et al. 2001; Lim et al. 2002; Yumino et al. 2002; McCormack et al. 2005; Cocheme and Murphy 2008; Pamplona 2008). Finally, α-synuclein is localized to the membrane where it also promotes membrane dysfunction cumulatively leading to enhance membrane conductance (Fig. 6, steps 1 and 2; Feng et al. 2010). It is likely that while α-synuclein itself significantly increased membrane conductance, the presence of oxidative stress further compromised a system already challenged by α-synuclein-induced toxicity disrupting membrane integrity beyond the buffering capacity of the system leading to increased cell vulnerability (Fig. 6, steps 1–5). However, we cannot rule out that our methods (western blot analysis of cell lysates and immunocytochemistry) may not be sufficiently sensitive to detect subtle changes in individual α-synuclein conformers which presumably constitute a small percentage of total α-synuclein (Caughey and Lansbury 2003). In addition, HO-1 has been shown to induce proteasomal degradation of α-synuclein, which could in our model prevent oligomer accumulation (Fig. 6, step 6; Song et al. 2008, 2009). Notably, in our paradigm, we demonstrated a significant upregulation of HO-1 in the presence of oxidative stressors, which could account for the stable levels of SDS-resistant α-synuclein oligomers.

Nevertheless, despite the lack of increased soluble oligomeric α-synuclein, we observed increased oxidative stress, cell death, and membrane conductance suggesting that the multiple-hit MN9Dsyn cells have diminished membrane integrity in addition to α-synuclein leak channels. Despite our increasing understanding of Parkinson's disease, the cause of this debilitating disease remains largely unknown. Various genes and epidemiological factors have been associated with sporadic Parkinson's disease, however, no insult or risk factor alone is sufficient to initiate the pathogenic process (Cory-Slechta et al. 2005). The multiple hit hypothesis argues that a combination of stressors including genetic vulnerability and environmental insults together compromise the cellular compensatory mechanisms and converge upon substantia nigra dopamine neuronal cell death (Maguire-Zeiss and Federoff 2003; Carvey et al. 2006; Sulzer 2007; Mosharov et al. 2009). Indeed, patients are exposed to a variety of insults over their entire lifespan and each pathogenic process is undoubtedly an issue of great complexity. For instance, while chronic paraquat exposure may contribute to Parkinson's disease pathogenesis in some patients, many risk factors may come into play for other patients, such as, age, genetic polymorphisms, rural living, well water drinking, heavy metal exposure, or traumatic brain injury (Smith et al. 1992; Hubble et al. 1993; Gorell et al. 1998; Engel et al. 2001; Di Monte et al. 2002; Lai et al. 2002; McCormack et al. 2002; Elbaz et al. 2007; Migliore and Coppedè 2009a; Satake et al. 2009; Simon-Sanchez et al. 2009; Hamza et al. 2010; Tanner 2010). In conclusion, in support of the multiple hit hypothesis for Parkinson's disease our study provides a possible explanation for the oxidative stress-induced cell vulnerability in combination with α-synuclein expression, namely enhanced membrane conductance.

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