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Effect of Mutant α -Synuclein on Dopamine Homeostasis in a New Human Mesencephalic Cell Line*

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Mutations in α -synuclein have been linked to rare, autosomal dominant forms of Parkinson's disease. Despite its ubiquitous expression, mutant α -synuclein primarily leads to the loss of dopamine-producing neurons in the substantia nigra. α -Synuclein is a presynaptic nerve terminal protein of unknown function, although several studies suggest it is important for synaptic plasticity and maintenance. The present study utilized a new human mesencephalic cell line, MESC2.10, to study the effect of A53T mutant α -synuclein on dopamine homeostasis. In addition to expressing markers of mature dopamine neurons, differentiated MESC2.10 cells are electrically active, produce dopamine, and express wild-type human α -synuclein. Lentivirus-induced overexpression of A53T mutant α -synuclein in differentiated MESC2.10 cells resulted in down-regulation of the vesicular dopamine transporter (VMAT2), decreased potassium-induced and increased amphetamine-induced dopamine release, enhanced cytoplasmic dopamine immunofluorescence, and increased intracellular levels of superoxide. These results suggest that mutant α -synuclein leads to an impairment in vesicular dopamine storage and consequent accumulation of dopamine in the cytosol, a pathogenic mechanism that underlies the toxicity of the psychostimulant amphetamine and the parkinsonian neurotoxin 1-methyl-4-phenylpyridinium. Interestingly, cells expressing A53T mutant α -synuclein were resistant to amphetamine-induced toxicity. Because extravesicular, cytoplasmic dopamine can be easily oxidized into reactive oxygen species and other toxic metabolites, mutations in α -synuclein might lead to Parkinson's disease by triggering protracted, low grade dopamine toxicity resulting in terminal degeneration and ultimately cell death.

The main pathological hallmarks of Parkinson's disease (PD)¹ are a striking loss of dopamine (DA)-producing neurons

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¹ The abbreviations used are: PD, Parkinson's disease; DA, dopamine; ROS, reactive oxygen species; TH, tyrosine hydroxylase; DAT, dopamine transporter; VMAT2, vesicular monoamine transporter 2;

in the substantia nigra, causing reduced DA levels in the striatum, and the presence of cytoplasmic inclusions known as Lewy bodies (1, 2). Even though oxidative stress and mitochondrial dysfunction have been implicated in the disease process, the mechanisms underlying nigral cell death in PD are still unknown (3). Most cases of PD are sporadic, but rare, familial forms of the disease do exist. To date, early-onset PD has been linked to mutations in two genes, α -synuclein and *parkin* (4). Autosomal dominant forms of the disease result from missense mutations in α -synuclein, leading to either an alanine to threonine substitution at position 53 (A53T) (5) or to an alanine to proline conversion at amino acid 30 (A30P) (6). Although these mutations are not present in the majority of patients with familial PD, pathogenic mechanisms involved in α -synuclein-mediated DA cell loss may provide important clues about sporadic and familial forms of the disease.

First identified as a component of cholinergic vesicles in the electric ray *Torpedo californica* (7), α -synuclein is a highly conserved, "natively unfolded," 140-amino acid phosphoprotein belonging to a family of closely related members (8). The function of α -synuclein is still unknown, although several studies suggest it plays an important role in synapse maturation and maintenance. α -Synuclein is enriched in presynaptic terminals (9–11) and is expressed rather ubiquitously in the brain, particularly in the neocortex, hippocampus, striatum, thalamus, and cerebellum (12). Its expression is developmentally regulated, redistributing from neuronal cell bodies to synaptic terminals during periods of neuronal differentiation (13, 14). Its expression is up-regulated during periods of synaptic plasticity, *i.e.* song-learning in the zebra finch (10). Lastly, overexpression of A53T mutant human α -synuclein in mice results in massive axonal degeneration of spinal cord motor neurons (15–17).

If α -synuclein plays an essential role in synaptic function, why do its mutant forms primarily lead to the degeneration of nigral DA neurons? This phenomenon could be explained by the cytotoxic potential of DA, a neurotransmitter that readily auto-oxidizes in the presence of iron and can also be metabolically deaminated to yield toxic DA metabolites and reactive

AMPH, D-amphetamine; WT, wild-type; A53TSYN, A53T mutant human α -synuclein; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline; EGF, epidermal growth factor; CMV, cytomegalovirus; PDL, poly-D-lysine; db-cAMP, dibutyryl cyclic AMP; GDNF, glial cell line-derived neurotrophic factor; PVDF, polyvinylidene difluoride; SOD, superoxide dismutase; HPLC-EC, high-performance liquid chromatography coupled to electrochemical detection; GFP, green fluorescent protein; DHE, dihydroethidium; ANOVA, analysis of variance; EPSP, excitatory postsynaptic potential; IPSP, inhibitory postsynaptic potential; m.o.i., multiplicity of infection; 6-OHDA, 6-hydroxydopamine; PLD2, phospholipase D isoform 2.

oxygen species (ROS), e.g. superoxide anions and hydroxyl radicals (18–20). Thus, a failure to properly store DA into synaptic vesicles may lead to abnormal elevations of cytosolic DA followed by generation of cytotoxic DA metabolites and ROS. This could, in turn, lead to oxidative stress, terminal degeneration, and eventually cell death.

The present study examined the possibility that expression of A53T mutant α -synuclein leads to DA cell loss by promoting DA-dependent oxidative stress, a mechanism that has been proposed to underlie the toxicity of both amphetamine and 1-methyl-4-phenylpyridinium (MPP⁺) (21–23). To test the hypothesis that mutant α -synuclein alters DA homeostasis, we first characterized a new human mesencephalic cell line, MESC2.10, and determined it to be a useful model system for studying the function of α -synuclein. In addition to expressing biochemical markers of mature DA neurons, MESC2.10 cells are electrically active, synthesize and release DA, and express wild-type (WT) human α -synuclein. Interestingly, lentiviral-induced expression of A53T mutant in MESC2.10 cells led to changes in DAT and VMAT2 protein levels, DA uptake and release, cytoplasmic DA immunofluorescence, intracellular superoxide production, formation of α -synuclein-positive inclusions, and response to amphetamine (AMPH)-induced toxicity, modifications that suggest alterations in DA function.

EXPERIMENTAL PROCEDURES

Generation of MESC2.10 Cells—Human mesencephalic cells were prepared from 8-week-old human embryonic ventral mesencephalic tissue (Lund University), which was procured in compliance with national laws and regulations, following permission from the Lund University Hospital Ethical Committee. Tissue fragments were maintained at 4 °C for ~72 h in Hanks' balanced salt solution (HBSS) without calcium and magnesium prior to mechanical dissociation in the presence of 3 mg/ml protease 23. Dispersed cells were then rinsed with HBSS containing 1 mg/ml trypsin inhibitor and 1 mg/ml bovine serum albumin prior to plating. Cells were seeded at a density of 5×10^4 cells/cm² in growth medium consisting of DMEM/F-12 and 10% fetal calf serum. Approximately 24 h later, the growth medium was replaced with DMEM/F-12 containing N2 supplement (Invitrogen, Gaithersburg, MD), 40 ng/ml human recombinant basic fibroblast growth factor (bFGF, Roche Molecular Biochemicals, Indianapolis, IN), 40 ng/ml human recombinant epidermal growth factor (EGF, Invitrogen), and 20 ng/ml platelet-derived growth factor A/B (PDGF, Roche Molecular Biochemicals, Indianapolis, IN).

Human mesencephalic cells were immortalized with a LINX v-myc retroviral vector (24). In this system, a tetracycline-controlled transactivator strongly activates transcription from a minimal CMV promoter, which, in turn, drives v-myc expression in the absence of tetracycline. A gene conferring neomycin resistance is also present in the vector allowing selection of v-myc-expressing cells. Mesencephalic cultures were retrovirally infected and G418-selected as previously described (25). After selection, cultures were maintained in N2 medium consisting of DMEM/F-12 high glucose, N2 supplement, 2 mM L-glutamine, 40 ng/ml bFGF, 40 ng/ml EGF, and 20 ng/ml PDGF. Confluent cultures were passaged by trypsin digestion. Clonal cell lines were isolated by two rounds of limited dilution in 96-well plates. Single colonies were expanded and passaged.

Differentiation of Human Mesencephalic Cells—MESC2.10 cells were propagated in N2 medium containing 40 ng/ml bFGF. After reaching confluency, cells were enzymatically dissociated with trypsin followed by trituration. Poly-D-lysine (PDL) pre-coated labware was treated overnight with 5 μ g/ml mouse laminin (dissolved in PBS with Ca²⁺) and washed three times prior to plating. Cells were seeded at a density of 2.5×10^4 cells/well in 8-well chamber slides, 7.5×10^4 cells/well in 24-well plates, or 2.0×10^5 cells/well in 12-well plates depending on the assay conducted. One day after plating, proliferation medium was replaced with N2 medium containing 1 μ g/ml tetracycline, 1 mM dibutyryl cyclic AMP (db-cAMP), and 2 ng/ml glial cell line-derived neurotrophic factor (GDNF, R&D Systems, Minneapolis, MN), heretofore called differentiation medium. Half of this medium was replaced every second day. Clone MESC2.10 was chosen for further characterization, because a high percentage of cells from this line expressed both microtubule-associated protein 2 and tyrosine hydroxylase (TH).

Western Immunoblotting—MESC2.10 cells were plated at a density

of 1×10^6 cells per PDL/laminin-coated P100 dish, allowed to become confluent, and differentiated for 0, 2, 4, or 6 days. Day 0 cells were lysed the same day that differentiation medium was added to prospective day 2–6 dishes. Briefly, cells were rinsed with ice-cold PBS and lysed in hypotonic buffer containing 2 mM EDTA, pH 7.6, 2 mM Hepes, 12 mM N-ethylmaleimide, and protease inhibitors (Sigma mixture). Cells were homogenized by passing them five to six times through a 27-gauge needle. After centrifuging at $800 \times g$ for 10 min at 4 °C, the supernatant was collected and SDS was added to 1% final concentration. An equal volume of 50 μ g of protein (as measured with a Bio-Rad DC protein assay) from day 0–6 samples was heated for 10 min at 55 °C in the presence of Laemmli buffer containing 4% β -mercaptoethanol. Proteins were separated on a 10–20% continuous gradient SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane. Blots were blocked in 5% nonfat milk/PBS with 0.05% Tween 20 and incubated overnight at 4 °C in blocking buffer containing the following primary antibodies: mouse anti-rat TH (1:1000, Chemicon International, Temecula, CA), mouse anti-human β -tubulin isotype III (1:2000, Sigma), mouse anti-human α -synuclein (1:100, Alexis Biochemicals, San Diego, CA), rabbit anti-human vesicular monoamine transporter 2 (VMAT2, 1:500, kind gift from Dr. Robert Edwards), rabbit anti-human DA transporter (DAT before dilution, 1:1000, Alpha Diagnostic International), rabbit anti-cow glial fibrillary acidic protein (before dilution 1:2000, DAKO A/S, Glostrup, Denmark), and rabbit anti-human myc (1:1000, Upstate Biotechnology Inc., Lake Placid, NY). After washing $3 \times$ for 10 min in PBS/Tween 20, blots were incubated with 1:5000 horseradish peroxidase-linked secondary antibodies (from donkey, in blocking buffer) recognizing mouse or rabbit species followed by detection using an ECL Plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ). Day 4 MESC2.10 cells transduced for 48 h with LV-GFP or LV-A53TSYN were lysed as previously described, and immunoblots were probed with antibodies against α -synuclein, DAT, VMAT2, and TH, rabbit anti-human Cu,Zn-superoxide dismutase (SOD, 1:3000, StressGen Biotechnology Corp., Victoria, British Columbia, Canada), and mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (1:300, Chemicon International). Band intensity was quantified using IMAGE software (National Institutes of Health, Bethesda, MD).

Electrophysiology—Electrical activity was measured using the perforated whole-cell configuration of the patch clamp technique, using an EPC-9 amplifier and Pulse software (version 8.50 or later, Heka Elektronik, Lambrecht, Germany). Recording electrodes were made from borosilicate glass capillaries coated with Sylgard close to the tips and fire-polished. The pipette resistance ranged between 3 and 6 megohms when filled with the intracellular solution specified above. The zero-current potential of the pipette was adjusted in the bath prior to gigaseal formation. Cells were continuously superfused with extracellular solution consisting of (in mM) 138 NaCl, 5.6 KCl, 1.2 MgCl₂, 2.6 CaCl₂, 25 D-glucose, and 5 Hepes (pH 7.4 with NaOH) at 32–34 °C. The pipette solution contained (in mM) 76 K₂SO₄, 10 NaCl, 10 KCl, 1 MgCl₂, and 5 HEPES (pH 7.35 with KOH), and 0.24 mg/ml of the pore-forming antibiotic amphotericin B (Sigma Chemical Co., St. Louis, MO).

Measurement of DA Content and Release by HPLC-EC—MESC2.10 cells were plated at a density of 2×10^5 /well in PDL/laminin-coated 12-well plates. After 24 h, cells were exposed to differentiation medium for 0–6 days, lysed in 200 μ l of 0.1 N perchloric acid containing 0.8 mg/ml glutathione by freeze/thawing, and the supernatants were collected for analysis of intracellular DA levels. Samples from day 0, 2, 4, and 6 cultures were frozen at –80 °C and analyzed by high-performance liquid chromatography coupled to electrochemical detection (HPLC-EC). For assays of DA release, day 6 MESC2.10 cells were treated with 60 mM KCl or 50 μ M AMPH for 20 min at 37 °C in 300 μ l of HBSS containing Ca²⁺ and 0.8 mg/ml glutathione. The extracellular medium was collected and filtered through a 0.2- μ m filter. A sample volume of 30 μ l was injected into an ESA Coulochem II electrochemical detector using a YMCaqua C18 column (Schermbek, Germany) set at a potential of 300 mV versus an Ag/AgCl reference electrode. The mobile phase was delivered by an LC 10 AD Shimadzu HPLC pump at 0.5 ml/min and contained 0.051 M NaH₂PO₄·H₂O with 0.92 mM octanesulfonic acid, 48 μ M Na₂EDTA, and 11% methanol, adjusted to pH 3.7 with 1 M phosphoric acid and degassed. Data was acquired using a Shimadzu (chromatographic CLASS LC-10) software package. The analyses were calibrated to a standard of 0.2 μ M DA. Assays were conducted in triplicate and experiments repeated three times.

Construction of Lentiviral Vectors—Mutant (A53T) human α -synuclein (A53TSYN) was isolated from an adeno-associated virus, rAAV-A53TSYN (kindly provided by Nina Rosenqvist, Lund University, Lund, Sweden), by PCR amplification using the following oligonucleotides: 5'-GTC AAG ATC TAT GGA TGT ATT CAT GAA AGG

ACT-3' and 5'-AGT CCT CGA GTT AGG CTT CAG GTT CGT AGT-3'. The pHR/CMV-A53TSYN-WHV construct was generated by ligating this PCR product with a lentiviral backbone obtained by excising GFP from pHR/CMV-GFP-WHV (kindly provided by Dr. Didier Trono, University of Geneva Medical School, Geneva, Switzerland) using *Bam*HI and *Xho*I. These restriction sites were introduced into the flanking sequences of A53TSYN by PCR. The construct was sequenced at the Center for Genomics Research, Karolinska Institute (Stockholm, Sweden) to ensure that no PCR-based mutations were generated.

Virus Production—Lentiviral vectors were produced as previously described (26, 27). Briefly, 293T cells were transiently transfected by a calcium phosphate method with a three-plasmid vector system consisting of: pHR/CMV-GFP-WHV or pHR/CMV-A53TSYN-WHR (transducing vector containing the transgene), pMD.G (vector encoding the envelope protein), and pCMV Δ R8.91 (packaging construct providing all viral proteins needed except for the envelope). All constructs were kindly provided by Dr. D. Trono. The supernatant was collected 2 and 3 days after transfection and ultracentrifuged at $141,000 \times g$ for 1.5 h. The pellet was resuspended in DMEM/10% FCS and frozen at -80°C . The virus titer was determined by infecting 293T cells with serial dilutions of virus and counting the number of GFP or α -synuclein-positive cells (as assessed by immunocytochemistry). Untransduced 293T cells did not express α -synuclein. The dilution resulting in $<30\%$ of GFP or α -synuclein-positive cells was used to calculate transducing units/cell. A multiplicity of infection (m.o.i.) = 10, corresponding to 10 transducing units/cell, was used at all times to infect MESC2.10 cells.

^3H JDA Uptake and Release—MESC2.10 cells were plated at a density of 7.5×10^4 cells/well in PDL/laminin-coated 24-well plates and transduced for 48 h with LV-GFP or LV-A53TSYN starting on day 4. On day 6, cells were rinsed with HBSS containing Ca^{2+} and incubated with $4.8 \mu\text{Ci/ml}$ [$7,8\text{-}^3\text{H}$]DA/HBSS + Ca^{2+} (Amersham Biosciences, Piscataway, NJ) for 20 min at 37°C . To measure intracellular [^3H]DA, the supernatant was removed and cells were lysed with 0.1 N perchloric acid by freeze/thawing. To measure basal [^3H]DA release, [^3H]DA-loaded cells were rinsed extensively with HBSS, and the supernatant was analyzed. For stimulation-induced [^3H]DA release, cells were treated with 60 mM KCl or 50 μM AMPH for 20 min at 37°C and extracellular [^3H]DA measured. All samples were analyzed with a 1214 Rackbeta liquid scintillation counter (LKB Wallac, Turku, Finland). Experiments were conducted in quadruplicate and repeated three times.

Immunocytochemistry—For DA antibody experiments, day 4 MESC2.10 cells were transduced with LV-GFP or LV-A53TSYN for 48 h and exposed on day 6 to 50 μM AMPH for 20, 40, or 60 min at 37°C . After fixation with 4% paraformaldehyde, cells were rinsed in PBS and preincubated with 5% normal goat serum/0.3% Triton-X/PBS for 1 h at room temperature. The following primary antibodies (in Triton X-100/PBS) were then added overnight at 4°C : mouse anti-human β -tubulin isotype III (1:2000, Sigma, St. Louis, MO), rabbit anti-rat TH (1:100, Pel-Freeze, Rogers, AR), mouse anti-human α -synuclein (1:1000, Zymed Laboratories, San Francisco, CA), rabbit anti-DAT (1:500, Alpha Diagnostic International, San Antonio, TX), and mouse anti-DA (1:500, Fitzgerald, Concord, MA). After rinsing $3 \times$ in PBS, cells were incubated in either goat anti-rabbit Alexa 594 or goat anti-mouse Alexa 488 for 1 h at room temperature, rinsed in PBS, and cover-slipped in PVA-DABCO. In some cases, cells were incubated in 1 $\mu\text{g/ml}$ Hoechst 33258 during the second to last wash before mounting. For assessment of α -synuclein-positive inclusions, day 6 MESC2.10 cells overexpressing GFP or A53TSYN were stained with a mouse anti-human α -synuclein antibody (see above). Cells were examined with a Bio-Rad MRC1024 confocal microscope using a $100\times$ objective. For DA antibody experiments, the mean cytoplasmic fluorescence intensity from ≥ 50 cells per experiment was quantified using Bio-Rad LaserSharp software (Hercules, CA).

Dihydroethidium Imaging—Production of ROS was monitored in MESC2.10 cells overexpressing GFP or A53TSYN exposed to AMPH using the superoxide-sensitive fluorophore dihydroethidium (DHE) as previously described (22, 23, 28). MESC2.10 cells were plated at a density of 2.5×10^4 cells/well in PDL/laminin-coated eight-well chamber slides and differentiated for 4 days. Cells were then transduced with LV-GFP or LV-A53TSYN for 48 h. On day 6 transduced MESC2.10 cells were exposed to 50 μM AMPH for 60 min or 2 h followed by 20-min incubation with 3 μM DHE at 37°C . Cells were then fixed with 4% paraformaldehyde and mounted. Fluorescence was measured at excitation = 488 nm and emission = 590 nm on a Bio-Rad MRC1024 confocal microscope using a $60\times$ objective. The mean cytoplasmic fluorescence from ≥ 50 cells/experiment was quantified using LaserSharp software. Experiments were repeated three to five times.

Determination of Cell Viability with Calcein-AM—MESC2.10 cells were plated at a density of 2.5×10^4 cells/well in PDL/laminin-coated

eight-well chamber slides and differentiated for 3 days. On day 3, cells were transduced with LV-GFP or LV-A53TSYN for 24 h and then exposed to 10, 50, or 100 μM AMPH for 48 h. On day 6, cells were incubated with 5 μM Calcein-AM for 20 min at 37°C . Five consecutive images per well were captured with a BMK 800 digital camera (Grundig Electronic, Nürnberg, Germany) using a $10\times$ objective. Calcein-AM-positive cells were counted and normalized to LV-GFP control cultures. Experiments were conducted in duplicate and repeated three to five times.

Statistical Analysis—All parameters are expressed as means \pm S.E. of three to five independent experiments, each treatment performed in three to four wells. The significance of effects between untreated and treated, or between LV-GFP- and LV-A53TSYN-expressing, cells, was determined by one- or two-way factor analysis of variance (ANOVA), as indicated, and post-hoc Dunnett's *t* test using a Statview package (Abacus Concepts, Berkeley, CA).

RESULTS

Differentiated MESC2.10 Express Markers of Mature DA Neurons—Eight-week-old human embryonic mesencephalic cells were immortalized with a retroviral vector containing the *v-myc* oncogene (Fig. 1) (29). In brief, the control elements of a tetracycline-resistance operon were fused to a human CMV promoter directing the expression of *v-myc* (24). In this system, addition of low, non-toxic concentrations of tetracycline abolish transcription activation by a tetracycline-controlled transactivator thus blocking *v-myc* expression (29). Constitutive expression of *v-myc* in the absence of tetracycline enabled MESC2.10 cells to proliferate continuously in culture. Addition of tetracycline in the presence of GDNF, a factor that promotes the survival and morphological differentiation of DA neurons (30), and dibutyryl cyclic AMP (db-cAMP), a known inducer of TH activity (31), arrested proliferation and promoted the differentiation of MESC2.10 cells. After 6 days in differentiation medium, a high percentage of MESC2.10 cells expressed both the microtubule-associated protein 2 and the rate-limiting enzyme in dopamine biosynthesis, tyrosine hydroxylase (TH) (29). Therefore, this clone was chosen for further characterization.

To determine which cell types were present in differentiated MESC2.10 cultures, the expression of various neuronal, astrocytic, and DA markers was assessed by Western immunoblotting and immunocytochemistry. Because microtubules play an essential role in neurite outgrowth, synapse formation, and axonal and dendritic transport, β -tubulin is often used as a marker of immature and mature neurons (31, 32). Immunoreactivity for β -tubulin isotype III was faintly present in lysates from undifferentiated MESC2.10 cultures and was up-regulated when cells were exposed to differentiation medium, reaching robust levels at days 4 and 6 (Fig. 2A). Similarly, day 6 cultures immunoprocessed with an antibody against β -III-tubulin exhibited long, branched processes characteristic of mature neurons. Double staining with the nuclear marker Hoechst 33258 showed that at least 80% of Day 6 cells were neurons (Fig. 2B). On the other hand, the astrocytic marker glial fibrillary acidic protein was modestly expressed in undifferentiated MESC2.10 cultures and was down-regulated in response to differentiation (Fig. 2A). Like β -III-tubulin, expression of α -synuclein was barely detected at day 0 by Western immunoblotting but increased markedly by day 6. This antigen was also readily detected by immunocytochemistry and was localized to the cell body and to a lesser extent to neurites (see Fig. 5C).

To assess the presence of a DA phenotype, blots were also probed for antigens expressed in mature DA neurons. Although completely absent in undifferentiated cells, TH was highly expressed at days 4 and 6. Double immunocytochemistry for TH and Hoechst 33258 revealed that $>80\%$ of day 6 MESC2.10 cells were TH-positive (see Fig. 4A). TH was expressed both in the cell soma and neuritic processes. Like TH, the plasma

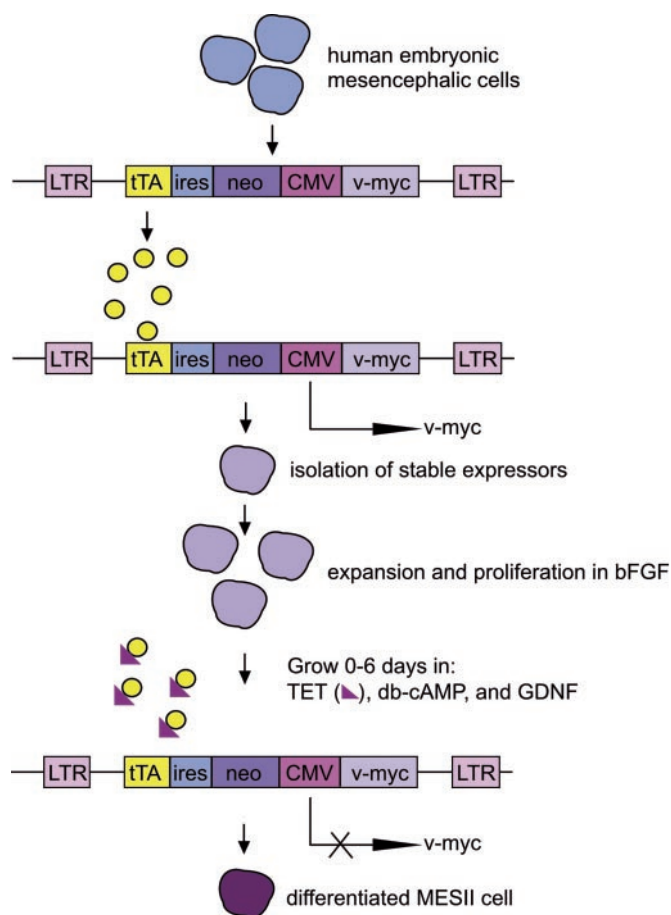


FIG. 1. Generation of MESC2.10 cells. First trimester human mesencephalic cells were dissociated and plated at a density of 5×10^4 cells/cm² in growth medium consisting of DMEM/F-12 and 10% fetal calf serum. Approximately 24 h later, the growth medium was replaced (see “Experimental Procedures”) and cells retrovirally infected with a LINX v-myc vector. In this system, a tetracycline-controlled transactivator (*tTA*, yellow circles) strongly activates transcription from a minimal CMV promoter in the absence of tetracycline. A gene conferring neomycin resistance is also present in the vector. Cells expressing myc were G418-selected in N2 medium consisting of DMEM/F-12 high glucose, N2 supplement, 2 mM L-glutamine, 40 ng/ml bFGF, 40 ng/ml EGF, and 20 ng/ml PDGF. Clonal cell lines were isolated by two rounds of limited dilution, and single colonies were expanded and passaged. Constitutive expression of v-myc in the absence of tetracycline allowed MESC2.10 cells to proliferate continuously in culture. Replacement with N2 medium containing 1 μ g/ml tetracycline (purple triangle) abolished transcription activation by tTA and blocked the production of v-myc. In addition to tetracycline, N2 medium also contained 1 mM db-cAMP and 2 ng/ml GDNF, factors that promoted the differentiation of MESC2.10 cells into DA-producing neurons (see “Results”).

membrane-bound DAT was absent in lysates from undifferentiated cultures but was present at day 2, 2 days before TH expression could be detected. Its robust expression did not change by day 6. Morphologically, DAT expression resembled the uniform pattern of TH expression (Fig. 4B). In contrast, expression of the vesicular monoamine transporter (VMAT2), which mediates vesicular storage of DA, was modestly expressed at day 0 and did not change in response to differentiation. As expected, tetracycline suppressed transcription activation of the CMV promoter and inhibited the production of myc, which became barely detectable at day 6 (Fig. 2A).

MESC2.10 Cells Are Electrically Active—The presence of neuronal (e.g. β -III-tubulin) and synaptic (e.g. α -synuclein) markers in Day 6 cultures suggested that differentiated MESC2.10 cells behave as functional neurons. To assess the electrophysiological properties of these cells, we applied the

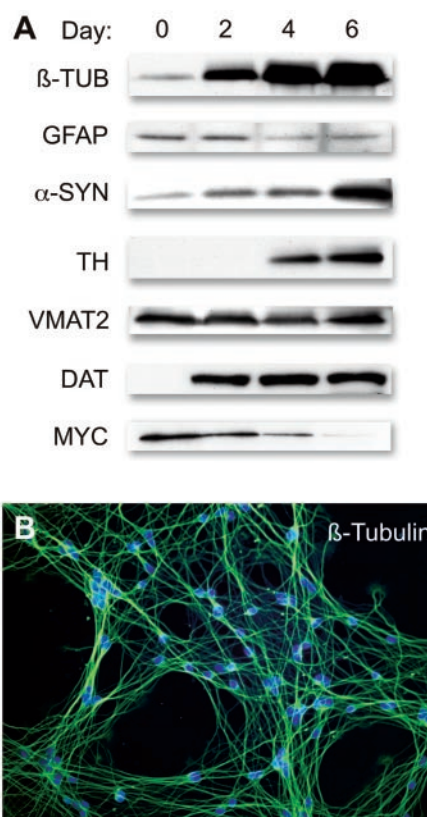


FIG. 2. Differentiated MESC2.10 cultures display markers of mature DA neurons. A, MESC2.10 cells were differentiated for 0–6 days in the absence of bFGF and in the presence of tetracycline, db-cAMP, and GDNF. Cultures were then lysed, and the expression of various proteins was assayed by Western immunoblotting. Briefly, equal amounts of protein were separated in 10–20% continuous gradient SDS-PAGE gels, transferred onto polyvinylidene difluoride membranes, and probed with antibodies against neuronal, astrocytic, synaptic, DA, and proliferative markers as listed under “Experimental Procedures.” Western blots for each marker were repeated three to five times. B, MESC2.10 cultures that had been differentiated for 6 days were fixed and immunoprocessed with an antibody recognizing anti-human β -tubulin isotype III (shown in green), a marker of immature and mature neurons. Cultures were co-stained with the nuclear dye Hoechst 33258 (shown in blue). Cells were examined by confocal microscopy using a 20 \times objective.

perforated whole-cell configuration of the patch-clamp technique to obtain membrane potential recordings from Day 6 MESC2.10 cells. 6 out of 16 studied cells showed spontaneous action potentials (Fig. 3A) that originated from a resting potential of -59 ± 2 mV and peaked at -12 ± 4 mV. The majority of the recorded cells (10 of 16) further displayed upward deflections from the resting potential consistent with excitatory postsynaptic potentials (EPSPs) and some (4 of 16) cells had inhibitory postsynaptic potentials (IPSPs) as well (Fig. 3B), indicating functional synapses. In several cases, EPSPs occurred close enough in time for summation (Fig. 3C).

MESC2.10 Cells Synthesize and Release DA upon Stimulation—To determine whether MESC2.10 cultures synthesize DA, intracellular DA levels were measured by HPLC-EC. Briefly, day 0–6 cultures were lysed with 0.1 N perchloric acid/0.8 mg/ml glutathione by freeze/thawing, and DA levels were analyzed by HPLC-EC. Intracellular DA content increased progressively in response to differentiation and was 9-fold higher in day 6 MESC2.10 cultures than in day 0 cultures (Fig. 4C). Our calculations revealed intracellular DA content to be 16.1 ± 0.1 pmol/TH-positive cell at day 6. We then tested whether day 6 MESC2.10 cells could release DA in response to high potassium stimulation. Exposure to 60 mM KCl for 20 min promoted

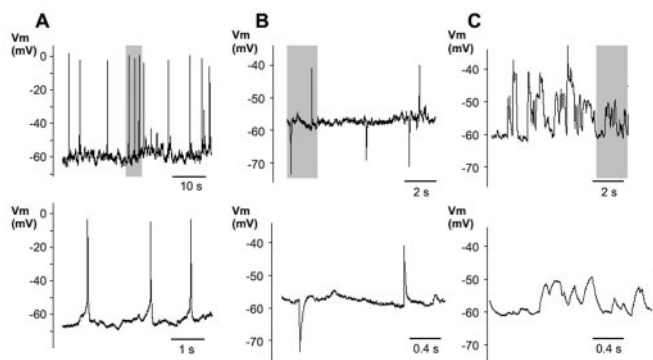


FIG. 3. MESC2.10 cells are electrically active. A–C, recordings of the membrane potential obtained in the perforated whole-cell configuration. A, typical action potentials; B, well resolved excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs); C, an example of the summation of EPSPs. The lower panels show the segments highlighted in gray on an expanded time scale.

an 8-fold increase in extracellular DA as measured by HPLC-EC, suggesting that the exocytic machinery of DA-synthesizing MESC2.10 cells is fully functional (Fig. 4D). This was also corroborated by patch-clamp capacitance measurements (data not shown). In addition to displaying exocytic release, differentiated MESC2.10 cells also extruded DA by reverse transport. The weak base psychostimulant AMPH has a high affinity for the DAT and is actively transported into DA neurons where it triggers the non-exocytic release of DA (33–36). Within the cell, AMPH blocks DA transport into vesicles by dissipating the transmembrane pH gradient, which provides the driving force for VMAT2-mediated uptake of DA (37). This leads to the accumulation of cytoplasmic DA that is either newly synthesized or has been taken up from the extracellular space. An increase in cytosolic DA concentration, in turn, promotes reverse transport of the neurotransmitter through the DAT, a phenomenon known as DA overflow (28, 38, 39). Therefore, AMPH can be used to measure non-exocytic, transporter-mediated DA release. Exposure of day 6 MESC2.10 cells to 50 μ M AMPH for 20 min elicited a 9-fold increase in DA release, suggesting that both DAT-mediated uptake and release in MESC2.10 cells is functionally intact (Fig. 4D).

Lentiviral-induced Overexpression of A53T Mutant α -Synuclein Leads to Changes in DA Homeostasis—The use of HIV-based recombinant retroviruses has proven to be a highly effective method for transferring foreign DNA into non-dividing and terminally differentiated cells. Due to its karyophilic properties, the lentiviral pre-integration complex allows viral sequences to be readily recognized by the nuclear transport machinery (40). Lentiviral vectors mediate efficient delivery, integration, and sustained long term expression into post-mitotic cells such as adult neurons (41, 42). In addition, deletion of $\geq 60\%$ of the viral genome, including all non-essential accessory proteins, has dramatically increased the biosafety of these retroviruses (43).

The goal of this study was to use a lentiviral transduction system to introduce A53TSYN into differentiated MESC2.10 cells to examine the effect of the mutant protein on intracellular DA homeostasis. The efficiency of lentiviral transfer was first tested by transducing undifferentiated and differentiated MESC2.10 cells with a lentivirus expressing the reporter green fluorescent protein (LV-GFP). Increasing dilutions of virus were used to determine the minimum m.o.i. needed to transduce $>80\%$ of MESC2.10 cells. Although LV-GFP used at an m.o.i. = 10 effectively transduced $>80\%$ of day 6 MESC2.10 cells, it did not infect undifferentiated MESC2.10 cells. The majority of LV-GFP-transduced cells showed high, uniform

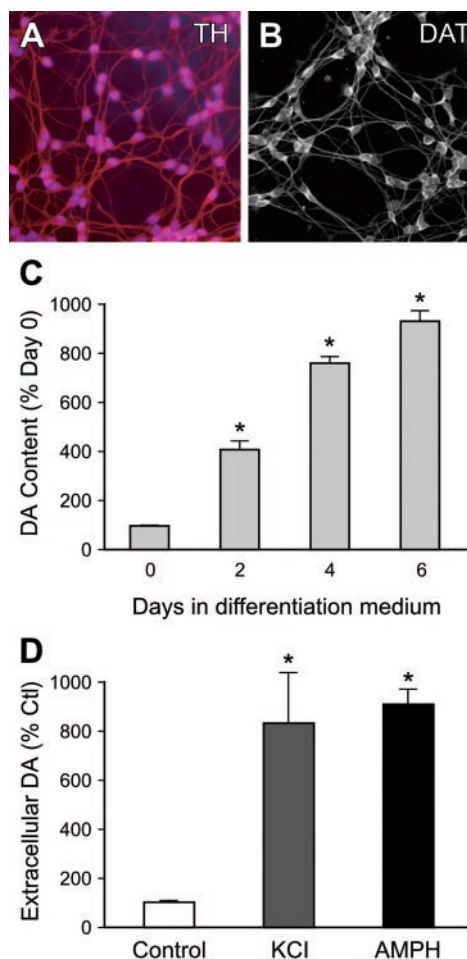


FIG. 4. Differentiated MESC2.10 cells synthesize and release DA. A, MESC2.10 cultures were differentiated for 6 days, fixed, and immunoprocessed with a rabbit antibody against TH (shown in red). Cells were co-stained with Hoechst 33258 to visualize nuclei, which appear pink due to co-localization with TH. B, Day 6 cultures were stained with a rabbit antibody recognizing DAT. Confocal images were taken with a 20 \times objective. C, MESC2.10 cultures differentiated for 0–6 days were lysed in 0.1 N perchloric with glutathione by freeze/thawing and the extracellular fluid collected for HPLC-EC analysis of DA. Values are expressed as a percentage of day 0. D, Day 6 MESC2.10 cells were exposed to 60 mM KCl or 50 μ M AMPH in HBSS/Ca²⁺ for 20 min at 37 $^{\circ}$ C, and the levels of extracellular DA were measured by HPLC-EC. Values are expressed as percent increase over control. All experiments were conducted in quadruplicate. The graphs represent the mean \pm S.E. of three independent experiments. *, $p < 0.01$ compared with day 0 (one-way ANOVA with post-hoc Dunnett's *t* test).

expression of GFP (arrowhead, Fig. 5A), although some displayed a more punctate pattern of fluorescence (arrow, Fig. 5A). Even though GFP was detected both in the cell body and in cellular processes resembling axons, a few cells expressed GFP primarily in the cell soma.

Having established that a lentiviral strategy can be used effectively to introduce foreign genes into differentiated MESC2.10 cells, a lentiviral vector containing human A53TSYN (LV-A53TSYN) was generated. In this study, we chose to concentrate on the effects of A53TSYN expression on DA homeostasis in human mesencephalic neurons. It can be argued that overexpression of WT human α -synuclein on MESC2.10 cells would have provided unequivocal proof that the effects seen in this study were indeed due to a pathogenic effect of the mutant protein and not to the overexpression of α -synuclein itself. In addition, it would have been helpful to compare all parameters in mutant α -synuclein-expressing cells, untransduced cells, and cells infected with an empty

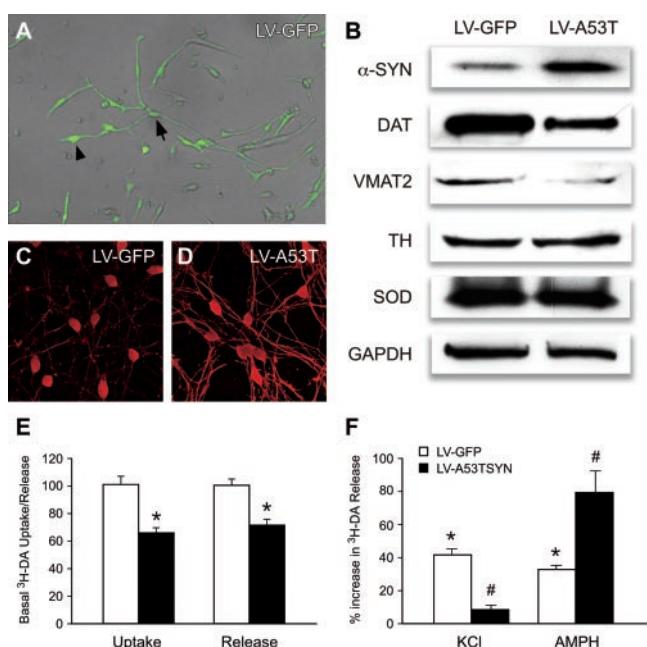


FIG. 5. Lentivirus-mediated overexpression of A53T mutant α -synuclein. *A*, day 6 MESC2.10 cells were transduced with a lentivirus overexpressing GFP for 48 h. Endogenous GFP fluorescence was visualized using a $20\times$ objective. Cells with strong, uniform GFP expression (arrowhead) and cells with lesser, granular expression (arrow) can be observed. *B*, Day 4 MESC2.10 cultures were transduced with LV-GFP or LV-A53T for 48 h and lysed, and the expression of different markers was assessed by Western immunoblotting. Briefly, proteins were separated in 10–20% continuous gradient SDS-PAGE gels, transferred onto polyvinylidene difluoride membranes, and probed with antibodies against α -synuclein, DAT, VMAT2, and TH; Cu,Zn-SOD; and glyceraldehyde-3-phosphate dehydrogenase, which was used to ensure that equal amounts of protein were loaded onto the gel. Blots are representative of three independent experiments. *C*, Day 4 MESC2.10 cells were transduced with LV-GFP or *D*) LV-A53T for 48 h and immunoprocessed with a mouse antibody against human α -synuclein (red). Confocal images were taken with a $40\times$ objective. *E*, Day 4 MESC2.10 cultures were transduced with LV-GFP or LV-A53T for 48 h, and basal [^3H]DA uptake and release were measured. Cells were loaded with [^3H]DA for 20 min at 37°C and rinsed extensively with HBSS/ Ca^{2+} . [^3H]DA uptake was then measured by lysing the cells immediately with 0.1 N perchloric acid/glutathione by freeze/thawing. Basal [^3H]DA release was measured by incubating cells in HBSS/ Ca^{2+} for 20 min at 37°C and measuring [^3H]DA in the supernatant. *F*, extracellular [^3H]DA release was induced by a 20-min exposure to 60 mM KCl or 50 μM AMPH. The supernatant was collected, and [^3H]DA was measured. All experiments were conducted in quadruplicate. Values are expressed as a percentage of LV-GFP control cells. The graphs represent the mean \pm S.E. of three independent experiments. *, $p < 0.01$ compared with LV-GFP control (paired t test). #, $p < 0.01$ compared with LV-A53T control (paired t test).

lentiviral vector. However, because we chose to examine a very large number of physiological parameters and MESC2.10 already express high levels of WT α -synuclein, we focused our efforts on studying the effects of A53TSYN expression. However, to control for the possible toxic effects of the lentiviral vector and of protein overexpression, we exposed control cultures to a lentivirus containing GFP, a reporter protein that is unlikely to have an effect on DA homeostasis. Transduction with LV-GFP led to a slight decrease in cell viability after 48 h (percentage of Calcein-AM-positive cells was $81.5 \pm 8.4\%$ compared with $100.0 \pm 2.6\%$ in untransduced cultures). Cell survival was not significantly different in cell cultures expressing A53TSYN ($74.3 \pm 8.1\%$ cell viability).

Unlike most cell lines and primary culture cells currently used to study the pathogenicity of mutant α -synuclein (e.g. PC12, N27, B103, BE (2)M17, and HEK293 cell lines, and rat

primary mesencephalic cells), differentiated MESC2.10 cells express high levels of WT human α -synuclein (Figs. 2A, 5B, C). Therefore we chose not to overexpress WT human α -synuclein in control cultures, even though the possibility that overexpression of the WT protein itself leads to pathogenicity cannot be excluded. Indeed, a recent study showed that adenoviral-induced overexpression of WT human α -synuclein in primary human mesencephalic cultures increased the rate of apoptosis in transduced DA neurons by 2-fold (44). In the present study, we used an m.o.i. that resulted in levels of A53TSYN that were only twice as high as those of endogenous WT α -synuclein to minimize a potential competitive effect between both mutant and WT proteins (Fig. 5B). Indeed, this 2-fold overexpression suggests that the ratio of mutant to WT protein is 1:1, as would be expected to occur in autosomal dominant forms of PD wherein one copy of the gene is mutant.

To examine the expression pattern of α -synuclein in differentiated MESC2.10 cells, Day 4 cells infected with either LV-GFP or LV-A53T for 48 h were immunoprocessed with an antibody recognizing human α -synuclein. As shown in Fig. 5C, endogenous expression of WT α -synuclein was localized primarily to cell bodies in a pattern resembling that of early postnatal substantia nigra pars compacta neurons from rodents and humans (14, 45). Weak staining was also detected in the nucleus and cellular processes (Fig. 5C). MESC2.10 cells transduced with A53TSYN showed a dramatic increase in the neuritic expression of α -synuclein, although intense staining was also detected in the cell body (Fig. 5D). No gross abnormalities in cell shape, size, or neurite length were detected although a “thickening” of processes was consistently seen (Fig. 5D). Expression of α -synuclein in LV-A53TSYN-transduced cultures was confirmed by Western blot analysis, which showed a 2-fold increase in α -synuclein compared with the level of α -synuclein in LV-GFP-transduced cultures (Fig. 5B).

The effect of A53TSYN on the DA system of MESC2.10 cells was determined by studying three parameters: 1) changes in the expression of different DA markers by Western analysis, 2) [^3H]DA uptake and release, and 3) changes in intracellular DA immunofluorescence. In all cases, MESC2.10 cells were transduced with LV-GFP or LV-A53TSYN for 48 h. Expression of A53TSYN led to a 50% decrease in the levels of both DAT and VMAT2 when compared with cells transduced with LV-GFP (Fig. 5B). Interestingly, the expression of TH remained unchanged (Fig. 5B). Consistent with this decrease in DAT expression, cells expressing A53TSYN showed a $34.7 \pm 4.4\%$ decrease in [^3H]DA uptake (Fig. 5E). We observed an equivalent decline in the spontaneous release of [^3H]DA into the culture medium in cells expressing A53TSYN ($28.4 \pm 3.7\%$), which could have resulted from a reduced capacity of MESC2.10 cells to initially take up [^3H]DA (Fig. 5E).

Next, MESC2.10 cells overexpressing A53TSYN were examined for their ability to release DA in response to stimulation. Briefly, day 6 MESC2.10 cells transduced with either LV-GFP or LV-A53TSYN were loaded with [^3H]DA for 20 min and then exposed to either 60 mM KCl or 50 μM AMPH. Whereas MESC2.10 cells expressing GFP showed a $40.2 \pm 4.8\%$ increase in [^3H]DA release during to high potassium stimulation, MESC2.10 cells expressing A53TSYN did not release [^3H]DA in response to depolarization (Fig. 5F). However, they displayed an increased capacity to extrude [^3H]DA in response to AMPH compared with GFP-expressing cells ($79.0 \pm 13.4\%$ versus $31.7 \pm 2.6\%$, Fig. 5F). Because AMPH promotes transporter-mediated release of cytoplasmic DA, an increase in AMPH-induced release coupled to a decrease in exocytic release suggests that vesicular sequestration of [^3H]DA into synaptic vesicles is impaired in cells expressing A53TSYN. This could be

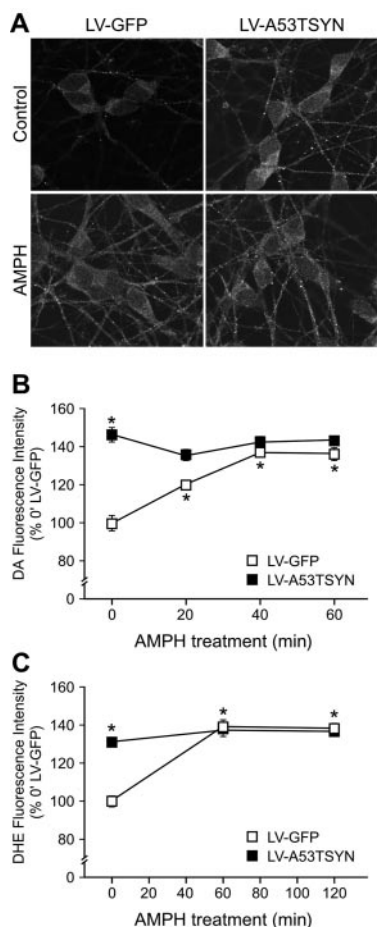


FIG. 6. MESC2.10 cells expressing A53T mutant α -synuclein have enhanced basal levels of cytoplasmic DA and superoxide. A, MESC2.10 cells were differentiated for 4 days and then transduced with either LV-GFP or LV-A53TSYN for 48 h. On day 6, cells were exposed to 50 μ M AMPH for 20, 40, or 60 min, fixed, and immunoprocessed with a mouse antibody against DA. Confocal images, which were taken with a 60 \times objective, show DA-stained cultures exposed to AMPH for 60 min. B, the cytoplasmic DA fluorescence from ≥ 50 cells was measured per time point and is expressed as a percentage of 0 min for LV-GFP- and LV-A53TSYN-transduced cells. The graphs denote the mean \pm S.E. of three independent experiments. C, same as in A except that transduced cells were exposed to 50 μ M AMPH for 1 or 2 h. Cells were then incubated with 3 μ M DHE, a superoxide-sensitive fluorophore, for 20 min at 37 $^{\circ}$ C and fixed with 4% paraformaldehyde for 30 min at 37 $^{\circ}$ C. Cytoplasmic DHE fluorescence was examined by confocal microscopy using a 60 \times objective. The fluorescence of ≥ 50 cells was measured per time point and is expressed as a percentage of 0 min for LV-GFP- and LV-A53TSYN-transduced cells. The graphs denote the mean \pm S.E. *, $p < 0.01$ compared with LV-GFP 0 min (two-way ANOVA with post-hoc Dunnett's t test).

due to either a defect in transmitter uptake or to a depletion of synaptic vesicles available for storage.

To examine the effect of A53TSYN on intracellular DA distribution, we measured cytoplasmic DA immunofluorescence using an antibody against DA. DA immunofluorescence studies have been used by others to reliably detect changes in intracellular DA following exposure to DA-enhancing or -depleting compounds (e.g. Ref. 46). Day 6 MESC2.10 cells expressing GFP showed a granular pattern of DA immunofluorescence mainly localized to the cell body (Fig. 6A). On the other hand, MESC2.10 cells expressing A53TSYN showed increased levels of DA immunofluorescence both in the cell soma and in neurites, with bright granules of DA often seen along axons and in the extracellular space (Fig. 6A). Quantification of cytoplasmic DA immunofluorescence by confocal microscopy revealed a $46.8 \pm 4.1\%$ increase in DA fluorescence intensity in MESC2.10

cells expressing A53TSYN compared with cells expressing GFP (Fig. 6B). This increase in cytoplasmic DA fluorescence is consistent with the impaired ability of A53TSYN-expressing cells to release [3 H]DA in response to depolarization and again indicates a redistribution of vesicular DA to the cytosol (Fig. 5F).

Next, we examined the effect of A53TSYN on MESC2.10 cells exposed to a DA-redistributing dose of AMPH. GFP-expressing cells treated with 50 μ M AMPH exhibited an increase in cytoplasmic DA immunofluorescence, which reached a plateau after 40 min of drug exposure (Fig. 6B, $37.8 \pm 3.1\%$ compared with untreated cells). The pattern of fluorescence resembled that of cells expressing A53TSYN. Interestingly, cells expressing the mutant protein did not display an increase in somal DA immunofluorescence in response to AMPH. In fact, fluorescence intensity decreased slightly in response to the psychostimulant (Fig. 6B), consistent with the greater ability of these cells to extrude DA through the DAT (Fig. 5F). MESC2.10 cells expressing A53TSYN did not exhibit an increase in DA immunofluorescence even after prolonged exposure to AMPH ($144.3 \pm 3.3\%$ versus $146.8 \pm 4.1\%$ in untreated cells after 2 h).

Due to its unstable catechol ring, cytoplasmic DA can be rapidly oxidized to yield both ROS-like superoxide anion as well as reactive DA metabolites like DA-quinone (18–20). Thus, to determine whether increased cytoplasmic DA leads to enhanced intracellular production of ROS in MESC2.10 cells expressing A53TSYN, we monitored both LV-GFP- and LV-A53TSYN-transduced cells with the redox-sensitive fluorophore DHE. Because DHE is oxidized into fluorescent ethidium by superoxide anions, changes in DHE fluorescence can be used as an index of superoxide formation (47–49). Day 6 MESC2.10 cells expressing A53TSYN exhibited $31.0 \pm 3.3\%$ higher levels of basal DHE fluorescence than cells expressing GFP, suggesting that elevations in cytoplasmic DA result in increased production of superoxide radicals (Fig. 6C). Because the emission spectrum of DHE is >590 nm and GFP emits at 509 nm, GFP fluorescence did not overlap with our DHE measurements (this was confirmed by looking at GFP-expressing cells through a DHE filter).

Given that AMPH-induced distribution of vesicular DA into the cytoplasm can promote free radical formation (28, 50), coupled to the observation that MESC2.10 cells appear to be under a heightened state of oxidative stress, we questioned whether AMPH-induced superoxide formation was enhanced by expression of A53TSYN. Whereas MESC2.10 cells expressing GFP exhibited a $39.1 \pm 3.6\%$ increase in DHE fluorescence in response to a 60-min exposure to 50 μ M AMPH, no changes were observed in response to A53TSYN expression, even after 2 h of drug exposure (Fig. 6C). Although quite surprising, these results suggest that MESC2.10 cells expressing A53TSYN are better able to deal with an AMPH challenge than cells expressing the WT protein. To test the possibility that A53TSYN-expressing cells scavenge DA-derived superoxide radicals more effectively than GFP-expressing cells when treated with AMPH, we examined the expression of cytoplasmic superoxide dismutase (Cu,Zn-SOD) in both types of cultures by Western immunoblotting. Protein levels of Cu,Zn-SOD in MESC2.10 cells expressing A53TSYN did not differ from those of GFP-expressing cells (Fig. 5B).

Expression of A53TSYN Confers Resistance to AMPH—Because MESC2.10 cells expressing A53TSYN exhibited higher basal levels of cytoplasmic DA and increased DHE fluorescence compared with cells expressing GFP, we questioned if they would be more sensitive to AMPH-induced toxicity. Thus, Day 3 MESC2.10 cells were exposed to LV-GFP or LV-A53TSYN for 24 h and then treated with 10–100 μ M AMPH for 2 days in the continuing presence of virus. Because $\geq 80\%$ of cells in Day 6

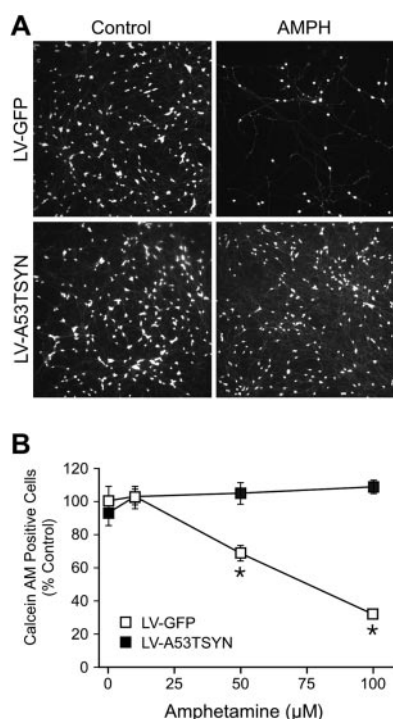


FIG. 7. MESC2.10 cells expressing A53T mutant α -synuclein are resistant to AMPH toxicity. *A*, Day 3 MESC2.10 cells were exposed to LV-GFP or LV-A53TSYN for 24 h and then treated with 10–100 μM AMPH for 2 days in the presence of virus. The *right panels* show cells exposed to 100 μM AMPH. On day 6, cell viability was assayed by incubating cells with 5 μM Calcein-AM for 20 min at 37 $^{\circ}\text{C}$. Cells were visualized with a 10 \times objective. *B*, the total number of Calcein-AM-positive cells from five consecutive fields was counted per well. All data are expressed as a percentage of LV-GFP control. The *graphs* denote the mean \pm S.E. of three independent experiments. *, $p < 0.01$ compared with LV-GFP control (two-way ANOVA with post-hoc Dunnett's *t* test).

cultures expressed TH, we assessed the viability using Calcein-AM, a membrane-permeable dye that is hydrolyzed in healthy cells by endogenous esterases into a membrane-impermeable fluorescent product. Transduction with LV-A53TSYN for 72 h did not lead to a significant decrease in cell viability compared with cells transduced with LV-GFP (Fig. 7*B*). Exposure of GFP-expressing cells to AMPH led to dose-dependent cell death, resulting in a $68.4 \pm 2.9\%$ loss of viability in cultures treated with 100 μM AMPH (Fig. 7, *A* and *B*). Surprisingly, MESC2.10 cells expressing A53TSYN were completely resistant to AMPH-induced toxicity (Fig. 7, *A* and *B*). Cell viability in LV-A53TSYN-transduced cultures treated with 100 μM AMPH was $91.5 \pm 4.3\%$ compared with $92.8 \pm 8.1\%$ in untreated cultures (Fig. 7*B*).

Lastly, because the appearance of protein aggregates has been inversely correlated to cell death both in PD and in Huntington's disease (51, 52), we looked for the presence of α -synuclein-positive inclusions in MESC2.10 cells expressing A53TSYN. We observed a dramatic increase in the number of MESC2.10 cells containing both cytoplasmic and intranuclear inclusions, the proportion of which was equal, in cultures transduced with LV-A53TSYN compared with cultures transduced with LV-GFP ($39.1 \pm 2.3\%$ versus $4.3 \pm 0.7\%$; Fig. 8, *A* and *B*). Moreover, a 2-h exposure to AMPH significantly increased the number of cells displaying α -synuclein-positive inclusions in LV-GFP and LV-A53TSYN-transduced cultures to a similar degree (Fig. 8*B*). The formation of intracellular aggregates in A53TSYN-expressing cells could contribute to their resistance to AMPH-induced toxicity.

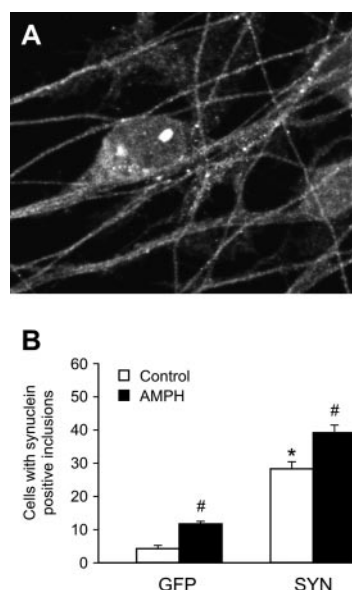


FIG. 8. A53T mutant α -synuclein leads to inclusion formation. *A*, MESC2.10 cells were differentiated for 4 days and then transduced with either LV-GFP or LV-A53TSYN for 48 h. On day 6, cells were exposed to 50 μM AMPH for 2 h, fixed, and immunoprocessed with a mouse antibody recognizing human α -synuclein. Shown is a confocal image taken with a 100 \times objective depicting both cytoplasmic and nuclear aggregates. *B*, the number of α -synuclein-positive inclusions from five consecutive fields was counted using a 60 \times objective. All data are expressed as a percentage of LV-GFP control. The *graphs* denote the mean \pm S.E. of three independent experiments. *, $p < 0.01$ compared with LV-GFP control (one-way ANOVA with post-hoc Dunnett's *t* test). #, $p < 0.01$ compared with LV-A53TSYN control (one-way ANOVA with post-hoc Dunnett's *t* test).

DISCUSSION

Linkage of α -synuclein mutations to rare, autosomal dominant forms of PD (5, 6) has spurred a great interest in the mechanisms by which mutant α -synuclein leads to PD. Unfortunately, transgenic mice overexpressing WT or mutant α -synuclein do not reproduce the pathology of PD, and *in vitro* studies examining the effects of WT and mutant α -synuclein overexpression have yielded conflicting results. These discrepancies could be explained, in part, by the presence of a threonine instead of an alanine at position 53 in WT rodent α -synuclein or by fundamental differences between the *in vitro* systems studied and human nigral dopaminergic neurons. Therefore, we chose to study the effect of mutant α -synuclein in a new human mesencephalic cell line, MESC2.10. In this relevant model system, we tested the hypothesis that mutant α -synuclein leads to oxidative stress in nigral neurons by altering intracellular DA homeostasis.

Even though mice overexpressing WT human α -synuclein under the direction of the PDGF- β promoter exhibit a loss of striatal TH-immunoreactive terminals, they do not display reduced numbers of nigral TH-immunoreactive neurons nor fibrillar α -synuclein-positive inclusions like those found in PD (47, 48). Moreover, transgenic mice expressing WT and mutant α -synuclein under the direction of the mouse prion promoter show no pathology in the substantia nigra despite abundant expression of the transgene in this region (16, 17). Targeted overexpression of either A53T or A30P mutant human α -synuclein in nigral neurons does not lead to inclusion formation, DA depletion, cell loss (55), or increased sensitivity to MPTP toxicity (56) in aged mice. *In vitro*, overexpression of WT human α -synuclein in cultured cells has yielded contradicting results. Depending on the cell type studied, WT human α -synuclein increases (44, 57), decreases (58), or does not affect

(59) naturally occurring apoptosis. In most of these studies, rodent or peripheral human cell lines that do not express human α -synuclein were used. Thus, to study the effect of its mutant form, WT α -synuclein had to be introduced as a control. The incongruity of these findings could therefore be attributed to the differing cellular backgrounds in which WT human α -synuclein was overexpressed. In our case, A53T mutant human α -synuclein was introduced into a human mesencephalic cell line (Fig. 1) that exhibits properties of mature dopaminergic neurons (Fig. 2), providing an excellent model system for studying the pathogenicity of this protein. Because our cells already expressed human α -synuclein (Fig. 5, B and C), we chose not to introduce the WT protein. However, we cannot exclude the possibility that overexpression of the WT protein would have resulted in DA-related toxicity, as suggested by a recent study (44).

In most cases, overexpression of A53T mutant α -synuclein increases sensitivity to toxin-induced cell death. For instance, it exacerbates susceptibility of HEK293 and SH-SY5Y cells to DA toxicity (60, 61), of primary DA neurons and N27 cells to 6-OHDA-induced apoptosis (59), and of SH-SY5Y cells to MPP⁺-mediated cell death (62). In contrast to these studies, MESC2.10 cells overexpressing A53TSYN were resistant to AMPH-induced toxicity (Fig. 7), a form of cell death thought to result from intracellular, DA-dependent oxidative stress (28, 50). Although the effect of WT or mutant α -synuclein on AMPH-induced toxicity has not been tested by other groups, recent studies suggest that cells overexpressing mutant forms of this protein are more sensitive to oxidative damage following addition of DA (60, 61) and 6-OHDA (59). The apparent discrepancy may be explained by high concentrations of applied DA and 6-OHDA acting as exogenous oxidant stressors not requiring the participation of endogenous transmitter stores or by differences in the cell types studied. In other words, application of exogenous dopamine may lead to both extracellular and intracellular formation of ROS, whereas AMPH primarily leads to the latter. Furthermore, we tested AMPH-induced toxicity in human mesencephalic neurons, while other groups measured DA- and 6-OHDA-mediated toxicity in either non-dopaminergic cells (e.g. HEK293) (54) or rat mesencephalic cells (53). Studies by Zhou and colleagues (44, 59) demonstrate that the pathogenicity of A53TSYN is not only dependent on the cell type used but on the species in which the mutant protein is expressed. For instance, although adenovirus-mediated overexpression of A53T mutant human α -synuclein in rat primary mesencephalic cultures led to a $\geq 60\%$ decrease in TH cell viability compared with cultures transduced with GFP (59), human primary mesencephalic cultures transduced with the same vector displayed only a $\leq 20\%$ loss of TH-positive cells (44).

Due to its unstable catechol ring, DA is readily oxidized in the cytoplasm into hydrogen peroxide, superoxide, and various DA metabolites (18). Intracellular accumulation of these toxic species can lead to detrimental cellular effects, including increased cytoplasmic calcium and protein and lipid damage (63). MESC2.10 cells overexpressing A53TSYN displayed 31% higher levels of superoxide compared with cells expressing GFP (Fig. 6C). Using the redox-sensitive dye dichlorofluorescein, Junn and Mouradian (61) also reported an increase in ROS production in SY-SH5Y cells overexpressing the mutant α -synuclein. One potential source of superoxide is unstored DA. Indeed, AMPH-induced elevations in somal DA immunofluorescence preceded increases in DHE fluorescence suggesting that superoxide radicals arose from the oxidation of cytoplasmic DA (Fig. 6, B and C). Furthermore, MESC2.10 cells expressing A53TSYN displayed a significant increase in basal DA immunofluorescence in the cell soma (Fig. 6, A and B) and an

increase in AMPH-stimulated DA release (Fig. 5F), which suggests that cells expressing the mutant protein are unable to properly store DA into synaptic vesicles.

A gradual increase in oxidative stress may have triggered adaptive mechanisms in MESC2.10 cells that made them resistant to AMPH-induced toxicity. Indeed, repeated administration of AMPH to rats leads to an adaptive up-regulation of various antioxidant enzymes (64). To test whether exposure to mild oxidative stress during the hours following transduction with LV-A53TSYN led to a compensatory increase in antioxidant capacity, expression of the cytoplasmic superoxide scavenger Cu,Zn-SOD was assessed in MESC2.10 cells transduced with LV-A53TSYN for 48 h. No changes in Cu,Zn-SOD expression were observed in comparison to cells expressing GFP (Fig. 5B). This does not exclude the possibility that other antioxidant enzymes may have been up-regulated. In addition, resistance to oxidative stressors could be mediated by other factors, including the redox-sensitive transcription factor NF- κ B (65). Interestingly, a similar resistance phenomenon has been observed during ischemic pre-conditioning, a condition whereby neurons exposed to sublethal cerebral ischemia become resistant to a subsequent lethal ischemic insult (66). In this case, enhanced mitochondrial calcium sequestration and/or calcium efflux is thought to be an important compensatory mechanism (67).

One possibility is that A53T mutant α -synuclein leads to an abnormal accumulation of cytoplasmic DA by reducing the number of vesicles in which neurotransmitter can be stored. Indeed, MESC2.10 cells expressing A53TSYN displayed a decrease in the levels of VMAT2, a marker of monoaminergic vesicles (Fig. 5B) and a reduction in depolarization-induced exocytotic DA release (Fig. 5F). The latter defect has also been observed in PC12 cells overexpressing the mutant protein (68). Even though α -synuclein binds to vesicles both *in vivo* (69) and *in vitro* (70), the A53T mutation does not affect its ability to bind to these structures (71, 72). Interestingly, α -synuclein is a potent inhibitor of phospholipase D isoform 2 (PLD2) (73), an enzyme that catalyzes the hydrolysis of phosphatidylcholine into choline and phosphatidic acid (74). *In vivo*, α -synuclein could modulate synaptic vesicle recycling via its regulation of PLD2 (75). This enzyme has been implicated in vesicle formation near the plasma membrane, given the ability of its product, phosphatidic acid, to recruit adaptor proteins and clathrin to membrane sites of vesicle budding (76). The possible role of α -synuclein in synaptic vesicle recycling is supported by a seminal study showing that suppression of WT α -synuclein by antisense oligonucleotides decreases the number of synaptic vesicles in the reserve pool (77).

Another mechanism whereby mutant α -synuclein could increase cytoplasmic DA might be through permeabilization of synaptic vesicles. Protofibrils of WT α -synuclein have been shown to bind and permeabilize phospholipid vesicles in a pore-like fashion, an effect that is enhanced by the A30P and A53T mutations (78, 79). In contrast, fibrillar α -synuclein oligomers are believed to be less cytotoxic (78), consistent with recent claims that fibrillar protein aggregates are harmless to cells (80). In addition, α -synuclein protofibrils appear to be stabilized by DA (81); increases in cytoplasmic DA could therefore promote further vesicle permeabilization. Alternatively, DA-induced oxidative damage of WT α -synuclein could also alter its ability to regulate PLD2 and worsen a mutant α -synuclein-mediated impairment in vesicle recycling.

A high proportion of MESC2.10 cells expressing A53TSYN exhibited aggregates immunoreactive for α -synuclein (Fig. 8). AMPH treatment also caused an increase in α -synuclein-positive inclusions (Fig. 8). Stressors such as ferric iron (82) or nitrating agents (83) promote α -synuclein aggregation, and the

increase in α -synuclein-immunoreactive aggregates observed in both cells overexpressing A53T α SYN and GFP exposed to AMPH could result from cytoplasmic DA-derived ROS. Interestingly, not only is α -synuclein a major component of Lewy bodies (84), but mutant α -synuclein is more prone to self-aggregation than the WT protein (85, 86). Because surviving nigral neurons in PD-containing Lewy bodies appear to display less apoptotic features than neighboring Lewy body-free neurons (51), it has been suggested that these cytoplasmic aggregates might actually protect DA neurons from cell death. A similar phenomenon has been observed in the striata of early grade Huntington's disease patients, where a much higher proportion of spared interneurons than dying projection neurons display perikaryal huntingtin aggregates (52). Similarly, in a cell culture model of Huntington's disease, blockade of inclusion formation leads to decreased cell survival (87), and in a transgenic mouse model of the disease the number of striatal inclusions correlated with resistance to excitotoxin-induced striatal damage (88). Therefore, protein aggregates may indicate the activation of mechanisms that, at least temporarily, protect against cell death in slow neurodegenerative diseases.

In conclusion, the present study suggests that α -synuclein may play an important role in DA homeostasis by regulating neurotransmitter sequestration into synaptic vesicles. Our results demonstrate that expression of A53T mutant α -synuclein in a human mesencephalic cell line leads to an AMPH-like redistribution of DA from vesicular stores to the cytoplasm, which is accompanied by an increase in ROS. Additional studies are needed to assess whether overexpression of the WT protein can also disrupt DA homeostasis and to clarify whether mutant α -synuclein impairs DA storage by curtailing the formation of new synaptic vesicles or by interfering with neurotransmitter uptake. Moreover, it would be interesting to examine whether the protective mechanism observed in this study is gradually overrun so DA neurons expressing A53T mutant α -synuclein eventually succumb to disrupted DA metabolism, as may be the case in PD.

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Addendum—A recent report by Xu *et al.* (89) shows that depletion of DA by a TH inhibitor blocked apoptosis induced by overexpression of WT and mutant α -synuclein in primary human mesencephalic cultures. This study supports the role of dopamine in the pathogenicity of mutant α -synuclein, although overexpression of the WT protein had a similar effect.

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