

A Blood-Brain Barrier (BBB) Disrupter Is Also a Potent α -Synuclein (α -syn) Aggregation Inhibitor

A NOVEL DUAL MECHANISM OF MANNITOL FOR THE TREATMENT OF PARKINSON DISEASE (PD)*

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Background: α -syn aggregation is a main pathology of PD.

Results: Mannitol interferes with α -syn aggregation *in vitro* and *in vivo*, whereas no adverse effects were observed in control animals.

Conclusion: In addition to its BBB-disrupting properties, mannitol, a chemical chaperon, may serve as a potential drug.

Significance: mannitol may serve as a basis for a dual mechanism therapeutic agent for treating PD.

The development of disease-modifying therapy for Parkinson disease has been a main drug development challenge, including the need to deliver the therapeutic agents to the brain. Here, we examined the ability of mannitol to interfere with the aggregation process of α -synuclein *in vitro* and *in vivo* in addition to its blood-brain barrier-disrupting properties. Using *in vitro* studies, we demonstrated the effect of mannitol on α -synuclein aggregation. Although low concentration of mannitol inhibited the formation of fibrils, high concentration significantly decreased the formation of tetramers and high molecular weight oligomers and shifted the secondary structure of α -synuclein from α -helical to a different structure, suggesting alternative potential pathways for aggregation. When administered to a Parkinson *Drosophila* model, mannitol dramatically corrected its behavioral defects and reduced the amount of α -synuclein aggregates in the brains of treated flies. In the mThy1-human α -synuclein transgenic mouse model, a decrease in α -synuclein accumulation was detected in several brain regions following treatment, suggesting that mannitol promotes α -synuclein clearance in the cell bodies. It appears that mannitol has a general neuroprotective effect in the transgenic treated mice, which includes the dopaminergic system. We therefore suggest mannitol as a basis for a dual mechanism therapeutic agent for the treatment of Parkinson disease.

An important challenge in the treatment of many neurodegenerative disorders, such as Parkinson disease (PD),⁴ is the

need to deliver therapeutic agents to the brain through the blood-brain barrier (BBB), despite its poor permeability to drugs (1, 2). Several approaches are in use, including local invasive delivery (3), local exposure to high intensity focused ultrasound, and permeability enhancement (4). The latter can be achieved for example by hyperosmotic solutions, such as mannitol, which induce osmotic opening of the BBB (5). Mannitol, a 6-carbon polyol, is a cell impairment, nonmetabolized Food and Drug Administration (FDA)-approved osmotic diuretic agent (6). In addition to its BBB-disrupting properties, hyperosmotic mannitol therapy is widely used in the clinic (7–9).

Here, we examined the ability of mannitol to serve as a therapeutic agent for PD. PD is a neurodegenerative illness with no current disease-modifying therapy (10). The neuropathological hallmarks are progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SN) accompanied by inclusions termed Lewy bodies and dystrophic Lewy neuritis in surviving neurons (11). The main constituent of the Lewy bodies is the α -synuclein protein (α -syn) (12, 13). α -syn is a protein whose dysfunction has been associated with several neurodegenerative diseases in addition to PD (14, 15).

Increasing evidence indicates that the majority of neurodegenerative disorders involve abnormal aggregation and deposition of specific misfolded proteins in inclusion bodies, such as α -syn in Lewy bodies (16, 17). Therefore, it has been suggested that aggregation of these proteins is pathogenic. The etiology of these proteins is thought to involve major conformational changes leading to their misfolding followed by production of β -sheet structures that have a strong tendency to aggregate into small oligomers and protofibrils that elongate into mature fibrils (18). Reducing or inhibiting the neurotoxic amyloido-

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⁴ The abbreviations used are: PD, Parkinson disease; α -syn, α -synuclein; BBB, blood-brain barrier; SN, substantia nigra pars compacta; ThT, thioflavin T;

TEM, transmission electron microscopy; Bis-Tris, 2-(bis(2-hydroxyethyl)-amino)-2-(hydroxymethyl)propane-1,3-diol; tg, transgenic; mThy1, murine Thy1; GFAP, glial fibrillary acidic protein; TH, tyrosine hydroxylase; ANOVA, analysis of variance; BG, basal ganglia.

Mannitol, a BBB Disrupter, Inhibits α -syn Aggregation

genic aggregation is intensively studied as a potential therapeutic approach for neurodegenerative disorders.

Several osmolytes such as polyols (including mannitol), trimethylamines such as trimethylamine *N*-oxide, and amino acids, which accumulate under stress conditions, were shown to have a very potent effect on the stabilization of protein structure (19–21). Such compounds are termed “chemical chaperones” due to the similarity of their effect to that achieved by molecular chaperones. Thus, chemical chaperones may aid in reducing neurotoxic protein aggregation in amyloidogenic diseases (22).

Usually, *in vitro* studies of protein folding are performed in diluted solutions. However, in the cellular context, proteins are exposed to a very crowded environment. In recent years, new evidence has indicated that molecular crowding can influence protein folding stability and aggregation propensity (23, 24). Chemical chaperones are often regarded as crowders, and their stabilizing capability is attributed to their crowding effect due to the high concentrations used.

In this present work, we demonstrate that mannitol inhibits the aggregation of α -syn monomers into fibrils *in vitro*. Interestingly, circular dichroism (CD) analysis indicated that this chaperone affects the structure of α -syn oligomers, and perhaps directs them to an alternative pathway of aggregation, which remains to be elucidated. To begin examining its efficacy *in vivo*, we fed mannitol to PD model *Drosophila* flies, which express a highly aggregative variant of α -syn (A53T) in their nervous system. We found that mannitol dramatically corrected their behavioral defects. This was subsequently corroborated *in vivo* in PD model mice (25).

EXPERIMENTAL PROCEDURES

Expression and Purification of α -syn— α -syn was expressed in pT7-7 BL21 *Escherichia coli* bacteria as described by Volles and Lansbury (26). Briefly, bacterial cultures were grown to a logarithmic phase for 3 h. The bacterial pellet was resuspended in TEN buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 150 mM NaCl) and frozen at -80°C until purification using a non-chromatographic method. For purification, the frozen samples were boiled and centrifuged. The supernatant was removed to a fresh tube, and streptomycin sulfate (136 μl of 10% solution/ml of supernatant) and acetic acid (glacial, 228 μl /ml of supernatant) were added followed by additional centrifugation for 2 min. The supernatant was removed again and then precipitated with ammonium sulfate (saturated ammonium sulfate at 4°C was used 1:1 v/v with supernatant). The precipitated protein was collected by centrifugation, and the pellet was washed once with 1 ml of 50% ammonium sulfate solution (4°C ; 1:1 v/v saturated ammonium sulfate (4°C):water). The washed pellet was resuspended in 900 μl of 100 mM ammonium acetate (to form a cloudy solution) and precipitated by adding an equal volume of 100% ethanol at room temperature. Ethanol precipitation was repeated once more followed by a final resuspension in 100 mM ammonium acetate, overnight dialysis to water at 4°C , freezing in liquid nitrogen, and lyophilization.

ThT Fluorescence Assay— α -syn was dissolved to a final concentration of 100 μM in 100 mM Tris buffer (pH 7.4). To begin the fibrillation process, the protein was first filtered through a

100-kDa Centricon. Although monomers and some dimers pass the filter, high molecular weight oligomers are retained on the upper side of the membrane. The monomeric protein was immediately mixed with or without increasing concentrations of mannitol. The samples were incubated at 37°C with vigorous agitation as described by Tsigelny *et al.* (27) to allow the formation of amyloid fibrils. The rate of fibrillation was monitored twice a day using thioflavin T (ThT) fluorescence assay (excitation at 450 nm, 2.5-nm slit, and emission at 480 nm, 5-nm slit). ThT was added to a 500-fold diluted sample, and fluorescence was measured using a Jobin Yvon Horiba FluoroMax 3 fluorometer. Mannitol fluorescence was measured as control and subtracted from the test samples.

Transmission Electron Microscopy—Samples (10 μl) of α -syn incubated in the presence or absent of mannitol were taken at the end of the ThT fluorescence assay and placed on 400-mesh copper grids covered with carbon-stabilized Formvar film (SPI Supplies, West Chester, PA). After 1.5 min, excess fluid was removed, and the grids were negatively stained with 10 μl of 2% uranyl acetate solution for 2 min. Finally, excess fluid was removed, and the samples were viewed by a JEOL 1200EX electron microscope operating at 80 kV.

Determination of Soluble Oligomer Formation—To examine the inhibitory effect of mannitol on the early stages of α -syn aggregation, monomeric α -syn was dissolved to a final concentration of 100 μM in 100 mM Tris buffer (pH 7.4) and was immediately mixed with increasing concentrations of mannitol, as described above for the ThT assay. After the samples were agitated at 37°C for several days, 10 μl of the samples were centrifuged at 13,000 rpm for 10 min, and the supernatant (soluble fraction) was collected and mixed with native loading buffer without β -mercaptoethanol or boiling. The samples were analyzed using Western blot with anti α -syn antibody, diluted 1:1,000 (Sc-7011-R, Santa Cruz Biotechnology) in 5% milk in TBS (0.3% Tween) followed by rabbit anti-mouse IgG (Fc-specific)-HRP-conjugated antibody diluted 1:5,000 in 5% milk diluted in TBS (0.3% Tween). Blots were developed after thorough TBS (0.3% Tween) washes, using an enhanced chemiluminescence system (ECL) according to the manufacturer's manual.

CD Analysis—To analyze the secondary structure of soluble and insoluble α -syn aggregates, 100 μM α -syn samples from the ThT fluorescence assay were taken at the end of the experiment (after several days of incubation) and centrifuged at 10,000 rpm for 10 min to separate soluble oligomers from the insoluble fibrils. The insoluble precipitates were resuspended in 100 mM Tris (pH = 7.4). Samples were placed in a 0.1-mm cuvette without dilution, and CD spectra in the range of 190–250 nm were recorded on a Chirascan spectrometer. Background was subtracted from the CD spectra.

Fly Keeping—Flies were reared on standard cornmeal-molasses medium and were kept at 25°C . As *Drosophila* females can store sperm cells in their bodies, crosses were conducted using virgin females collected no longer than 8 h after eclosion at 25°C or 18 h after eclosion at 18°C . The crosses were performed at 29°C . Adult offspring (F1) from the crosses were collected up to 9 days after the beginning of their eclosion at 25°C to avoid offspring from the next generation (F2).

Fly Crossing—Male flies carrying the pan-neuronal driver *elav^{c155}-Gal4* on their X chromosome were crossed to females carrying the UAS-regulated A53T α -syn transgene located on their X chromosome (a kind gift from Prof. Mel Feany, Harvard Medical School). This resulted in first generation (F1) female offspring expressing A53T α -syn in their nervous system, which served as our PD fly model. In parallel, male flies carrying the driver *elav^{c155}-Gal4* on their X chromosome were crossed to wild type (Oregon-R) females and the resultant F1, carrying only the *elav^{c155}-Gal4* driver, served as control. The crosses were performed at 29 °C, and F1 offspring were kept at 25 °C.

Special Fly Feeding—Mannitol was added to standard molasses medium at a concentration of 75 mM. The vials were kept at 4 °C until use. Crosses were performed either on regular *Drosophila* medium (control) or on medium supplemented with mannitol. Animals fed on the appropriate medium from the beginning of the larval stage onwards during adult life. After eclosion, adult offspring were transferred into a fresh vial containing regular *Drosophila* medium on top of which a solution of 0.75 mg/ml peptide was dripped every other day.

Locomotion (Climbing) Assay—Vials of each of the following four F1 classes: 1) females expressing A53T α -syn reared on regular medium; 2) females expressing A53T α -syn reared on medium supplemented with mannitol; 3) control females carrying only the *elav^{c155}-Gal4* driver, reared on regular medium; and 4) control females carrying only the *elav^{c155}-Gal4* driver, reared on medium supplemented with mannitol, each containing 10 flies, were tapped gently on the table and were allowed to climb for 18 s. The percentage of flies that climbed about 1 cm from the bottom of the test tube during that time period was then calculated. Each class had four independent vial repeats. The climbing ability of the offspring flies was monitored for 27 days.

Statistical Analysis—*p* values were calculated for comparison of female flies expressing A53T α -syn reared on regular medium with female flies expressing A53T α -syn on medium supplemented with mannitol and with control flies using one-tailed ANOVA test. *p* < 0.05 was considered significant.

Immunostaining of Adult Fly Brains—Fourteen-day-old adult flies were dissected, and their brains were removed. After incubation in paraformaldehyde, the whole brains were transferred to formic acid and blocked with 0.5% Triton in 5% BSA. Following an overnight incubation with anti α -syn antibody (Santa Cruz Biotechnology) diluted 1:200, the brains were stained with Cy5-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). After being thoroughly washed with PBS, cells were mounted using ProLong antifade (Invitrogen). Images were taken with LSM510 confocal microscope (Zeiss).

Transgenic α -Synuclein Mouse Model and Administration of Mannitol—This study was conducted in heterozygous tg mice overexpressing human wild-type α -syn under the murine Thy-1 promoter (mThy1- α -syn/Line 61) (25). This well characterized tg model was selected because of its noted behavioral deficits in tests of sensorimotor function. The robustness of these behavioral deficits is preserved across frequent rounds of repeated testing (28, 29). These mice also display abnormal accumulation of detergent-insoluble α -syn and develop α -syn-immunoreactive inclusion-like structures in the brain.

Although some nuclear staining has been observed in this model, distinct cytoplasmic inclusion-like structures have been consistently identified by confocal and electron microscopy (25, 30). The mThy1- α -syn mice were created and are maintained on a hybrid C57BL/6-DBA/2 background (25). Animals were maintained on this background by mating N5 females hemizygous for the transgene with male wild-type (WT) mice on the hybrid background obtained from Charles River Laboratories, Inc (28, 29). For these experiments, 16-week-old mice were utilized. mThy1- α -syn tg mice and non-tg age-matched littermates received daily intraperitoneal injections with mannitol (1 g/kg) (*n* = 8 mice per group) or saline as vehicle (*n* = 8 mice per group) for 4 weeks.

Mice Tissue Processing—Following National Institutes of Health guidelines for the humane treatment of animals, under anesthesia, mice were sacrificed, and brains were removed. The right hemibrain was immersion-fixed in 4% paraformaldehyde in PBS, pH 7.4, and serially sectioned at 40 μ m with a vibratome (Leica, Deerfield, IL) for subsequent analysis of neurodegeneration. The left hemibrain was kept at -80 °C for biochemical analysis.

Immunocytochemical and Neuropathological Analysis of tg Mice—To investigate the effects of mannitol administration in mThy1- α -syn tg mice, 40- μ m vibratome sections were immunolabeled overnight with antibodies against the neuronal marker, NeuN (1:1000, Chemicon), the astroglial marker, glial fibrillary acidic protein (GFAP, 1:500, Millipore), the dopaminergic marker tyrosine hydroxylase (TH; 1:200; Chemicon), murine HSP-70 (Santa Cruz Biotechnology), and human α -syn (1:1000; Chemicon) followed by incubation with species-appropriate secondary antibodies (1:2000, Vector Laboratories). Sections were transferred to SuperFrost slides (Fisher Scientific) and mounted under glass coverslips with anti-fading medium (Vector Laboratories). The immunolabeled blind-coded sections were analyzed with the digital bright field microscope (B54, Olympus). Stereological analysis of NeuN immunoreactive cells in the hippocampus and neocortex was conducted with the Stereo-Investigator software (MBF Biosciences). Images were collected according to the Optical Disector method and expressed as cells per unit volume. TH, GFAP, and α -syn immunoreactivity levels were assessed by obtaining optical density measurements using the Image Quant 1.43 program (National Institutes of Health) and corrected against background levels; this is presented as optical density where the background signal has been subtracted from the optical density measurements.

Western Blot Analysis of α -syn Immunoreactivity in tg Mice—Samples from left hemibrain were fractionated by ultracentrifugation into cytosolic and insoluble fractions. Twenty micrograms of total protein per mouse were loaded onto 4–12% Bis-Tris (Invitrogen) SDS-PAGE gels and transferred onto Immobilon membranes, washed, blocked in BSA, and then incubated with antibodies against total human α -syn (1:1000, Chemicon) or murine HSP-70 (Santa Cruz Biotechnology). After an overnight incubation with the primary antibodies, membranes were incubated in the appropriate secondary antibodies, reacted with ECL, and developed on a VersaDoc gel-

Mannitol, a BBB Disrupter, Inhibits α -syn Aggregation

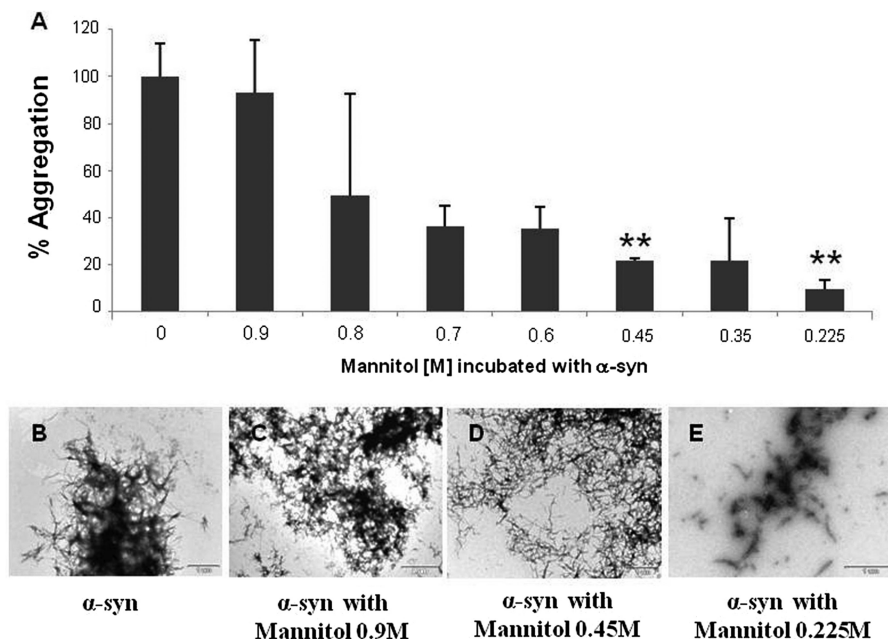


FIGURE 1. *In vitro* analysis of α -syn fibrillation. The inhibition of α -syn fibrillation in the presence or absence of mannitol was detected. A, after 6 days of incubation at 37 °C with shaking, ThT analysis was performed for all samples, and the formation of fibrils was quantified. A reversed dose-dependent inhibition was noted in the presence of different concentrations of mannitol with 0.225 M as the most effective inhibitory dose. For 0.225 and 0.45 M mannitol, **, $p < 0.005$. B–E, TEM images of α -syn fibrils in the presence of several mannitol concentrations: α -syn alone, α -syn with 0.9 M mannitol, α -syn with 0.45 M mannitol, α -syn with 0.225 M mannitol. Scale bars of B, D, and E = 1 μ m. Scale bar of C = 2 μ m.

imaging machine (Bio-Rad). Anti- β -actin (1:1000; Sigma) antibody was used to confirm equal loading.

RESULTS

Mannitol Inhibition of α -syn Fibril Formation as Determined by ThT Analysis—To determine and quantify the ability of mannitol to interfere with α -syn fibril formation, we used ThT binding analysis. Various concentrations of mannitol were incubated with α -syn at 37 °C for several days while shaking. The fibrillation process was monitored after 6 days of incubation. Fig. 1A demonstrates that although the formation of amyloid fibrils was significantly reduced in the presence of low concentrations of mannitol, a high concentration of 0.9 M mannitol did not have any significant inhibitory effect on the amount of fibrils formed. The IC_{50} of mannitol inhibition was determined as ~ 0.8 M.

Mannitol Reduction of α -syn Aggregation as Determined by TEM Analysis—Transmission electron microscopy (TEM) analysis was performed on the samples of three selected concentrations of α -syn incubated with and without mannitol from the ThT experiment (presented in Fig. 1A). In agreement with the ThT analysis, although the fibrils formed by α -syn alone were large and mature (Fig. 1B), only short or no fibrils were detected in the presence of 0.225 M mannitol (Fig. 1C). In the presence of 0.45 M mannitol, fibrils were detected, but at a lower amount and intensity than observed without mannitol (Fig. 1D). In the presence of 0.9 M mannitol, no reduction in the fibrils formed was visible. When incubated with preformed fibrils, mannitol did not dissolve the fibrils *in vitro*.

Mannitol Inhibits α -syn Oligomer Formation—To examine the ability of mannitol to inhibit the early stages of α -syn aggregation, soluble fractions of α -syn were collected after incuba-

tion with and without mannitol at different concentrations. The reaction mixtures were separated by SDS-PAGE followed by Western blot analysis using a specific anti α -syn antibody (Santa Cruz Biotechnology) (Fig. 2). Unlike the inhibitory effect of 0.45 and 0.225 M mannitol on the fibrillation process, in these concentrations, mannitol did not prevent formation of high molecular weight oligomers. Furthermore, 0.225 M mannitol increased the oligomerization process. In comparison, 0.9 M mannitol significantly decreased the formation of tetramers and high molecular weight oligomers, suggesting different potential pathways for α -syn oligomer and fibril formation.

Mannitol Affects Secondary Structure of α -syn Aggregates—Samples containing α -syn aggregates formed in the presence or absence of mannitol were analyzed using CD to gain information about the secondary structural changes occurring within the fibrils (Fig. 3A) and oligomers (Fig. 3B) caused by mannitol. Samples obtained after 6 days of incubation at 37 °C with shaking were centrifuged. The supernatant, containing the soluble oligomers, was removed, and the pellet, containing the insoluble fibrils, was resuspended in an equal volume. Because the concentration of the samples after centrifugation was not identical due to the different amount of oligomers and fibrils in each tube, we could not refer to the amplitude of the peak but only to its shift on the horizontal wavelength axis. Naive α -syn fibrils exhibited a strong positive peak around 195 nm and a negative peak around 220 nm, indicating a β -sheet conformation. When assembled in the presence of mannitol, no clear shift in the peak of the fibrils was notable (Fig. 3A). In comparison, naive α -syn oligomers exhibited a positive peak around 190 nm and two negative peaks around 210 and 230 nm, indicating α -helix conformation. When assembled in the presence of mannitol, a shift

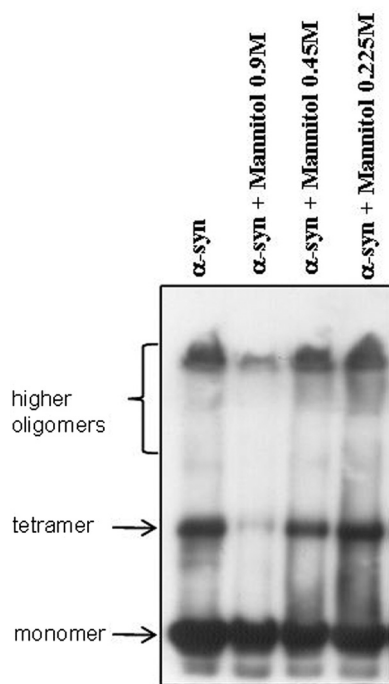


FIGURE 2. **Inhibition of α -syn oligomerization by mannitol.** Monomeric α -syn was incubated with mannitol in different concentrations for 6 days at 37 °C with shaking. The soluble fraction was collected after centrifugation, and the inhibition of oligomer assembly was detected by Western blot analysis, using a specific anti- α -syn antibody.

in the spectrum of the oligomers was evident with increasing concentrations of mannitol, indicating a change in the structure of the oligomers.

Mannitol Ameliorates Behavioral Deficits of *Drosophila* Model of PD—The locomotion (climbing) assay, commonly used for assessing behavior of *Drosophila* flies expressing amyloidogenic proteins in their brain (31–34), was used to evaluate the effect of mannitol on a fly model of PD, expressing mutant A53T α -syn in their nervous system via the Gal4-UAS system. Although normal flies tend to climb up the side of a tube, A53T α -syn-expressing flies remain at the bottom (35). Wild-type (Oregon-R) flies, which served as control, and A53T α -syn-expressing flies were grown on regular *Drosophila* medium or on medium supplemented with 75 mM mannitol. The climbing ability of the flies was monitored for 27 days (Fig. 4). Due to their aging, control flies retained 72% of their climbing ability by day 27. In comparison, only 38% of A53T α -syn-expressing flies of the same age climbed along the tube, displaying a severe motor dysfunction. When reared on a medium supplemented with mannitol, the PD model flies gained almost complete phenotypic recovery, with 70% climbing ability at day 27. Mannitol had no significant effect on locomotion of the control flies. One-tailed ANOVA statistics showed *, $p < 0.05$ at days 12 and 14 and ***, $p < 0.0005$ at days 16, 23, and 27 (Fig. 4).

Mannitol Reduces α -syn Accumulation in Brains of PD Flies—To assess the effect of mannitol on the accumulation of α -syn in the brains of the PD model flies, brains were removed from A53T α -syn-expressing flies grown either on a regular medium or on a medium supplemented with mannitol and were immunostained with anti- α -syn antibody (Fig. 5). The level of aggregated α -syn in the brains of the treated flies (Fig. 5B) was sig-

nificantly lower than in the brains of the untreated PD flies (Fig. 5A), displaying ~70% reduction in the accumulated protein. Student's *t* test analysis showed ***, $p < 0.0005$ (Fig. 5C).

Mannitol Reduces α -syn Accumulation in the mThy1- α -syn tg Mouse Model—As reported previously (25) the saline-treated mThy1- α -syn tg mice displayed an accumulation of α -syn in the neuronal cell bodies and neuropil in the neocortex (Fig. 6A), hippocampus (Fig. 6B), SN (Fig. 7, A and C), and basal ganglia (BG) (Fig. 7, A and B) in contrast to saline-treated non-tg mice (in the corresponding figures mentioned). Interestingly, mannitol administration resulted in decreased α -syn immunoreactivity in the hippocampus (Fig. 6B), BG (Fig. 7, A and B), and SN (Fig. 7, A and C) of the mThy1- α -syn tg mice as compared with vehicle-treated tg mice, but had no significant effect in the neocortex (Fig. 6A). To further investigate the effect of mannitol on α -syn accumulation, immunoblot analysis in soluble and insoluble brain fractions was conducted. In comparison with non-tg vehicle-treated mice, in mThy1- α -syn tg mice, there was a significant increase in accumulation of α -syn in both soluble and insoluble fractions. Remarkably, treatment with mannitol highly reduced the accumulation of α -syn in the tg mice in both fractions (Fig. 8). Notably, no significant change in mRNA expression levels of the human α -syn transgene was detected following mannitol administration.

Mannitol Ameliorates Neuropathological Deficits in the tg Mice—Immunohistochemical and stereological analysis of NeuN-positive neurons showed a subtle but significant decrease of neurons in the neocortex (Fig. 6C) and hippocampus (Fig. 6D) in the mThy1- α -syn vehicle-treated tg as compared with non-tg vehicle-treated controls. In contrast, mannitol administration ameliorated the loss of NeuN-positive neurons in the neocortex and hippocampus of the mThy1- α -syn tg mice, whereas it had no effect on the non-tg controls. In the mThy1- α -syn tg mice, loss of NeuN-positive cells is accompanied by astrogliosis in the neocortex (Fig. 6E) and hippocampus (Fig. 6F). mThy1- α -syn tg mice treated with mannitol showed a reduction in the levels of GFAP in the neocortex as compared with vehicle-treated tg mice (Fig. 6E); however, no effects were observed in the hippocampus (Fig. 6F).

To investigate the effects of mannitol in the dopaminergic system, levels of TH, the rate-limiting enzyme in the conversion of tyrosine to dopamine, were assessed. Saline-treated mThy1- α -syn tg mice display significantly reduced levels of TH immunoreactivity in the BG in comparison with the saline-treated non-tg mice (Fig. 7, D and E). Mannitol administration was able to restore TH immunoreactivity in the mThy1- α -syn tg mice back to levels comparable with vehicle-treated non-tg mice (Fig. 7, D and E). Mannitol had no effect on BG TH levels (Fig. 7, D and E) in the non-tg mice or on levels of TH in the SN of mThy1- α -syn or non-tg mice (Fig. 7, D and F).

Mannitol Administration Up-regulates HSP-70—To explore the mechanism underlining α -syn reduction in the brain tissue following mannitol treatment, we investigated whether mannitol induces a clearance mechanism in the cell bodies. In agreement with the *in vitro* results, mannitol, added to brain sections taken from α -syn tg mice, had no direct α -syn-dissolving effect as compared with vehicle treatment (data not shown). However, and in accordance with the literature (36), mannitol

Mannitol, a BBB Disrupter, Inhibits α -syn Aggregation

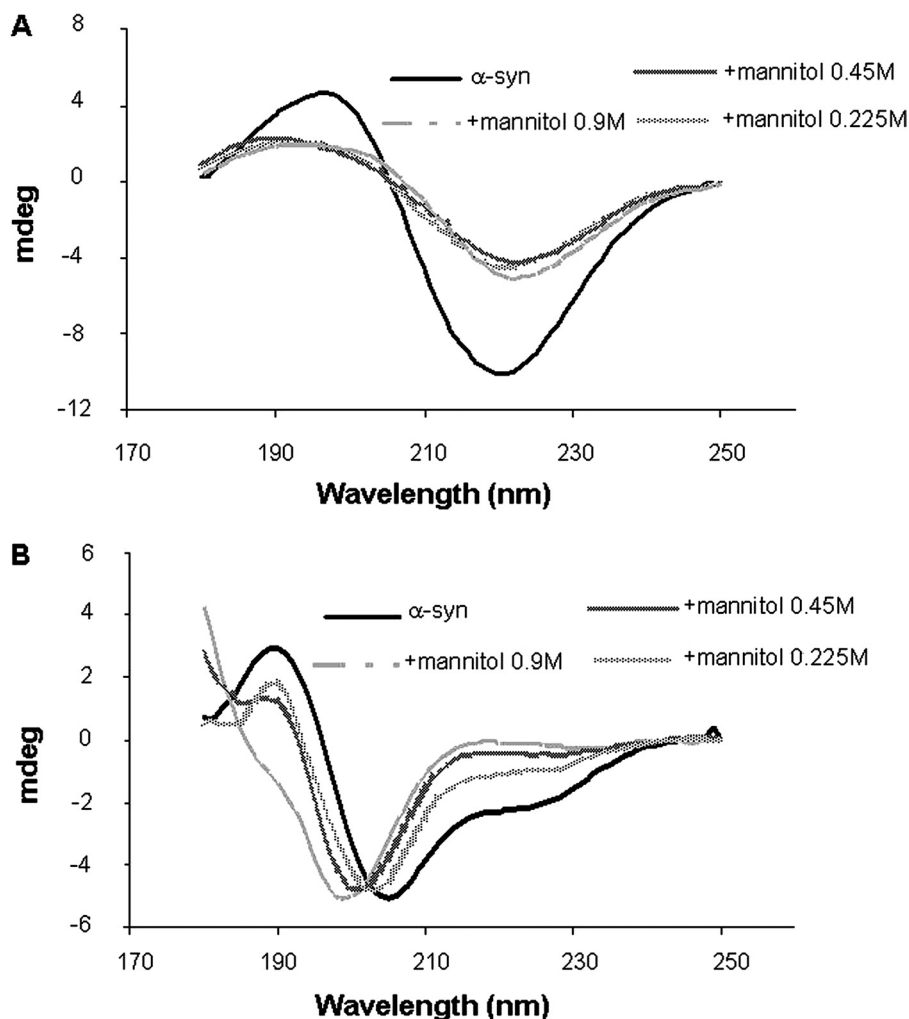


FIGURE 3. **CD analysis of α -syn assemblies in the presence of mannitol.** The secondary structure of the fibrils and oligomers of α -syn was monitored following incubation with mannitol. Samples were centrifuged and analyzed. *A*, the pellet containing the insoluble fibrils was resuspended, and the structure was identified as β -sheet. No change in structure was detected in the presence of mannitol. *mdeg*, millidegrees. *B*, the supernatant, containing the soluble oligomers, revealed different structures in the presence or absence of mannitol.

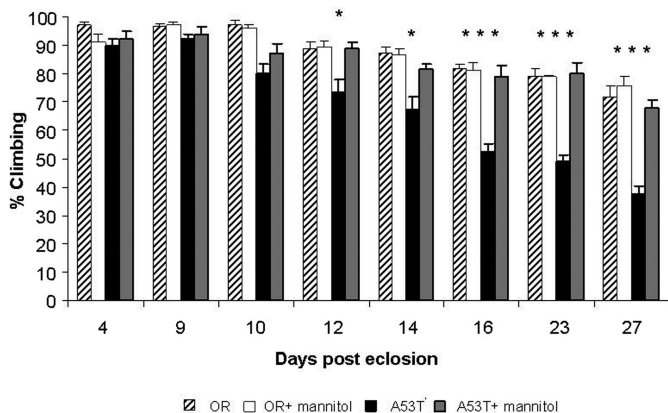


FIGURE 4. **Locomotive behavior of A53T PD model flies following mannitol administration.** The climbing ability of the flies was monitored for 27 days. The results of four classes of flies are presented: control flies (Oregon-R (OR)), not expressing α -syn A53T, grown on a regular medium (cross-hatch); control flies (Oregon-R), not expressing α -syn A53T, grown on medium containing 75 mM mannitol (white); flies expressing α -syn A53T in their brain grown on a regular medium (black); and flies expressing α -syn A53T grown on medium containing 75 mM mannitol (gray). At days 12 and 14, $p < 0.05$, and at days 16, 23, and 27, $***, p < 0.0005$.

administration in mice was followed by a significant increase in the level of the HSP-70 protein (Fig. 9).

DISCUSSION

The pathology of PD is characterized by the loss of neuromelanin-containing dopaminergic neurons in the SN with the presence of Lewy body inclusions, mainly composed of abnormally accumulated and aggregated α -syn within the surviving neurons (12, 37). To date, no disease-modifying treatment is available for PD or other synucleinopathies.

One of the main obstacles in drug development is the inability of most drugs to pass across the BBB into the central nervous system (CNS). As reported previously (6), mannitol, a nonmetabolized FDA-approved osmotic diuretic mediator, can also be used to open the BBB by producing osmotic shrinkage of the endothelial cells and mechanical separation of the tight junctions that form the BBB. Therefore, it can be used to deliver various drugs directly to the brain (38).

In this study, we sought to assess the potential of mannitol as a therapeutic agent for PD. We analyzed *in vitro* the ability of mannitol to interfere with the formation of soluble and insoluble

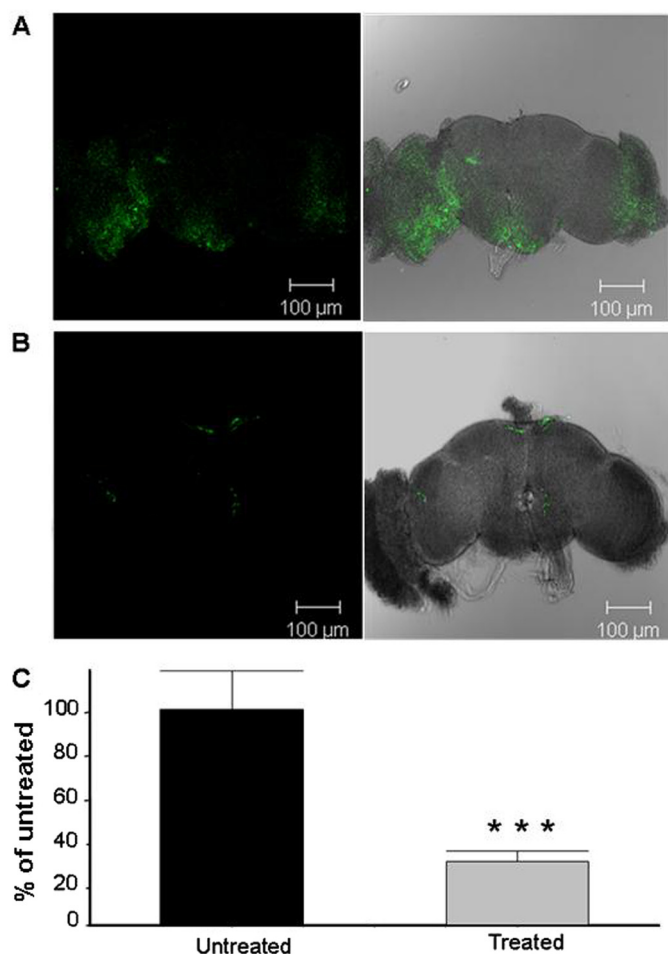


FIGURE 5. Immunostaining of α -syn accumulation in brains of α -syn-expressing flies. Brains of 10 mannitol-treated and 10 untreated A53T PD model flies were extracted and immunostained with anti- α -syn antibody followed by Cy5-conjugated antibody. α -syn is indicated in green. *A*, staining of untreated flies expressing α -syn A53T grown on a regular medium. *B*, staining of flies expressing α -syn A53T-treated with mannitol. *C*, quantification of treated and untreated brains. The Student's *t* test analysis showed ***, $p < 0.0005$.

ble aggregates of α -syn. We found that mannitol inhibits the fibrillation of α -syn at the lower concentrations tested (0.225 and 0.45 M), but not at the higher concentration (0.9 M). These results were further validated by TEM analysis. However, analysis of the soluble fractions revealed that only a high concentration of mannitol (0.9 M) significantly decreased the amount of tetramers and high molecular weight oligomers. It appears *in vitro* that mannitol acts as a chemical chaperone on α -syn folding. CD analysis indicated that although the lower concentrations of mannitol did not inhibit the formation of α -syn oligomers, they affected their secondary structure. Therefore, we presume that mannitol directs the oligomers to an alternative pathway of aggregation that remains to be elucidated.

In vivo studies reveal that mannitol has also effect on PD fly and mouse models. Mannitol dramatically corrected the behavioral defects of PD model *Drosophila*. This was accompanied by ~70% reduction in α -syn aggregates in the brains of mannitol-treated flies as compared with the untreated group.

In the mThy1- α -syn tg mouse model, daily intraperitoneal injections of mannitol (1 g/kg) reduced α -syn accumulation in

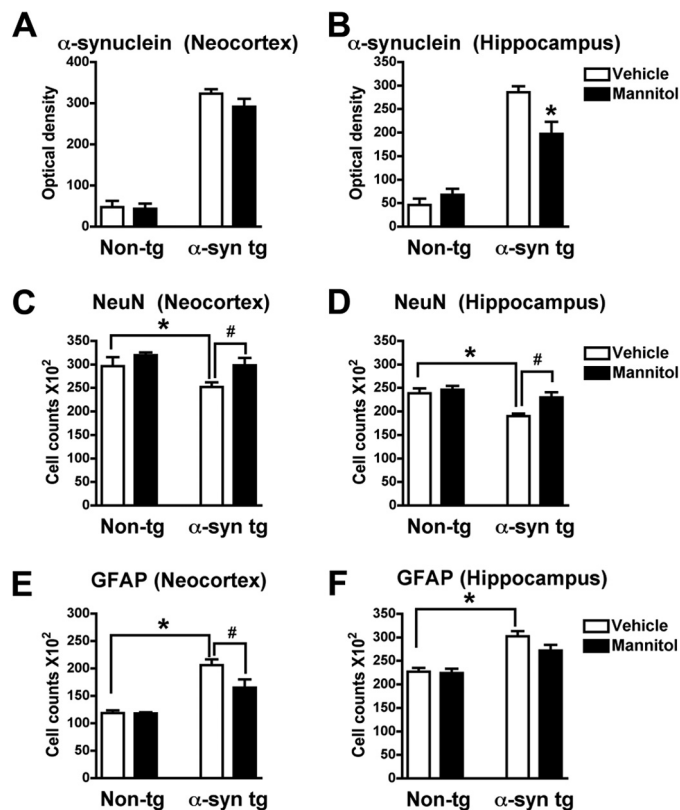


FIGURE 6. Immunocytochemical and general neuropathological analysis of mannitol-treated tg mice. *A* and *B*, density of α -syn-positive staining in the neocortex (*A*) or hippocampus (*B*) of α -syn-immunostained 40- μ m sections of saline- or mannitol-treated non-tg or tg mice. *C* and *D*, quantification of NeuN-positive neurons in the neocortex (*C*) or hippocampus (*D*) of sections of saline- or mannitol-treated non-tg or tg mice. *E* and *F*, quantification of GFAP-positive neurons in the neocortex (*E*) or hippocampus (*F*) of sections of saline- or mannitol-treated non-tg or tg mice. # and * indicate a significant difference between the two groups compared ($p < 0.05$, one way ANOVA and post hoc Fisher). Values are mean \pm S.E., $n = 5$.

the hippocampus, BG, and SN but not in the neocortex. In addition, biochemical analysis revealed that treatment with mannitol highly reduced the total accumulation of α -syn in the tg mice brains in both soluble and insoluble fractions in comparison with untreated tg mice. These results suggest that mannitol promotes α -syn clearance in the cell bodies. In accordance with the literature, an increase in the amount of HSP-70, a marker of cellular stress and/or injury, was detected following mannitol administration, possibly due to BBB disruption. It appears that mannitol also has a general neuroprotective effect because it reduces the loss of NeuN-positive neurons in the neocortex and hippocampus of the mThy1- α -syn tg mice and decreases astrogliosis in the neocortex, although not in the hippocampus.

Mannitol also exhibited specific neuroprotective qualities in the dopaminergic system of the treated mice, where it was able to restore TH immunoreactivity in the mThy1- α -syn tg mice back to levels comparable with vehicle-treated non-tg mice in the BG. Importantly, mannitol had no general neuroprotective effect on the control non-tg mice.

Mannitol seems to have a preventive ability to inhibit α -syn aggregation, but not a reversible ability. Mannitol was not able to dissolve preformed α -syn aggregates *in vitro* nor to dissolve

Mannitol, a BBB Disrupter, Inhibits α -syn Aggregation

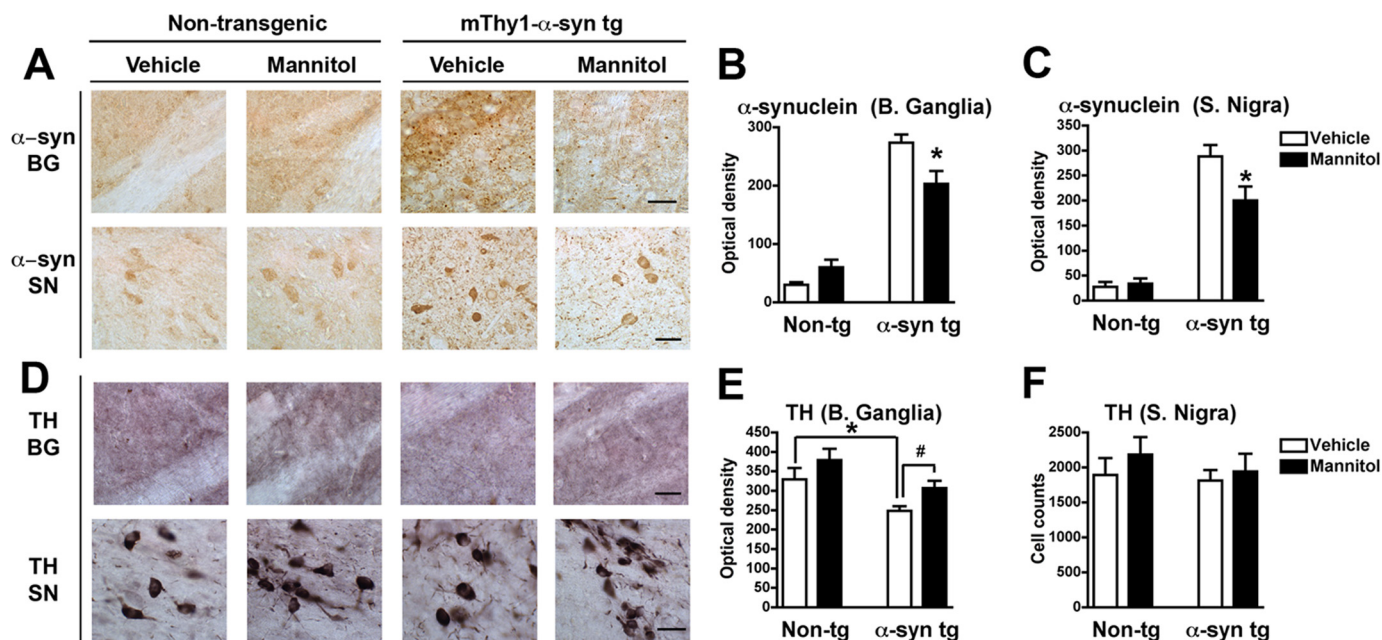


FIGURE 7. Immunocytochemical and neuropathological analysis of mannitol-treated tg mice in the dopaminergic system. *A*, representative images of BG and SN of α -syn-immunostained 40- μ m sections of saline- or mannitol-treated non-tg or tg mice. *B* and *C*, density of α -syn-positive staining in the BG (*B*) or SN (*C*) of α -syn-immunostained sections of saline- or mannitol-treated non-tg or tg mice. *B. ganglia*, basal ganglia; *S. Nigra*, substantia nigra. *D*, representative images of BG and SN of TH-immunostained sections of saline- or mannitol-treated non-tg or tg mice. *E* and *F*, density of TH-positive staining in the BG (*E*) or SN (*F*) of TH-immunostained sections of saline- or mannitol-treated non-tg or tg mice. Scale bar = 50 μ m. # and * indicate a significant difference between the two groups compared ($p < 0.05$, one-way ANOVA and post hoc Fisher). Values are mean \pm S.E., $n = 5$.

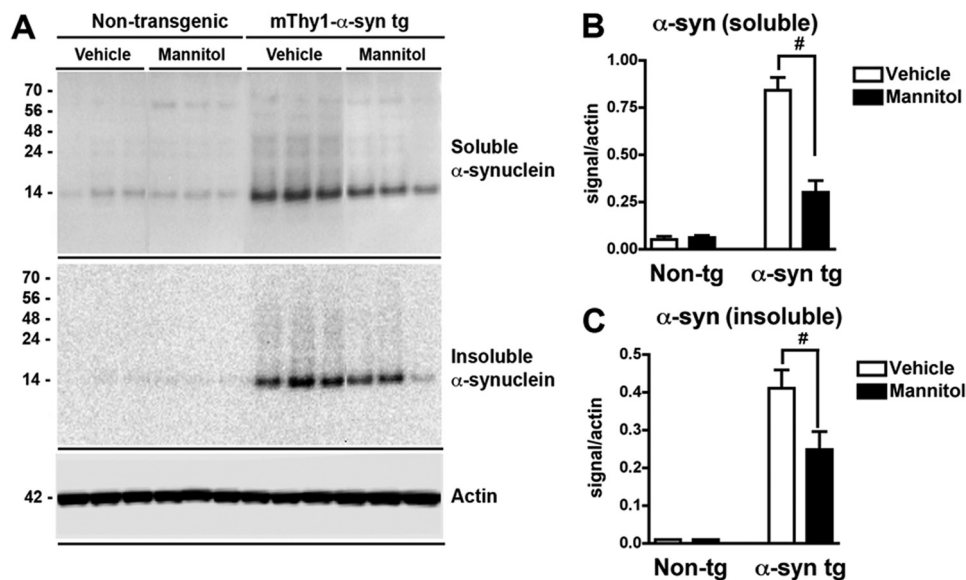


FIGURE 8. Analysis of α -syn levels in tg mice. *A*, brain lysates of saline- or mannitol-treated non-tg or tg mice were fractionated into cytosolic and insoluble fractions followed by separation on SDS-PAGE gels and immunoblot against human α -syn. Anti- β -actin antibody was used to confirm equal loading. *B* and *C*, quantification of the signal recorded in the Western blot of the soluble (*B*) and insoluble (*C*) fractions. # Indicates a significant difference between the two groups compared ($p < 0.05$, one-way ANOVA and post hoc Fisher).

α -syn aggregates when dripped on brain sections taken from α -syn tg mice.

Abnormal protein misfolding and aggregation are key features in many neurodegenerative disorders. Interestingly, a decline in the intracellular level of molecular chaperones was shown to increase the levels of abnormally folded proteins inside the cell (39). Therefore, it was proposed that the cell toxicity in neurodegenerative disorders may result from an imbalance between normal chaperone capacity and the pro-

duction of misfolded protein species (40). Hence, the addition of chemical and molecular chaperones, which are able to stabilize misfolded proteins, was suggested as a therapeutic approach in neurodegenerative disorders (22). In addition to its BBB-disrupting properties, mannitol was previously suggested to function as a chemical chaperone, demonstrating a very potent effect on the stabilization of protein structure (19–21).

Here, we have demonstrated that mannitol interferes with α -syn aggregation *in vitro* and *in vivo*, whereas no adverse

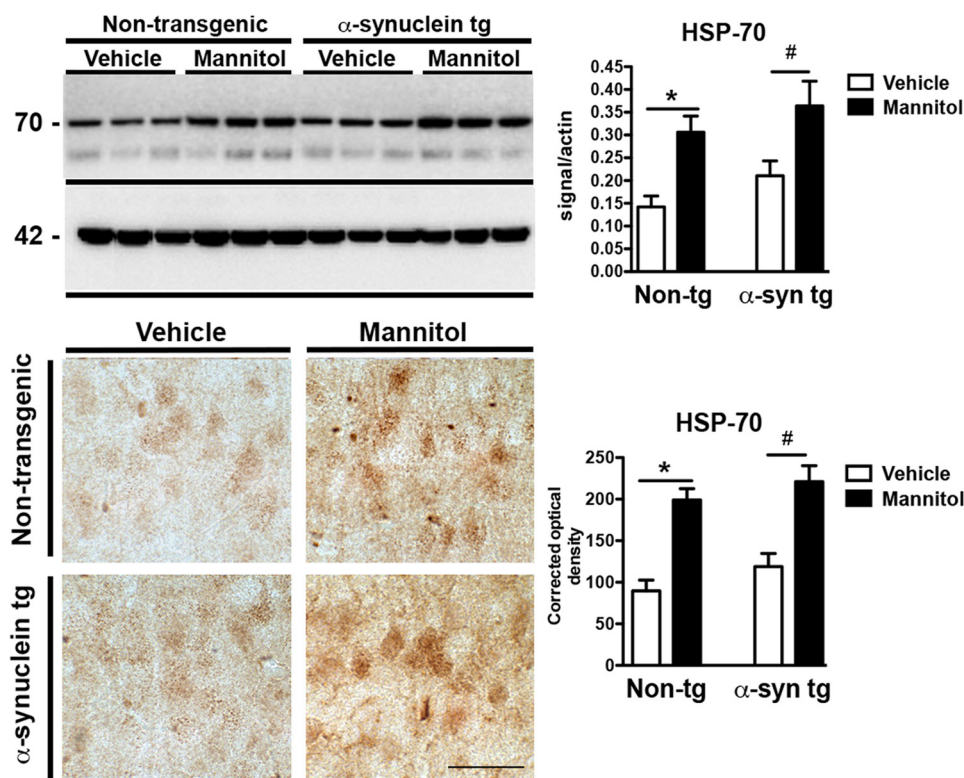


FIGURE 9. Analysis of HSP-70 levels in tg mice. Top panels, brain lysates of saline- or mannitol-treated non-tg or tg mice were separated on SDS-PAGE gels and immunoblotted against murine HSP-70 (top left panel), and the signal recorded was quantified (top right panel). Anti- β -actin antibody was used to confirm equal loading. Lower left panel, representative images of HSP-70 immunostained sections of saline- or mannitol-treated non-tg or tg mice. Lower right panel, density of HSP-70-positive staining in the lower left panel. # and * Indicates a significant difference between the two groups compared ($p < 0.05$, one-way ANOVA and post hoc Fisher).

effects were observed in control-treated flies or mice. In addition to its osmotic diuretic effect, mannitol is known for its BBB-disrupting properties (41). To the best of our knowledge, mannitol has not been tested or used until now in the clinic for drug delivery into the brain. Therefore, we suggest that mannitol administration in combination with other drugs could be a promising new approach for treating PD and other brain-related diseases such as Alzheimer disease. This prediction is based on its chemical chaperone properties, its vast protective cellular capabilities, and its BBB-disrupting properties.

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