

Neuropeptide Treatment with Cerebrolysin Enhances the Survival of Grafted Neural Stem Cell in an α -Synuclein Transgenic Model of Parkinson's Disease

Supplementary Issue: Molecular and Cellular Mechanisms of Neurodegeneration

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ABSTRACT: Neuronal stem cell (NSC) grafts have been investigated as a potential neuro-restorative therapy in Parkinson's disease (PD) but their use is compromised by the death of grafted cells. We investigated the use of Cerebrolysin (CBL), a neurotrophic peptide mixture, as an adjunct to NSC therapy in the α -synuclein (α -syn) transgenic (tg) model of PD. In vehicle-treated α -syn tg mice, there was decreased survival of NSCs. In contrast, CBL treatment enhanced the survival of NSCs in α -syn tg groups and ameliorated behavioral deficits. The grafted NSCs showed lower levels of terminal deoxynucleotidyl transferase dUTP nick end labeling positive cells in the CBL-treated mice when compared with vehicle-treated α -syn tg mice. No evidence of tumor growth was detected. Levels of α -syn were similar in the vehicle in CBL-treated tg mice. In conclusion, CBL treatment might be a potential adjuvant for therapeutic NSC grafting in PD.

KEYWORDS: Parkinson's disease, dementia with Lewy bodies, stem cells, neurotrophic factors, α -synuclein

SUPPLEMENT: Molecular and Cellular Mechanisms of Neurodegeneration

CITATION: Rockenstein et al. Neuropeptide Treatment with Cerebrolysin Enhances the Survival of Grafted Neural Stem Cell in an α -Synuclein Transgenic Model of Parkinson's Disease. *Journal of Experimental Neuroscience* 2015;9(S2) 131–140 doi:10.4137/JEN.S25521.

TYPE: Original Research

RECEIVED: October 12, 2015. **RESUBMITTED:** December 14, 2015. **ACCEPTED FOR PUBLICATION:** December 15, 2015.

ACADEMIC EDITOR: Lora Talley Watts, Editor in Chief

PEER REVIEW: Three peer reviewers contributed to the peer review report. Reviewers' reports totaled 1021 words, excluding any confidential comments to the academic editor.

FUNDING: This work was partially supported by NIH grant AG05131 and by a grant from EVER Pharma. The authors confirm that the funder had no influence over the study design, content of the article, or selection of this journal.

COMPETING INTERESTS: The authors Stefan Winter, Hemma Brandstaetter, and Dieter Meier are employed by EVER Neuro Pharma, which provided the CBL used for this study.

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Introduction

Lewy body disease (LBD) is a heterogeneous group of disorders with α -synuclein (α -syn) accumulation,^{1–4} clinically characterized by parkinsonism, behavioral and cognitive impairments, and dysautonomia. Included under the LBD rubric are idiopathic Parkinson's disease (PD), PD dementia, and dementia with Lewy bodies (DLB).⁵ α -syn is a 140 amino acid molecule that concentrates in the synaptic terminals⁶ and, under physiological conditions, plays a role in synaptic transmission and vesicle release.⁷ In DLB and PD, α -syn accumulates as oligomers, protofibrils, and fibrils⁸ that are neurotoxic and can propagate from cell to cell in a prion-like manner.⁹ Overexpression of wild-type and mutant α -syn in invertebrates, rodents, and primates has been shown to result in the formation of α -syn aggregates resembling Lewy pathology and neurodegeneration and behavioral deficits.¹⁰ Among them, we have shown that overexpression of α -syn under the mThy1 promoter (line 61) results in behavioral, biochemical, and pathological alterations that resemble some aspect of PD/DLB.^{10–12}

In PD and DLB, larger, toxic, fibrillar intraneuronal α -syn aggregates are found in neurons and denominated Lewy bodies and Lewy neurites, these α -syn aggregates accumulate in the synapses and neurons leading to neurodegeneration.¹³ The neurodegenerative process in patients with PD and DLB is characterized by synaptic loss¹⁴ and selective loss of cholinergic, glutamatergic, and DOPA-ergic neurons.^{15–17} In addition, more recent studies suggest that alterations in adult neurogenesis may also participate in the neurodegenerative process in PD/DLB.¹⁸ Studies in both PD patients and α -syn transgenic (tg) mice have shown that neurogenesis is decreased in the hippocampus and olfactory bulb and might be associated with α -syn toxicity.^{18–22}

At present, only limited therapeutic strategies are available to manage the parkinsonism and nonmovement alterations in patients with PD/DLB, and no disease-modifying therapies are currently available.^{23,24} However, given the advanced clinical stage at which several patients with PD and DLB present, with over 75% loss of dopaminergic neurons,²⁵ alternative therapies have been considered, including replacement therapy with fetal neuronal stem cell (NSC) grafts.



However, only limited results have been obtained with fetal engrafting in PD patients,^{26,27} and recent studies have shown that complications, such as dyskinesias and α -syn propagation to the grafted cells, are a potential problem.^{28–30} Likewise, experimental studies in α -syn tg mice have shown that neural stem cell (NSC) (3131) or fetal dopaminergic cells (3232) grafting into the hippocampus or striatum, respectively, results in α -syn propagation to the transplanted cells accompanied by apoptotic cell death and dysfunction.

Therefore, adjuvant therapies that enhance the survival of grafted stem cells might be important. Among them, we have proposed combining stem cells with Cerebrolysin™ (CBL),³³ a peptide mixture with neurotrophic-like properties, that improves cognition in patients with mild-to-moderate Alzheimer's disease (AD).^{34–38} CBL is neuroprotective in models of excitotoxicity³⁹ and stroke.^{40–42} Furthermore, CBL is neurotrophic in amyloid precursor protein (APP) tg models of AD by promoting synaptic formation and neurogenesis.^{43–48} In recent studies, we have shown that CBL enhances the survival of NSC when grafted into the brains of young and old APP tg mice,³³ supporting the notion that CBL might be a putative adjuvant therapy when combined with NSC grafting in models of neurodegeneration.

In this context, the main goal of this study was to investigate whether CBL is capable of improving the survival of NSCs grafted into the striatum of the mThy1- α -syn tg model of DLB/PD-like pathology.¹² We found that NSC survival progressively declined with age in α -syn tg mice when compared with controls and that combined support therapy with CBL improved the survival of the grafted NSC. This study supports the notion that CBL might be a potentially useful adjuvant therapy in combination with NSCs in PD/DLB.

Materials and Methods

Generation of α -syn tg mice, grafting, and CBL treatment. For these experiments, α -syn tg mice expressing wild-type human α -syn under the regulatory control of the mouse (m) Thy-1 promoter (mThy1- α -syn; line 61)¹² were used. These mice have been extensively characterized,^{10,11} and we have previously shown that these mice display loss of synaptic contacts,¹² defects in neurogenesis,⁴⁹ high levels of α -syn oligomers,⁵⁰ and behavioral deficits.^{51,52} Genomic DNA was extracted from tail biopsies and analyzed by PCR amplification. Transgenic lines were maintained by crossing heterozygous tg mice with non-transgenic (non-tg) C57BL/6 \times DBA/2 F1 breeders. All mice were heterozygous with respect to the transgene.

Mice were divided into two groups. The first group was evaluated after one month of grafting and the second group after three months of grafting. Mice were six months old at the start of the study. A total of 64 mice were used, $n = 32$ for the one-month study and $n = 32$ for the three-month study. The groups of 32 mice were divided into four subgroups that

included (A) non-tg ($n = 8$) vehicle, (B) non-tg ($n = 8$) CBL, (C) α -syn tg ($n = 8$) vehicle, and (D) α -syn tg ($n = 8$) CBL. Mice were treated with either vehicle or CBL (IP: 5 mL/kg) two weeks prior to the grafts.

Two weeks after either vehicle or CBL treatment, all mice received bilateral NSC grafts (~120,000 cells per side) into the striatum utilizing³³ a Kopf stereotaxic apparatus with coordinates 1.0, \pm 1.5, and -3.0 as previously described. Mouse cortical neuronal progenitor cells (Millipore) were grown on F12/DMEM basal media supplemented with B27 and were kept in proliferative status without induction of neuronal differentiation. Cells were labeled 48 hours prior to grafting into the mouse by infection with lentiviral constructs containing the GFP coding sequence at MOI = 50. Additional pulse labeling with BrdU (10 μ M) was carried out 24 hours prior to grafting. Following the grafting, mice continued to receive vehicle or CBL for one or three months.

The CBL neuropeptide mixture was provided by EVER Pharma in preprepared ampoules, in which each milliliter of CBL contains 215.2 mg of the active CBL concentrate in an aqueous solution.⁵³ Mass spectrometry analysis has shown that CBL comprises amino acids (80%) and small (<10 Da) peptides (20%). Previous work had shown that the small peptides mimic the effect of neurotrophic factors, including ciliary neurotrophic factor, fibroblast growth factor 2, and insulin-like growth factor.⁵⁴ Mice were injected daily with saline alone or CBL (ip, 5 mL/kg, CBL Batch #92382008) for the duration of the experiment for a total of one or three months. By the end of the experiment for each corresponding group, mice were seven and nine months old.

All the experiments described have been approved by the committee of the University of California at San Diego (UCSD) and were performed according to the NIH guidelines for animal use.

Tissue processing. All procedures conformed to NIH guidelines on the humane treatment of animals. Mice were anesthetized using chloral hydrate and flush-perfused transcardially with 0.9% saline. The brains were removed and divided sagittally. The left hemibrain was postfixed in phosphate-buffered saline with 4% paraformaldehyde (pH 7.4) at 4°C for 48 hours, and a Vibratome 2000 (Leica, Germany) was used to section the hemisphere at 40 μ m; the right hemibrain was snap frozen and stored at -70°C .

Immunohistochemical analysis and TUNEL assay. Briefly, as previously described,⁵⁵ blind-coded vibratome sections were immunolabeled with mouse monoclonal antibodies against BrdU (1:500; Millipore), proliferation cell nuclear antigen (PCNA; 1:500; Millipore), GFAP (1:500, Millipore), NeuN (1:500; Millipore), doublecortin (DCX; 1:500; Millipore), and α -syn (1:500, SYN-1 from BD Biosciences) followed by horse anti-mouse biotinylated secondary antibody and developed with diaminobenzidine. All sections were processed blind coded under the same standardized conditions. The immunolabeled sections were imaged with

an Olympus OX54 digital photomicroscope. Briefly, for each section, a total of eight images at 630 \times were analyzed. A threshold was set utilizing the ImageQuant system; for each image, the threshold was set within a dynamic range that was consistent for all cases. The levels of pixel intensity or optical density for the area were ascertained with the measuring tools of the system. An adjacent unstained area was used as background to correct against. An average area of 1024 \times 1024 pixels was analyzed for each image. This area included on average at least four to five neurons that were analyzed. The average integrated pixels of optical density were estimated for all neurons within a given area and then averaged by the 16 images captured. This average was corrected to the background and is expressed as an overall mean by mouse per group. Control experiments were performed where sections were incubated overnight in the absence of primary antibody (deleted) or with preimmune serum or with primary antibody alone in order to confirm the specificity of primary antibodies.

For detection of apoptosis, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) detection method using the ApopTag In Situ Apoptosis Detection Kit (Chemicon) was adopted with modifications for free floating sections as described previously.⁴⁶ Detection was performed with DAB.

Double immunolabeling and confocal microscopy. To determine the colocalization between TH and BrdU in grafted NSCs, double-labeling experiments were performed. Briefly, vibratome sections were immunolabeled with antibodies against TH (1:250; Millipore) and BrdU (1:500; Millipore). The TH was detected with an FITC-tagged secondary antibody (1:75; Vector Laboratories) while BrdU with the Tyramide Signal AmplificationTM-Direct system (1:100; NEN Life Sciences). Sections were imaged with a Zeiss 63 \times objective on an Axiovert 35 microscope (Zeiss) with an attached MRC 1024 laser scanning confocal microscope (BioRad).

Unbiased stereology. Stereological methods were used to estimate the number of BrdU immunoreactive cells utilizing an optical fractionator unbiased sampling design. A total of four alternating sections separated by \sim 250 μ m, which contain the hippocampus were outlined using a 4 \times objective on an Olympus BX51 microscope. All anatomical nomenclature used is consistent with that in Paxinos and Franklin (2012).⁵⁶ StereoInvestigator 8.21.1 software (Micro-BrightField) was used in order to perform systematic sampling of the outlined areas, which was made from a random starting point. Counts were taken at predetermined intervals ($x = 152$, $y = 152$), and a counting frame (70 \times 70 μ m = 4900 μ m²) was superimposed on the live image of the tissue sections. Sections were analyzed using a 60 \times 1.4 Plan Apo oil-immersion objective with a 1.4 numerical aperture. Section thickness was determined by first focusing on the top of the section, zeroing the z -axis, and then focusing on the bottom of the section. The average

section thickness was 18 μ m. The dissector height was set at 12 μ m, allowing for a 2- μ m top guard zone and at least a 2- μ m bottom guard zone. Forbidden zones were not included in the cell counting procedure. Immunoreactive neurons were only included in the analyses if their first recognizable profile came into focus within the counting frame. This method allowed for a uniform, systematic, and random design.

Round beam testing. Round beam data were collected using a custom-built apparatus that consists of removable 2 Delrin[®] acetal plastic rods (3 and 1 cm diameter) on a smooth acrylic frame elevated 17.5–22.5 cm above a testing bench. Each animal was tested consecutively for three trials on each 1 m beam A (3 cm) and D (1 cm) with a brief break in-between each trial. Using a manual counter, each obvious foot slip past the marked line was counted by the experimenter. In addition, forward distance traveled (assessed using marked 10 cm sections on the side of the beam and then assigned a score) and the latency to fall (60 seconds max.) for each trial was recorded for each animal. The trial ended when the animal fell off the beam, reached the maximum allowed time (60 seconds), or traversed the full distance. The forward distance score of 0–10 was based on the distance traveled forward, where 0–10 cm = 1, 11–20 cm = 2, 21–30 cm = 3, 31–40 cm = 4, 41–50 cm = 5, 51–60 cm = 6, 61–70 cm = 7, 71–80 cm = 8, 81–90 cm = 9, and 91–100 cm = 10.

Statistical analysis. Statistical analysis was performed with the GraphPad Prism software package (GraphPad). All the results are expressed as mean \pm SEM. The analyses were carried out with the StatView 5.0 program (SAS Institute Inc.), unless otherwise indicated. Differences among the means of multiple groups were assessed by one-way analysis of variance (ANOVA) with post hoc Dunnett's test when compared to the control group; comparisons among the treatment groups were performed with the Tukey's post hoc test.

Results

Effects of CBL on the survival of grafted NSCs in the brains of α -syn tg mice. To investigate the survival of grafted cells in α -syn tg mice, mouse NSCs labeled with BrdU were injected into the striatum of six-month-old mice and analyzed at one and three months postgrafting. Immunocytochemical analysis with an antibody against BrdU showed that the grafted cells distributed homogeneously around caudoputamen region, within a radius of 300–400 μ m (Fig. 1A–C). After one month, in the vehicle-treated non-tg mice, an average of 17,000 cells were found, which was equivalent to \sim 14.1% of the grafted cells (Fig. 1A and B). When compared to the non-tg vehicle control group, in the vehicle α -syn tg mice, there was a significant reduction in the survival of the grafted cells at one month postgraft (7400 cells or 6.1%; Fig. 1A and B). In contrast, in the non-tg CBL group \sim 21,000 cells and in the CBL α -syn tg mice 24,000 cells were found, which was equivalent to \sim 17.5% and 21% of the grafted cells, respectively (Fig. 1A and B).

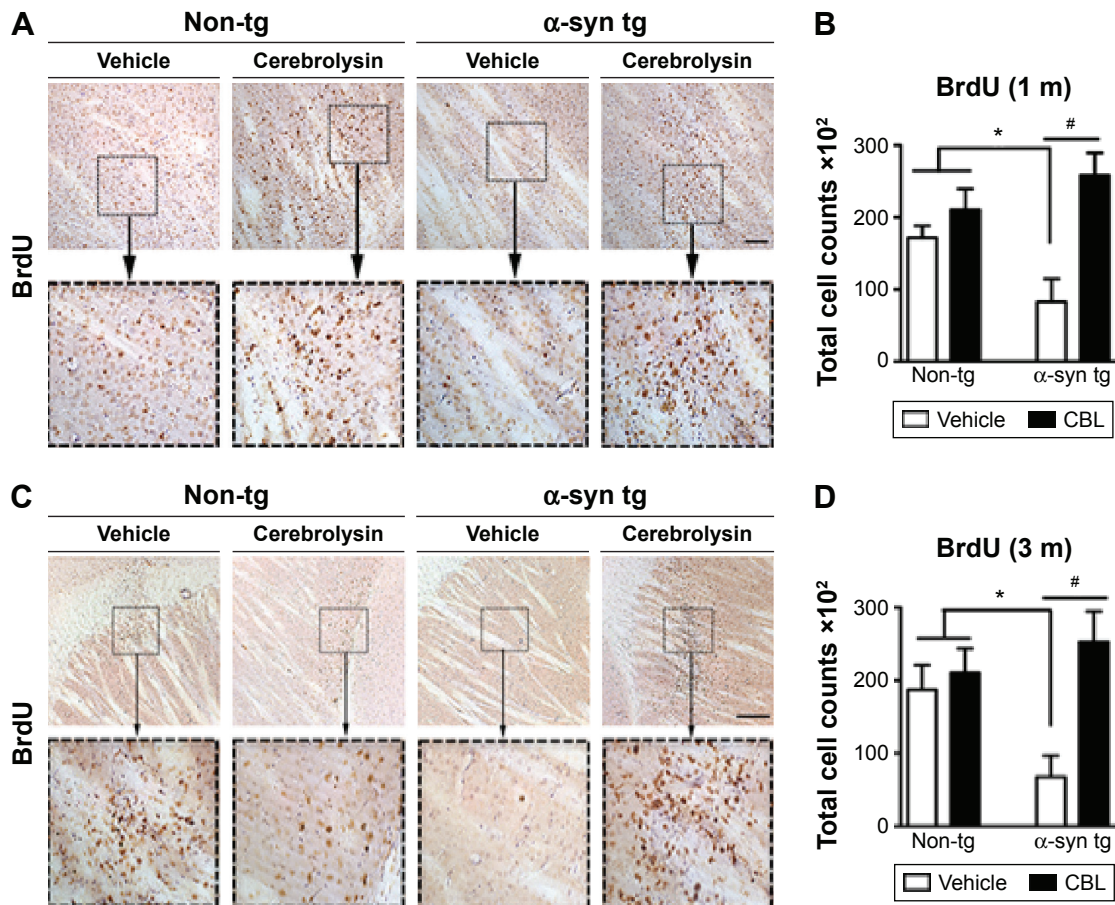


Figure 1. Immunocytochemical analysis of BrdU-labeled grafted NSCs in CBL-treated α -syn tg mice. Cortical NSCs labeled with BrdU were transplanted into the striatum of non-tg and α -syn tg mice (three months old) treated with vehicle or CBL, and immunocytochemical analysis was performed with vibratome sections at one month (A) and three months (C) postgraft. Upper panel representative images at low power (20 \times) at one or three months postgraft. Lower panel is higher magnification (400 \times) of the area in the upper panel marked by an open dashed square. (B, D) Computer-aided image analysis of the numbers of BrdU-positive cells in the striatum at one and three months postgraft, respectively. All results are presented as mean \pm SEM, each group includes $n = 8$ vehicle and $n = 8$ CBL per time point. * $P < 0.05$ between vehicle-treated non-tg and α -syn tg by one-way ANOVA and post hoc Dunnett's test. # $P < 0.05$ between vehicle-treated and CBL-treated α -syn tg groups by one-way ANOVA and post hoc Tukey–Kramer test. Bar = 250 μ m for low power panel and bar = 50 μ m for high power panel.

After three months of the transplant, the number of surviving NSCs were comparable between the non-tg vehicle (19,000 cells or 15.8%) and CBL groups (21,000 cells or 17.4%). In the vehicle-treated α -syn tg mice, only 4950 cells were identified (4.1%; Fig. 1C and D). However, after three months in the CBL-treated α -syn tg mice, 25,000 NSCs were found (21%; Fig. 1C and D). These results support the notion that grafted NSCs display poor survival in the brains of vehicle-treated α -syn tg mice and that adjuvant treatment with CBL reverts this effect.

Effects of CBL on NSCs' markers of neurogenesis, proliferation, and apoptosis in α -syn tg mice. Next, to better understand how the neuropeptide mixture rescued the NSCs in the α -syn tg mice, we investigated the effects of CBL on the expression of markers of neurogenesis (DCX), proliferation (PCNA), and apoptosis (TUNEL) in the grafted NSCs. DCX is a microtubule-associated protein expressed by neuronal precursor cells and immature. NSCs express DCX while

actively dividing. Downregulation of DCX occurs when cells differentiate into mature neurons. After one and three months, in the vehicle-treated non-tg mice, abundant DCX-positive grafted NSCs were identified in the striatum (Fig. 2A–C). When compared to the non-tg vehicle control group, in the α -syn tg vehicle-treated mice, there was a moderate reduction in DCX-positive grafted cells at one month postgraft (Fig. 2B) and a more extensive reduction at three months after grafting (Fig. 2A and C). In contrast, CBL treatment resulted in preservation of DCX-positive NSCs in the α -syn tg mice at one month (Fig. 2B) and three months after grafting (Fig. 2A and C). Levels of PCNA in the grafted NSCs were increased in the CBL-treated groups (Fig. 2D); however and probably due to the variability in the sample, the differences were not significant ($P = 0.08$; Fig. 2E and F). Although a trend toward increased proliferation was detected, no evidence of tumor formation was observed after one month (Fig. 2D) or three months (Fig. 2D) postgrafting. Consistent with the BrdU (Fig. 1) and

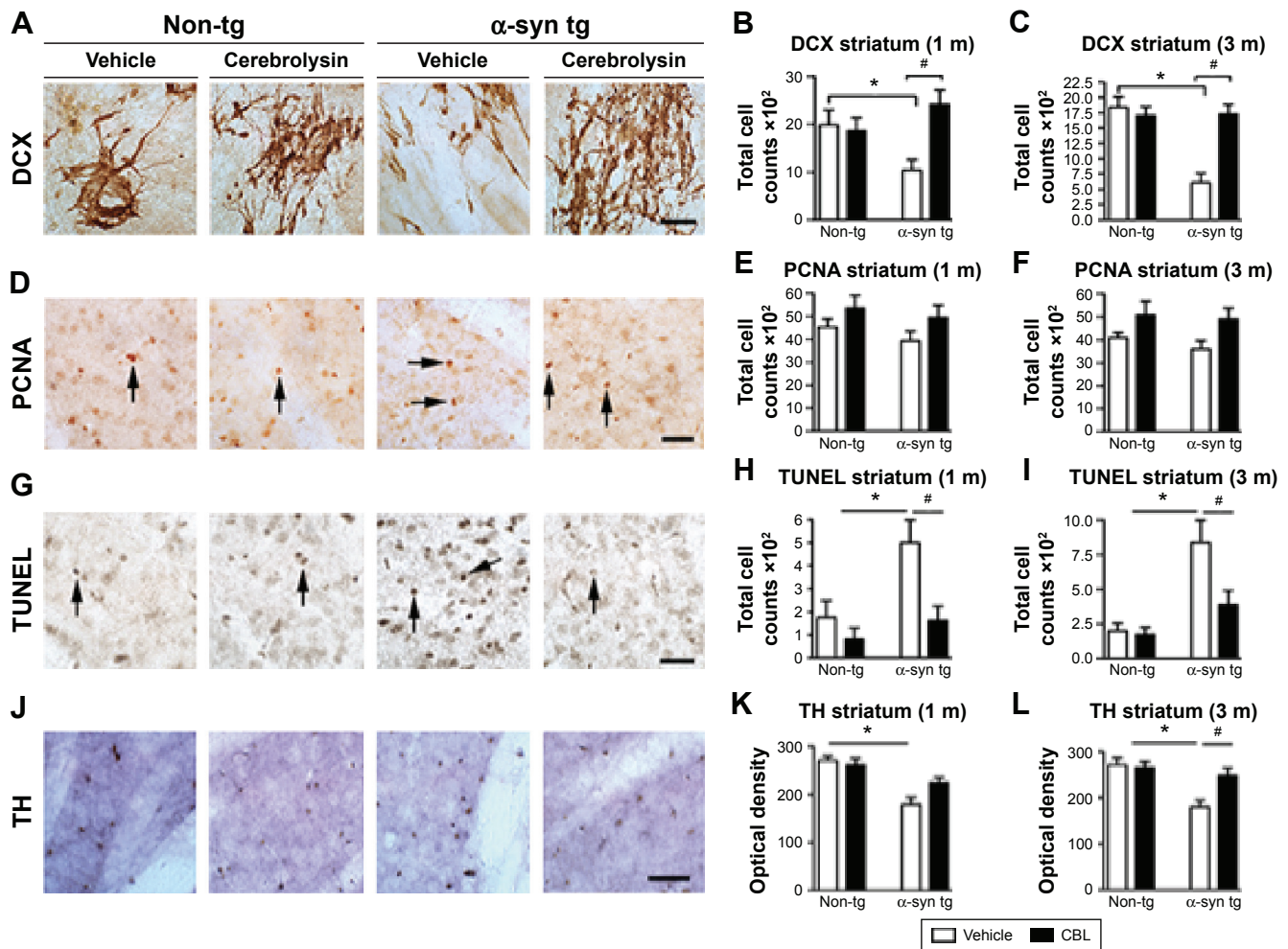


Figure 2. Immunocytochemical analysis of markers of NSC grafted cell survival and cell death. Vibratome sections from non-tg and α -syn tg mice treated with vehicle or CBL and grafted with NSCs were immunolabeled with antibodies against DCX (A), PCNA (B), histochemistry for TUNEL (C), or immunostained for TH (D) and imaged by digital video microscopy. (A, D, G, J) Representative images at high power (400 \times) at three months postgraft of DCX, PCNA, TUNEL, and TH, respectively. Arrows indicate positive grafted NSCs. (B, E, H, K) Computer-aided image analysis of the NSCs with DCX, PCNA, and TUNEL, respectively, and TH in the striatum neuropil at one month postgraft. (C, F, I, L) Computer-aided image analysis of the NSCs with DCX, PCNA, and TUNEL, respectively, and TH in the striatum neuropil at three months postgraft. All results are presented as mean \pm SEM, each group includes $n = 8$ vehicle and $n = 8$ CBL mice per time point. $^*P < 0.05$ between vehicle-treated and CBL-treated α -syn tg mice by one-way ANOVA and post hoc Tukey–Kramer test. Bar = 25 μ m.

DCX (Fig. 2A–C) findings, levels of TUNEL staining (Fig. 2G) were higher in the grafted NSCs in the vehicle-treated α -syn tg mice when compared with non-tg mice at one month (Fig. 2H) and three months (Fig. 2G and I) posttransplantation. Likewise, treatment with CBL significantly reduced this marker of apoptosis in the grafted NSCs in the α -syn tg mice at one month (Fig. 2H) and three months (Fig. 2G and I) posttransplantation. Finally, we investigated whether NSCs converted to dopaminergic cells or if TH levels were changed following grafting and CBL treatment. Immunocytochemistry with an antibody against TH did not show conversion of grafted NSCs to dopaminergic cells (Fig. 2J). However, compared to non-tg controls, vehicle-treated (NSC-grafted) α -syn tg mice showed decreased TH immunostaining in the striatum (Fig. 2J–L). This effect was partially reversed at one month in

CBL (NSC-grafted) α -syn tg mice and completely reversed at three months post CBL treatment (Fig. 2J–L). Double immunolabeling and confocal microscopy showed that the grafted BrdU+ cells were present in-between the TH + fibers in the non-tg (Fig. 3A) and α -syn tg mice (Fig. 3B) treated with vehicle or CBL; overall TH did not colocalize with the BrdU+ grafted cells in either group (Fig. 3).

Taken together, these results support the notion that poor survival of the NSC grafts is related to increased cell death and decreased neurogenesis but not decreased proliferation. However, CBL protective effects on the NSCs might be related to reduced cell death, but not increased proliferation.

Effects of combined NSC grafting and CBL on α -syn pathology in tg mice. To confirm the presence of PD-like

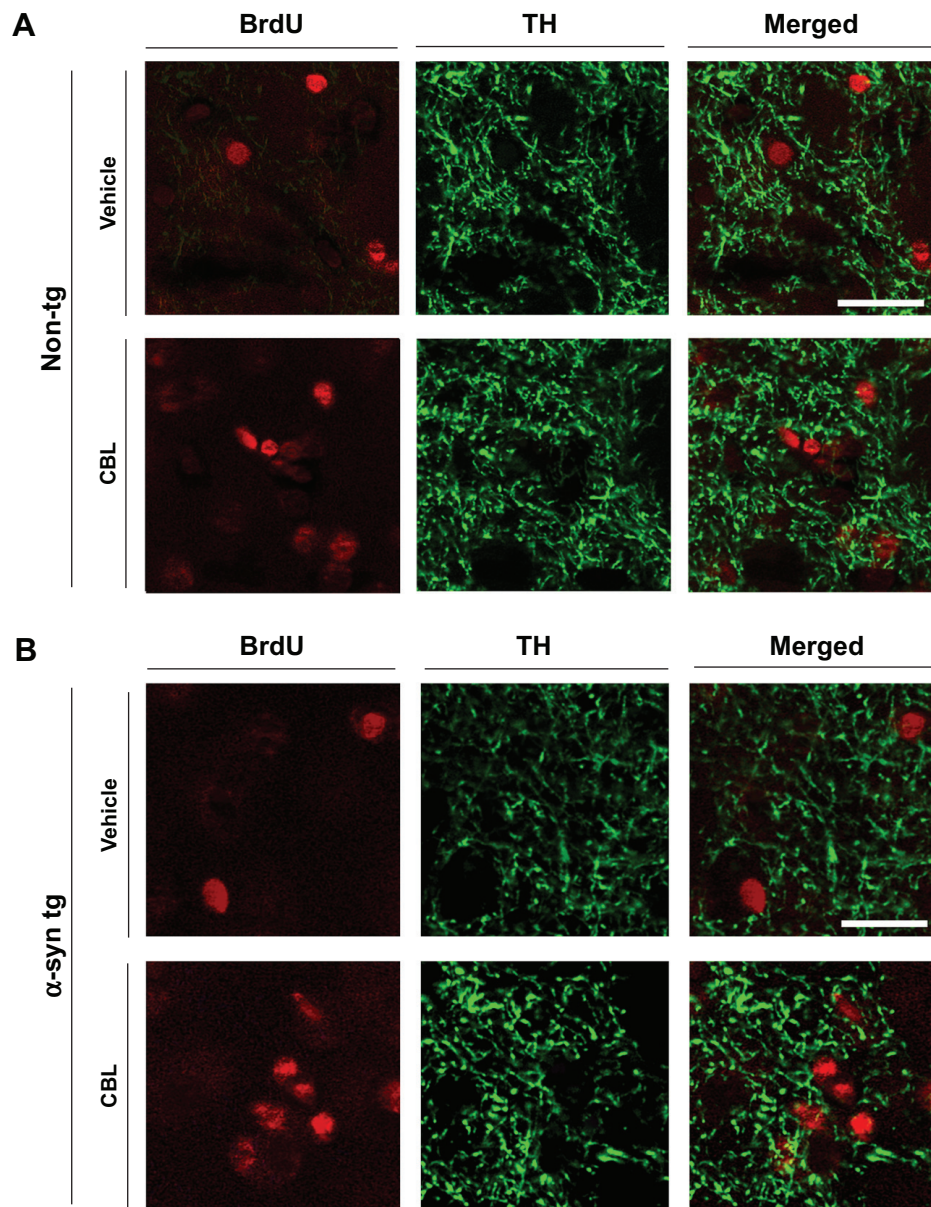


Figure 3. Double immunolabeling for BrdU and TH in grafted cells. Vibratome sections from non-tg and α -syn tg mice treated with vehicle or CBL and grafted with NSCs were double immunolabeled with antibodies against BrdU (red) and TH (green) and imaged with the laser scanning confocal microscope. **(A)** Representative merged and individual confocal images at high power (900 \times) showing the TH immunoreactive fibers and the grafted BrdU-labeled NSCs in the non-tg mice treated with vehicle and CBL. **(B)** representative merged and individual confocal images at high power (900 \times) showing the TH immunoreactive fibers and the grafted BrdU-labeled NSCs in the α -syn tg mice treated with vehicle and CBL. Bar = 25 μ m.

neuropathology in the brains of α -syn tg mice and evaluate the impact of the combined effects of NSC grafting and CBL treatment, sections were immunolabeled with antibodies against α -syn. As previously reported,¹² compared to the non-tg group the vehicle-treated α -syn tg mice displayed extensive neuronal accumulation of α -syn in the neocortex and hippocampus and accumulation of α -syn in the nerve fibers (but not neuronal cell bodies) in the striatum (Fig. 4A). The α -syn tg mice treated with CBL displayed comparable levels of total α -syn accumulation in the neuropil of the striatum when compared with vehicle controls at one month (Fig. 4B) and three months (Fig. 4A and C) postgrafting. In addition,

in the grafted area in the striatum, we identified cells that contained α -syn immunoreactive material, which is consistent with the possibility of transmission of α -syn from the neuropil to the grafted cells (Fig. 4A). Image analysis showed that at one month (Fig. 4D) and three months (Fig. 4A and E), comparable numbers of α -syn-positive cells were detected in the grafted area in the tg mice treated with vehicle or CBL. No α -syn-positive cells were detected in the grafted striatum of vehicle or CBL non-tg mice (Fig. 4A). These studies suggest that CBL's protective effects on the grafted NSCs might be related to reduced cell death and enhanced survival rather than by reducing levels of α -syn accumulation or transmission.

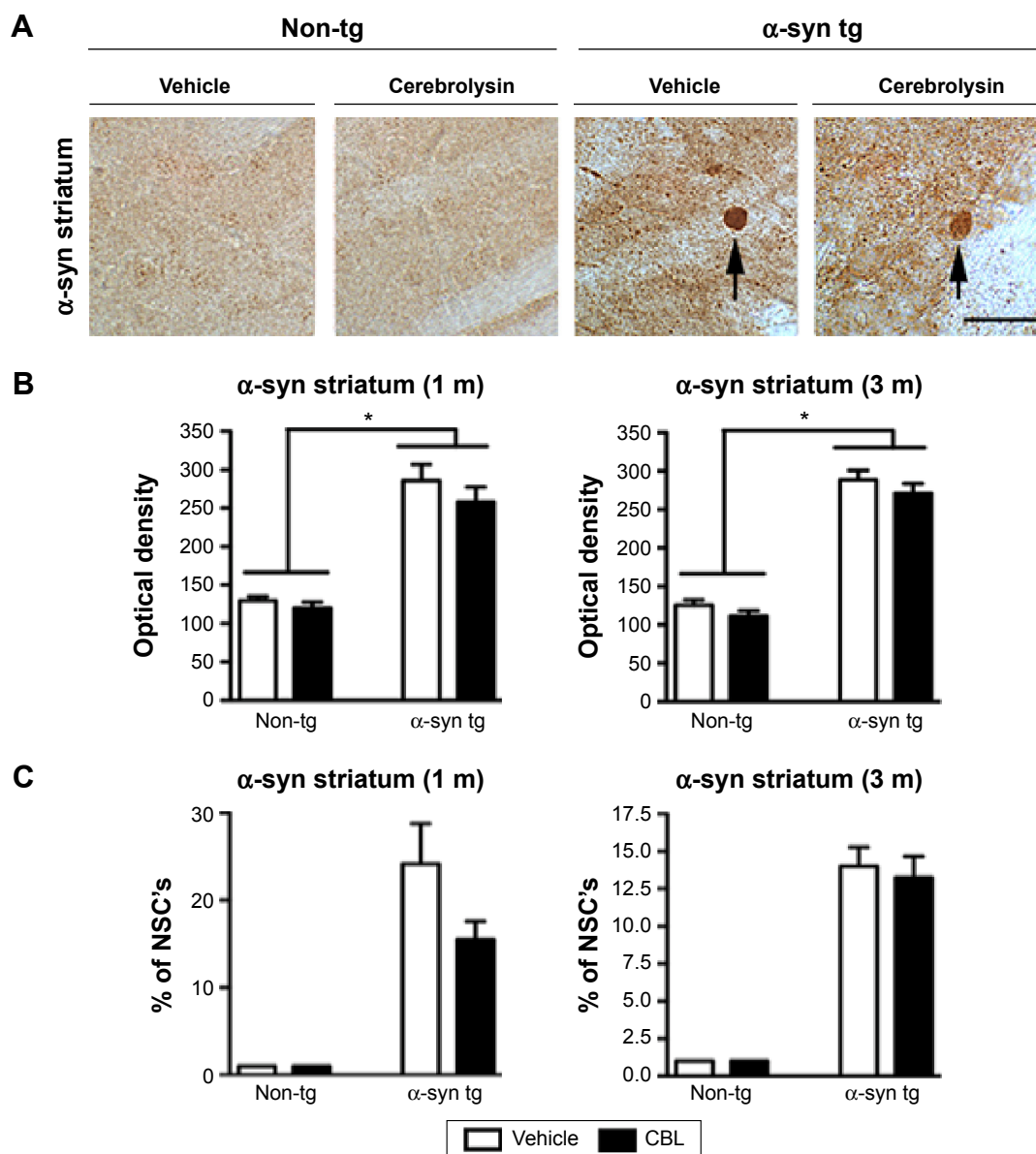


Figure 4. Immunohistochemical analysis of levels of α -syn immunoreactivity in the striatum of NSC-grafted and CBL-treated α -syn tg mice. Vibratome sections from non-tg and α -syn tg mice treated with vehicle or CBL and grafted with NSCs were immunolabeled with an antibody against total α -syn (cross-reactive human and mouse) and imaged with the digital video microscope. (A) Representative images at three months postgraft of the neuropil showing higher levels of α -syn accumulation in tg mice as well as the accumulation of α -syn in the NSCs (arrow). (B) Computer-aided image analysis of the levels of α -syn immunoreactivity in the striatum at one and three months postgraft. (C) Computer-aided image analysis of the % of NSCs displaying α -syn immunoreactivity in the striatum at one and three months postgraft. All results are presented as mean \pm SEM, each group includes $n = 8$ vehicle and $n = 8$ CBL mice per time point. $^{\#}P < 0.05$ between vehicle-treated and CBL-treated α -syn tg mice by one-way ANOVA and post hoc Tukey–Kramer test. Bar = 25 μ m.

Behavioral effects of combined NSC grafting and CBL on α -syn tg mice. Given that previous studies have shown that the α -syn accumulation in the striatum of these mice is associated with motor deficits detected in the horizontal beam test,⁵⁷ we used this test to evaluate the functional effects of NSC grafting and CBL treatment in the non-tg and α -syn tg mice (Fig. 5A). Mice from the three-month postgraft group were chosen for this test. As expected, all mice in the four groups performed comparably in terms of time (Fig. 5B) and distance (Fig. 5C) that were required to negotiate the test horizontal

beam. The main difference was in the number of errors, with the vehicle-treated α -syn tg mice displaying significantly more errors when compared with the non-tg mice (Fig. 5D), whereas, the α -syn tg mice treated with CBL displayed comparable number of errors as the non-tg controls and less than that of the vehicle-treated α -syn tg mice (Fig. 5D).

Discussion

The present study demonstrated that vehicle-treated α -syn tg mice exhibit decreased survival of grafted NSCs, while CBL

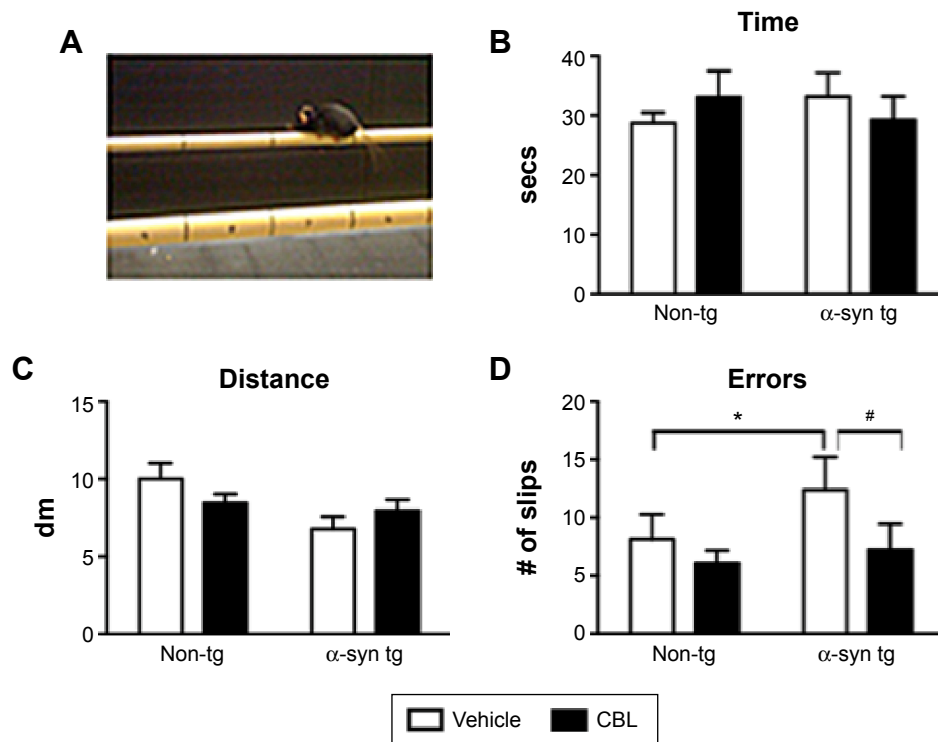


Figure 5. Behavioral motor analysis of the grafted CBL-treated α -syn tg mice. The non-tg and α -syn tg mice that were NSC grafted and treated with vehicle or CBL for three months postgraft were included for horizontal beam analysis. (A) Representative image of a tg mouse in the horizontal beam apparatus. (B) Analysis of time taken to traverse the test beam. (C) Analysis of time taken to traverse the test beam and (D) analysis of errors when walking in the beam, showing more errors in the vehicle α -syn tg mice and reversal with CBL treatment. All the results are presented as mean \pm SEM, each group includes $n = 8$ vehicle and $n = 8$ CBL mice per time point. $\#P < 0.05$ between vehicle-treated and CBL-treated α -syn tg mice by one-way ANOVA and post hoc Tukey–Kramer test.

treatment enhanced the survival of grafted NSCs in the brains of these mice. This is consistent with our previous studies which showed that the toxic microenvironment in the brain of tg mice with other neurodegenerative conditions such as AD in the APP tg mice might compromise the survival of both endogenous and transplanted NSCs and that CBL treatment improved the survival of the grafted NSCs.³³

Studies in experimental animal models of PD and clinical reports⁵⁸ have both shown a potential use for NSCs, particularly those coming from the mesencephalon to differentiate into dopaminergic cells in the adult CNS and ameliorate some of the symptoms of the disease.^{59,60} Therefore, grafting NSCs or fetal DA neurons provide a potential therapeutic option for late-stage PD.²⁸ In particular, striatal transplantation of fetal dopaminergic neurons or NSCs has been shown to improve the PD-like symptoms in animal models, but the low rate of cell survival, differentiation, and integration in the host brain limits the therapeutic value.^{28,61,62} As recently noted, poor viability of transplanted cells could be an important problem associated with stem cell-based therapy for PD⁶³ and other neurodegenerative disorders.

In an attempt to overcome some of these problems and enhance the therapeutic effects of cell transplantation, a recent study cogenerated mesencephalic NSCs engineered to express

glial-derived neurotrophic factor together with fetal mesencephalic neurons into the 6-hydroxydopamine (6-OHDA) rat model of PD.⁶⁴ This approach was shown to reduce the apomorphine-induced rotation, improve survival of NSCs in vivo, and promote greater differentiation of NSCs into DA neurons when compared with separate transplantation of NSCs or fetal DA neurons alone.⁶⁴ Moreover, a recent study showed that combining a collagen hydrogel incorporating an integrin-binding protein complex as a carrier for neural stem cells improve the viability after transplantation into the striatum.⁶³ These studies are in agreement with our study in that enhanced survival of grafted NSCs was achieved in the context of providing an exogenous source of neurotrophic factors to support the transplanted cells in the noxious environment of neurodegenerative pathology. The advantage of this transgenic model is that it displays extensive α -syn accumulation and related deficits; the main disadvantage is that it only develops limited degeneration of the dopaminergic system.

The mechanisms through which CBL might enhance the survival of grafted cells in the α -syn tg model of PD/DLB are not completely clear. One possibility is that CBL might reduce the accumulation or toxicity of α -syn that can potentially damage the grafted NSC. However, we did not find a significant effect of CBL in the overall levels of α -syn after one or

three months of treatment, neither did we observe that CBL blocked or reduced the transmission of α -syn to the grafted NSCs. Another possibility is that CBL might have promoted the differentiation of NSCs to dopaminergic neurons in the striatum. However, we did not detect new TH-positive cells when analyzing the grafted area. Finally, it is possible, as we have shown before, that CBL might enhance the survival of the grafted NSCs without increasing the differentiation into a mature neuronal phenotype.^{33,46} In such a scenario, grafted NSCs have been shown to promote survival by producing neurotrophic factors such as BDNF.³³ Consistent with the possibility that in α -syn tg mice CBL enhanced the survival of the grafted cells, we showed that the grafted cells in the CBL-treated animals displayed an increase in BrdU- and DCX-positive cells and a decrease in TUNEL+ cells.

The mechanisms through which CBL enhances survival of NSCs and exerts its antiapoptotic effects are complex. However, we have recently shown that CBL increased furin levels, which in turn resulted in increased processing of pro-BDNF into BDNF in the grafted cells.³³ This is consistent with the previous studies showing that CBL might increase the levels of NGF by promoting the processing of pro-NGF to its mature form via furin.⁶⁵ Previous studies have shown that NSCs are capable of producing BDNF that in turn can protect neighboring host cells.^{66,67} Along these lines, a recent study found that NSCs rescue cognitive and motor dysfunction in a tg mouse model of DLB through a BDNF-dependent mechanism.⁶⁸

Limitations of our study are that we included only two time points and that more extensive behavioral testing is necessary. Moreover, more detailed analysis as to the potential mechanisms and implications of the combined effects of CBL and NSCs are necessary given the potential for proliferation and transformation as to prolonged time points. In summary, the results of the present study suggest that CBL is capable of protecting the grafted NSCs in an α -syn tg mouse model and as such may be a potential adjuvant therapy when combined with cell-based therapy for neurodegenerative disorders.

Author Contributions

Analyzed the data: ER, PD, KU, MM, JF. Wrote the first draft of the manuscript: EM, KU. Contributed to the writing of the manuscript: KU, PD, ER. Agree with manuscript results and conclusions: ER, PD, KU, MM, JF, AA, SW, HB, DM, HM, EM. Jointly developed the structure and arguments for the paper: ER, PD, KU. Made critical revisions and approved final version: KU, SW, HB, DM, HM. All authors reviewed and approved of the final manuscript.

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