GDNF signaling in subjects with minimal motor deficits and Parkinson’s disease

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**A B S T R A C T**

The failure of glial cell derived neurotropic factor to be efficacious in blinded clinical trials for Parkinson’s disease may be due to alterations in signaling receptors and downstream signaling molecules. To test this hypothesis, brain sections were obtained from older adults with no motor deficit (n = 6), minimal motor deficits (n = 10), and clinical diagnosis of Parkinson’s disease (n = 10) who underwent motor examination proximate to death. Quantitative unbiased stereology and densitometry were performed to analyze RET and phosphorylated ribosomal protein S6 expression in nigral neurons. Individuals with no motor deficit had extensive and intense RET and phosphorylated ribosomal protein S6 immunoreactive neurons in substantia nigra. The number and staining intensity of RET-immunoreactive neurons were reduced moderately in subjects with minimal motor deficits and severely reduced in Parkinson’s disease relative to no motor deficit group. The number and staining intensity of phosphorylated ribosomal protein S6 was more markedly reduced in both subjects with minimal motor deficits and Parkinson’s disease. Reductions in levels of RET and phosphorylated ribosomal protein S6 were recapitulated in a non-human primate genetic Parkinson’s disease model based on over-expression of human mutant a-synuclein (A53T). These data indicate that for neurotrophic factors to be effective in patients with minimal motor deficits or PD, these factors would likely have to upregulate RET and phosphorylated ribosomal protein S6 immunoreactive neurons in substantia nigra.

1. Introduction

The progressive loss of dopamine neurons in the nigrostriatal system leads to motor symptoms in Parkinson’s disease (PD). Although dopamine substitution therapy and deep-brain stimulation can potently lead to evaluating neuroprotective efficacy of GDNF and NRTN in PD brain. However to date, neither GDNF nor NRTN delivery to PD brains exhibited significant improvements in motor function in randomized placebo controlled studies (Lang et al., 2006; Marks Jr. et al., 2010; Olanow et al., 2015; Whone et al., 2019). Nevertheless, there is interesting evidence from brain imaging suggesting biological activity from intrastitial GDNF delivery in patients (Heiss et al., 2019).

Recent morphological study on postmortem brains with NRTN gene delivery revealed that the localized areas of NRTN-expression displayed intense and dense tyrosine hydroxylase (TH) immunopositive fibers in putamen and healthy dopaminergic neurons in substantia nigra (Chu et al., 2020), suggesting that NRTN can induce regeneration of putaminal dopaminergic terminals and protect nigral dopaminergic neurons in PD. Although these clinic trials were limited by small numbers of patients, it highlights the need for further studies to examine the alterations of GDNF signaling pathway during the development of PD. One factor that should be considered is whether the failure of these trials is

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related to impairment in the functional status of residual nigrostriatal neurons is the subject population. Our previous study demonstrated that compared with normal aging, the density of melanized and TH-positive neurons was reduced by 54.6% and 64.1% in subjects with minimal motor deficits (MMD) and 73.9% and 85.8% in PD cases respectfully (Chu et al., 2018). Whether the remaining melanized nigral neurons are continuously expressing GDNF receptors and downstream signaling molecules is still unknown. To address this issue, the present proposal investigated whether GDNF and NRTN’s signaling receptor RET and its downstream molecule phosphor-ribosomal protein S6 (p-pS6) expression is intact in elders with MMD and patients with clinic and pathological diagnostic PD relative to age-matched controls. In this and previous studies, we consider this population with MMD to be prodromal PD.

2. Materials and methods

2.1. Subjects

We analyzed brain tissues from older adults with no motor deficit (NMD; n = 6), minimal motor deficits (MMD; n = 10) or a clinical and pathologic diagnosis of PD (n = 10; Table 1). Each subject signed an informed consent for clinical assessment prior to death and an anatomical gift act for donation of brain at the time of death. Both subjects with NMD and MMD were participants in the Religious Orders Study, a community-based cohort study of chronic conditions of aging who agreed to brain autopsy at the time of death and were examined by neurologist or geriatrician at the Rush Alzheimer’s Disease Center. All adults with sporadic PD were diagnosed by movement disorder specialists in the department of Neurological Sciences at Rush University Medical Center. The Human Investigation Committee at Rush University Medical Center approved this study.

All subjects with NMD and MMD were assessed within 1 year prior to their death by trained nurse investigators with a modified version of the UPDRS using a 0–5 rating scale (Louis et al., 2003; Buchman et al., 2012). Four established parkinsonian signs (26 items) including gait, rigidity, bradykinesia, and tremor were considered, and a global parkinsonian score was derived from the UPDRS scores (Bennett et al., 2012). MMD was defined as having 2 or more of these parkinsonian signs with a score of 1 but without clinical features sufficient to meet the definition of PD based on U.K. Brain Bank criteria (Supplementary table 2A). NMD was defined as having 1 or none of these parkinsonian signs (Supplementary Table 1A; Buchman et al., 2012; Chu et al., 2018). All PD cases were diagnosed by a board-certified movement disorders specialist using a battery of tests including the full UPDRS and met U.K. Brain Bank criteria. In order to compare with MMD, the 26 items included in the modified version of the UPDRS were extracted from the full UPDRS in patients with PD (Supplementary Table 3). The ratio (the raw score/the maxima possible score) was multiplied by 100. Exclusion criteria included familial PD, the Lewy body variant of Alzheimer’s disease or the combination of PD and Alzheimer’s disease.

2.2. Tissue processing

All brains were processed as described previously (Chu et al., 2006). Briefly, each brain was cut into 2 cm coronal slabs and then hemisected. The slabs were fixed in 4% paraformaldehyde for 5 days at 4 °C. After 24 hours, the brain blocks were sampled from one side of the brain for pathologic diagnoses, the remaining brain slabs were dried and subjected to 0.1 M phosphate buffered saline (PBS; pH 7.4) containing 2% dimethyl sulfoxide, 10% glycerol for two days followed by 2% dimethyl sulfoxide, and 20% glycerol in PBS for at least 2 days prior to sectioning. The fixed slabs were then cut into 18 adjacent series of 40 μm thick sections on a freezing sliding microtome. All sections were collected and stored at −20 °C in a cryoprotectant solution prior to processing.

A complete neuropathological evaluation was performed (Schneider et al., 2006). Dissection of diagnostic blocks included a hemisection of brain. Lewy bodies were examined with H&E staining and further identified with antibodies to alpha-synuclein (α-syn) on sections from midfrontal, midtemporal, inferior parietal, anterior cingulate, entorhinal and hippocampal cortices, basal ganglia and midbrain. McKeith criteria (McKeith et al., 1996) were modified to assess the categories of Lewy body disease. Bielschowsky silver stain was used to visualize neurofibrillary tangle in the frontal, temporal, parietal, entorhinal cortex, and the hippocampus. Braak stages were based upon the distribution and severity of neurofibrillary tangle pathology. The neuritic plaques density was scored as recommended by Consortium to establish a Registry for Alzheimer’s Disease (CERAD).

2.3. Viral vector delivery

Adeno associated vectors (AAV) of a 1/2 serotype were designed such that expression was driven by a chicken beta actin (CBA) promoter hybridized with the cytomegalovirus (CMV) immediate early enhancer sequence. In addition, a woodchuck post-transcriptional regulatory element (WPRE) and a bovine growth hormone polyadenylation sequence (bGH-polyA) were incorporated to further enhance transcription following transduction. AAV1/2 is a chimeric vector where the capsid expresses AAV1 and AAV2 serotype proteins in a 1:1 ratio and uses the inverted terminal repeats (ITRs) from AAV2 according to the following scheme: CMV/CBA promoter-human ASAT α-synuclein (h-ASAT-α-syn) WPRE-bGH-polyA-ITR (Hauck et al., 2003). The vectors were produced in GeneDetect Ltd., Auckland, New Zealand. Viral titers were determined by quantitative PCR (Applied Biosystems 7900 QPCR) with primers directed to the WPRE region, thus representing the number of functional physical particles of AAV in the solution containing the genome to be delivered (5.1 × 1012 vg/ml). Full details of h-ASAT-α-syn vector design can be found in Koprich et al., 2010. The h-ASAT-α-syn was tagged with hemagglutinin (HA) on the carboxy terminal end.

Six cynomolgus macaques ranging 8 to 13 years of age (4 male and 2 female) were used in this study. Animals received two injections of h-ASAT-α-syn (n = 3) or sham surgery (n = 3) into left substantia nigra. Coordinates for stereotoxic injection were based upon MRI guidance. Prior to surgery, monkeys were anaesthetized with an intramuscular injection of ketamine (3 mg/kg) and dexamethomidine (0.03 mg/kg). Once in an anesthetic plane, the monkeys were placed in a specially designed MRI compatible stereotoxic frame modelled after a Kopf

Table 1

| Measure | No Motor Deficit (NMD) | Minimal Motor Deficit (MMD) | PD |
|---------|-----------------------|----------------------------|----|
| Case number | 6 | 5 | 5 |
| Age at death (yr) | 83.33 ± 4.19 | 91.50 ± 14.90 | 76.40 ± 10.13 |
| Sex, M/F | 2/4 | 5/5 | 6/4 |
| Mini-Mental status examination (0–30) | 28.83 ± 0.75 | 14.90 ± 11.03 | 6.13 ± 2.94 |
| Global parkinsonism score (0–100) | 0.83 ± 0.98 | 1.40 ± 0.51 | 32.98 ± 6.60 |
| Parkinsonia Gait score (0–100) | 4.93 ± 5.50 | 45.90 ± 19.41 | 42.91 ± 21.06 |
| Rigidity (0–100) | 2.50 ± 6.13 | 22.00 ± 27.20 | 37.00 ± 17.35 |
| Bradykinesia (0–100) | 5.83 ± 8.01 | 17.16 ± 16.22 | 44.58 ± 6.05 |
| Tremor (0–100) | 3.50 ± 2.94 | 5.90 ± 6.22 | 8.21 ± 14.19 |
| Postmortem interval (hrs) | 7.48 ± 3.86 | 7.98 ± 4.49 | 6.52 ± 3.06 |

* P < 0.01 compared with no motor deficit.
** P < 0.001 compared with no motor deficit.
### P < 0.005 compared with minimal motor deficit.
#### P < 0.001 compared with minimal motor deficit.
primate stereotactic frame. The angle of the head was established by measuring the height of an incisor tooth using a standard micromanipulator and a modified electrode holder. Then the monkey was transferred to a 3.0 T MRI unit (GE Healthcare). Coronal T1-weighted images were acquired at a 2-mm slice thickness with 1 mm interpolation. The coordinates for injection were measured separately for each animal using the MRI unit’s built-in software. The rostral injection (15 μl) was targeted to the substantia nigra at the level of the root of third cranial nerve. The second injection (15 μl) was targeted to the substantia nigra at the level of decussation of the cerebellar peduncle. Monkeys were housed one per cage on a 12-h on/12-h off lighting cycle with ad libitum access to food and water. The quality of the animal care exceeded the recommended NIH guidelines. Institutional Animal Care and Use Committee at Rush University Medical Center approved this study.

At eight month post-injection, animals were sacrificed for histochemical analysis. Monkeys were pretreated with ketamine (20 mg/kg, intramuscularly) and then were deeply anaesthetized with sodium pentobarbital (25 mg/kg, intravenously). Prior to perfusion, monkeys were injected with 1 ml of heparin (20,000 IU) into the left ventricle of the heart. Animals were then perfused through the ascending aorta with physiological saline, followed by 1 l of 4% ice-cold paraformaldehyde. The brains were then removed from the calvaria, post-fixed in the same fixative solution overnight, progressively transferred through 10%, 20%, and 30% sucrose, and sectioned on a freezing microtome at 40 μm in the coronal plane. All sections were collected and stored in order in a cryoprotectant solution (30% sucrose and 30% ethylene glycol in 0.1 M phosphate buffered saline, pH 7.4) before processing.

2.4. Immunohistochemistry

An immunoperoxidase labeling method was used to visualize nigral RET-positive cells with RET antibody (AF1485, R&D system) and p-rpS6-positive cell with phosphosp-S6 ribosomal protein antibody (#5364, Cell Signaling). To keep the immunostaining processes consistent, sections from each subject were all stained at the same time under the same experimental conditions including buffers, concentration of chemicals and antibodies, and incubating time and temperature. To increase the signal to noise ratio, tissue was processed using a modified antigen retrieval protocol (Shi et al., 2001; Chu et al., 2002). Sections from each brain were heated in 0.01 M sodium citrate buffer, pH 6.0, for 20 min at 95 °C using a water bath. Endogenous peroxidase was quenched by 20 min incubation in 0.1 M sodium periodate, and background staining was blocked by 1 h incubation in a solution containing either 2% bovine serum albumin and 5% normal rabbit or goat serum. Tissue sections were immunostained for RET (1:1000) and p-rpS6 (1:1000) at room temperature overnight. After 6 washes, sections were sequentially incubated for 1 h in biotinylated rabbit anti-goat IgG (1:200; Vector, Burlingame, CA) for RET and goat anti-rabbit IgG (1:200; Vector, Burlingame, CA) for p-rpS6 followed by the Elite avidin–biotin complex (1:500; Vector) for 75 min. The immunohistochemical reaction was completed with 0.05% 3, 3-diaminobenzidine (DAB) and 0.005% H2O2 at room temperature for eight minutes. Sections were mounted on gelatin-coated slides, dehydrated through graded alcohol, cleared in xylene, and coverslipped with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI). For control, additional sections were processed identically except for the omission of the primary antibody.

2.5. Evaluating densities of RET and p-rpS6 immunoreactive neurons

The density of RET immunoreactive (RET-ir) and p-rpS6 immunoreactive (p-rpS6-ir) neurons was evaluated from each subject using an optical fractionator unbiased sampling design and Cavalieri’s principle to assess the volume within substantia nigra pars compacta (Chu et al., 2018; Gundersen and Jensen, 1987). In each subject, we evaluated the substantia nigra pars compacta from the level of midbrain at the exit of the 3rd nerve to the decussation of the superior cerebellar peduncle.

Approximately 5 equispaced sections were sampled from each brain. The section sampling fraction (ssf) was 1/0.055. The distance between sections was approximately 0.72 mm. The substantia nigra pars compacta was outlined using a 1.25× objective. A systematic sample of the area occupied by the substantia nigra pars compacta was made from a random starting point (StereoInvestigator v10.40 software; MicroBrightField, Colchester, VT). Counts were made at regular predetermined intervals (x = 313 μm, y = 313 μm), and a counting frame (70 × 70 μm = 4900 μm2) was superimposed on images obtained from tissue sections. The area sampling fraction (asf) was 1/0.05. These sections were then analyzed using a 60× Planapo oil immersion objective with a 1.4 numerical aperture. The section thickness was empirically determined. Briefly, as the top of the section was first brought into focus, the stage was zeroed at the z-axis by software. The stage then stepped through the z-axis until the bottom of the section was in focus. Section thickness averaged 16.21 ± 2.3 μm in the midbrain. The dissector height (counting frame thickness) was 10 μm. This method allowed for 1 μm top guard zones and at least 2 μm bottom guard zones. The thickness sampling fraction (ssf) was 1/0.62. Care was taken to ensure that the top and bottom forbidden planes were never included in the cell counting.

Most dopaminergic neurons contain neuromelanin (NM) in human midbrain. NM provides an easily discernible endogenous marker for dopaminergic neurons, allowing for an easy assessment of colocalization with RET-ir and p-rpS6-ir products in dopaminergic neurons. The number of RET-ir/NM-laden, p-S6-ir/NM-laden or NM-laden only neurons within the substantia nigra pars compacta was estimated separately using the following formula: N = ΣQ/1/asf/1/asf/1/tsf. ΣQ was the number of raw counts. The coefficients of error (CE) were calculated according to the procedure of Gunderson and colleagues as estimates of precision (West and Gundersen, 1990). The values of CE were 0.10 ± 0.02 (range 0.08 to 0.12) in NMD and MMD and 0.12 ± 0.05 (range 0.10 to 0.15) in PD.

2.6. Double-label immunofluorescence

A double-label immunofluorescence procedure was employed to determine whether RET expression was affected in remaining dopaminergic neurons with and without α-syn inclusions and whether RET was co-localized with TH and p-rpS6 expression. Tissue from each subject was treated with antigen retrieval protocol (see Immunohistochemistry section). After background staining was blocked for 1 h, sections were incubated in the first primary antibody (RET, 1: 500) overnight and the donkey anti-goat antibody coupled to DyLight 488 (1:200, Jackson ImmunoResearch) for 1 h. After blockade for 1 h, the sections were then incubated in the second primary antibodies (α-syn, 1:500; TH, 1:5000; p-rpS6, 1:1000) overnight, and the donkey anti-mouse (for TH) and donkey anti-rabbit (for α-syn and p-rpS6) antibodies coupled to DyLight 649 (1:200, Vector) for 1 h. The sections were mounted on gelatin-coated slides, dehydrated through graded alcohol, cleared in xylene, and covered using DPX (Sigma-Aldrich, St Louis, MO).

2.7. Measurements of RET, TH, and p-S6 immunofluorescence intensity

Fluorescence intensity measurements were performed according to previously published procedures (Chu et al., 2018). All immunofluorescence double-labeled images were scanned with an Olympus Confocal Fluoroview microscope equipped with argon, helium-neon lasers, and transparent optics. With a 20× magnification objective and a 488 and 633 nm excitation source, images were acquired at each sampling site in the substantia nigra and were saved to a Fluoview file. Following acquisition of an image, the stage moves to the next sampling site to ensure a completely non-redundant evaluation. To maintain consistency of the scanned image for each slide, the laser intensity, confocal aperture, photomultiplier voltage, offset, electronic gain, scan speed, image size, filter, and zoom were set for the background level whereby autofluorescence was not visible with a control section. These
settings were maintained throughout the entire experiment. The intensity mapping sliders ranged from 0 to 4095; 0 represented a maximum black image and 4095 represented a maximum bright image. The RET, TH, and p-rpS6 immunopositive perikarya within neurons were identified, outlined, and measured separately. Five equispaced nigral sections were sampled and evaluated. Over 150 cells were quantified in each subject. To account for differences in background stainig intensity, 5 background intensity measurements lacking immunofluorescent profiles were taken from each section. The mean of these 5 measurements constituted the background intensity that was then subtracted from the measured optical density of each individual neuron to provide a final optical density value.

2.8. Digital illustrations

Conventional light microscopic images were acquired using an Olympus microscope (BX61) attached to a Nikon (Tokyo, Japan) digital camera DXM1200 and stored as tiff files. Confocal images were exported from the Olympus laser-scanning microscope with Fluoview software and stored as tiff files. All figures were prepared using Photoshop 7.0 graphics software (Adobe Systems, San Jose, CA). Only minor adjustments of brightness were made.

2.9. Data analyses

Neuronal estimates and optical density measurements were compared across groups with one-way Kruskal–Wallis test followed by Dunn’s post hoc tests for multiple comparisons (Prism 4, GraphPad Software, Inc.). Correlation between optical density measurements of RET and p-rpS6 was performed using Spearman’s rank correlation. The level of significance was set at 0.05 (two-tailed).

3. Results

3.1. Parkinsonian signs in subjects with and without a clinical diagnosis of PD

We analyzed brain tissues from 6 older adults with NMD, 10 older adults with MMD, and 10 adults with a clinical diagnosis of PD. The diagnosis of subjects with MMD and PD was confirmed pathologically in each case and there was no evidence of an atypical Parkinsonism (e.g. PSP or MSA) in any of these subjects. Demographics are provided in Table 1. NMD subjects displayed low global scores as well as individual scores on gait, bradykinesia, rigidity, and tremor. In contrast, subjects with PD exhibited significantly higher scores on each of these measures. For the MMD cases, the parkinsonian gait and mini-mental status examination scores were significant higher as compared with NMD. Other scores were intermediate but no statistical differences between groups were observed. There was no difference on tremor score among groups. Detail clinical examinations from individual present in supplementary Table 1A, 2A, and 3.

3.2. Pathological examination in subjects without a clinical diagnosis of PD

Lewy body, nigral neuronal loss, neurofibrillary tangle, and neuritic plaques were examined from individual with NMD and MMD. Subjects with NMD displayed undetectable Lewy body, no nigral loss, but some neurofibrillary tangle and low density of neuritic plaque in limbic regions and neocortex (supplementary Table 1B). Subjects with MMD (supplementary Table 2B), Lewy bodies were examined not only in substantia nigra but also limbic system or neocortex. There was mild to moderate neuronal loss in the substantia nigra. The distribution of neurofibrillary tangles and density of nritic plaque were similar to the NMD group. Subjects with PD had nigral Lewy bodies and moderate or severe nigral neuronal loss (Braak et al., 2003; Buchman et al., 2012).

The specificity of RET antibody has been established previously in human tissue (Chu et al., 2020). RET staining in NM-laden neurons appeared a dark blue-black product (Fig. 1B, D, F) distributed throughout perikarya and proximal processes. The RET stained perikarya and processes were distributed throughout the full thickness of the sections. The fact that RET antibody had penetrated the full depth of sections was confirmed by a series of 1-μm disectors from the top to the bottom of RET-stained sections. In NMD group, intense and extensive RET-labeling was observed in substantia nigra pars compact (Fig. 1A). Virtually all of NM-laden neurons were RET-immunopositive (Fig. 1B). A few non-NM-laden neurons were also RET-immunopositive. The RET-labeling intensity in the substantia nigra from MMD group (Fig. 1C) was slightly reduced relative to NMD group (Fig. 1A). The majority of nigral neurons in the MMD cases showed intense RET staining (Fig. 1D). In contrast, RET labeling was remarkably decreased in PD group (Fig. 1E), which was expected as nigral neuronal loss is a defining pathological feature in PD. However, some remaining NM-laden neurons still displayed RET immunostaining and others were RET-immunonegative (Fig. 1F). Stereological analyses revealed that densities of RET-ir neurons were reduced 55.62% in MMD (1505.16 ± 438.61/mm²) but this was not significant difference as compared with NMD (2706.15 ± 550.33/mm²) group (P > 0.05, Fig. 1G). A total of 85.53% of remaining NM-laden neurons were RET-positive in MMD group (Fig. 1H). The density of RET-ir neurons was significantly declined 34.27% in PD (937.15 ± 364.80/mm²) group (Fig. 1G) relative to NMD group (P < 0.01). Interestingly, similar to MMD group, 82.59% of remaining NM-laden neurons were RET-positive in PD group (Fig. 1H). In NMD group, 98.13% of NM-laden neurons were RET positive that was significantly higher relative to both MMD (P < 0.05) and PD groups (P < 0.01, Fig. 1H). These results indicated that the number of RET-immunopositive neurons was severely reduced but the remaining nigral NM-laden neurons were continuously expressing RET.

3.3. Morphologic features and stereological estimate of RET-immunoreactive neurons in substantia nigra

The specificity of RET antibody has been established previously in human tissue (Chu et al., 2020). RET staining in NM-laden neurons appeared a dark blue-black product (Fig. 1B, D, F) distributed throughout perikarya and proximal processes. The RET stained perikarya and processes were distributed throughout the full thickness of the sections. The fact that RET antibody had penetrated the full depth of sections was confirmed by a series of 1-μm disectors from the top to the bottom of RET-stained sections. In NMD group, intense and extensive RET-labeling was observed in substantia nigra pars compact (Fig. 1A). Virtually all of NM-laden neurons were RET-immunopositive (Fig. 1B). A few non-NM-laden neurons were also RET-immunopositive. The RET-labeling intensity in the substantia nigra from MMD group (Fig. 1C) was slightly reduced relative to NMD group (Fig. 1A). The majority of nigral neurons in the MMD cases showed intense RET staining (Fig. 1D). In contrast, RET labeling was remarkably decreased in PD group (Fig. 1E), which was expected as nigral neuronal loss is a defining pathological feature in PD. However, some remaining NM-laden neurons still displayed RET immunostaining and others were RET-immunonegative (Fig. 1F). Stereological analyses revealed that densities of RET-ir neurons were reduced 55.62% in MMD (1505.16 ± 438.61/mm²) but this was not significant difference as compared with NMD (2706.15 ± 550.33/mm²) group (P > 0.05, Fig. 1G). A total of 85.53% of remaining NM-laden neurons were RET-positive in MMD group (Fig. 1H). The density of RET-ir neurons was significantly declined 34.27% in PD (937.15 ± 364.80/mm²) group (Fig. 1G) relative to NMD group (P < 0.01). Interestingly, similar to MMD group, 82.59% of remaining NM-laden neurons were RET-positive in PD group (Fig. 1H). In NMD group, 98.13% of NM-laden neurons were RET positive that was significantly higher relative to both MMD (P < 0.05) and PD groups (P < 0.01, Fig. 1H). These results indicated that the number of RET-immunopositive neurons was severely reduced but the remaining nigral NM-laden neurons were continuously expressing RET.

3.4. Co-localization and quantitative analyses of RET and TH expression in nigral neurons

Our previous study demonstrated that in PD, about 43.83% of remaining nigral NM-laden neurons were TH immunonegative (Chu et al., 2006). The present study revealed that 82.59% of nigral NM-laden neurons were RET immunopositive demonstrating discordance between TH and RET expression in PD brains. To evaluate this, we performed RET and TH fluorescent double labeling to examine co-existence of two markers in nigral neurons. Co-localization analyses revealed that the RET and TH immunofluorescent labeling were fully co-existed in nigral neurons in subjects with NMD (Fig. 2A–2C). The NM-laden neurons featuring intense RET immunostaining (Fig. 2A) displayed strong TH-immunoreactivity (Fig. 2B). In cases with MMD, remaining nigral neurons displayed intense RET-immunoreactivities (Fig. 2D) but weak TH-immunoreactivities (Fig. 2E). Interestingly, some nigral neurons exhibited only RET-labeling but no TH-labeling (Fig. 2D–2F). In PD, both RET and TH immunoreactivities were remarkably reduced (Fig. 2G–2I). Remaining NM-laden nigral neurons with light RET labeling (Fig. 2G) displayed TH-immunonegative (Fig. 2H). The continuing expression of RET in remaining nigral neurons would explaining that the percentage of RET-labeling cells was higher than TH-labeling cells in PD. Quantitative analyses of RET and TH immunofluorescent intensities were obtained from NM-laden neurons. A Kruskal-Wallis test revealed significant differences in RET expression across these experimental groups (Fig. 2J; P < 0.001). Post hoc analyses further revealed statistically significant decreases in optical densities of RET.
Fig. 1. Patterns of RET immunoreactivity in substantia nigra from NMD (A, B), MMD (C, D), and PD (E, F) subjects. Intense and extensive RET immunoreactive staining was observed throughout substantia nigra in subjects with NMD (A, B). The RET immunoreactive products were distributed throughout the perikarya and processes of nigral melanized neurons. There was a slight reduction of RET immunoreactive neurons in subjects with MMD (C, D) compared with NMD (A, B). PD case (E) displayed severe reduction of RET immunoreactivity relative to NMD (B) and MMD (D). Some remaining nigral melanized neurons exhibited RET immunoreactivities while others displayed undetectable RET immunoreactivity (arrows; F). Scale bar = 100 μm in F (applies to B, D); 500 μm for A, C, E. (G) Stereological analyses revealed that the density of RET-positive neurons was gradually reduced from MMD (55.62%) to PD (34.27%) relative to NMD group. (H) Majority of remaining melanized neurons displayed RET-immunopositive in NMD (98.13%), MMD (85.53%), and PD (82.59%). * P < 0.05, ** P < 0.01, compared with NMD.
Fig. 2. Confocal microscopic images of substantia nigra from NMD (A–C), MMD (D–F), and PD (G–I) illustrating RET (green; A, D, G), TH; (red; B, E, H), and colocalization of RET and TH (merged; C, F, I). In NMD, intense RET (A) and TH (B) immunoreactivities were co-localized in the nigral neurons (C). In MMD, some remaining nigral neurons displayed intense Ret (arrow; D) and TH (arrow; E) that were co-localized in nigral neurons (arrow; F) and others exhibited RET only (arrowheads; D) but no TH (arrowheads; E). In PD, both RET (G) and TH (H) immunoreactivities were severely reduced. Some nigral neurons displayed light RET labeling (arrowheads; G) but no detective TH (arrowheads; H) and others showed undetectable of both RET and TH (curled arrows; G–I). Scale bar in I = 100 μm (applies to all). Quantitation of immunofluorescent intensities revealed that the optical density values of RET (J) were decreased slightly in MMD group (no statistic deference, \( P > 0.05 \)) and significantly in PD (\( ***P < 0.001 \)) and TH (K) were significantly reduced in MMD (\( *P < 0.05 \)) and PD (\( **P < 0.001 \)) as compared with NMD group. The optical densities of RET and TH immunofluorescent intensities were measured from nigral neurons with neuronal melanin. AFU = arbitrary fluorescence units.
immunofluorescence signals in PD ($P < 0.001$) but not in MMD ($P > 0.05$), compared with NMD. The optical density of TH immunofluorescence signals were significantly different among the three groups analyzed (Fig. 2K; $P < 0.001$). Post hoc analyses further revealed a significant decline in the optical density of the TH immunofluorescence signal in MMD ($P < 0.05$) and PD ($P < 0.001$) compared with NMD group. Taken together, results from double labeling analysis demonstrated that the levels of RET expression were progressively reduced from MMD to PD and some NM-laden nigral neurons exhibited still RET but not TH.

3.5. Co-localization and quantitative analysis of RET nigral neurons with α-synuclein inclusions

α-syn inclusion is a major pathologic feature in PD (Spillantini and Goedert, 2018). Thus we examined whether the reduction of RET expression in subjects with MMD and PD is associated with α-syn accumulation by double labeling anti-α-syn and anti-RET and obtained the optical density measurements for RET from neurons with or without α-syn immunoreactive inclusions. Co-localization studies revealed that neurons with α-syn-immunoreactive inclusions had lower optical density of RET immunoreactivity (Fig. 3F, I). In MMD subjects, neurons without α-syn-immunoreactive inclusions exhibited intense RET expression and neurons with α-syn-immunoreactive inclusions displayed light RET expression (Fig. 3D–F). RET immunoreactivity was severely reduced in both nigral neuron with (Fig. 3G–3I) or without (Fig. 3G–3I) α-syn inclusions in subjects with PD. To unequivocally determine whether decreases in levels of RET were associated inclusions, we quantified the relative intensities of RET-labeling neurons that did or did not contain α-syn-positive inclusions. Kruskal–Wallis test revealed a statistically significant difference in optical density of RET-immunoreactive intensity across these groups (Fig. 3J; $P < 0.001$). In MMD subjects, post hoc analyses revealed a significant decrease of RET-immunoreactive optical density in nigral neurons with α-syn inclusions ($P < 0.001$) but not the neurons without absent α-syn inclusions ($P > 0.05$). In PD group, the RET-immunoreactive optical density was significantly reduced in both neurons with ($P < 0.001$) or without ($P < 0.05$) α-syn inclusions compared with NMD. These data indicate that in nigral neurons of cases with MMD and PD,

3.6. Phosphorylated S6 ribosomal protein immunoreactivity in remaining nigral neuron

Growth factors and mitogens induce the activation of p70S6 kinase 1 and the subsequent phosphorylation of the p-rS6. The p-rS6 is an indicator of the upstream activation of the neuroprotective pathway and correlates with an increase in translation of mRNA transcripts (Peterson and Schreiber, 1998; Ruvinsky et al., 2005), and is a valuable hallmark of neuronal activity (Biever et al., 2015). To understand whether the GDNF receptor RET activate downstream molecules in brain with motor deficits, we further observed p-rS6 expression in remaining nigral neurons. Morphological analyses revealed that the p-rS6 immunoreactive active products were distributed into perikarya and main processes (Fig. 4B). In NMD group, intense and extensive p-rS6-immunopositive neurons were wildly distributed throughout substantia nigra (Fig. 4A). The majority of NM-laden neurons were p-rS6-immunopositive (Fig. 4B). The p-rS6-immunoreactivity was obviously reduced in MMD group (Fig. 4C) relative to NMD group (Fig. 4A). In MMD subjects, numerous NM-laden neurons displayed p-rS6-immunonegativity (Fig. 4D). In PD group, the p-rS6-immunoreactivity was severely reduced (Fig. 4E). Although many NM-laden neurons could be identified, most were p-rS6-immunonegative (Fig. 4F). Some p-rS6 immunopositive products were detected in non-NM-laden cells (Fig. 4F). Stereological analyses revealed that densities of p-rS6-immunoreactive neurons were significantly reduced in both MMD (1103.45 ± 741.87/mm$^3$) and PD (329.40 ± 204.90/mm$^3$) groups as compared with NMD (2579.51 ± 289.82/mm$^3$) group ($P < 0.05$; Fig. 4J). A total of 45.30% of remaining NM-laden neurons were p-rS6-positive in MMD group (Fig. 4H) and 27.09% in PD group (Fig. 4H). In NMD group, 82.07% of NM-laden neurons were p-rS6 positive that was significantly higher relative to both MMD ($P < 0.01$) and PD ($P < 0.001$) groups.

To further understand whether the RET and p-rS6 are co-existed in nigral neurons, RET and p-rS6 were examined with fluorescence double labeling in all participant cases. Confocal microscopic analysis revealed that major NM-laden neurons displayed both RET and p-rS6 staining in NMD group (Fig. 5C). The p-rS6 immunoactivities displayed different levels in MMD group (Fig. 5E). Some nigral neurons with strong RET staining exhibited light or undetected p-rS6 labeling (Fig. 5E, F) and others had both RET and p-rS6 labeling (Fig. 5F). In PD group, majority of remaining NM-laden neurons displayed only light RET staining but not detectable p-rS6 (Fig. 5G–5I). Quantitative analyses of p-rS6 and RET immunofluorescent intensities were obtain from NM-laden neurons. A Kruskal-Wallis test revealed significant difference in p-rS6 levels across these experimental groups (Fig. 5J; $P < 0.001$). Post hoc analyses further revealed statistically significant decreases in optical densities of p-rS6 immunofluorescence signals in MMD ($P < 0.001$) and PD ($P < 0.01$) compared with NMD. A regression analysis demonstrated a positive correlation between RET and p-rS6 labeling neuronal optical densities across groups ($r = 0.74$; $P < 0.001$; Fig. 5K).

3.7. Targeted viral over-expression of α-synuclein in monkey

Numerous pathological events occur in PD brain. To further test the hypothesis that α-syn accumulation specifically alters RET and p-rS6 expression, we overexpressed α-syn specifically within the substantia nigra using viral vectors. A specifically antibody (LB509) which recognizes human α-syn protein (Giasson et al., 2000) was used to examine the expression of α-syn in h-A53T gene injected monkey. An intense α-syn immunostaining was observed in the substantia nigra of monkeys received rAAV-A53T (Fig. 6A). α-syn immunoreactivity was diffusely distributed throughout the cells including the soma and processes (Fig. 6E). α-syn immunoreactive dystrophic neurites were also observed in the substantia nigra and striatum (data not show). Target over-expression of α-syn resulted in reduction of TH expression (Fig. 6B). The remaining nigral neurons displayed light TH-immunostaining (Fig. 6F). Quantitative observation revealed that overexpression of α-syn caused significant reduction of TH immunoreactive neurons (16–39%) in substantia nigra and intensities in striatum (19–26%) relative to the controls.

3.8. Qualitative and quantitative analysis of RET and p-rS6 immunoreactivity in substantia nigra of monkey with viral overexpression of α-synuclein

To examine whether the α-syn overexpression affects RET and p-rS6 expressions, the colocalization analyses of RET/α-syn and p-rS6/α-syn was performed and the levels of RET and p-rS6 were quantified in nigral neurons with or without α-syn expression. In control animals, robust RET and p-rS6 immunoreactivities were observed in substantia nigra (Fig. 7A, 8A). Both RET and p-rS6 immunostaining profiles were distributed in soma and proximal processes similar to what was seen in the human brain. In substantia nigra with h-A53T gene delivery, there was no obvious reduction of RET expression in nigral neurons (Fig. 7B). Double labeling revealed that nigral neurons with α-syn immunoreactivity still displayed RET labeling (Fig. 7F). Fluorescence intensity measurements revealed that neurons with α-syn accumulation exhibited mild reduction of RET immunofluorescence intensity but was not statistically different among groups ($P > 0.05$; Fig. 7G).

Double labeling of p-rS6 and α-syn revealed three population neurons in h-A53T–gene-injected substantia nigra: 1) Neurons that did not display α-syn immunoreactivity and exhibited intense p-rS6 labeling (Fig. 8B). 2) Neurons with α-syn immunoreactivity that displayed...
Fig. 3. Laser confocal microscopic images of substantia nigra from NMD (A–C), MMD (D–F), and PD (G–I) illustrating RT (green; A, D, G), phosphorylated α-synuclein (α-syn; red; B, E, H), and the co-localization of RT and α-syn (merged; C, F, I). Note that some nigral neurons with α-syn inclusions displayed RT labeling (arrowheads; D–F) while others were severely diminished (arrow; D–F) in subjects with MMD. The RT immunoreactivities were remarkably reduced in both neurons with (arrows; G–I) or without (arrowheads; G–I) α-syn inclusions in subjects with PD. Scale bar in I = 100 μm (applies to all). Quantitative measurement further revealed that the optic density of RT immunoreactivities was much severely decreased in the neurons with α-syn immunopositive inclusions than the neurons with absent α-syn immunonegative inclusions as compared with NMD group (J). *P < 0.05, **P < 0.001, compared with NMD; #P < 0.05 compared without α-syn inclusions in PD group; *P < 0.01 compared without α-syn inclusions in MMD group. AFU = arbitrary fluorescence units.
Fig. 4. Photomicrographs of substantia nigra from subjects with NMD (A, B), MMD (C, D), and PD (E, F) illustrating the patterns of phosphorylated ribosomal protein S6 (p-rpS6) immunoreactivities. In NMD group, majority of melanized neuron displayed intense p-rpS6 immunoreactivities in the soma and main processes through substantia nigra (A, B). In contrast, the p-rpS6 immunoreactivity was severely reduced in subjects with MMD (C) and some melanized neurons displayed p-rpS6-immunopositive (arrows; D) and others were p-rpS6-immunonegative (arrowheads; D). In subjects with PD, most of remaining neuromelanin-laden neurons showed no detectable p-rpS6 labelling (arrowheads; F). However, non-melanized cells exhibited strong p-rpS6 immunoreactivity in the soma and processes (arrow; F). Scale bar in F = 100 μm applies to B and D; 500 μm for A, C, E. (J) Stereological analyses revealed that the density of p-rpS6-positive neurons was significantly reduced in MMD (42.80%) and PD (12.67%) relative to NMD group. (H) Bar graph shown the percent of remaining melanized neurons displaying RET-immunopositive in NMD (82.07%), MMD (45.30%), and PD (27.09%). *P < 0.05, **P < 0.01, ***P < 0.001 compared with NMD group. #P < 0.05 compared with MMD group.
a marked reduction in p-rpS6 marker (Fig. 8F). 3) Neurons with strong α-syn labeling showed undetectable p-rpS6 (Fig. 8F), and colocalization of RET and p-rpS6 (merged; C, F, I). In NMD, intense RET (A) and p-rpS6 (B) immunoreactivities were co-localized in the nigral neurons (C). In MMD, some remaining nigral neurons displayed intense RET (arrows; D, F) but no p-rpS6 (arrows; E, F). In PD, both RET (G) and p-rpS6 (H) immunoreactivities were severely reduced. Some nigral neurons displayed light RET labeling (arrows; G, I) but no detective p-rpS6 (arrows; H, I). Scale bar in I = 100 μm (applies to all). Quantitation of immunofluorescent intensities revealed that the optical density values of p-rpS6 (J) were significantly decreased in MMD group (*P < 0.05) and PD (***P < 0.01) as compared with NMD. Regression analysis revealed a positive correlation of optic density in RET- and p-rpS6-immunoreactive neurons across groups (K). AFU = arbitrary fluorescence units.

Fig. 5. Confocal microscopic images of substantia nigra from NMD (A–C), MMD (D–F), and PD (G–I) illustrating RET (green; A, D, G), phosphorylated ribosomal protein S6 (p-rpS6; red; B, E, H), and colocalization of RET and p-rpS6 (merged; C, F, I). In NMD, intense RET (A) and p-rpS6 (B) immunoreactivities were co-localized in the nigral neurons (C). In MMD, some remaining nigral neurons displayed intense RET (arrows; D, F) but no p-rpS6 (arrows; E, F). In PD, both RET (G) and p-rpS6 (H) immunoreactivities were severely reduced. Some nigral neurons displayed light RET labeling (arrows; G, I) but no detective p-rpS6 (arrows; H, I). Scale bar in I = 100 μm (applies to all). Quantitation of immunofluorescent intensities revealed that the optical density values of p-rpS6 (J) were significantly decreased in MMD group (*P < 0.05) and PD (***P < 0.01) as compared with NMD. Regression analysis revealed a positive correlation of optic density in RET- and p-rpS6-immunoreactive neurons across groups (K). AFU = arbitrary fluorescence units.

4. Discussion

In present study, utilizing normal aging, subjects with minimal motor deficits or clinically and pathologically diagnosed PD, as well as non-human primate PD model allowed for the analysis of RET and its downstream molecule p-rpS6 expressions during PD development. The
results obtained from this study demonstrate that RET expression is highly localized to nigral dopaminergic neurons in the ventral midbrain, continued and strong RET expressed in majority of the dopaminergic neurons in the normal aging (NMD group). These RET-immunopositive neurons have strong p-rpS6 and TH expressions suggesting that GDNF signal pathway is well functioning in normal aging. In MMD group, the density of RET-immunopositive neurons and the levels of RET expression were slightly decreased but was not statistically difference when compared to the NMD group. However, the number and staining intensity of p-rpS6-immunoreactive neurons were significantly diminished in MMD cases. Interestingly, several nigral NM-laden neurons with RET expression displayed undetectable p-rpS6 and TH indicating that the GDNF signaling pathway is not functioning in some remaining nigral neurons and would need to be upregulated by the trophic factor to effect neuroprotective or neurorestorative effects. In clinical and pathological diagnostic PD, although there were remaining NM-laden nigral neurons, RET expression was much lower and p-rpS6-immunoreactivity was scarcely detected in remaining neurons indicating that GDNF signal pathway is extremely dysfunctional. These data demonstrated that dysfunction of GDNF signaling pathway begins early in the PD pathological process. If we can confirm that the subjects with MMD are truly prodromal PD, their attenuated loss of RET and p-rpS6 might make this population a better choice for GDNF delivery. Reductions in levels of RET and p-rpS6 were recapitulated in the non-human primate model of familial PD based on over-expression of human mutant α-syn (A53T) displayed significant reduction of TH and p-rpS6 but not RET expressions which recapitulated the pathological features of MMD. 

In subjects with MMD, Lewy body pathologies were found not only in substantia nigra but also in limbic regions or neocortical areas and there was mild or moderate neuronal loss in the substantia nigra. Additionally, mild to moderate neurofibrillary tangles and neuritic plaques were observed in brain especially limbic regions. So the subjects with MMD had minimal motor deficits and mild cognitive impairments. Among parkinsonian signs, the score of parkinsonian gait impairment was similar to what was seen in the PD group. The mild motor deficits were thought to be associated with nigral neuronal loss and Lewy body rather than Alzheimer’s pathologies (Bennett et al., 1996; Louis et al., 2003). Regression models suggested that nigral neuronal loss and Lewy body were related to increasing signs of parkinsonian gait impairment, rigidity, and bradykinesia but not related to tremor (Buchman et al., 2012). Subjects with MMD had moderate tangle and plaque pathology. Whether these tangle and plaque pathology contributed mild cognitive impairment is uncertain. However, the subjects with NMD had neurofibrillary tangles and neuritic plaques but no Lewy body in limbic regions and had no cognitive impairment. It is possible that emerging Lewy body pathology in limbic system could accelerate neurodegeneration resulting in cognitive impairment (Kalaitzakis et al., 2009). More studies are needed to elucidate pathology in PD with cognitive decline. Previous studies (Chu et al., 2018) revealed that there was moderate nigral neuron loss and Lewy body pathology and present study verified that the GDNF signaling was downregulated in nigral neurons in subjects with MMD. We hypothesize that subject with MMD is putative prodromal PD.

RET activation is necessary for the GDNF family ligands to support the survival of dopaminergic neurons (Baloh et al., 1997; Durbec et al., 1996; Trupp et al., 1996; Oo et al., 2003). Therefore, GDNF and NRTN would be effective as treatment for PD if the surviving nigral dopaminergic neurons continue to express RET and the RET activate GDNF signaling pathway (Airaksinen and Saarma, 2002). Many papers indicate neurotrophic factors have great potential protective and regenerative effects on the nigrostriatal dopaminergic neurons which undergo progressive degeneration in PD (Sullivan and Toulouse, 2011; Hegarty et al., 2014; Sullivan and O’Keeffe, 2016). However, all randomized controls clinical trial of GDNF or NRTN have failed. Several postmortem studies verified that AAV-NRTN gene therapies can enhance dopaminergic terminal branches in putamen and protect dopaminergic neurons in substantia nigra (Bartus et al., 2011, 2015; Chu et al., 2020). This enhancement of dopaminergic terminal branches was still observed after
Fig. 7. Laser confocal microscopic images of substantia nigra from monkeys with sham surgery (A, C, E) or human mutant (A53T) α-synuclein gene delivery (h-A53T, B, D, F) illustrating RET (green; A, B), α-synuclein (red; C, D), and colocalization of RET and α-synuclein (merged; E, F). Note that there was no obvious difference of RET immunoreactivities between monkeys received h-A53T (B) and sham surgery (control; A). Nigral neurons with α-synuclein immunoreactivity (arrows; D) were continually expressing RET (arrows; B, F). The neurons with absent α-synuclein labeling (arrowheads; B, F) in monkeys received h-A53T displayed intense RET labeling similar to the controls (A, E). Scale bar in F = 100 μm (applies to all). Quantitation of immunofluorescent intensities revealed that the optical density values of RET immunostaining (G) were slightly decreased in nigral neurons with present α-synuclein labeling (α-syn⁺) but no statistic difference (P > 0.05) as compared with control monkeys. The optical density of RET immunofluorescent intensities from nigral neurons with absent α-synuclein labeling (α-syn⁻) was similar to the control monkeys. AFU = arbitrary fluorescence units.
Fig. 8. Laser confocal microscopic images of substantia nigra from monkeys with sham surgery (control; A, C, E) or human mutant A53T α-synuclein gene delivery (h-A53T; B, D, F) illustrating p-rpS6 (green; A, B), α-synuclein (red; C, D), and colocalization of p-rpS6 and α-synuclein (merged; E, F). Note that the p-rpS6 immunoreactivities were diminished in the substantia nigra with h-A53T delivery (B) relative to control (A). Nigral neurons with α-synuclein staining displayed undetectable p-rpS6 labeling (arrows, B, F) or light p-rpS6 labeling (arrowheads; B, F). The neurons with absent α-synuclein immunoreactivity exhibited intense p-rpS6 labeling (cured arrows; B, F) similar to the nigral neurons in control (A). Scale bar in F = 100 μm (applies to all). Quantitation of immunofluorescent intensities revealed that the optical density values of p-rpS6 immunostaining (G) were significantly decreased in nigral neurons with present α-synuclein labeling (α-syn$^+$; $P < 0.05$) but not in the neurons with absent α-synuclein labeling (α-syn$^-$; $P > 0.05$) as compared to the control monkeys. AFU = arbitrary fluorescence units.
8–10 years post-neurturin gene delivery (Chu et al., 2020). These studies suggest that neurturin gene therapy takes far longer to fulfill its promise neuroanatomically in advanced PD than previously suspected, for a very long time appears to be required for amplification with enhanced dopaminergic structures. However, the NRTN distribution was located in a small portion of putamen (about 12.40% of putamen) and the enhancement of dopamine terminals was only occurred in area with NRTN expression and failure to sufficiently deliver enough of the trophic factor, in addition to the protein changes observed here, likely are responsible for these failures.

P-\(\text{rpS6}\) is one of RET’s downstream molecule in GDNF signaling pathway. In the field of neuroscience, \(\text{rpS6}\) phosphorylation is commonly used as readout of the mammalian target of rapamycin complex1 signaling activation or as a marker for neuronal activity (Sieber et al., 2015). Nevertheless, its biological role in nigral dopamine neurons still remains puzzling. Inhibiting \(\text{rpS6}\) phosphorylation result in neuronal death (Xu et al., 2014; Barialai et al., 2020) and promoting \(\text{rpS6}\) phosphorylation protects neurons against neurotoxicity (Xie et al., 2019) indicating that \(\text{rpS6}\) is necessary for neuronal function. GDNF-mediated rescue of the nigral neurons may depends on inducing the activation of \(\text{p70S6K}\) kinase 1 and the subsequent phosphorylation of the \(\text{rpS6}\) (Quintino et al., 2019) and mRNA translation (Pruigerman et al., 2017). The present study revealed that the expression of \(\text{p-rpS6}\) was more severely reduced than RET in subjects with MMD and PD. Many nigral melanized neurons displayed RET-immunopositive but no detectable p-rpS6. These data suggested that the remaining RET could not activate its downstream molecule. Whether the level of RET expression is too low, the GDNF expression is absent, or nigrostriatal axonal transport of GDNF is interrupted need further investigation. A recent study revealed that NRTN gene delivery to substantia nigra induced p-rpS6 expression in nigral neurons in a patient with PD suggesting that the decrease of p-rpS6 expression is associated with a deficit in neurotrophic factor function and indicated that the remaining neurons with RET expression can still respond to neurotrophic therapy. It had previously been reported in animals models that over-expression of \(\alpha\)-syn downregulates RET (Volakakis et al., 2015; Decressac et al., 2011). Subjects with MMD had large numbers of \(\alpha\)-syn-positive Lewy neurites in the putamen and Lewy bodies in the substantia nigra (Chu et al., 2018). In the present study, we observed that in PD, the RET-expressing neurons were reduced in both neurons with or without \(\alpha\)-syn inclusions. Interestingly, some NM-laden neurons with \(\alpha\)-syn inclusions still had RET expression in MMD group (Fig. 3F). Though many NM-laden neurons were lost in MMD and PD brain, about 80% (PD) to 85% (MMD) of remaining NM-laden neurons were RET-positive which is similar to the early reports (Walker et al., 1998). These findings suggest that RET expression persist in majority of NM-laden neuron during the development of PD. In previous studies of NRTN gene therapy in PD subjects, we observed robust RET and TH staining in neurons even though they contained \(\alpha\)-syn positive aggregates (Chu et al., 2020). In addition, more p-rpS6-positive neurons were only observed in direct nigral delivery of NRTN suggesting that NRTN activate it’s signaling pathway. These findings suggest that as long as RET expression in the remaining nigral neurons, synucleinopathy does not prevent GDNF or NRTN from expressing trophic effects on nigrostriatal dopaminergic neurons in PD. It should also be noted that gene delivery of \(\alpha\)-syn in rat models produces \(\alpha\)-syn levels 8–10 times than typically seen in PD patients (Decressac et al., 2011). This high dose of \(\alpha\)-syn produced by AAV- \(\alpha\)-syn gene delivery may also down regulate the normal function in down regulating GDNF signaling pathway. The downregulation of RET receptor expression by \(\alpha\)-syn was thought to be the reason for the lack of neuroprotective action of GDNF. However, in a letter to the editor, another group has urged caution in this interpretation (Su et al., 2017) and this letter also reported no downregulation of RET in transgenic \(\alpha\)-syn mice. To further understand that \(\alpha\)-syn accumulation affects RET expression, we examined the levels of RET expression in nigral neurons with or without \(\alpha\)-syn overexpression in monkey with targeted h-A53T gene delivery. Our results demonstrated that nigral neurons with \(\alpha\)-syn expression were still expressing RET. The levels of RET in neurons with \(\alpha\)-syn immunoreactivity were slightly but not significantly reduced compared to controls. Whether the dysfunction in the GDNF signaling pathway results in \(\alpha\)-syn accumulation and aggregation is needed to study. It is possible that without GDNF support, proteasomal and lysosomal dysfunction impaired protein clearance and protein accumulation and aggregation. Post-mortem studies must be interpreted with caution as factors such as disease heterogeneity and post-mortem interval can influence the results. To support our findings, we injected monkeys with viral vectors inducing the over-expression of human \(\alpha\)-syn. The h-A53T-gene caused moderate reduction of TH expression in nigrostriatal system which is similar to MMD. Interestingly, both h-A53T-gene-injected animals and MMD exhibited severe reductions in p-rpS6 rather than RET expression. These pre-clinical data support the accuracy of the human post-mortem findings. Results from h-A53T-gene-injected animals and subjects with MMD make us to speculate that synucleinopathy may attack the transitive system first and then reduce cellular physiological function. However, the remaining nigral neurons still expressed RET. In this patient population, there may remain opportunities to restore cellular function using neurotrophic factors such as NRTN or GDNF.

In summary, present and our other published data (Chu et al., 2018, 2020) suggest that the prodromal PD have about 55% of remaining nigral neurons comparing with normal age control and 85% of remaining nigral neurons displayed RET-immunopositive and relative high levels of RET expression relative to PD. To prevent neuro-degeneration and restore neuronal function, individuate with minimal motor deficits may be candidate for neurotrophic factor therapy. Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnbd.2021.105298.

Declaration of Competing Interest

The authors report no competing interests.

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References

Airaksinen, M.S., Saarma, M., 2002. GDNF family: signalling, biological functions and therapeutic value. Nat. Rev. Neurosci. 3, 383–394. https://doi.org/10.1038/nrev812.
Balog, R.H., Tansey, M.G., Golden, J.P., Creedon, D.J., Heuckeroth, R.O., Keck, C.L., Zimonicj, D.B., Popescu, N.C., Johnson Jr., E.M., Milbrandt, J., 1997. Tmn2, a novel receptor that mediates neuritin and GDNF signaling through Ret. Neuron 18, 793–802. https://doi.org/10.1016/S0896-6273(00)80218-5.
Barialai, L., Streckerm, M.I., Luger, A.L., Jäger, M., Bruns, I., Sittig, A.C.M., Mildenberger, I.C., Heller, S.M., Delaídellí, A., et al., 2020. AMPK activation protects astrocytes from hypoxia-induced cell death. Int. J. Mol. Med. 45, 1385–1396. https://doi.org/10.3892/ijmm.2020.4528.
Bartus, R.T., Herzog, C.D., Chu, Y., Wilson, A., Brown, L., Siffert, J., Johnson Jr., E.M., Olano, C.W., Mufson, E.J., Kordower, J.H., 2011. Bioactivity of AAV2-neurturin gene therapy (CERE-120): differences between Parkinson’s disease and nonhuman primate brains. Mov. Disord. 26, 27–36. https://doi.org/10.1002/mds.23442.
Bartus, R.T., Kordower, J.H., Johnson Jr., E.M., Brown, L., Kruegel, B.R., Chu, Y., Baumann, T.L., Lang, A.E., Olano, C.W., Herzog, C.D., 2015. Post-mortem assessment of the short and long-term effects of the trophic factor neuritin in patients with \(\alpha\)-synucleinopathies. Neuropathol. Dis. 78, 162–171. https://doi.org/10.1016/j.jnd.2015.03.023.
Bennett, D.A., Beckett, L.A., Murray, A.M., Shannon, K.M., Goetz, C.G., Pilgrim, D.M., Evans, D.A., 1996. Prevalence of parkinsonian signs and associated mortality in a community population of older people. N. Engl. J. Med. 334 (2), 71–76. https://doi.org/10.1056/NEJM19960111340202.
Bennett, D.A., Schneider, J.A., Arvanitakis, Z., Wilson, R.S., 2012. Overview and findings from the religious orders study. Curr. Alzheimer Res. 9, 628–645. https://doi.org/10.2174/156720512801322573.
Chu, Y., Le, W., Kompoliti, K., Jankovic, J., Mufson, E.J., Kordower, J.H., 2006. Nurr1 in Parkinson’s disease. J. Neurosci. 26, 10114–10122. https://doi.org/10.1523/JNEUROSCI.23-12-05141.2003.

Chen, E.Y., Chu, Y., et al., 2008. Transgene expression, bioactivity, and safety of delivery of neurturin by AAV2(CERE-120)-mediated gene transfer to the monkey striatum. Mol. Ther. 16, 1073–1078. https://doi.org/10.1038/s41434-018-0049-0.

Chu, Y., Bartus, R.T., Manfredsson, F.P., Olanow, C.W., Kordower, J.H., 2020. Long-term post-mortem studies following neurutrin gene therapy in patients with advanced Parkinson’s disease. Brain 143, 960–975. https://doi.org/10.1093/brain/awaa230.

Decressac, M., Ulsaky, A., Mattsson, B., Georgievskaja, B., Romero-Ramos, M., Kikid, D., Björklund, A., 2011. GDNF fails to exert neuroprotection in a rat α-synuclein model of Parkinson’s disease. Brain 134, 2302–2311. https://doi.org/10.1093/brain/awr200.

Deng, M., Fan, Q., Wang, D., et al., 2008. Increased Akt/mTOR activity in the striatum of α-synuclein overexpressing transgenic mice: potential role for autophagy. Proc. Natl. Acad. Sci. U. S. A. 105, 14184–14189. https://doi.org/10.1073/pnas.0805123105.

D’Agnillo, V., Boveris, A., 1973. Mitochondrial function in the aging process and tissue aging: a review. Mech. Ageing Dev. 1, 13–29. https://doi.org/10.1016/0047-6374(73)90020-6.

Djebali, M., Bada, L., Moyer, J.T., et al., 2013. Carbon monoxide reduces α-synuclein aggregates in dopaminergic neurons. J. Neurosci. 33, 10794–10803. https://doi.org/10.1523/JNEUROSCI.2875-13.2013.

Dugan, J.E., Gas, D.N., Kuzyk, A., et al., 2009. α-Synuclein mRNA is not increased in sporadic PD and α-synuclein accumulation depends on the degree of degeneration. Gene Ther. 26, 57–64. https://doi.org/10.1038/s41434-018-0049-0.

Ellenberger, T., 2008. The role of GABA release in the control of the dopaminergic system. Prog. Neurobiol. 85, 183–199. https://doi.org/10.1016/j.pneurobio.2008.07.002.

Kalaitzakis, M.E., Christian, L.M., Moran, L.B., Graeber, M.B., Pearce, R.K., Gentleman, S. et al., 2001. Translation control: connecting mitogens and the in vivo control of cell size and glucose homeostasis. Genes Dev. 15, 2199–2211. https://doi.org/10.1101/gad.351605.

Kalaitzakis, M.E., Christian, L.M., Moran, L.B., Graeber, M.B., Pearce, R.K., Gentleman, S. et al., 2001. Translation control: connecting mitogens and the in vivo control of cell size and glucose homeostasis. Genes Dev. 15, 2199–2211. https://doi.org/10.1101/gad.351605.

Kalaitzakis, M.E., Christian, L.M., Moran, L.B., Graeber, M.B., Pearce, R.K., Gentleman, S. et al., 2001. Translation control: connecting mitogens and the in vivo control of cell size and glucose homeostasis. Genes Dev. 15, 2199–2211. https://doi.org/10.1101/gad.351605.

Kalaitzakis, M.E., Christian, L.M., Moran, L.B., Graeber, M.B., Pearce, R.K., Gentleman, S. et al., 2001. Translation control: connecting mitogens and the in vivo control of cell size and glucose homeostasis. Genes Dev. 15, 2199–2211. https://doi.org/10.1101/gad.351605.

Kalaitzakis, M.E., Christian, L.M., Moran, L.B., Graeber, M.B., Pearce, R.K., Gentleman, S. et al., 2001. Translation control: connecting mitogens and the in vivo control of cell size and glucose homeostasis. Genes Dev. 15, 2199–2211. https://doi.org/10.1101/gad.351605.

Kalaitzakis, M.E., Christian, L.M., Moran, L.B., Graeber, M.B., Pearce, R.K., Gentleman, S. et al., 2001. Translation control: connecting mitogens and the in vivo control of cell size and glucose homeostasis. Genes Dev. 15, 2199–2211. https://doi.org/10.1101/gad.351605.

Kalaitzakis, M.E., Christian, L.M., Moran, L.B., Graeber, M.B., Pearce, R.K., Gentleman, S. et al., 2001. Translation control: connecting mitogens and the in vivo control of cell size and glucose homeostasis. Genes Dev. 15, 2199–2211. https://doi.org/10.1101/gad.351605.

Kalaitzakis, M.E., Christian, L.M., Moran, L.B., Graeber, M.B., Pearce, R.K., Gentleman, S. et al., 2001. Translation control: connecting mitogens and the in vivo control of cell size and glucose homeostasis. Genes Dev. 15, 2199–2211. https://doi.org/10.1101/gad.351605.