Molecular Mechanism of Regulation of MTA1 Expression by Granulocyte Colony-stimulating Factor*

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Parkinson disease (PD) is a neurodegenerative disorder with loss of dopaminergic neurons of the brain, which results in insufficient synthesis and action of dopamine. Metastasis-associated protein 1 (MTA1) is an upstream modulator of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, and hence MTA1 plays a significant role in PD pathogenesis. To impart functional and clinical significance to MTA1, we analyzed MTA1 and TH levels in the substantia nigra region of a large cohort of human brain tissue samples by Western blotting, quantitative PCR, and immunohistochemistry. Our results showed that MTA1 and TH levels were significantly down-regulated in PD samples as compared with normal brain tissue. Subsequently, immunohistochemistry analysis for MTA1 in substantia nigra sections revealed that 74.1% of the samples had a staining intensity of <6 in the PD samples as compared with controls, 25.9%, with an odds ratio of 8.54. Because of the clinical importance of MTA1 established in PD, we looked at agents to modulate MTA1 expression in neuronal cells, and granulocyte colony-stimulating factor (G-CSF) was chosen, due to its clinically proven neuroprotective effects. Treatment of the human neuronal cell line KELLY and acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model with G-CSF showed significant induction of MTA1 and TH with rescue of phenotype in the mouse model. Interestingly, the observed induction of TH was compromised on silencing of MTA1. The underlying molecular mechanism of MTA1 induction by G-CSF was proved to be through induction of c-Fos and its recruitment to the MTA1 promoter.

Parkinson disease (PD) is an idiopathic degenerative disorder of the central nervous system characterized by slow and decreased movement, pill-rolling tremor, and postural instability, and it is primarily caused by dopamine deficiency (1, 2). The rate-limiting enzyme for dopamine synthesis is tyrosine hydroxylase (TH) (3). Currently, there is no cure for PD, and patients are usually given drugs that provide symptomatic relief (4). Recently, it was shown that G-CSF enhances recovery in the mouse model of PD, and hence the G-CSF receptor might be a novel target for modifying the disease process in PD (5, 6). It is well documented that a consistent abnormality in PD is degeneration of dopaminergic neurons in SN leading to a reduction of striatal dopamine levels (7). TH catalyzes the formation of L-hydroxyphenylalanine, the rate-limiting step in the biosynthesis of dopamine. Thus, efficient treatment strategy for PD could be based on correcting or bypassing the TH enzyme deficiency or its downstream enzymes essential for catecholamine synthesis (8). For this, elucidation of the molecular mechanism of human TH gene regulation is very essential. Also, epigenetic profiling of the human TH promoter suggests that chromatin remodeling could have a significant impact in conferring tissue-specific gene expression of the human TH gene (9); however, its specific role in TH transcription remains poorly understood. Dynamic regulation of gene expression demands the participation of transcription factors, their coregulators, and multiprotein chromatin remodeling activity at target genes. Previously, it was shown that androgen receptor, DJ-1, Pitx3, and Nurrol regulate TH transcription (10–13). Recently, it was reported that MTA1 is an upstream modifier of TH and that the MTA1-tyrosine hydroxylase pathway plays an essential role in regulating the production of the dopamine precursor L-3,4-dihydroxyphenylalanine and its functions in movement disorders (14). However, the clinical significance of this nuclear receptor coregulator MTA1 in Parkinson disease brain samples and the ways to interfere with the development of this disease in experimental model systems have not been explored.

Granulocyte colony-stimulating factor (G-CSF) is commonly used in clinics to increase hematopoietic stem cells in bone marrow donors, and it has been shown to have neuroprotective, anti-apoptotic, and anti-inflammatory effects (15–18). Several studies have evaluated the effect of G-CSF in the treatment of PD in established mouse models (19–23). Despite several studies reporting the beneficial effects of G-CSF in PD, it is still not clear about the precise mechanism of G-CSF action at the molecular level. In this study, we established the functional and clinical significance of MTA1 in PD and demonstrated the molecular mechanism of G-CSF action.

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4 The abbreviations used are: PD, Parkinson disease; TH, tyrosine hydroxylase; qPCR, quantitative PCR; IHC, immunohistochemistry; SN, substantia nigra; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ANOVA, analysis of variance.

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Experimental Procedures

Cell Line and Reagents

Human neuroblastoma cell line KELLY (a kind gift from Prof. Christer Einvik, Department of Pediatrics, University Hospital of North-Norway) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. Short tandem repeat profiling validation was performed for KELLY cells by Eurofins, Germany. The following reagents were purchased: antibodies to MTA1 (A300–911A) and MTA1 (IHC-0026) (Bethyl Laboratories); STAT3 (9139), pSTAT3 (9131), MAPK (9107), pMAPK (9106), MTA1 (5641), and c-Fos (4384) (Cell Signaling); TH (AB 152) (Millipore); GAPDH (G8795) and vinculin (V9131) (Sigma); MTA1 siRNA (sc-35981), control siRNA-A (sc-37007), and ImmunoCruz™ mouse ABC staining kit (Santa Cruz Biotechnology); c-Fos siRNA (Dharmacon, Inc.); 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Sigma); bovine serum albumin (BSA) and citrate (trisodium salt (Sisco Research Laboratories, India). Unless mentioned, all other chemicals and reagents used were of analytical grade.

RNA and Protein Extraction from Brain Tissue

Ethically approved Parkinson disease (n = 26) and normal (n = 4) SN tissue samples were procured from the Parkinson Disease Society Tissue Bank, Imperial College, London. RNA and protein were isolated as per the protocol described previously (24).

Quantitative PCR (qPCR)

cDNA was generated from 1 μg of isolated RNA, and qPCR was performed as per the manufacturer’s instruction using human GAPDH as control (Applied Biosystems). Relative gene expression (Ct) was calculated using the ΔCt method.

Immunohistochemistry

Clinical Samples—SN sections of controls (n = 22) and PD cases (n = 25) were obtained from Queen Square Brain Bank for Neurological Disorders, Institute of Neurology, University College London. Immunohistochemistry for MTA1 and TH was done on serial sections of paraffin-embedded tissues on coated slides. A standard procedure was used for both the antigens. Briefly, the slides were dewaxed in xylene and subsequently hydrated in graded alcohols. Antigen retrieval was done using the heated citrate buffer method. The sections were blocked using normal goat serum, washed in PBST, and incubated with the respective primary antibodies overnight at 4 °C. The sections were then washed in PBST and incubated with the universal kit for secondary antibody (Biogenex Laboratories), counter-stained, and mounted using DPX mountant. Slides were scored using the Quick score method adapted from a protocol previously used (25) by an experienced pathologist. The statistical analysis of the categorized data was done using the SPSS software (version 16.0).

Criteria for the Categorization of MTA1 and TH Levels (Values)—Based on the receiver operating characteristic curve, the cutoff points for MTA1 and TH levels/values were calculated. There is a greater sensitivity and specificity for MTA1 at 6, and it is 9 for TH level. Based on these cutoff levels, we categorized the overall MTA1 and TH values of <6 and >6 and <9 and >9, respectively, for the purpose of categorical analysis and also to find out the odds ratio to quantify the risk of Parkinson disease for normal individuals.

G-CSF Treatment Studies—Cells were seeded in a 60-mm plate, and G-CSF (Neupogen, Amgen Inc.) treatment (100 ng/ml) was given, 24 h post-serum starvation for the time points 30 min and 1 and 2 h. Cells were lysed using RIPA buffer for Western blotting, and RNA was extracted using the TRizol method.

siRNA Transfection—Cells at 40% density were transfected with siRNA 24 h post-seeding, using Oligofectamine (Invitrogen) according to manufacturer’s instructions. 48 h post-transfection, cells were serum-starved for 24 h followed by G-CSF treatment for 2 h, and lysates were prepared in RIPA buffer.

MTA1 Promoter Construction and Luciferase Activity—Human MTA1 promoter (from −2951 to +216) was cloned from human genomic DNA by PCR amplification, followed by insertion into HindIII and Xhol site of pGL3 basic vector (Promega, Madison, WI). The c-Fos binding site deletion constructs were generated by PCR. Wild type (FL) and deletion construct (DEL) promoter plasmids were transfected into neuroblastoma cell line KELLY using FuGENE according to the manufacturer’s protocol. c-Fos expression plasmid was used with the full-length as well as the deletion construct to check for its effect on the promoter. 24 h after transfection with the full-length promoter, cells were serum-starved for 24 h treated with Statistic (20 μM) and U0126 (10 μM) for 1 h and 15 min, respectively, followed by treatment with G-CSF.

Chromatin Immunoprecipitation—24 h post-serum starvation, KELLY cells were treated with or without G-CSF and cross-linked with formaldehyde (1% final concentration, Sigma), and chromatin fragmentation was done by sonication on ice to yield an average length of less than 500 bp. Supernatants of the fragmented lysates were diluted 10-fold with chromatin dilution buffer (EMD Millipore, MA). An equal amount of chromatin was used for both control and G-CSF-treated samples and were immunoprecipitated with c-Fos antibody at 4°C overnight. Protein A/G PLUS–agarose beads were added to the lysate to isolate the antibody–bound complexes. The eluate was reverse cross-linked by heating at 65 °C for 4 h. RNase treatment was given to digest the RNA contamination. Samples were then treated with proteinase K for 1 h at 45 °C to digest the proteins that were immunoprecipitated, and finally, the DNA was extracted using the phenol-chloroform method. PCR was performed with a primer specific for the MTA1 promoter with c-Fos-binding site.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared for both G-CSF-treated and untreated KELLY cells using Nonidet P-40 lysis method. EMSA was performed with Light Shift chemiluminescent EMSA kit (Pierce) according to the manufacturer’s instruction. The binding of c-Fos transcription factor was performed using annealed biotin-labeled oligonucleotide probes in a 20-μl reaction mixture for 20 min at room temperature. Samples were run on a non-denaturing 5% polyacrylamide gel and transferred to a nylon membrane, cross-linked in UV light with the membrane face down on a
transilluminator and processed and imaged by exposing to x-ray film (Amersham Biosciences, Buckinghamshire, UK).

Western Blotting—100 μg of protein lysates per sample was run on 8% SDS-PAGE and electrobotted onto a nitrocellulose membrane. The membranes were then blocked in 5% milk for 1 h and then probed with the appropriate primary antibody at 4 °C overnight. The blots were probed with corresponding secondary antibody for 1 h at room temperature the following day. Protein bands were visualized using an enhanced chemiluminescence detection system (Bio-Rad).

Animal Experiments

Animal Study—Experimental animals were procured from Biogen, Hosur, India. Experimental protocol was approved by the Institutional Animal Ethics Committee, Sri Ramachandra University, Chennai, India. Six male Swiss Albino mice (20–23 g) were divided into two groups with three mice in each: group I received vehicle and group II received G-CSF (Pegfilgrastim (Imupeg), Abbott Healthcare Pvt. Ltd., India) in a single dose at 120 μg/kg, s.c. 4 h after treatment, the experimental animals were euthanized using carbon dioxide, and brains were quickly excised out and snap-frozen in liquid nitrogen; SN regions were identified using Paxinos and Franklin (26) mouse brain atlas and were dissected from frozen brain sections. The whole lysate of SN tissue was prepared using RIPA buffer.

MPTP Model Study—Male C57BL6/j mice (25–32 g b.wt) were obtained from the central animal facility, Sri Ramachandra University, Chennai, India. Animals were housed in polycarbonate cages with five animals/cage in a well ventilated room (air cycles, 12–15 exchanges/h; recycle ratio, 55:45) under an ambient temperature of 22 ± 3 °C and 30–70% relative humidity, with a 12-h light/dark artificial light cycle. They were provided with rodent feed (procured from Provimi Animal Nutrition India Pvt. Ltd., India) and purified water ad libitum. Animals were acclimatized at least for 7 days to the laboratory conditions prior to experimentation. Guidelines (see Ref. 27) were strictly followed throughout the experiment. The study protocol was approved by the Institutional Animal Ethics Committee, Sri Ramachandra University, Chennai, India (IAEC/XXVII/SRU/345/2014).

Experiment Design—Mice were selected based on their motor performance on beam walk and stride length tests on day 0. Animals were divided into three groups with 10 in each group. Group I served as negative control; group II served as positive control, and group III served as G-CSF. On days 1 and 3, the experimental groups II and III received MPTP at 60 mg/kg in four divided doses (4 × 15 at 2-h intervals, i.p) (with slight modifications). Development of motor disturbance was assessed on days 4 and 8 after MPTP intoxication. One animal from group II and two from the group III were removed from the study as they did not showed signs of motor dysfunction. G-CSF (120 μg/kg, s.c) or PBS (10 ml/kg, s.c) were injected from days 5 to 8 in group III. On day 8, 24 ± 1 h after the last dose of G-CSF/saline, the animals were euthanized for motor function. Following the motor function tests, animals were euthanized (by CO2 exposure), and brains were harvested and stored in buffered formalin for IHC.

Dopamine Measurement—Mice (n = 3) treated as above, were euthanized (by CO2 exposure), and brains were harvested and stored at −80 °C for dopamine estimation by HPLC.

Motor Functional Analysis

Beam Walk Experiment—Beam walk test was performed according to the Schallert (29) method with slight modifications. Mice were trained, before MPTP injection, to traverse a narrow beam of 100 cm length to reach an enclosed escape platform. A bright light (20 lux) was placed above the narrow beam to create an aversive stimulus. This encourages the mice to traverse the beam to the dark enclosed goal box. On days 4 and 8, mice were placed individually at the start of the beam, and the time taken for mice to traverse from the start of the beam to the enclosed escape platform, the number of foot slips, and the immobility period were recorded. The individual who scored the behavior was blinded to treatments.

Stride Length—Stride length test was performed according to the Fernagut et al. (30) method with slight modifications. Apparatus consisted of a runway (in cm) 4.5 (width) × 40 (length) × 9.5 (height) and a black wooden box 20 (width) × 14.5 (length) × 6.5 (height) with a hole (45 mm in diameter) placed at one end of the runway. The runway was illuminated with two halogen lamps (50 watts each), so as to create an averse stimulus. The fore paws and hind paws of the mice were wet with non-toxic color ink (red and green color ink, respectively) and placed at the other end of the runway that was covered with a strip of white paper. The time taken by the mice to cross the runway and the stride length between the limbs were measured manually. The individual who scored the behavior was blinded to treatments.

Rota Rod Test—Rota rod test was used to measure the locomotor function in experimental mice following the standard protocol with minor modifications. On day 8, 2 h before the test, the mice were exposed to a rota rod run for 5 min set at 15 rpm with three trials with a 10-min gap between the sessions. During the test, the animals were placed on the rod for 3 min, and the latency to fall off (mean of three falls) from the rotating rod was measured and compared between the experimental groups.

Mouse Tissue Sections—Immunohistochemistry for TH and MTA1 was performed in substantia nigra pars compacta (~ Bregma −3.16 mm, interaural 0.64 mm) region in mouse brains using the Panninos and Franklin (26) mouse brain atlas. From the 10% neutral buffered formalin-fixed brains, 5-μm-thick paraffin sections through matched coronal levels of the SN pars compacta was stained with rabbit anti-TH (Santa Cruz Biotechnology, 1:50) and rabbit anti-MTA1 (Cell Signaling, 1:50) using standard immunoperoxidase techniques. Briefly, paraffin sections of mouse midbrains were deparaffinized and hydrated. Antigenic sites were exposed by incubating the sections in antigen retrieval solution (trisodium citrate, pH 6.2) for 20 min at 90 °C using a microwave oven. Following retrieval, slides were cooled in distilled water for 5 min. Phosphate-buffered saline (PBS) was used for washing between each steps. Endogenous peroxidase activity was quenched by treating the sections with 3% hydrogen peroxide. Nonspecific binding was
blocked by 1 h of incubation in 1.5% BSA. TH and MTA1 immunoreactivity were detected with a biotinylated rabbit anti-TH (Santa Cruz Biotechnology, 1:50) and rabbit anti-MTA1 (Cell Signaling, 1:50) and ImmunoCruz™ rabbit ABC staining kit (Santa Cruz Biotechnology). All slides were counterstained with Mayer’s hematoxylin and visualized in light microscopy (Motic DMB1–2MP, China).

The expressions of immunopositivity in sections were evaluated at a magnification of ×400 under light microscopy. The person who scored the sections was blinded to the treatment groups. In each specimen, immunopositive cells in 10 random high-power fields (0.12 mm² each) were counted.

Data and Statistical Analysis—Data were expressed as means ± S.E. of the mean (S.E.). Mean differences between the groups were analyzed by Student’s unpaired t test or ANOVA using GraphPad Prism 5.0 (San Diego) software. p ≤ 0.05 was considered to be statistically significant. Pearson’s correlation analysis was performed using SYSTAT 11 to identify the correlation between TH, MTA1, and motor functions.

Study Approval—All the human samples procured and animal experiments performed were done after getting appropriate ethical clearance from respective committees.

Results

MTA1 Expression Is Dysregulated in Parkinson Disease

MTA1 and TH mRNA and Protein Levels Are Down-regulated in SN of PD Samples—As this study is intended to directly implicate the role of MTA1 in human PD, we analyzed MTA1 and TH levels in SN regions of brain samples of 4 normal and 26 PD patients by qPCR. The results showed that MTA1 and TH levels were significantly down-regulated in PD samples as compared with normal. Box plot (Fig. 1A) shows that 75% of the MTA1 levels in PD samples are less than 0.5, and for TH all the
PD sample values are less than 0.3; whereas for the normal samples, MTA1 and TH levels are much higher than the PD cases. We performed the non-parametric Mann-Whitney U test to verify the difference. Our results showed that the difference between the normal and PD groups for MTA1 and TH levels was statistically significant at $p = 0.014$ and 0.011, respectively.

We further analyzed the expression levels of MTA1 and TH in SN regions of brain from 4 normal and 29 Parkinson cases by Western blotting. Results from Western blotting analysis showed that 27 and 28 out of 29 Parkinson samples had either no expression or much less expression of MTA1 and TH, respectively, as compared with normal (Fig. 1B).

**IHC Analysis for MTA1 and TH in SN of PD and Control Samples**—IHC analysis for MTA1 showed diffuse cytoplasmic expression of MTA1 (Fig. 2A) with a mean staining intensity of 4.92 with a standard deviation (S.D.) of 1.95 in the SN sections of the PD samples, whereas the mean staining intensity in the SN sections of the control samples for MTA1 was 7.04 with a S.D. of 2.64. The scoring results showed that MTA1 levels were significantly down-regulated in the SN region of PD as compared with the controls. An independent $t$ test showed significant difference ($p = 0.014$) in the staining intensity between the controls and the PD samples.

IHC analysis for TH showed granular cytoplasmic staining in cell bodies of the dopaminergic neurons as well as their neuronal processes (Fig. 2A). The mean staining intensity for TH was observed to be 8.36 with a S.D. of 3.52 in the SN sections of the PD samples, whereas the mean staining intensity in the SN sections of the control samples for TH was 10.36 with a S.D. of 1.94. The scoring results show that the TH levels are low in the PD samples as compared with the controls. Independent $t$ test showed significant difference ($p < 0.05$) in TH expression between the controls and the PD samples. The values for both MTA1 and TH in all the samples are presented in Table 1.

Further categorization of the samples ($n = 47$) for MTA1 positivity in SN sections showed that 74.1% of the samples had a staining intensity of less than 6 in the PD cases as compared with controls (25.9%), whereas only 25% of the PD cases had a staining intensity of more than 6 as compared with controls (75%). $\chi^2$ test showed significance with a $p$ value of 0.001 and an odds ratio of 8.54. This indicates that low levels of MTA1 in SN region could be an independent risk factor for PD. For TH, the distribution of samples with a staining intensity of less than 9 and more than 9 did not show any significance (Table 2). Although the $\chi^2$ value is not significant ($p = 0.163$), we could observe the trend in sample distribution in the TH staining. In this study, 61% of the PD samples have a $Q$ score less than 9 and 57% of normal samples have a $Q$ score more than 9, which is an indication that the sample size of this study might not be sufficient to get a significant odds ratio for the TH level in Parkinson disease and normal individuals. An expanded study with more samples would probably yield a $p$ value that would be less than 0.05. The choice for staining intensity values of 6 or 9 was chosen and categorized based on the receiver operating characteristic curve of the entire sample pool of 47 (PD cases (25) and controls (22)), where 50% of the samples had that score; hence...
the choice was not random or biased to show statistical significance.

Furthermore, we used image analysis software (Optika View 2, Italy) to count immunopositive cells in the SN sections of controls and PD and to measure the immunopositive area in 100 mm². In comparison with the control group, the number of MTA1 and TH immunopositive cells were significantly ($p < 0.01$) less in PD subjects. The average number of MTA1-immunopositive cells in the controls was 89 as compared with 20 in the PD samples, although it was 114 and 16 for TH (Fig. 2B).

**MTA1 Level Modulation as a Therapeutic Intervention in PD**

**G-CSF Stimulation of MTA1 and TH Expression in Cell Line and Mouse Models**—Previous reports (31, 32) have shown that MTA1 is induced by growth factors such as Heregulin and Neuregulin, and the fact that MTA1 is an upstream regulator of TH (14) encouraged us to explore MTA1 level modulation as a possible treatment strategy for Parkinson disease. However, physiological imbalance of growth factors may tend to push the cells toward oncogenicity. Existing and emerging data suggest that growth factor G-CSF is a safe and potential agent for neuroprotection in the Parkinson disease model, although the underlying mechanisms of action remain to be clearly elucidated (6,

![G-CSF treatment in vitro and in vivo leads to corresponding up-regulation of MTA1 and TH expression.](image)

**TABLE 1**

Mean staining intensity of MTA1 and TH levels in SN sections of PD and controls

| Antigen | Group          | Samples | Mean (S.D.) | $p$ value |
|---------|----------------|---------|-------------|-----------|
| MTA1    | Normal         | 22      | 7.04 (2.64) | 0.003     |
|         | Parkinson      | 25      | 4.92 (1.95) |           |
| TH      | Normal         | 22      | 10.36 (1.94) | 0.022     |
|         | Parkinson      | 25      | 8.36 (3.52) |           |

**TABLE 2**

Percent distribution of staining intensity in PD and controls

|                  | Parkinson disease samples | Normal samples | Total | $p$ value | Odds ratio |
|------------------|---------------------------|---------------|-------|-----------|------------|
|                  | %                         | %             |       |           |            |
| MTA1             | Staining intensity <6     | 20 (74.1)     | 7 (25.9) | 27 | 0.001 | 8.54 |
|                  | Staining intensity >6     | 5 (25)        | 15 (75) | 20 |       |     |
| TH               | Staining intensity <9     | 16 (61.5)     | 10 (38.5) | 26 | 0.163 | 2.13 |
|                  | Staining intensity >9     | 9 (42.9)      | 12 (57.1) | 21 |       |     |

**FIGURE 3.** G-CSF treatment *in vitro* and *in vivo* leads to corresponding up-regulation of MTA1 and TH expression. A, neuroblastoma cell line KELLY was treated with G-CSF (100 ng/ml) for indicated time points, and expressions of MTA1, TH, and cyclin D3 were analyzed. Vinculin was used as internal loading control and cyclin D3 as positive control for G-CSF treatment. B, real time PCR analysis of MTA1 and TH expression in G-CSF-treated KELLY cells. Graph represents mean ± S.E. *p < 0.05 compared with untreated cells. C, treatment of mice with pegylated G-CSF (120 µg/kg) for the indicated time point (for 4 h) and analyzed for expression of MTA1, TH, and cyclin D3. Expression data represent three mice per group. D, expression of MTA1 and TH after siRNA-mediated down-regulation of MTA1, followed by G-CSF treatment in KELLY cells.
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19–23). This prompted us to test G-CSF as an alternative agent to stimulate MTA1 and to explore the molecular mechanism behind G-CSF’s neuroprotective action. To this effect, treatment of the neuroblastoma cell line KELLY with G-CSF revealed time-dependent stimulation of MTA1 mRNA as well as protein levels. We also observed that there is also a simultaneous significant increase in the levels of TH (Fig. 3, A and B). The presence of the G-CSF receptor on KELLY cells was confirmed by RT-PCR (data not shown). Furthermore, to evaluate G-CSF’s modulatory effect on MTA1 in vivo, we treated WT-Swiss albino mice with a pegylated derivative of granulocyte colony-stimulating factor (pegfilgrastim), wherein we observed a significant increase in the levels of MTA1 and TH protein in the substantia nigra region of brain (Fig. 3C). Furthermore, to delineate the significance of MTA1 in the action of G-CSF, we silenced MTA1 using MTA1-specific siRNA and analyzed for levels of TH. Results showed that G-CSF is not able to induce TH efficiently in the absence of MTA1 (Fig. 3D). All these results provide a proof-of-principle to our hypothesis of G-CSF-mediated MTA1 modulation as a potential intervention option for Parkinson disease.

Validation of G-CSF Effect in Acute MPTP Mouse Model—Based on the above results, we next checked the effect of G-CSF on a conventional MPTP mouse model of Parkinsonism. In the beam walk test, saline-treated MPTP mice showed a significant increase ($p < 0.05$) in the time taken to cross the marked zone, the immobility period, and the number of foot slips on days 4 and 8, when compared with negative control group mice. These alterations in time taken and immobility period was found to be worsen on day 8 in MPTP group. Treatment with G-CSF significantly decreased ($p < 0.05$) the time taken, the immobility period, and the number of foot slips on day 8 when compared with MPTP mice (Fig. 4A). Correlation analysis revealed that TH and MTA1 possess a strong negative correlation with time taken ($r = -0.751$ and $-0.724$, respectively), immobility period ($r = -0.725$ and $-0.636$, respectively), and number of foot slips ($r = -0.725$ and $-0.657$, respectively).

Consistently, in the stride length test, saline-treated MPTP mice showed a significant increase in time taken to cross the runway on day 4 ($p < 0.01$) when compared with normal mice. On day 8, a significant decrease ($p < 0.05$) in the average distance between each fore and hind limb was observed in MPTP mice in comparison with normal mice. G-CSF administration significantly increased ($p < 0.05$) the average distance between each fore and hind limb on day 8 when compared with MPTP mice (Fig. 4B). Correlation analysis revealed that TH and MTA1 possess a strong negative correlation with time taken to cross the runway ($r = -0.754$ and $-0.641$, respectively). A strong positive correlation was observed between TH and MTA1 with that of forelimb ($r = 0.693$ and 0.745, respectively) and hind limb ($r = 0.861$ and 0.839, respectively) distance.

Furthermore, immunohistochemical analysis showed that MTA1- and TH-immunopositive cells were found to be significantly decreased ($p < 0.01$) in MPTP mouse brains when compared with normal mouse brains (Fig. 5, A–C). G-CSF administration (120 μg/kg body weight) rescued the TH- and MTA1-immunopositive cells in comparison with MPTP mouse brains (Fig. 5, B and C). Correlation analysis revealed that TH was found to possess a strong positive correlation ($r = 0.879$) with MTA1 in MPTP mouse brains (Fig. 5D). We also measured for the dopamine levels by HPLC and found that dopamine was significantly decreased ($p < 0.01$) in MPTP mouse brains when compared with normal mouse brains. G-CSF administration...
resulted in an increase in dopamine levels compared with MPTP mouse brains (Fig. 5E). Consistently, in the rotarod test, the falling latency of the saline-treated MPTP was significantly shorter when compared with the saline-treated normal mice. Treatment with G-CSF showed a non-significant improvement in the rotarod performance (Fig. 5F).

**Molecular Mechanism of MTA1 Induction by G-CSF**—To understand the mechanism by which G-CSF induces MTA1, we cloned the 3.0-kb MTA1 promoter (−2951 to +216) and examined for promoter activity either with or without G-CSF. Results showed that there is a significant induction of MTA1 promoter activity with G-CSF treatment (Fig. 6A), indicating that regulation of MTA1 by G-CSF is at the transcriptional level. This prompted us to investigate the mechanism of regulation of MTA1 transcription by G-CSF. Previous reports (33–35) have shown that c-Fos acts as a downstream effector transcription factor of STAT3 and MAPK signaling cascades upon G-CSF treatment. Sequence analysis of the MTA1 promoter revealed a putative c-Fos binding consensus sequence TGACTCAC (36) at regions −2121 to −2114. As G-CSF role in activation of c-Fos with G-CSF and observed an induction in c-Fos and MTA1 protein levels in a time-dependent manner (data not shown), which corroborated with the MTA1 induction observed earlier. Furthermore, we observed a significant increase in the MTA1 promoter activity upon cotransfection with c-Fos (Fig. 6B). These results clearly indicate that c-Fos regulates MTA1. In addition, to establish the molecular cooperation between G-CSF and c-Fos in the regulation of the MTA1 promoter, we examined the recruitment of c-Fos onto the MTA1 promoter region encompassing the c-Fos-binding element using chromatin immunoprecipitation (ChIP). Results illustrated that c-Fos was recruited onto the MTA1 promoter at a region between −2121 and −2114 (Fig. 6C), and there is a significant increase in the recruitment upon treatment with G-CSF. Furthermore, to examine the direct recruitment of c-Fos to the MTA1 promoter, we performed electrophoretic mobility shift assay (EMSA) using the oligonucleotides containing the c-Fos consensus sequence TGACTCAC, and binding of the c-Fos was analyzed. Results showed the formation of DNA-protein complexes, and the observed complex was significantly enhanced with G-CSF treatment (Fig. 6D).
To investigate the functional significance of this c-Fos binding consensus region in the transcriptional regulation of MTA1 by c-Fos and G-CSF, we constructed an MTA1 promoter construct deleting the c-Fos binding region and performed reporter luciferase assay. Results showed that either G-CSF or c-Fos failed to induce MTA1 promoter activity upon deletion of c-Fos binding consensus region (Fig. 7, A and B). In addition, depletion of c-Fos using siRNA significantly decreased G-CSF’s ability to induce MTA1 promoter activity and protein levels (Fig. 7, C and D). From the above results, it is clear that the recruitment of c-Fos to human MTA1 promoter in KELLY cells. The ChIP analysis shows the recruitment of c-Fos to human MTA1 promoter using the wild type biotin-labeled probe encompassing c-Fos-binding site using Kelly cell nuclear lysate. * indicates the specific band of interest.

Collectively, it is evident from the given model (Fig. 7E) that G-CSF might be regulating MTA1 via c-Fos through both the MAPK and STAT3 pathway, as both these pathways are essential for cellular functions.

Discussion

One of the hallmarks of Parkinson disease is the depletion of dopamine in substantia nigra, which is caused by degeneration of TH-positive neurons leading to reduced TH expression, thereby making TH one of the molecular players in PD. Based on several studies (37–41) on TH in PD pathogenesis, therapeutic strategies aimed to improve TH expression or its downstream enzymes in PD have received wide attention. Evidence exists to show that using drugs or other treatment methods, such as gene replacement therapy to increase nigrostriatal TH
expression, is an effective therapeutic modality for PD (41). Because of its importance, an extensive investigation of TH regulatory mechanisms is warranted to provide additional therapeutic avenues.

In this study, we have clinically validated and established MTA1 as a bona fide target in PD after studying its expression pattern in clinical cohort of samples. Various methods adopted to study the expression of MTA1 and TH revealed that their levels are significantly reduced in PD patients. Comprehensively, to our knowledge this is the first report attributed to the expression pattern of TH and its regulatory gene MTA1 in a total of 54 PD samples and 26 normal samples. The genuine implication for evaluating the expression of MTA1 in such a large sample pool is to eventually translate the knowledge gained from this study into a pre-clinical and clinical setting. In this regard, the target molecule MTA1 is a well studied nuclear receptor coregulator in malignant disorders and has been shown to be induced in response to growth factors (32). Several recent studies have reported the tissue-specific functions of MTA1 and have been shown to act both as a coactivator and a corepressor depending on the tissue type (42). In view of this, we tested the idea whether MTA1 could be up-regulated in the dopaminergic neurons by growth factors, thereby increasing the levels of TH. Recent data showed that neurotrophic growth factor G-CSF has been tested in several preclinical studies in PD and has been shown to increase TH levels and also the neurotransmitter dopamine in dopaminergic neurons (6, 19–23). The advantage of using this growth factor is its distinct pharmacological efficacy and its clinical acceptance over several years (19–23). In support of this, it was reported that pegfilgrastim (pegylated G-CSF) penetrates through the blood–brain barrier, and patients maintain high circulating levels of pegfilgrastim over prolonged duration of time without any alterations in liver or kidney function or disturbances in serum electrolytes (6). This encouraged us to choose G-CSF to test whether it mediated its actions in the target dopaminergic neurons to up-regulate TH through MTA1. Recent investigations on the underlying mechanism of G-CSF action showed that the ERK was activated following G-CSF treatment. G-CSF also prevents dopaminergic neurons from 6-hydroxydopamine-induced toxicity via ERK pathway followed by inhibiting the apoptosis-execution process (43). Our cell line and animal model studies showed that G-CSF up-regulates MTA1 and concurrently TH with rescue of phenotype, and interestingly, the observed induction of TH was compromised in the absence of MTA1. Taken together, these studies point out that TH levels in dopaminergic neurons are controlled at least in part by MTA1 and that G-CSF mediated actions in dopaminergic neurons are mediated by MTA1. This was further supported by our finding that silencing of MTA1 compromises G-CSF’s ability to induce...
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TH. At a molecular level, our proposed mechanism of G-CSF action in inducing MTA1 via c-Fos is supported by the findings that G-CSF triggers a de novo induction of c-Fos, and overexpression of c-Fos is sufficient to stimulate TH gene transcription (44, 45). Furthermore, this induction of c-Fos by G-CSF was shown to be modulated by both MAPK and STAT3 signaling pathways (34, 46). Thus, our data unravel the plausible molecular mechanism of G-CSF's action via c-Fos, MTA1, and TH in dopaminergic neurons and also reiterate that G-CSF is a promising therapeutic opportunity for the treatment of Parkinson disease.

Author Contributions—A. S. K., S. J., and A. S. conducted the experiments. P. D. performed the pathological scoring. H. B., S. B. C., and G. V. designed the study, interpreted the data, and wrote the manuscript. All authors analyzed the results and approved the final version of the manuscript.

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