

RESEARCH ARTICLE

Molecular Features of Parkinson's Disease in Patient-Derived Midbrain Dopaminergic Neurons

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ABSTRACT: Background: Despite intense efforts to develop an objective diagnostic test for Parkinson's disease, there is still no consensus on biomarkers that can accurately diagnose the disease.

Objective: Identification of biomarkers for idiopathic Parkinson's disease (PD) may enable accurate diagnosis of the disease. We tried to find molecular and cellular differences in dopaminergic (DA) neurons derived from healthy subjects and idiopathic PD patients with or without rest tremor at onset.

Methods: We measured the expression of genes controlling dopamine synthesis, sequestration, and catabolism as well as the levels of corresponding metabolites and reactive oxygen species in midbrain DA neurons differentiated from induced pluripotent stem cells (iPSCs) of healthy subjects and PD patients with or without rest tremor.

Results: Significant differences in DA-related gene expression, metabolites, and oxidative stress were found between midbrain DA neurons derived from healthy subjects and patients with PD. DA neurons derived from PD patients with or without rest tremor at onset exhibited significant differences in the levels of some of these transcripts, metabolites, and oxidative stress.

Conclusion: The unique combination of these quantifiable molecular and cellular traits in iPSC-derived midbrain DA neurons can distinguish healthy subjects from idiopathic PD patients and segregate PD patients with or without rest tremor at onset. The strategy may be used to develop an objective diagnostic test for PD. © 2021 International Parkinson and Movement Disorder Society

Key Words: Parkinson's disease; molecular features; induced pluripotent stem cells; dopamine; metabolism

The diagnosis of Parkinson's disease (PD) is based on clinical observations and lacks an objective test.¹ The defining locomotor symptoms of PD are caused by a progressive loss of nigral dopaminergic (DA) neurons.² More than 90% of PD cases are idiopathic, on which there is little mechanistic understanding. A well-recognized categorization of PD is based on whether rest tremor is present at onset.³ Patients with PD who

present with rest tremor at onset generally have slower progression and a better prognosis than PD patients without rest tremor at onset.⁴ The discovery of induced pluripotent stem cells (iPSCs)⁵ has enabled us to generate patient-specific iPSCs to study the vulnerabilities of midbrain DA neurons from PD patients with parkin mutations in previous studies.^{6–9} Because the degeneration of nigral DA neurons causes motor symptoms that

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define PD, we generated iPSC-derived midbrain DA neurons to identify molecular and cellular features that differ between neurons derived from healthy subjects and idiopathic PD patients in the present study.

Dopamine synthesis starts from tyrosine or phenylalanine, two essential amino acids from food. Phenylalanine is converted to tyrosine by phenylalanine hydroxylase (PAH), which requires the cofactor tetrahydrobiopterin (BH₄). The conversion of tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa) by tyrosine hydroxylase (TH) also requires BH₄, whose level is maintained by regeneration and de novo synthesis from Guanosine-5'-triphosphate (GTP). Once DA is synthesized in the cytosol (DA_c) from L-dopa by aromatic L-amino acid decarboxylase (AADC), it is sequestered by vesicular monoamine transporter 2 (VMAT2) into the lumen of synaptic vesicles, where the low pH prevents oxidation of vesicular dopamine (DA_v) by O₂. The catabolism of DA_c produces H₂O₂ and many free radicals that play a key role in the selective degeneration of nigral DA neurons in PD.¹⁰ Dopaminergic transmission is shaped by controlled release of DA_v to the extracellular compartment (DA_e) and selective reuptake of released DA_e by dopamine transporter (DAT).

Using iPSC-derived midbrain DA neurons from six healthy subjects and 14 idiopathic PD patients (seven with rest tremor and seven without rest tremor at onset), we found significant differences between healthy subjects and patients with PD in the expression of genes involved in dopamine synthesis, sequestration, and catabolism, with consistent changes in metabolites and oxidative stress. There were also significant differences between PD patients with or without rest tremor in the expression levels of some of these genes and the amounts of metabolites and oxidative stress. The unique combination of various parameters that differ significantly in iPSC-derived midbrain DA neurons may be used to develop an objective diagnostic test of PD.

Methods

Derivation of iPSCs from Skin Fibroblasts

With approvals from the Health Sciences Institutional Review Board of the State University of New York at Buffalo and the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine, skin biopsies were collected from healthy subjects and patients with PD for the derivation of skin fibroblasts⁶ and iPSCs.¹¹

Directed Differentiation of iPSCs to Midbrain DA Neurons

The iPSCs were differentiated to midbrain DA neurons according to our published method.⁶ Neurons at 70 or more days of differentiation were used in the

study. All differentiation experiments were performed at least three times. The numbers of independent measurements are given in the legends of figures 1-4.

Cytoarray Study of Genomic Stability

Infinium HumanCytoSNP-12 BeadChip (Illumina, San Diego, CA) analysis was performed at the Genomic Shared Resource at Roswell Park Comprehensive Cancer Center. Data analysis was performed in Illumina's GenomeStudio (v2.0) Genotyping Module (v2.0.4). Illumina's BlueFuse Multi Analysis Software (v4.5) was used for cytogenetic visualization.

Whole Exome Sequencing

Genomic DNA (50 ng) isolated from the 14 lines of PD iPSCs were used for whole exome sequencing at the University at Buffalo Genomics and Bioinformatics Core. Single nucleotide polymorphisms (SNPs) and insertion or deletion of bases (Indels) were detected by FreeBayes (version v1.3.2, GitHub.com). Variants with base quality scores Q > 30 and read depths between 20 and 300 were retained for screening against pathogenic PD SNPs and Indels from ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) and Leiden Open Variation Database (LOVD)¹² (<https://www.lovd.nl/>) (genes *DNAJC6*, *FBXO7*, *GIGYF2*, *MAPT*, *PRKAG2*, *SYNJ1*, and *VPS13C* are included with curator permission) by in-house code. Read counts for exons were estimated by ExomeDepth (version 1.1.15, GitHub.com) with hg38 exon annotations from the University of California Santa Cruz (UCSC) genome browser and were used for detecting copy number variations (CNVs) by ExomeDepth in all samples. CNVs were screened against PD-linked pathogenic CNV genes by in-house code and manually inspected for overlaps between CNVs detected from samples and curated pathogenic CNVs from ClinVar and LOVD.

Real Time–Quantitative Polymerase Chain Reaction

Real-time–quantitative polymerase chain reaction (RT-qPCR) measurement of gene expression was performed as described previously.⁶ Each sample was run in triplicate from at least three independent differentiations.

Dopamine Release and Uptake

Measurements of dopamine release and uptake were performed as described previously.⁶

Measurements of Metabolites

Metabolites involved in DA synthesis and degradation are measured by high-performance liquid chromatography (HPLC) as described previously.⁶ The dopamine aldehydes 3,4-dihydroxyphenylacetaldehyde and 3-methoxy-4-hydroxyphenylacetaldehyde are too

unstable to be detected by HPLC; they are quickly converted to other metabolites.¹³

Detection of Reactive Oxygen Species

Live iPSC-derived neurons treated with or without dopamine (75 μ M for 2 hours) were incubated at 37°C for 15 minutes with 1 mM of CM-H₂DCFDA, which is converted to a fluorescent compound by reactive oxygen species (ROS).¹⁴ Fluorescence images were acquired with a single 300-millisecond exposure, followed by the acquisition of a Differential Interference Contrast (DIC) image of the same field. Unretouched images were merged using the SPOT software (Diagnostic Instrument, Sterling Heights, Michigan). Fluorescence intensity for each cell was measured in a 60 \times 60 pixel box using Image J (National Institutes of Health, Bethesda, MD). Values from at least 900 neurons in six wells from three independent experiments (two wells for each experiment) were averaged for each line of iPSC-derived neurons.

Statistical Analyses

All statistical analyses were performed using the software Origin (OriginLab, Northampton, MA). Data were expressed as mean \pm SEM (standard error of measurement). Statistical significance was determined using one-way analysis of variance (ANOVA).

Results

Generation of iPSCs and Their Differentiation to Midbrain DA Neurons

We reprogrammed skin fibroblasts from 20 subjects (six unaffected spousal controls [C group], seven idiopathic PD patients without rest tremor at onset [N group], and seven idiopathic PD patients with rest tremor at onset [T group]; see Table S1 for information on the subjects) to iPSCs using well-established methods.¹¹ All lines of iPSCs exhibited morphology indistinguishable from human embryonic stem cells and expressed pluripotency markers, such as octamer-binding transcription factor 4 (OCT4), Tír na nÓg (NANOG), and stage-specific embryonic antigen 3 (SSEA3) (Figure S1). Analyses of genomic DNA isolated from these iPSCs on the Illumina HumanCytoSNP-12 Beadchip platform showed normal genomic stability in these cells (Figure S2). We performed whole exome sequencing of genomic DNA isolated from the 14 lines of idiopathic PD iPSCs. Compared with the known PD pathogenic SNPs (Table S2), Indels (Table S3), and CNVs (Table S4), the genomic variants identified in each of the 14 samples (Table S5) did not contain any PD pathogenic variants. Using a previously published method,⁶ we differentiated all 20 lines of iPSCs

(Figure S3A–C for three representative lines) for 70 days or longer to midbrain DA neurons, which expressed markers for mature (microtubule associated protein 2, MAP2) dopaminergic (TH, DAT, VMAT2) neurons with synaptic connections (synaptophysin), as well as markers for midbrain neurons (nuclear receptor related 1 (Nurr1), Engrailed-1 (En1), and Forkhead Box A2 (FoxA2)) (Figure S3). Manual counting⁶ of TH⁺ neurons showed no significant variations in the percentages of TH⁺ neurons among all 4',6-diamidino-2-phenylindole (DAPI⁺) cells ($38.3 \pm 2.3\%$ for the C group, $37.1 \pm 3.5\%$ for the N group, and $37.0 \pm 2.9\%$ for the T group; $P > 0.7$, $n = 6$ or 7 lines for each group) and the percentages of Tuj1⁺ neurons in all cells ($67.2 \pm 3.7\%$ for the C group, $69.6 \pm 3.0\%$ for the N group, and $69.8 \pm 4.2\%$ for the T group; $P > 0.4$, $n = 6$ or 7).

Significant Differences in Expression Levels of Genes Controlling DA Synthesis and Sequestration

Using RT-qPCR (primer sequences in Table S6), we found that mRNA levels of PAH (Fig. 1A) and TH (Fig. 1B) were significantly increased in patients with PD (N or T group, $n = 7$ lines for each) compared with control subjects (C group, $n = 6$ lines) ($P < 0.05$, one-way ANOVA for all data). The N group expressed significantly higher levels of PAH and TH than the T group ($P < 0.05$) (Fig. 1A,B). Gene expression levels were normalized against *GAPDH*, which was expressed at very similar levels across the 20 lines of neurons (Fig. 1F). Abbreviations of genes and metabolites are listed in Table S7. Summary of RT-qPCR data, fold changes, and statistical analyses are in Table S8. PAH converts phenylalanine to tyrosine. TH catalyzes the conversion of tyrosine to L-dopa, which is converted to dopamine by AADC (see the diagram in Fig. 5). There was no significant change in the expression of AADC (Fig. 1C). Once DA is synthesized in the cytosol, it is sequestered in synaptic vesicles by VMAT2, which was significantly decreased both in the N and the T groups of patients with PD compared with controls (Fig. 1D). There was a significant difference in VMAT2 levels between the N and the T groups (Fig. 1D). The expression levels of DAT were not significantly different between the three groups (Fig. 1E), attesting to the lack of significant line-to-line variations in the differentiation of iPSCs to midbrain DA neurons. Western blotting confirmed that the amount of TH protein was indeed significantly increased in patients with PD (N or T group, $n = 6$ lines for each) compared with control subjects (C group, $n = 6$ lines) ($P < 0.05$). No significant difference in TH protein levels was seen between the N and T groups of patients with PD (Figure S4). This

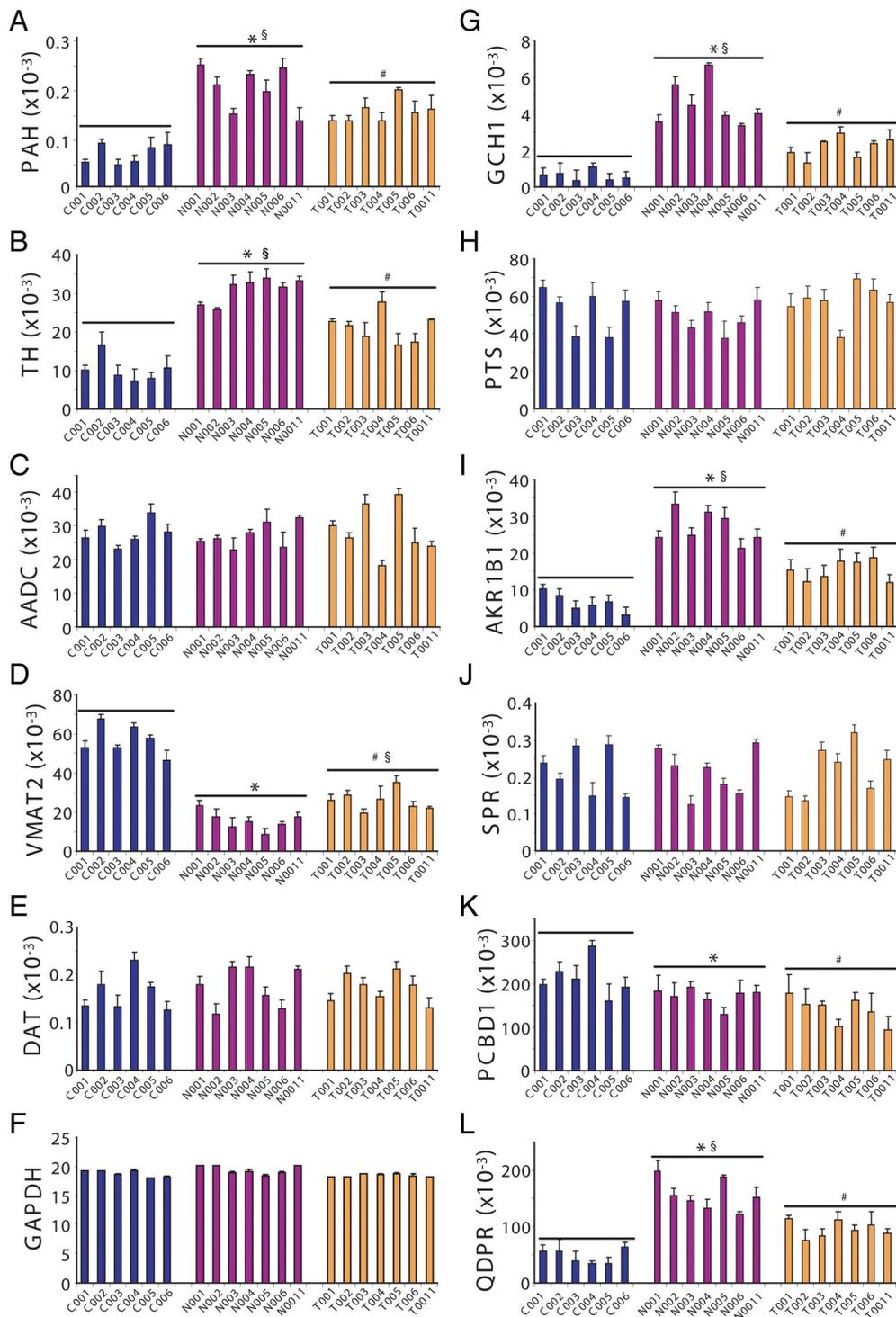


FIG. 1. Genes involved in dopamine synthesis and sequestration were differentially expressed in iPSC-derived neurons from healthy subjects and idiopathic PD patients with or without rest tremor at onset. **(A–F)** RT-qPCR measurements of mRNA levels of genes controlling dopamine synthesis **(A–C)**, vesicular sequestration **(D)** and reuptake **(E)**, and the housekeeping gene *GAPDH* **(F)** in iPSC-derived midbrain dopaminergic neurons from the three groups of subjects. **(G–L)** mRNA levels of genes controlling the de novo synthesis **(G–J)** and regeneration **(K–L)** of tetrahydrobiopterin (BH₄). Error bars present mean \pm SEM. AADC, aromatic L-amino acid decarboxylase; AKR1B1, aldo-keto reductase family 1, member B; DAT, dopamine transporter; GCH1, GTP cyclohydrolase I; PAH, phenylalanine hydroxylase; PCBD1, pterin-4 α -carbinolamine dehydratase 1; PTS, 6-pyruvoyltetrahydropterin synthase; QDPR, quinoid dihydropteridine reductase; SPR, sepiapterin reductase; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter 2. * $P < 0.05$, N group versus C group; # $P < 0.05$, T group versus C group; § $P < 0.05$, N group versus T group; $n \geq 9$ for each line, one-way ANOVA. [Color figure can be viewed at wileyonlinelibrary.com]

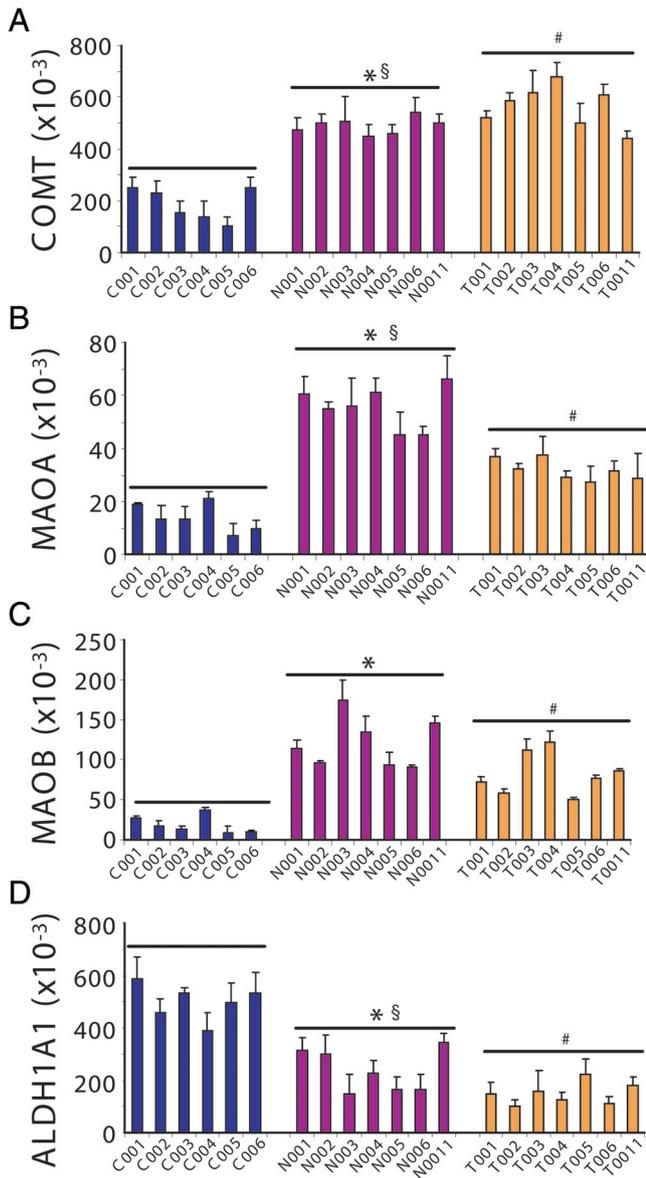


FIG. 2. Altered expression of genes controlling dopaminergic catabolism in iPSC-derived neurons from patients with Parkinson's disease. (A–D) RT-qPCR measurements of mRNA levels of COMT (A), MAOA (B), MAOB (C), and ALDH1A1 (D) in iPSC-derived midbrain dopaminergic neurons from the three groups of subjects. Error bars present mean \pm SEM. ALDH1A1, aldehyde dehydrogenase 1 family member A1; COMT, catechol-O-methyl transferase; MAOA, monoamine oxidase A; MAOB, monoamine oxidase B. * $P < 0.05$, N group versus C group; # $P < 0.05$, T group versus C group; § $P < 0.05$, N group versus T group; $n \geq 9$ for each line, one-way ANOVA. [Color figure can be viewed at wileyonlinelibrary.com]

might be caused by increased degradation of the TH protein in the presence of higher ROS levels in neurons of the N group (see Figure 4). AADC protein levels had no significant difference among the three groups nor the amounts of actin protein (as loading controls) (Figure S4). The protein levels of VMAT2 and DAT were too low to be reliably quantified by Western blotting.

Both PAH and TH require BH₄ as a coenzyme. The de novo synthesis of BH₄ from GTP requires the enzymes GCH1, PTS, AKR1B1, and SPR¹⁵ (Fig. 5). There were prominent and significant increases in the expression levels of GCH1 (Fig. 1G) and AKR1B1 (Fig. 1I) in patients with PD ($P < 0.05$). In contrast, there was no significant change in the expression of PTS (Fig. 1H) and SPR (Fig. 1J). In addition to de novo synthesis, BH₄ is regenerated in reactions catalyzed by PCBD1 and QDPR¹⁶ (Fig. 2). PCBD1 levels were moderately but significantly decreased in patients with PD ($P < 0.05$) (Fig. 1K). There was a significant increase in QDPR expression in patients with PD ($P < 0.05$) (Fig. 1L). Furthermore, levels of GCH1 (Fig. 1G), AKR1B1 (Fig. 1I), and QDPR (Fig. 1L) were significantly higher in the N group than in the T group of idiopathic PD patients ($P < 0.05$).

Significant Differences in Expression Levels of Genes Controlling Dopamine Catabolism

Dopamine is degraded in the cytosol by two alternative pathways that use monoamine oxidase (MAO) A and B, catechol-O-methyltransferase (COMT), and aldehyde dehydrogenase 1A1 (ALDH1A1)¹⁷ (Fig. 5). There was a significant increase in the expression of COMT (Fig. 2A), MAOA (Fig. 2B), and MAOB (Fig. 2C) in patients with PD ($P < 0.05$). ALDH1A1 levels (Fig. 2D) were significantly decreased in idiopathic PD patients ($P < 0.05$). Furthermore, COMT (Fig. 2A), MAOA (Fig. 2B), and ALDH1A1 (Fig. 2D) were expressed at significantly different levels between the N group and the T group ($P < 0.05$).

Significant Differences of Metabolites in Agreement with Altered Gene Expression Patterns

To substantiate the significant differences in the expression of genes involved in dopamine metabolism, we measured the amounts of various metabolites involved in the synthesis and degradation of dopamine by HPLC coupled with electrochemical detection⁶ in iPSC-derived midbrain DA neurons from 12 of the 20 subjects (four in the C group, four in the N group, and four in the T group). Consistent with the increased expressions of PAH (Fig. 1A) and TH (Fig. 1B) in patients with PD, tyrosine (Fig. 3A) and L-dopa (Fig. 3B) levels were significantly increased in patients with PD ($P < 0.05$). The increase of BH₄ in patients with PD ($P < 0.05$) (Fig. 3C) is consistent with the increased expressions of GCH1 (Fig. 1G) and AKR1B1 (Fig. 1I) in the de novo synthesis of BH₄ and the increased expression of QDPR (Fig. 1L) in the regeneration of BH₄. There was no significant change in the levels of dopamine (Fig. 3D). This may be related to the lack of significant change in AADC (Fig. 1C) or the

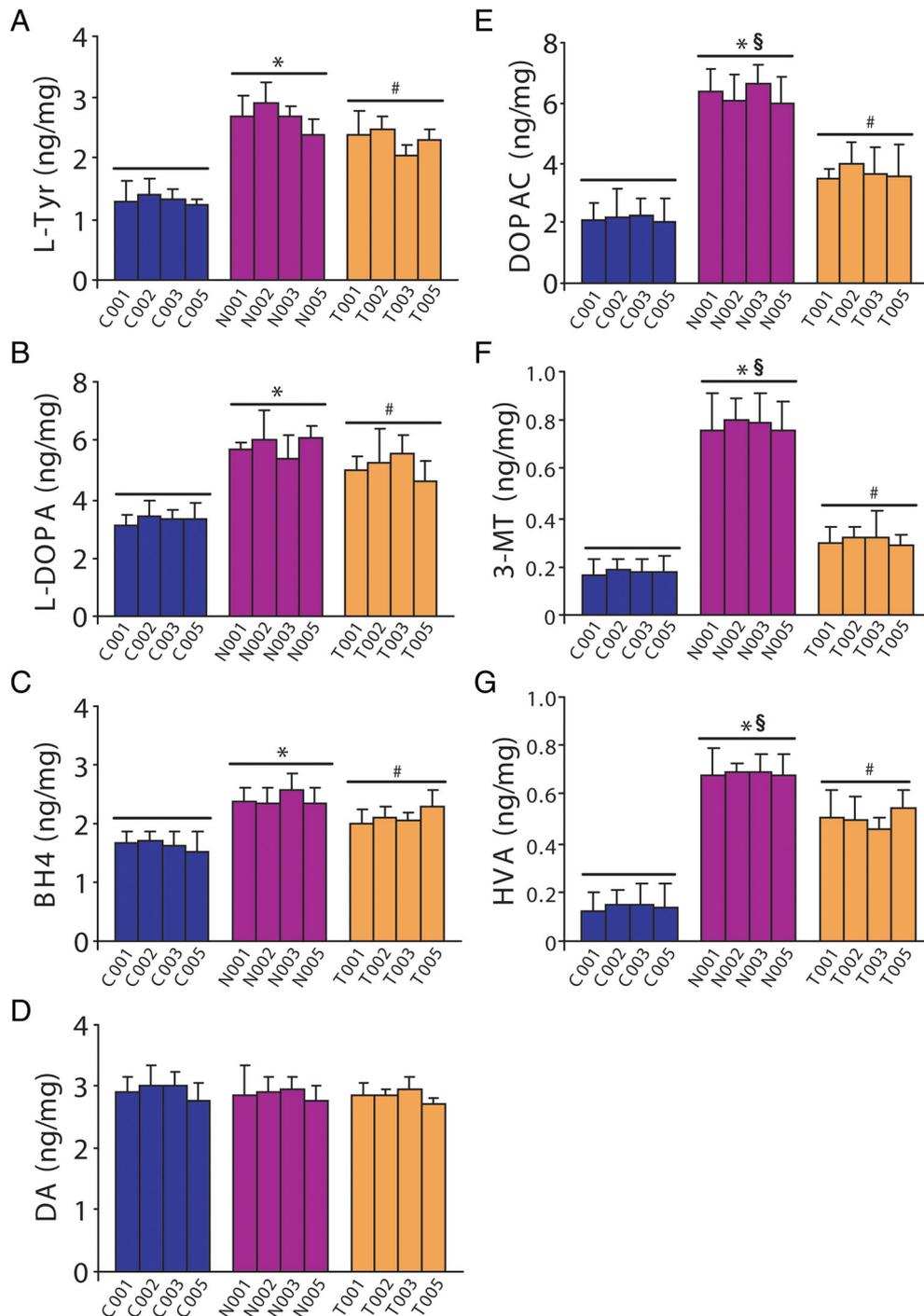


FIG. 3. Altered levels of metabolites in dopaminergic synthesis and catabolism in ipSC-derived neurons from patients with Parkinson's disease. **(A–D)** HPLC measurements of tyrosine **(A)**, L-DOPA **(B)**, BH₄ **(C)**, and dopamine **(D)** in the biosynthesis of dopamine in ipSC-derived midbrain dopaminergic neurons from the three groups of subjects. **(E–G)** Levels of DOPAC **(E)**, 3-MT **(F)**, and HVA **(G)** in dopamine catabolism in the three groups of ipSC-derived neurons. Error bars present mean \pm SEM. * $P < 0.05$, N group versus C group; # $P < 0.05$, T group versus C group; § $P < 0.05$, N group versus T group; $n \geq 9$ for each line, one-way ANOVA. [Color figure can be viewed at wileyonlinelibrary.com]

complex homeostatic regulation of TH activity.¹⁸ Consistent with the increased expressions of COMT (Fig. 2A), MAOA (Fig. 2B), and MAOB (Fig. 2C) in patients with PD, there were significant increases in the levels of DOPAC (Fig. 3E), 3-MT (Fig. 3F), and HVA

(Fig. 3G) in patients with PD ($P < 0.05$). Furthermore, the significant decreases in DOPAC (Fig. 3E) and HVA (Fig. 3G) in the T group in comparison with the N group are consistent with the reduced expression of MAOA (Fig. 2B), MAOB (Fig. 2C), and ALDH1A1

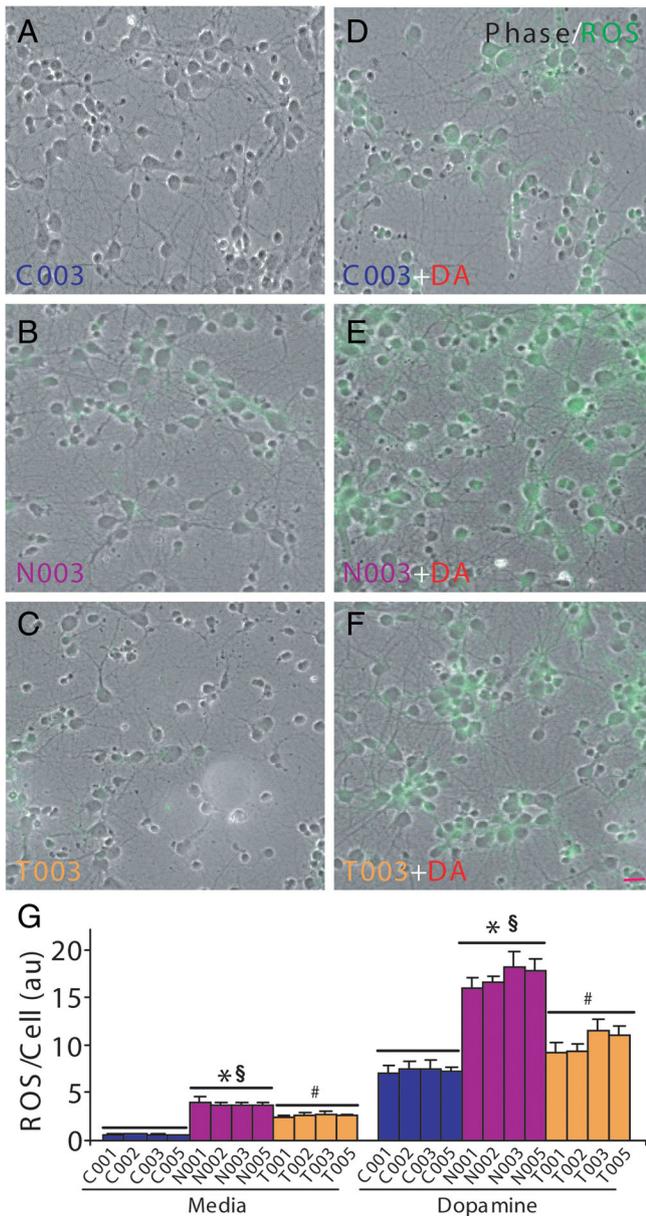


FIG. 4. Increased oxidative stress in iPSC-derived neurons from patients with Parkinson's disease. (A–F) Merged phase-contrast and fluorescence (green, for ROS) images of iPSC-derived neurons from C003 (A,D), N003 (B,E), or T003 (C,F) treated with media (A–C) or media containing 75 μM dopamine for 2 hours (D–F) and live stained with CM-H₂DCFDA (1 μM for 15 minutes), a membrane-permeable fluorescence indicator of ROS. ROS in the cell converts the nonfluorescent dye into fluorescein, which emitted green fluorescence. Bar, 10 μm. (G) Average fluorescence intensity per cell was quantified for the indicated lines of iPSC-derived neurons. Error bars present mean ± SEM. **P* < 0.05, N group versus C group; #*P* < 0.05, T group versus C group; §*P* < 0.05, N group versus T group; n > 900 cells in six wells from three independent experiments; one-way ANOVA. [Color figure can be viewed at wileyonlinelibrary.com]

(Fig. 2D), despite the slight but significant increase of COMT expression (Fig. 2A) in the T group versus the N group. The levels of 3-MT were significantly lower in the T group compared with the N group (Fig. 3F). This is in conflict with the slight but significant increase in

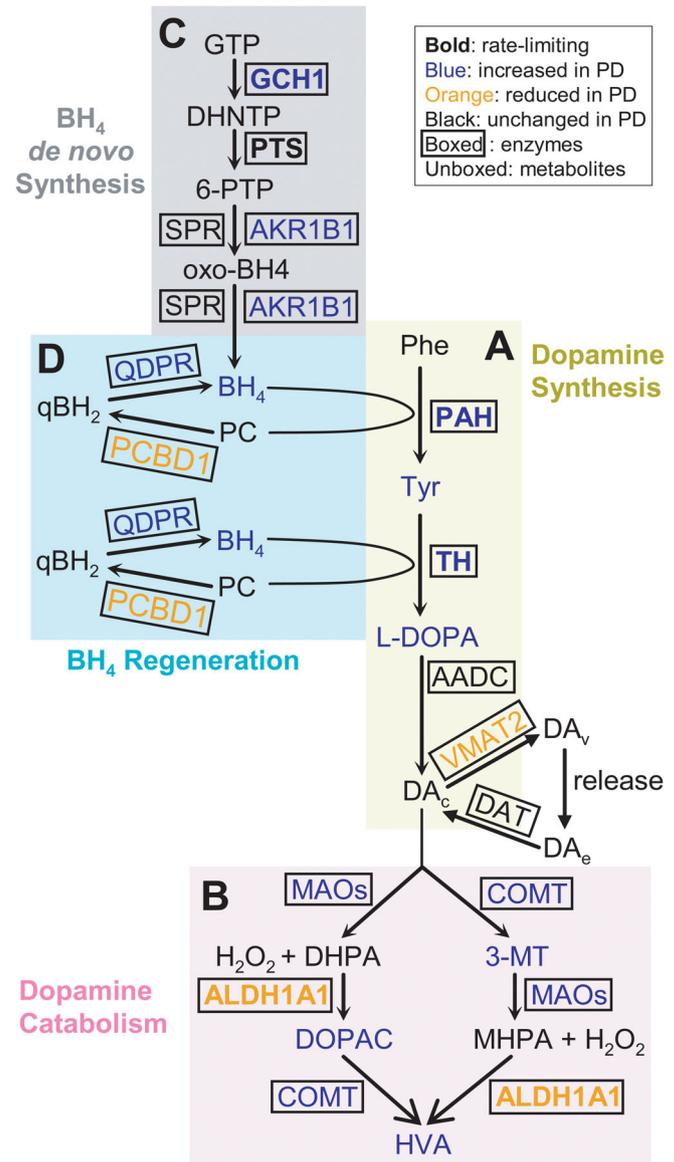


FIG. 5. Altered dopamine metabolism distinguished iPSC-derived mid-brain DA neurons from healthy subjects and patients with PD. (A–D) Changes in dopamine synthesis and vesicular sequestration (A), dopamine catabolism (B), de novo synthesis of BH₄ (C), and regeneration of BH₄ (D) are summarized in the metabolic diagram. mRNAs (boxed) and metabolites (unboxed) that were increased in PD are marked blue; those that were decreased in PD are marked orange; those that were unchanged are marked black. Rate-limiting enzymes are labeled in bold. In iPSC-derived midbrain DA neurons, increased expression of PAH and TH (A) was facilitated by higher levels of BH₄, which was caused by elevated expression of GCH1 and AKR1B1 in the de novo synthesis of BH₄ (C) and increased expression of QDPR to regenerate BH₄ (D). Consequently, both Tyr and L-dopa were increased. Although the total amount of DA was not significantly changed, the decrease of VMAT2 may increase cytosolic DA. Increased expression of MAOs and COMT, in conjunction with decreased expression of ALDH1A1, elevated the levels of 3-MT, DOPAC, and HVA (D), leading to increased oxidative stress (see Fig. 4). Definitions of abbreviations for genes and metabolites are listed in Table S7 [Color figure can be viewed at wileyonlinelibrary.com]

COMT levels in the T group versus the N group (Fig. 2A). One possibility could be that the two alternative dopamine catabolic pathways (Fig. 5) are used

differentially in the N and the T groups, with less dopamine being first methylated by COMT in the T group and more dopamine being first diverted to MAOs. The amounts of various metabolites are listed in Table S9.

Significantly Increased Oxidative Stress

The significant increases in the expression of genes for the oxidative deamination of dopamine (Fig. 2), which were corroborated by increased DA metabolites (Fig. 3), suggest elevated oxidative stress in neurons derived from patients with PD. We measured the amount of ROS in live iPSC-derived midbrain neurons using the membrane-permeable ROS probe CM-H₂DCFDA, which becomes fluorescent when oxidized inside the cell.¹⁴ We randomly selected four lines of iPSC-derived neurons from each of the C, N, and T groups. Merged fluorescence and phase-contrast images for a representative line of each group (C003, N003, and T003) at basal condition (Fig. 4A–C) showed significantly increased ROS levels in neurons from patients with PD ($P < 0.05$) (Fig. 4G and Figure S5). Dopamine treatment (75 μ M for 2 hours) greatly increased ROS levels in all lines of iPSC-derived neurons (Fig. 4D–F); however, the amount of ROS was significantly higher in patients with PD (Fig. 4G and Figure S5). ROS levels were significantly lower in the T group than in the N group of patients with PD, both at the basal condition and after dopamine treatment ($P < 0.05$) (Fig. 4G). The amounts of ROS at different conditions are listed in Table S10.

Unchanged Dopamine Release and Uptake

The lack of significant change in dopamine content (Fig. 3D) led us to examine DA release and reuptake. There was no significant change in spontaneous DA release, which was significantly but uniformly increased by treatment with KCl (56 mM) ($P < 0.05$). KCl-evoked DA release in the absence of extracellular Ca²⁺ was not significantly different among the three groups. Thus, Ca²⁺-dependent DA release was not significantly different among the three groups (Figure S6A). Consistent with the lack of significant change in DAT expression (Fig. 1E), there was no significant change in specific DA uptake (Figure S6B).

Discussion

Using iPSC-derived midbrain DA neurons from healthy subjects and patients with PD, the present study identified a set of cellular traits that significantly and consistently differ between healthy subjects and patients with PD, as summarized in Figure 5. First, there was a significant and coordinated increase in the expression of genes responsible for dopamine synthesis (PAH and TH) (Fig. 5A), which requires BH₄, either through de novo synthesis (GCH1 and AKR1B1) (Fig. 5C) or

regeneration (QDPR) (Fig. 5D). The slight but significant decrease in the expression of PCBD1 (N/C = 0.75 fold; T/C = 0.61 fold; Table S8) apparently was unable to offset the much higher increase in the expression of QDPR (N/C = 2.97 fold; T/C = 1.87 fold; Table S8), which was consistent with a significantly higher level of BH₄ in PD neurons (Fig. 5D). Both increased synthesis and regeneration of BH₄ would ensure that the coenzyme matches higher expression levels of PAH and TH, which led to the increased synthesis of Tyr and L-dopa (Fig. 5A). Interestingly, the total dopamine content of the neurons did not differ significantly between healthy subjects and patients with PD (Fig. 3D). Cellular dopamine levels are regulated by complex and dynamic processes to maintain homeostasis.¹⁸ These include regulation of TH by phosphorylation/dephosphorylation,¹⁹ nitration,²⁰ feedback inhibition²¹ (which is itself relieved by phosphorylation²²), inclusion in protein complexes,²³ neuronal activities,²⁴ and so on. Decreased expression of VMAT2 (Fig. 5A) suggests that vesicular sequestration of dopamine may be reduced, which may increase the concentration of cytosolic dopamine. Homeostatic regulation of DA release by neuronal activities, autoreceptors, and so on²⁵ may explain the lack of significant changes in spontaneous or evoked dopamine release (Figure S6A). Consistent with the lack of change in DAT expression, DA reuptake was not significantly different (Figure S6B). Significantly increased expression of genes responsible for the oxidative deamination of dopamine (MAOA, MAOB, and COMT) (Fig. 5B) and the consistent decrease of ALDH1A1, which degrades dopamine aldehydes (Fig. 5B), synergistically elevated oxidative stress (Fig. 4) and dopamine catabolites (Fig. 3E–G).

In addition to the significant differences between neurons derived from healthy subjects and idiopathic PD patients, some of these differences also significantly distinguish neurons derived from PD patients with or without rest tremor at onset, for example, in the expression levels of PAH, TH, VMAT2, GCH1, AKR1B1, QDPR (Fig. 1), and MAOA (Fig. 2); the levels of DOPAC, 3-MT, and HVA (Fig. 3); and the amount of ROS (Fig. 4). Intriguingly, these differences parallel the slower progression and better prognosis of PD patients with tremor than those without tremor at onset.⁴ It appears that differing expression levels of genes controlling dopamine synthesis, sequestration, and degradation may give rise to varying vulnerabilities in midbrain DA neurons, which may correlate with differential clinical symptoms in PD patients with or without tremor at onset. Future studies, for example, metabolomic profiling of these neurons under different treatments to perturb key nodes of dopamine metabolism, are needed to understand whether the molecular and clinical features are coincidental or causal. This descriptive study lays the foundation for further investigations that may reveal mechanistic insights into idiopathic PD. Upon validation in much larger cohorts, the

statistically different range of values for the parameters in the C, N, and T groups may be used to develop criteria to assign a sample to a particular group. Because iPSC derivation can be conducted at any time, the test may be of predictive value before the onset of symptoms. Further development of stem cell and bioengineering technologies may enable assaying a small number of neurons directly converted from patient cells and thus allow validation of these molecular features in a larger, independent cohort.

As the iPSC-derived neurons embody the intrinsic properties of their *in vivo* counterparts at the embryonic stage, our results share several similarities and differences with previous studies on postmortem brain tissues or cerebrospinal fluid (CSF) and imaging studies of live patients with PD, which reflect much later stages of the disease and can have conflicting results. For TH mRNA levels in PD brains, there are reports of decreases,^{26,27} or selective increases in non-melanized DA neurons,²⁸ in contrast to increased TH expression in Figure 1B. Reductions in the mRNA and protein levels of AADC²⁷ are different from our results showing unchanged expression (Fig. 1C). Decreased BH₄ levels²⁷ and GCH1 activities,²⁷ increased expression of SPR, and decreased expression of AKR1B1 and PTS in PD brain tissues²⁹ are different from our data (Fig. 1G–J and Fig. 3C). We are not aware of previous studies on the expression levels of PAH, QPDR, and PCBD1 in PD tissue samples. The significant reduction in VMAT2-binding sites in positron emission tomography imaging studies^{30,31} is consistent with our results (Fig. 1D). Decreases in DAT-binding sites in PD brains³² are different from the unchanged DAT expression in our study (Fig. 1E). CSF levels of DA, L-dopa, and DOPAC are significantly reduced in patients with PD,^{33–35} with no significant change in HVA levels in CSF.³⁶ These results are different from our data (Fig. 3). We are not aware of any publications on the levels of 3-MT, COMT, or MAOA in PD tissue samples, although increased COMT mRNA levels are observed in two PD patients with parkin mutations,³⁷ which is consistent with our results (Fig. 2A). Increased MAOB levels in postmortem PD brains³⁸ are consistent with our results (Fig. 2C). Reduced expression of ALDH1A1 in the substantia nigra of patients with PD³⁹ is consistent with our results (Fig. 2D). Increased serum levels of 8-hydroxyguanine⁴⁰ and higher CSF levels of lipid peroxidation and nitric oxide in patients with PD⁴¹ are consistent with the significantly higher ROS levels in our study (Fig. 4). It seems likely that increased ROS may render iPSC-derived PD neurons more vulnerable to degeneration than control neurons. However, it is impossible to culture these neurons for many years to compare their rates of degeneration.

Conclusion

The study identified significant differences in the expression levels of genes controlling the synthesis, sequestration, and catabolism of dopamine between iPSC-derived DA neurons from healthy subjects and idiopathic PD patients. Consistent changes in dopamine-related metabolites and increased oxidative stress distinguished PD neurons from normal neurons. Neurons derived from PD patients with or without rest tremor at onset exhibited significant differences in the levels of some of these transcripts, metabolites, and oxidative stress. The unique combination of these quantifiable cellular traits may be used to develop an objective diagnostic test for PD, which would significantly facilitate therapeutic development and clinical trials. ■

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Data Availability Statement

All data are included in the manuscript and supplemental materials.

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

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(1) Research Project: A. Conception, B. Organization, C. Execution; (2) Statistical Analysis: A. Design, B. Execution, C. Review and Critique; (3) Manuscript: A. Writing of the First Draft, B. Review and Critique.

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