# **Cell Reports**

# **Molecular Features Underlying Neurodegeneration** Identified through In Vitro Modeling of Genetically **Diverse Parkinson's Disease Patients**

### **Graphical Abstract**



### **Highlights**

- A model of PD was established by generation of human mDA neurons from patient iPSCs
- Evidence of neurodegeneration was observed in PD-derived mDA neurons
- Transcriptome analysis identified dysregulated molecular features in PD model
- RBFOX1, a splicing factor, is elevated in PD and led to disease-related expression patterns

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### In Brief

Lin et al. show that neurons derived from patient-specific iPSCs recapitulate several Parkinson's disease-related phenotypes. Gene expression profiling provides molecular insights into the disease and shows that elevated RBFOX1, a gene previously linked to neurodevelopmental diseases, underlies alternative RNA-splicing changes in PD neurons.

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# Cell Reports

## Molecular Features Underlying Neurodegeneration Identified through In Vitro Modeling of Genetically Diverse Parkinson's Disease Patients

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### SUMMARY

The fact that Parkinson's disease (PD) can arise from numerous genetic mutations suggests a unifying molecular pathology underlying the various genetic backgrounds. To address this hypothesis, we took an integrated approach utilizing in vitro disease modeling and comprehensive transcriptome profiling to advance our understanding of PD progression and the concordant downstream signaling pathways across divergent genetic predispositions. To model PD in vitro, we generated neurons harboring disease-causing mutations from patientspecific, induced pluripotent stem cells (iPSCs). We observed signs of degeneration in midbrain dopaminergic neurons, reflecting the cardinal feature of PD. Gene expression signatures of PD neurons provided molecular insights into disease phenotypes observed in vitro, including oxidative stress vulnerability and altered neuronal activity. Notably, PD neurons show that elevated RBFOX1, a gene previously linked to neurodevelopmental diseases, underlies a pattern of alternative RNA-processing associated with PD-specific phenotypes.

### INTRODUCTION

Parkinson's disease (PD) is caused by the specific and progressive loss of dopaminergic (mDA) neurons in the midbrain substantia nigra. The degeneration of mDA neurons results in a deficiency of dopamine in the striatum and neuronal denervation, giving rise to movement-related disorders and cognitive problems (Shulman et al., 2011). It is recognized that PD is a complex, age-related disease that results from a mixture of genetic predisposition and environmental factors. There are a dozen genes identified associated with familial PD cases. Among the most important genetic influences, mutations in *SNCA* and *LRRK2* are inherited in an autosomal dominant pattern (Paisán-Ruíz et al., 2004; Polymeropoulos et al., 1997; Zimprich et al., 2004), while mutations in *PARKIN*, *DJ-1*, and *PINK1* are inherited in an autosomal recessive pattern (Bonifati et al., 2003; Kitada et al., 1998; Valente et al., 2004). Intriguingly, although there are a number of definitive genetic mutations identified in familial PD, the mechanisms underlying progressive neurodegeneration due to these mutations remain unclear. The fact that different genetic mutations contribute to a similar spectrum of clinical features and that selective mDA neuronal degeneration implicates the involvement of similar downstream signaling pathways across divergent genetic predispositions.

Recent advances have been made in recapitulating PDrelated phenotypes by directed differentiation of patient-specific induced pluripotent stem cells (iPSCs) (Byers et al., 2011; Cooper et al., 2012; Devine et al., 2011; Imaizumi et al., 2012; Liu et al., 2012; Nguyen et al., 2011; Reinhardt et al., 2013; Sánchez-Danés et al., 2012; Seibler et al., 2011). These human disease models produced PD phenotypes, including increased sensitivity to oxidative stress (Byers et al., 2011; Nguyen et al., 2011) and mitochondrial deficits (Cooper et al., 2012). Nevertheless, each of these studies focused on the dysfunction caused by a single mutation and failed to investigate the common downstream pathways that contribute to PD pathogenic mechanisms. Moreover, strong evidence indicates that not only the genetic variants but also the pre- and post-transcriptional regulatory mechanisms are involved in PD pathogenesis.

We report here a combination of iPSC-based disease modeling of PD with comprehensive transcriptome analysis to interrogate the common molecular features underlying the early stages of mDA neuron degeneration. This work reveals dysregulated signaling networks and dynamic splicing alterations, thus providing mechanistic insights into the development and progression of PD.



### RESULTS

## Differentiation of Midbrain Dopaminergic Neurons from Human iPSCs

We recruited a total of 12 iPSC lines in this study (Table S1): three lines (GM23280, GM23279, and IR1.7) derived from healthy individuals; six lines carrying heterozygous or homozygous G2019S mutation in LRRK2 (ND40597, ND40599, ND41181, ND41180 heterozygotes and ND40018, ND35367 homozygotes); one line with SNCA triplication (ND34391); one line with c.255delA mutation in PARKIN (ND38477); and one sporadic case (ND39896) (see Table S2 for patient details). Among these cell lines, three wildtype (WT) cell lines and three PD cell lines (ND38477, ND35367, and ND34391) carrying different PD genetic mutations were employed for the phenotypic characterization and RNA-sequencing (RNA-seq). The additional PD cell lines were employed along with the original cell lines to validate the main phenotypic and transcriptome findings. Point mutations in PARKIN and LRRK2 were confirmed by sequencing (Figures S1A and S1B), and SNCA copy number was verified by gPCR analysis of genomic DNA (Figure S1C). Immunostaining demonstrated that all iPSC lines expressed the pluripotent markers NANOG, OCT4, SSEA4, and TRA1-60 (Figure S1D), and all had apparently normal karyotypes (Figure S1E). Non-specific in vitro differentiation demonstrated that the iPSC lines were capable of generating all three germ layers (Figure S1F). Enriched populations of neural progenitor cells (NPCs) were derived from WT and PD iPSCs within 7 days by treating monolayer cultures with the small molecule inhibitors CHIR99021, SB431542, and Compound E to modulate key signaling pathways (Li et al., 2011) (Figures 1A and S2A). gRT-PCR at different time points during differentiation showed reduced expression by of pluripotency markers, whereas the neuroectoderm makers NESTIN, PAX6, and VIMENTIN were upregulated (Figure S2B). NPCs were immunopositive for the neural markers, but not for pluripotency marker (Figure S2C). Subsequently, NPCs were cultured in the presence of bFGF and EGF supplemented with reduced dosage of CHIR99021 and SB431542 (Li et al., 2011) for more than ten passages with persistent expression of neural stem cell markers (Figure S2D).

Since PD is defined pathologically by progressive degeneration of mDA neurons, NPCs were subsequently differentiated into this neuronal type with a combination of patterning and maturation factors with modifications of published methods (Doi et al., 2014; Kriks et al., 2011; Li et al., 2011) (Figure 1A). Exposure to FGF8 and purmorphamine (days 0-14) drove the NPCs toward midbrain floor plate progenitors, as indicated by expression of CORIN and FOXA2 (Figure 1B). Quantification of CORIN-positive population by fluorescence-activated cell sorting was performed in both WT and PD cell lines at day 16 after differentiation showing yields of 40~60% mDA precursors (Figure S2E). Further maturation was facilitated by  $\gamma$ -secretase inhibitor DAPT plus neurotropic factors glial cell line-derived neurotrophic factor (GDNF) and BDNF for 14-16 days. Neurons generated carried the characteristics of DA neurons regionally specific in substantia nigra pars compacta as indicated by expression of dopamine active transporter (DAT), G-proteinactivated inward rectifier potassium channel 2 (KIR3.2), vesicular monoamine transporter 2 (VMAT2), and floor plate marker FOXA2 (Figure 1C). Confirming their regional identity, the neurons were co-stained for midbrain transcription factors PITX3 and NURR1 (Figure 1D). The mDA neurons also expressed mature neuronal markers synaptophysin (SYP) and postsynaptic density protein 95 (PSD95) (Figure 1D). qRT-PCR analysis further illustrated the upregulation of neuronal markers: TUJ1, MAP2, SYP, and mDA-specific markers: TH, NURR1, EN1, and FOXA2 (Figure 1F). To assess the robustness of the differentiation protocol, emerging mDA neurons from both WT and PD cell lines were characterized for co-expression of the panneuronal marker TUJ1 and DA neuronal marker TH (Figures 1E and S2G) and the expression of FOXA2 (Figure S2F). Approximately half of the differentiated neurons were TH<sup>+</sup> (Figures 1G and S2H). The efficacy of mDA differentiation showed no significant difference between WT and PD cell lines.

We next performed functional studies to investigate the functional properties of these neurons. First, release of dopamine was confirmed by ELISA (Figure S2I). Second, spontaneous action potentials were recorded in a 256-channel microelectrode array (MEA), indicating that the mDAs neurons were electrically active (Figure 1H). Furthermore, channel properties expected of mDA neurons were validated by introducing pharmacological agents. DL-2-amino-5-phosphonovaleric acid (APV), a selective NMDA receptor antagonist, significantly reduced the firing rates.

### Figure 1. Characterization of mDAs Generated from NPCs

<sup>(</sup>A) Schematic representation of the mDA differentiation protocol: NPCs were generated by introducing small molecule inhibitors into monolayer iPSC culture for 7 days; NPCs were maintained in growth medium supplemented with growth factors for further passaging; NPCs were patterned into ventral midbrain lineage with FGF8b and purmorphamine for 14 days; FP progenitors were further differentiated into mature neurons with DAPT, GDNF, and BDNF.

<sup>(</sup>B) 14 days post-initiation of patterning of NPCs, live cells were stained for the floor plate surface marker: CORIN. Scale bar, 200  $\mu$ m; fixed cells were stained for the floor plate marker: FOXA2. Scale bar, 100  $\mu$ m.

<sup>(</sup>C) 30 days post-initiation of patterning of NPCs, neurons were stained for the mDA marker DAT, KIR3.2, FOXA2 (scale bar, 100  $\mu$ m) and VMAT2 (scale bar, 200  $\mu$ m).

 <sup>(</sup>D) Neurons were co-stained for the mature neuronal markers SYP, PSD95 (scale bar, 200 μm) and mDA neuronal markers NURR1, PITX3 (scale bar, 100 μm).
(E) Expression of pan-neuronal marker TUJ1 and DA neuron markers TH in iPSC-derived 35-day neurons by immunostaining. Scale bar, 200 μm.

<sup>(</sup>F) qRT-PCR analysis showing the expression of neuron markers: *TUJ1*, *MAP2*, *SYP*, and mDA-specific markers: *TH*, *NURR1*, *EN1*, and *FOXA2*. Data represent mean ± SEM (n = 3).

<sup>(</sup>G) Quantification of mDA differentiation efficacy based on TH and TUJ1 colocalization, in 30-day neurons derived from WT and PD lines. Data represent mean ± SEM (n = 3).

<sup>(</sup>H) Electrophysiological recordings of spontaneous action potentials from WT and PD mDA neuron cultures by microelectrode arrays (MEAs); effects of neuromodulators applied to WT and PD mDA neurons. APV, an NMDA antagonist; Risperidone, dopamine D2 receptor antagonist. See also Figure S2.



Decreased firing was also induced by risperidone, indicating that the differentiated neurons possessed functional dopamine (DA)-D2 receptors (Figure 1I). Thus, the in vitro generated mDA neurons were functional in terms of synthesizing and releasing dopamine, emitting spontaneous synaptic activities, and responding appropriately to pharmacological blockade.

### mDA Neurons Derived from PD iPSCs Exhibit Disease-Related Phenotypes

Previously, a progressive degenerative phenotype in NPCs was reported for a LRRK2 iPSC model of PD, which recapitulated aging-associated molecular processes in vivo (Liu et al., 2012). Aberrant nuclear architecture was observed upon prolonged passaging, and late-passage NPCs showed deficiency in proliferation and ability to generate mature neurons. Consistent with this report, we found that the mDA neurons derived from latepassage PD NPCs (P12) exhibited morphological abnormality in neurite length. Cultures of mDA neurons derived from three WT cell lines and three PD cell lines were co-stained with TUJ1 and TH to visualize the neurite morphology (Figure 2A). Quantification of neurite length showed significant (p < 0.001) reduction in the extension of mDA neurites in ND35367 and ND34391 cell lines (Figure 2B). Noticeably, mDA neurons derived from ND35367 (LRRK2 G2019S) displayed damaged neurites as indicated by blebbing in neuronal projections and axonal fragmentation. These morphological signs of neurodegeneration were not observed in WT mDA neurons (Figure 2C). Next, it was determined that PD mDA neuron cultures showed increased apoptosis. At day 28, subtle increases of caspase-3 expression were detected by gPCR in both WT and PD mDA neurons compared to NPC stage (Figure 2D). However, after further culturing (day 49), caspase-3 expression was significantly (p < 0.001) elevated in all three PD cell lines (Figure 2D). To further assess the amount of apoptosis within the cultures at initial stages, neurons at day 40 were co-stained for MAP2 and cleaved caspase-3. PD cultures showed significantly higher percentage of cells that were positive for cleaved caspase-3, and also showed fragmented neuronal structures and formation of apoptotic bodies (Figures 2E and 2F). Thus, our results indicate that PD mDA neurons in culture display degenerative morphologies and undergo apoptosis.

Another clinical feature implicated in PD is the abnormal accumulation of  $\alpha$ -synuclein in protein aggregates within Lewy bodies (Baba et al., 1998; Chung et al., 2001; Farrer et al., 2001; Kalia et al., 2015). First, SNCA mRNA expression changes were deter-

mined by qPCR with further prolonged culture of mDA neurons at days 28, 35, 42, and 49 (Figure 3A). Two-way ANOVA tests were performed to verify the progressive increase in SNCA expression in a few PD cell lines. On the other hand, ND34391, which carries the SNCA triplication mutation, showed enhanced SNCA expression at NPC stage (Figure S3A). Accumulation of a-synuclein protein was further evaluated by detecting phosphorylated form of  $\alpha$ -synuclein (P<sup>S129</sup> $\alpha$ -synuclein), the predominant type within Lewy bodies (Fujiwara et al., 2002; Sato et al., 2013; Sugeno et al., 2008). Western blots showed that PD mDA neurons robustly expressed  $P^{S129}\alpha$ -synuclein compared to WT (Figures 3B and 3C), especially in ND34391, which carries the SNCA triplication mutation. The solubility of  $P^{S129}\alpha$ -synuclein was further examined by protein fractionation, indicating that such phosphorylated species were highly enriched in SDSsoluble fraction from PD mDA neuronal cultures after further culturing, but not present in WT (Figures S3B and S3C). Protein lysates collected from WT (GM23280 and GM23279), ND38477 PARKIN, and ND34391 SNCA mDA neurons at day 44 were treated with calf-intestinal alkaline phosphatase to verify the presence of phosphorylated  $\alpha$ -synuclein (Figure S3D). Overall, in the cell line with PARKIN c.255delA mutation, an enrichment of  $\mathsf{P}^{S129}\alpha\text{-synuclein}$  was observed, corresponding to the Lewy body pathology in Parkin-related early-onset Parkinsonism and the related iPSC model (Farrer et al., 2001; Imaizumi et al., 2012). A mild  $P^{S129}\alpha$ -synuclein phenotype was present in most of the LRRK2 G2019S cell lines, whereas ND34391 (SNCA triplication) from a patient with cognitive impairment/dementia and diffuse Lewy Body (Table S2) showed significant  $P^{S129}\alpha$ -synuclein accumulation.

Besides morphological and neurochemical phenotypes, electrophysiological differences between PD and WT neurons were also observed. MEAs with 256 electrodes embedded within a cell culture well measured field potentials from multiple areas within mDA neuron cultures. In this experiment, we selected the ND35367 (*LRRK2* G2019S) cell line for examination since it presented the most pronounced degeneration-related phenotypes (Figure 2). It was notable that the simultaneous recording of cultured neurons revealed synchronized firing networks in WT cultures (indicated by arrow), while such synchronized firing was not observed in PD mDA neurons with *LRRK2* G2019S mutation (Figure 3D). Moreover, quantification of electrical activity within the mDA neuron cultures indicated that there was significant decrease in the number of active channels and their firing rates in the PD cell line (Figure 3E). These results suggest that

### Figure 2. Neurite Shortening in mDAs Derived from PD iPSC Lines

(C) Representative images of loss of neuronal projections particularly in LRRK2 G2019S. Arrows indicate noticeable neurite fragmentation. Scale bar, 10 µm.

(D) Caspase-3 gene expression was detected by qRT-PCR on mDA neuron cultures differentiated for 28 and 49 days. Data represent mean  $\pm$  SD (n = 4). Comparisons are by two-way ANOVA followed by Bonferroni posttests. ns, non significant; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

(E) Immunofluorescence analysis of mDA neurons differentiated from WT and PD iPSCs for 40 days, co-stained for MAP2 and cleaved caspase-3. Scale bar, 100 μm.

(F) Quantitative analyses of the percentage of neurons with positive staining for cleaved caspase-3. Data represent mean  $\pm$  SEM (n = 3). Comparisons are by one-way ANOVA followed by Dunnett's test. \*p < 0.05.

<sup>(</sup>A) Representative images of neurites double labeled with TUJ1 and TH at day 40 post-differentiation of NPCs. Axonal degenerative morphology is obviously present in mDA cultures derived from iPSCs with *LRRK2* G2019S mutation (ND35367). Scale bar, 100 μm.

<sup>(</sup>B) Quantification TH<sup>+</sup> neurites in mDA cultures from wild-type (WT) and PD cell lines. Data represent mean  $\pm$  SEM (n = 3). ns, non-significant; \*\*\*p < 0.001. Comparisons are by one-way ANOVA followed by Dunnett's test.



Figure 3. Abnormal Accumulation of pS129  $\alpha$ -Synuclein Species and Defective Electrical Activity in PD mDA Cultures (A) SNCA gene expression was detected by qRT-PCR on mDA neuron cultures differentiated for 28, 35, 42, and 49 days. Data represent mean  $\pm$  SD (n = 4). Comparisons are by two-way ANOVA followed by Bonferroni posttests. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. (B) Representative western blot illustrating P<sup>S129</sup> $\alpha$ -synuclein protein abundance in WT and PD mDA neuron cultures with different genetic backgrounds. PSD95, a

mature neuron marker, and total SNCA was plotted to show neuronal abundance and total SNCA expression.

(C) Quantification of  $P^{S129}\alpha$ -synuclein protein abundance in two WT cell lines and four PD cell lines from three biological replicates at days 44–46 after NPC differentiation. Data represent mean  $\pm$  SEM (n = 3). Comparisons are by one-way ANOVA followed by Dunnett's test. \*p < 0.05, \*\*p < 0.01.

PD mDA neurons display electrophysiological impairment analogous to other PD models observed in vivo and in vitro (Bilbao et al., 2006; Woodard et al., 2014; Yee et al., 2014).

### Increased Oxidative Stress Susceptibility in PD mDA Neurons

It has been suggested in previous reports that exposure to neurotoxins can cause mitochondrial functional impairment and the release of reactive oxidative species (ROS), leading to a number of cellular responses including apoptosis and increased risk of developing PD (Bové and Perier, 2012). To explore the cellular antioxidant capacity, we compared the neurotoxic sensitivity of mDA neurons derived from the three PD lines harboring divergent genetic mutations (Figure 4A). Antioxidant components in the cell culture were removed, and after 16 hr various neurotoxins were added individually. First, 6-OHDA (10 µM) and MG-132 (2 µM) were used to challenge the mDA neurons. At 24 hr, cells were stained for TUJ1 and TH to visualize the neuron morphology and to quantify the neurite length (Figures 4B and 4C). The morphology of mDA neurons from WT cell lines was unaltered, however, in PD neurons exhibited morphological changes in neurite length after treatment. TH-positive neurite length was significantly reduced in ND35367 (p < 0.01) and ND34391 (p < 0.001), while the decrease was not observed in WT cell lines (Figure 4D). For MG-132 treatment, the neurite length in PD cell lines also decreased compared to WT (Figure 4D).

To further measure the differential susceptibility of neurotoxininduced cell death, neurons were stained for TUJ1 and cleaved caspase-3 after treatment (Figures S4A and S4B). WT mDA neurons had no significant increase in cell death after 6-OHDA treatment, while enhanced apoptosis was noted in the mDA neurons derived from ND35367 and ND34391 (Figure S4A). In MG-132treated cells, such apoptotic populations became more pronounced (Figure S4B). The apoptotic neurons were quantified based upon the cleaved caspase-3 and normalized by the total number of nuclei in the field, indicating a statistically significant increase in cell death in ND35367 and ND34391 cell lines compared with WT (Figure S4C).

The herbicide paraquat has been identified as an environmental factor for PD that leads to destruction of mDA neurons in humans (McCormack et al., 2002). To test the effect of paraquat, neurons were treated with paraquat (400  $\mu$ M for 20 hr) and subsequently co-stained for TUJ1 and TH (Figure 4E). The majority of mDA neurons derived from WT cell lines preserved the morphology of extended neurites with paraquat incubation, while in mDA neuron cultures derived from PD cell lines, showed impaired neurites, especially evident for ND35367 (*LRRK2* G2019S). It was notable that there were no intact mDA neurons in cultures generated from ND35367 after paraquat treatment. Moreover, although PD mDA neurons displayed a native neurite shortening phenotype indicated in the DMSO control, the defective neurite further deteriorated after paraquat treatment (Figure S5C). Subsequently, neurons were stained for NURR1 and cleaved caspase-3 after neurotoxin treatment (Figure S5A). The apoptotic neurons in the culture showed a statistically significant enhancement in PD cultures based on two-way ANOVA (Figure S5B).

In summary, these findings demonstrate that neurotoxin exposure induces a degenerative impact on neurite extensions, leading to apoptosis of PD mDA neurons, while the WT cell lines resist the cytotoxicity and display only minor degenerative phenotypes. Overall, these observations indicate that PD mDA neurons are prone to the neurotoxin-induced oxidative stress and apoptosis.

# Identifying Transcriptome Dysregulation in PD mDA Neurons

Having cultured mDA neurons that display salient characteristics of neural degeneration provided a unique opportunity to explore the molecular changes associated with early events and progressive stages of PD pathology. To this end, comprehensive gene expression profiling was performed on both NPC and mDA neurons derived from six iPSC lines, including three WT cell lines and three PD cell lines carrying different PD genetic mutations (ND38477 PARKIN c.255delA, ND35367 LRRK2 G2019S, ND34391 SNCA triplication) (Figure 5A). RNA-seq was performed on two biological replicates for each condition at a depth of  $\sim$ 40 million reads per sample. For a global view of expression differences in WT and PD cell types, we applied principal component analysis (PCA) (Figure 5B). NPCs derived from all iPSC lines clustered (PC1) closely together (Figure 5B, upper left), indicating that, compared to mDA neuron cultures, the transcriptomes of NPCs were similar across different cell lines. Furthermore, the subtle variance between WT and PD samples across two cell stages was captured based on PC3 with an overlap between a few samples (Figure 5B, upper right). Since NPCs gave rise to more homogeneous population across PD and WT cell lines, such uniformed cell population largely affects PCA analysis done with both NPC and mDA samples in terms of isolating the difference between PD and WT. When NPCs samples were removed from the PCA analysis, a clear separation between PD and WT mDA neurons was observed in PC2 (Figure 5B, lower). A total of 908 genes were differentially expressed (p < 0.01 and absolute fold change >2) between PD and WT mDA neurons, of which 511 were protein-coding and 188 were long non-coding RNA (Figures 5C and S6A). Enrichment for groups of genes related within biological pathways and biochemical functions, based on Gene Ontology (GO), was conducted using Consensus-PathDB (http://cpdb.molgen.mpg.de). Interestingly, functional implications of the unifying upregulated protein-coding genes in PD mDA neurons indicated enriched clustering of gene functions involved in synaptic signaling transmission, providing

<sup>(</sup>D) Raster plot of spontaneous spiking activity by using 248-channel MEAs at day 38 post-differentiation from NPCs. Synchronized networks in WT cultures are presented as vertical bands of spikes network; MEA recordings reveal less synchronized neuron networks in PD DA cultures.

<sup>(</sup>E) Quantitative analysis of the numbers of firing neurons and firing rates was calculated in one WT cell line (GM23279) and one PD cell line (ND35367) in two biological replicates. In each biological replicate, multiple field potentials were recorded indicated by N. Data represent mean ± SD. See also Figure S3.



molecular evidence of a unifying dysregulated neuronal activity in PD mDA neurons (Figures 5D and 5E).

To verify the RNA-seq result, independent differentiation was performed with the original cell lines and six additional PD lines, which either carried familial mutations or sporadic cases of unknown genetic causes (Table S1). The recruitment of additional cell lines in validation increases confidence that such dysregulation could be the unifying regulators for pronounced disease phenotypes. In total, 12 cell lines were differentiated into mDA neurons to validate gene expression differences. Based on the ranking of p values and fold changes, 38 protein-coding genes and 22 long non-coding RNAs identified from RNA-seq were selected for further analysis by qRT-PCR. We observed a certain level of heterogeneity, reflecting the diversity in genetic background. However, for the majority of selected genes, the differential expression was confirmed in a consistent pattern in most of the PD cell lines (Figure 6A).

### **RBFOX1** Contributes to Alternative Splicing Events in PD mDA Neurons Enriched in Mitochondrial-Related Genes and Neuronal Activity

The RNA-seq data comparing WT and PD neurons represent a rich resource of information to ascertain molecular changes associated with the onset and progression of the disease. We sought to determine how dysregulated gene expression might contribute to the pathologies observed for PD mDA neurons. Among the most consistently upregulated genes in PD neurons was RBFOX1 (RNA-binding protein fox-1 homolog), a neuronspecific splicing factor known to target the binding motif (U) GCAUG and regulate alternative exon usage (Jin et al., 2003; Shibata et al., 2000). RBFOX1 regulates neuronal splicing networks and hence controls neuronal excitation. Knockout of RBFOX1 in mice alters the splicing of exons for multiple transcripts affecting neuronal excitation and calcium homeostasis resulting in neuronal hyperexcitation (Fogel et al., 2012; Gehman et al., 2011). We found similar expression of RBFOX1 in PD and WT cell lines at NPC stage, but a robust elevation in PD mDA neurons (Figures S6B and S7A). Additionally, protein levels of RBFOX1 correlated with the disease-related phenotypes (Figure S3D).

To investigate a potential role of *RBFOX1* and globally measure differential alternative splicing events in the PD mDA neurons, we generated RNA-seq data with longer pair-end reads for two WT cell lines (GM23280 and IR1.7) and two PD cell lines (ND35367 and ND40018), which displayed the highest *RBFOX1* expression (Figure S7B). RNA samples were collected from two biological replicates of each cell line. Reads that mapped to splice junctions were quantified to reveal alternative splicing events in both WT and PD mDA neurons (Figure 6B): 645 splicing events involving 439 protein-coding genes were identified (fold change >2 and p value  $\leq$ 0.05). Gene ontology analysis revealed that genes showing significant differential splicing in PD mDA neurons are robustly associated with mito-chondrial function, cell apoptosis, and cellular redox metabolism (Figures 6C and 6D). Such dysregulated antioxidant network by alternative splicing may affect the threshold of neuronal degeneration triggered by external stimuli, which serves as a possible explanation of the increased vulnerability in PD mDA neurons toward oxidative-stress-induced apoptosis triggered by neurotoxins.

To identify the differentially spliced genes specifically mediated by *RBFOX1*, the 439 genes were overlapped with predicted *RBFOX1* targets (Gehman et al., 2011; Weyn-Vanhentenryck et al., 2014), which generated a short list of 41 genes (Figures 6E and S7E). GO analysis showed a significant enrichment in GO terms related to neuron projection and neuronal activity (Figure S7F). In summary, the genome-wide identification of alternative splicing events in mDA neuron cultures enabled us to identify the splicing variants in PD mDA neurons associated with differential expression of *RBFOX1*.

# Dysregulated Splicing Events Mediated by *RBFOX1* in PD mDA Neurons Highlight Disturbed Neuronal Activity

Given the potential importance of dysregulated alternative splicing in PD (Dredge et al., 2001), two genes previously identified as under the influence of RBFOX1 were studied in our in vitro model (Gehman et al., 2011). GRIN1 belongs to the glutamate receptor channel superfamily that forms functional heterotetramers with other NMDA receptor gene families. Two (U)GCAUG motifs within GRIN1 mediate exon 5 inclusion by RBFOX1 (Figure 7A). The alternative splicing events in GRIN1 was evaluated by isoform-specific RT-PCR (Figure 7B). Total GRIN1 transcripts were detected with primers spanning exons 3 and 4, which are present in all splice variants. The overall expression of GRIN1 did not vary between PD and WT mDA neurons. Another isoform with exon 21 inclusion also did not show much difference and thus serves as a useful endogenous control. In contrast, the expression of a specific isoform with exon 5 inclusion showed significant increase in PD mDA neurons, demonstrating that differential splicing events in PD mDA neurons are regulated by RBFOX1 (Figures 7B and S7C).

Another gene that showed significantly altered isoform expression is the known RBFOX1 target gene SNAP25

Figure 4. Neurite Degenerative Effect of Neurotoxins on mDA Neuron-Derived PD iPSCs

(A) Schematic representation of neurotoxin assay in mDA neuron cultures.

<sup>(</sup>B and C) Representative images of neurites double labeled with TUJ1 and TH at D34 with DMSO control or 6-OHDA treatment (B) and MG-132 treatment (C). Scale bar, 100 µm.

<sup>(</sup>D) Quantification TH+ neurite length in mDA neuron cultures from WT and PD cell lines with 6-OHDA or MG-132 treatment, normalized to DMSO control. Data represent mean  $\pm$  SEM (n = 3). WT samples were pooled for comparisons with individual PD cell lines by one-way ANOVA followed by Dunnett's test. ns, non-significant; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

<sup>(</sup>E) Paraquat-induced defective neurites in mDA neurons derived from late-passage PD NPCs. Representative images of neurites double labeled with TUJ1 and TH at D34 with DMSO or paraquat treatments. Scale bar, 200 µm. Zoom-in images presenting local fields of mDA morphology. Scale bar, 100 µm. See also Figures S4 and S5.



(legend on next page)

(Figure S5E). SNAP-25 (synaptosomal-associated protein of 25 kDa) is a component of the SNARE (Soluble NSF Attachment protein REceptor) complex, interacting with syntaxin and synaptobrevin to facilitate neurotransmitter exocytosis. It was notable that RBFOX1 binding motifs have been spotted in this mutually exclusive exon pair, resulting in two isoforms, SNAP-25a and b (Figure 7C). From our RNA-seq data, a differential distribution of reads in exon 5a and b was observed in WT and PD mDA neurons (Figure S7D). Further verification was performed in additional PD cell lines, indicating a consistent dysregulation of SNAP-25 alternative splicing in PD mDA neurons (Figure 7D). To further prove that RBFOX1 is the regulator of such alternative splicing events, short hairpin RNA (shRNA) knockdown of RBFOX1 was performed in mDA neurons from three WT and three PD cell lines (ND35367, ND35391, and ND41181). The efficiency of RBFOX1 knockdown was determined by gRT-PCR, showing approximately 70% reduction of mRNA expression in both WT and PD mDA neuron cultures (Figure 7E). Subsequently, the splicing events of SNAP-25 with either scrambled shRNAs or RBFOX1 shRNAs transduction were measured. The expression profiling of SNAP-25 isoforms in PD mDA neurons with RBFOX1 knockdown indicated marked shifts of SNAP-25b expression (Figure 7F). Thus, we validated the dysregulated RBFOX1depenent splicing events in PD mDA neurons. Consistent with the phenotypic characterization and what we validated in RBFOX1 downstream splicing targets, a splicing-regulatory network for controlling neuronal activity was apparent, implying substantial importance of dysregulated splicing events in our in vitro model of PD.

### DISCUSSION

In this report, we highlight that mDA neurons generated from PD-specific iPSC lines display disease-like characteristics in vitro. It is noteworthy that a consistent vulnerability was observed in different PD cell lines in response to neurotoxin treatments, indicating enhanced vulnerability to stress in PD mDA neurons compared to WT. It should be noted that there was heterogeneity among different PD cell lines. For example, ND38477 cell line, which carried *PARKIN* c.255delA mutation, showed only mild degenerative phenotypes compared to other cell lines. Such phenotypic diversity in vitro is unsurprising given the complex nature of PD progression associated with various genotypes and the underlying individual-to-individual genome variation, which manifests in patients as a broad time frame of disease initiation and development.

To date, only a few studies on transcriptome profiling of iPSC-derived neurons have been discussed in the context of neurodegenerative diseases. Among these studies, researchers interrogated the alterations in monogenic PD (Reinhardt et al., 2013; Woodard et al., 2014). However, the phenotypic similarity of neuronal degeneration across PD genetic mutations firmly implies the sharing of common downstream signaling pathways. Toward this end, a collection of iPSC lines with distinct PD mutations were recruited for gene expression profiling to identify molecular regulators that could be potentially important for early events in PD progression. Although the reprogramming procedures have become more efficient and standard in recent years, it has been observed that iPSCs generated from different sources or even same starting cell population yield variable epigenetic signatures (Bar-Nur et al., 2011; Lister et al., 2011). Thus, some gene expression differences we observed might be due to the genome variance in two-group comparison. In order to minimize the impact of inconsistent iPSC identities, larger numbers of iPSC cohorts for each type of genetic mutations and healthy individuals would need to be recruited, including multiple clones from the same patient. Another means to minimize genetic heterogeneity in patient samples is to generate isogenic iPSC lines using gene-editing technologies.

Intriguingly, we observed a consistent pattern of gene dysregulation in PD mDA neurons across different gene mutations. A key neuron-specific splicing factor RBFOX1 was found consistently over-represented in PD mDA neurons. RBFOX1 has been linked to various neurological disorders including epilepsy, mental retardation, and autism spectrum disorder (Bhalla et al., 2004; Martin et al., 2007; Voineagu et al., 2011; Wen et al., 2015), revealing an important role of alternative splicing events in regulating the homeostasis of neuronal function. Thus, the splicing dysregulation triggered by RBFOX1 in PD progression was further evaluated by RNA-seq. Notably, the abnormal splicing events in NMDA receptor GRIN1 and SNARE component SNAP-25 due to overrepresentation of RBFOX1 in PD mDA neurons provide apparently important clues to the decreased neuronal activity described in our cell-culture model. The alternative splicing of exon 5 in GRIN1 substantially contributes to the GRIN1 receptor activity (Vance et al., 2012). NMDA receptors consisting of GRIN1 subunits that contain exon 5 possess lower potency of NMDA receptor agonists and displays faster deactivation (Paoletti et al., 2013; Rumbaugh et al., 2000). Thus, the increase expression of exon-5-containing GRIN1 due to overrepresentation of RBFOX1 in PD mDA neurons could possibly explain the

Figure 5. Global Gene Expression in NPCs and mDA Neurons Derived from WT and PD iPSCs by RNA-sequencing

(D) Gene ontology analysis of upregulated genes in mDA neuron cultures derived from PD iPSC lines compared with WT.

(E) Heatmap depiction of the representative differentially expressed genes within gene ontology categories presented in Figure 5D.

See also Figure S6.

<sup>(</sup>A) Schematic diagram of RNA-seq experimental design and analysis workflow. Two biological replicates were sequenced in each condition.

<sup>(</sup>B) Principal component analysis (PCA) illustrating clustering of cell types based on the first principal component (PC1) and the separation of PD and WT samples based on the PC3. After removing the NPC samples, PCA shows the separation of PD and WT mDA samples in PC2, and a subtle batch effect of two biological replicates was observed in PC1.

<sup>(</sup>C) Transcriptome characterization by DESeq analysis identifies 908 differentially expressed transcripts in PD mDA neurons. Separate pie charts were plotted for upregulated and downregulated classes.



decreased neuronal firing rates that we observed by MEA recording. Alternative splicing of exon 5a or 5b in *SNAP-25* introduces nine amino acid substitutions in the C-terminal end of the first SNARE motif and the first part of the cysteine-containing linker (Nagy et al., 2005). The characteristics of exocytosis with different *SNAP-25* isoforms have been evaluated, varying in their ability to stabilize vesicles in the releasable pools (Sørensen et al., 2003). A balanced expression of the two isoforms is required for maintaining an operational neuronal network homeostasis. One could speculate that such dysregulated splicing events could be the preceding events affecting the neuron function in PD pathology.

From previous reports and the phenotypic characterization in our study, there is strong evidence of mitochondria-mediated oxidative stress implicated in PD mDA neurons (Byers et al., 2011; Nguyen et al., 2011; Reinhardt et al., 2013; Sánchez-Danés et al., 2012). Thus, the evaluation of altered cell responses to various neurotoxic substances highlights the overall vulnerability of PD mDA neurons toward oxidative stress, reflecting a proof of concept for the potential PD mechanisms between dysregulated mitochondrial splicing and neuronal degeneration. In short, these findings further validate iPSC-based modeling of PD with various genetic backgrounds. Moreover, splicing modulation by RBFOX1 could be induced by depolarization (Lee et al., 2009). Thereby, the enrichment of specific splice variants in PD mDA neurons could be gradually achieved along with advancing neurons, which corresponds well to the age dependence of neurodegenerative processes. Taken together, the expression profiling of PD iPSC-based model supports our initial hypothesis that a unifying pattern of molecular changes has already occurred in the target PD mDA neurons as early events in disease progression. The differential gene expression and defective splicing regulation in PD mDA neurons reveals a complex homeostatic crosstalk between transcriptional regulation and splicing network mediated by neuron-specific splicing factor RBFOX1. Further elucidation of the factors mediating the upregulation of RBFOX1, and how RBFOX1 impacts the function of downstream splicing targets in PD neurons, will have important relevance to our understanding of the early pathogenesis of PD. It will be interesting to test whether the dysregulation of RBFOX1 in WT cell lines could induce neurodegenerative phenotypes.

### **EXPERIMENTAL PROCEDURES**

### Maintenance and Characterization of Human iPSCs

WT iPSC lines GM23280A and GM23279A, PD iPSC lines ND34391, ND35367, ND38477, ND40018, ND41181, ND41180, ND40600, ND40597, ND40599, and

ND39896 were obtained from the Coriell Cell Repositories. WT iPSC line IR1.7 was a kind gift of Dr. Irene Aksoy. For feeder-free culture, cells were cultured with chemically defined mTeSR medium (STEMCELL Technologies) on Matrigel-coated tissue culture plates.

#### Differentiation of hPSCs into NPCs and mDA Neurons

NPCs were generated using an adapted protocol (Li et al., 2011). NPCs were regularly cultured in maintenance contained N2B27 medium supplemented with 3  $\mu$ M CHIR99021, 2  $\mu$ M SB431542, 5  $\mu$ g/ml BSA, 20 ng/ml bFGF, and 20 ng/ml EGF. Differentiation of NPCs into mDA neurons was initiated 1 day after passaging the NPCs on poly-L-ornithine hydrochloride/laminin-coated culture. NPC maintenance medium was substituted by mDA patterning medium consisting of N2B27 medium supplemented with FGF8, Purmorphamine, Dibutyryl cAMP (db-cAMP), and L-ascorbic acid (L-AA) for 14 days. From days 14, cells were fed with maturation medium consisting of GDNF, BDNF, DAPT, db-cAMP, and L-AA.

#### Immunofluorescence Microscopy

Cells were fixed in ice-cold 4% paraformaldehyde (PFA) for 25 min and subsequently permeabilized by PBS solution with the detergent Tween 20 (PBST) and blocked with 10% fetal bovine serum in PBST for 30 min. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C. Secondary antibodies conjugated with Alexa Fluor (Invitrogen) were diluted 1: 500 in blocking buffer and incubated for 45 min at room temperature. DAPI (0.5 µg/ml) was used to visualize cell nuclei. The respective primary antibodies and dilutions are provided in Table S5. Image acquisition and processing was performed in AxioVision software (Zeiss). Confocal images were taken using a Zeiss LSM 510 upright confocal microscope. Co-localization efficiency for plugin based on Pearson's coefficient of correlation. Neurite length quantification was performed by ImageJ NeuronJ plugin.

### **RNA-Sequencing and Data Analysis**

Total RNAs were extracted with Trizol reagent (Invitrogen) and purified with RNeasy Mini kit (QIAGEN), according to the manufacturers' instructions. Ribosomal-RNAs were depleted from 4  $\mu$ g total RNAs by using Ribo-Zero Gold rRNA Removal Kit (Human/Mouse/Rat) (Epicenter Illumina), and RNAsequencing libraries were constructed by using 20 ng ribosomal RNA (rRNA)-depleted total RNA samples with NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs). RNA-sequencing at a 51- or 76-bp single-end read scale was performed on an Illumina Platform HiSeq2000 (Genome Institute of Singapore), using rRNA-depleted total RNA from two biological replicates of the WT cell lines GM23280A, GM23279A, and IR1.7 and PD iPSC lines ND34391, ND35367, and ND38477 cell at NPC stage and mDA stage. Reads were uniformly trimmed down to 51 bp and mapped with Tophat2 to the human genome version hg19. Reads were counted using R, normalized reads were obtained using the regularized log transformation. Differentially expressed genes were identified using DESeq2 (Love et al., 2014). The PCA plot was calculated on the top 10,000 genes that showed the highest variance across the samples.

For detecting alternative splicing events, RNA-sequencing libraries were generated from intact poly(A)+ RNAs prepared by using Oligo d(T)25 Magnetic Beads (New England Biolabs). RNA-sequencing was conducted on an Illumina Platform HiSeq2000 at a 101-bp paired-end read scale. Reads were aligned using the same software and parameters as the other datasets. RNA-seq

Figure 6. RNA-Sequencing Identified Global Alternative Splicing Dysregulation of Oxidative-Stress-Responsive Genes

(D) Heatmap of normalized read counts at splice junctions of representative PD-specific alternative splicing events in 47 mitochondrial-related genes.

See also Figure S7.

<sup>(</sup>A) Selective list of genes showed consistent differential expression in PD cell lines. Results were plotted as fold differences of relative gene expression normalized to controls. Data represent mean  $\pm$  SD (n = 3); \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

<sup>(</sup>B) Scheme of RNA-seq analysis for detecting alternative splicing variants.

<sup>(</sup>C) Gene ontology analysis of genes with differentially alternative splicing in PD mDA neurons relative to WT mDA neurons.

<sup>(</sup>E) Venn diagram showing the overlap of alternative splicing events in PD versus WT with predicted RBFOX1 targets. Significance of the overlap was estimated using the hypergeometric test.



### Figure 7. Differential Alternative Splicing of GRIN1 and SNAP-25 Mediated by RBFOX1 in PD mDA Neurons

(A) Alternative splice variants in human *GRIN1* mainly involved with exon 5 and exon 21. Hatched box below indicated the exon 5. Yellow boxes represent (U) GCAUG motifs.

(B) Relative expression levels of various GRIN1 isoforms in WT and PD mDA neurons. Data represent mean ± SD (n = 3).

(C) SNAP-25 gene structure: hatched boxes indicate the open reading frame of SNAP-25. Yellow boxes represent (U)GCAUG motifs. Arrows indicate the locations of primers for detection of different isoforms.

(D) qRT-PCR verified the relative expression levels of SNAP-25a and SNAP-25b in WT and PD mDA neurons normalized to total transcripts of SNAP-25. Data represent mean  $\pm$  SD (n = 3). \*\*\*p < 0.001.

(E) Knockdown efficiency of *RBFOX1* shRNAs was determined by qPCR in WT and PD mDA neurons relative to scramble shRNA controls. WT cell lines and PD cell lines were pooled for comparisons. Data represent mean  $\pm$  SD (n = 3).

(F) Relative expression levels of SNAP-25a and SNAP-25b after scramble shRNA knockdown and RBFOX1 knockdown in WT and PD mDA neurons normalized to total SNAP-25 transcripts. WT cell lines and PD cell lines were pooled for comparisons. Data represent mean  $\pm$  SD (n = 3). ns, non-significant; \*p < 0.05. See also Figure S7.

reads that mapped to two exons (junction reads) were used to quantify splicing events (Lu et al., 2013). For each gene, DESeq2 was used to identify exon junctions that significantly differed between WT and PD samples relative to the total junction read count for that gene.

### **Statistical Analysis**

Statistical analysis was performed using Statistical Package GraphPad Prism v.5.0c (GraphPad Software). Two-tailed unpaired Student's

t test was universally used for the comparison of two groups. Statistical testing also involved one-way ANOVA with Bonferroni's multiple comparison test or Dunnett's test for multiple comparisons, as indicated in the figure legend. Data are expressed as means  $\pm$  SEM or means  $\pm$  SD) as indicated. Significance was considered for \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

Additional details are provided in the Supplemental Experimental Procedures.

### **ACCESSION NUMBERS**

The accession number for the RNA-seq data reported in this paper is ArrayExpress: E-MTAB-4586.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.022.

#### **AUTHOR CONTRIBUTIONS**

L.L. and L.W.S. designed the study and wrote the paper. L.L. performed experiments, analyzed data, and prepared the manuscript. J.G. and E.C. performed the computational analyses and modified the manuscript. M.R.D. performed the MEA recordings and interpreted the data. A.M.J.V. participated in the analysis and interpretation of MEA data and contributed to the experimental discussion.

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