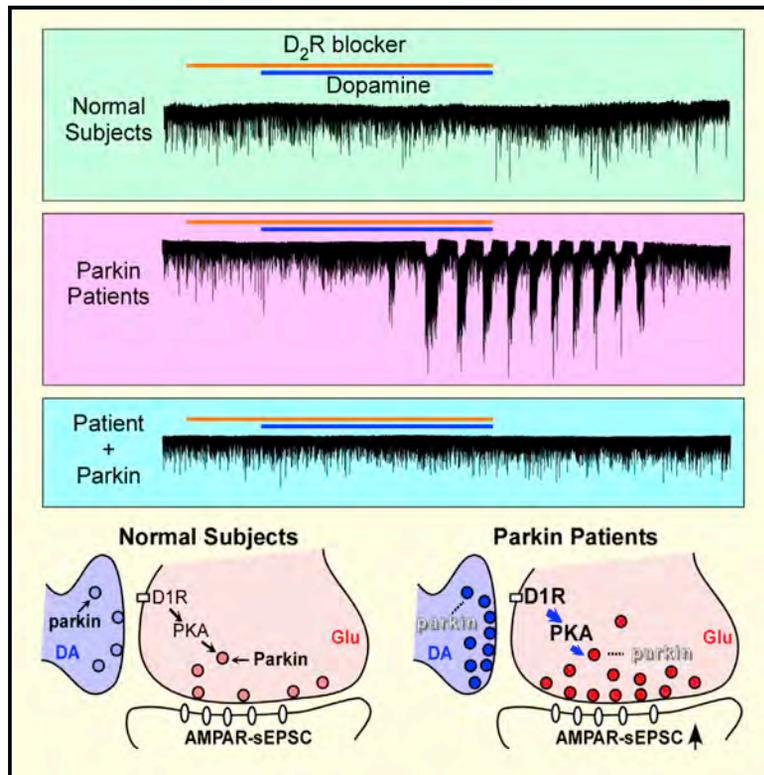


Dopamine Induces Oscillatory Activities in Human Midbrain Neurons with Parkin Mutations

Graphical Abstract



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In Brief

In midbrain neurons derived from induced pluripotent stem cells of Parkinson's disease patients with parkin mutations, Zhong et al. find that activation of dopamine D1-class receptors induces oscillatory activities reminiscent of synchronized and rhythmic neuronal activities seen uniquely in the brains of Parkinson's disease patients.

Highlights

- Dopamine D1 receptors elicit oscillatory activities in neurons from parkin patients
- No oscillatory activity is found in iPSC-derived neurons from normal subjects
- Wild-type parkin rescues oscillatory activities in neurons from parkin patients
- Mutant parkin fails to rescue oscillatory activities in Parkinson's patient neurons



Dopamine Induces Oscillatory Activities in Human Midbrain Neurons with Parkin Mutations

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SUMMARY

Locomotor symptoms in Parkinson's disease (PD) are accompanied by widespread oscillatory neuronal activities in basal ganglia. Here, we show that activation of dopamine D1-class receptors elicits a large rhythmic bursting of spontaneous excitatory post-synaptic currents (sEPSCs) in midbrain neurons differentiated from induced pluripotent stem cells (iPSCs) of PD patients with parkin mutations, but not normal subjects. Overexpression of wild-type parkin, but not its PD-causing mutant, abolishes the oscillatory activities in patient neurons. Dopamine induces a delayed enhancement in the amplitude of spontaneous, but not miniature, EPSCs, thus increasing quantal content. The results suggest that presynaptic regulation of glutamatergic transmission by dopamine D1-class receptors is significantly potentiated by parkin mutations. The aberrant dopaminergic regulation of presynaptic glutamatergic transmission in patient-specific iPSC-derived midbrain neurons provides a mechanistic clue to PD pathophysiology, and it demonstrates the usefulness of this model system in understanding how mutations of parkin cause movement symptoms in Parkinson's disease.

INTRODUCTION

Parkinson's disease (PD) is a movement disorder characterized by the loss of nigral dopaminergic (DA) neurons. Its defining locomotor symptoms, tremor, rigidity, bradykinesia, and postural instability, are caused by the degeneration of nigral DA neurons and the ensuing dysfunction of basal ganglia motor circuits (Lang and Lozano, 1998). Glutamatergic inputs from motor cortex are processed by the basal ganglia motor circuits, whose output is relayed through thalamus to motor cortex to enable voluntary motor activities (Wichmann et al., 2011). DA input to all parts of basal ganglia, particularly to striatum, is essential to the processing of cortical glutamatergic inputs. In PD, dimin-

ished DA inputs to striatum due to the loss of nigrostriatal DA neurons disrupt the balanced actions of dopamine on striatal neurons (Obeso et al., 2010). Thus, understanding the impact of dopamine on glutamatergic neurotransmission would reveal significant insights into the mechanisms of PD.

The complexity of idiopathic PD makes it necessary to analyze how mutations of single genes cause PD. Among the PD-linked genes, parkin (Kitada et al., 1998), which encodes for a protein-ubiquitin ligase (Shimura et al., 2000), is most frequently mutated in recessively inherited PD (Hardy, 2010). Unlike LRRK2 mutations, which are dominant, more frequent, but have incomplete penetrance and a strong founder effect (Paisán-Ruiz et al., 2013), parkin mutations are fully penetrant and independently arisen in diverse genetic backgrounds (Nuytemans et al., 2010). The excellent human genetics data on parkin (Nuytemans et al., 2010) and the absence of robust PD phenotypes in parkin knockout mice (Perez and Palmiter, 2005) and rats (Dave et al., 2014) have led us to generate induced pluripotent stem cells (iPSCs) from PD patients with parkin mutations to study the pathogenic mechanism of PD caused by parkin mutations (Jiang et al., 2012). Using iPSC-derived midbrain DA neurons, we found that parkin mutations increased the spontaneous release of dopamine (Jiang et al., 2012). Consistent with this, many previous studies linked parkin to synaptic vesicles. Parkin monoubiquitinates synaptic vesicle proteins endophilin A, synaptotagmin 1, and dynamin (Trempe et al., 2009; Cao et al., 2014), as well as proteins involved in vesicle recycling, such as CASK (Fallon et al., 2002), PICK1 (Joch et al., 2007), and Eps15 (Fallon et al., 2006). It polyubiquitinates synaptic vesicle proteins CDCrel-1 (Zhang et al., 2000) and synaptotagmin XI (Huynh et al., 2003).

In the present study, we found that dopamine induced a delayed increase in the amplitude of spontaneous excitatory post-synaptic currents (sEPSCs) in iPSC-derived midbrain neurons from PD patients with parkin mutations, but not from normal subjects. It was accompanied by a significant increase in quantal content in response to dopamine, as miniature EPSCs were very similar between normal and patient neurons, irrespective of dopamine treatment. This modest action of dopamine became very striking with the concomitant inhibition of dopamine D2-class receptors or selective activation of dopamine D1-class receptors alone, either of which elicited a large rhythmic bursting of sEPSCs in neurons from parkin patients, but not from normal subjects. The phenotype was rescued by



overexpression of wild-type parkin, but not its PD-causing mutant. The rhythmic bursting of sEPSCs in parkin-deficient neurons is reminiscent of the oscillatory neuronal activities seen in basal ganglia of PD patients and animal models of PD (Galvan and Wichmann, 2008), but it differs from the *in vivo* observations in its frequency and how dopamine impacts on the oscillation (Brittain and Brown, 2014). Our results suggest that presynaptic glutamatergic transmission is strongly potentiated by D1-class dopamine receptors when parkin is mutated. The patient-specific iPSC-derived midbrain DA neurons provide a novel platform to study the molecular mechanism of the abnormal rhythmic bursting of neuronal activities in basal ganglia, which has long been associated with movement symptoms of PD.

RESULTS

Differentiation of Patient-Specific iPSCs to Midbrain Neurons

We differentiated iPSCs from three PD patients with different parkin mutations and three normal subjects (see Table S1 for information on the six subjects) to midbrain DA neurons using an improved rosette-based protocol (Jiang et al., 2012). These iPSCs (Figure 1A) were first differentiated to embryoid bodies (EBs) (Figure 1B), which were differentiated to neuroepithelial cells resembling a cross section of the neural tube (Figure 1C). Cells from the rosettes were cultured in suspension to form neurospheres, which were dissociated for further differentiation to neurons (Figure 1D). When we stained the iPSC-derived neurons for various markers, it was clear that the neuronal culture contained a mixed population of GABAergic neurons (35.6% ± 4.7% of DAPI⁺ cells) (Figures 1E–1G), glutamatergic neurons (33.8% ± 4.0% of DAPI⁺ cells) (Figures 1H–1J), and DA neurons (30.5% ± 2.5% of DAPI⁺ cells) (Figures 1E–1J). Almost all DAPI⁺ cells were neurons; 23.5% ± 5.8% of the neurons in the culture expressed D1 dopamine receptors (Figures 1K–1M), while 18.2% ± 5.1% expressed D2 dopamine receptors (Figures 1N–1P). Of TH⁺ neurons, 72.2% ± 8.5% coexpressed the midbrain marker FoxA2 (Figures 1Q–1S), corroborating with our previous finding that TH⁺ neurons generated with this differentiation protocol are mainly midbrain DA neurons (Jiang et al., 2012).

Dopamine Induces a Delayed Increase of sEPSC in Midbrain Neurons from Parkin Patients

We examined the effect of dopamine on sEPSCs in mature iPSC-derived neurons that were cultured for at least 100 days since the start of differentiation, as our previous study showed that electrophysiological properties of iPSC-derived neurons mature over time (Jiang et al., 2012). Neurons from different patients exhibited similar responses and were thus grouped together. The same was true for neurons from different normal subjects. The number of neurons recorded for each figure is listed in Table S2, which shows a generally even distribution of recorded neurons among different subjects. These small numbers mask the large number of neurons that we had to record in order to obtain a successful trace that lasted 20–30 min for us to fully document oscillatory activities. For each one neuron reported in Table S2,

we had to try about five coverslips, recording six to seven neurons on each coverslip. Thus, of ~30 neurons recorded, we obtained one recording that lasted 20–30 min. Since oscillatory neuronal activities in PD patients are widespread in many types of neurons, not limited to DA neurons (Wichmann and Dostrovsky, 2011), our electrophysiological recording was performed on all types of neurons in the culture. Variations in sEPSCs among individual neurons were reflected in error bars, which were quite small and suggest fairly uniform behaviors in different types of neurons. A saturating concentration (100 μM) of dopamine was used to fully activate all dopamine receptors, as described in previous papers (Otani et al., 1999; André et al., 2011; Shen and Johnson, 2012).

In iPSC-derived midbrain neurons from parkin patients, the application of dopamine induced an initial decrease (21.7% ± 1.8%; n = 7; p < 0.05, Kolmogorov-Smirnov [K-S] test) of sEPSC amplitude (Figure 2A, phase 1), which was followed by a significant increase (36.7% ± 2.4%; n = 7; p < 0.05, K-S test) (Figure 2A, phase 2). However, in iPSC-derived neurons from normal subjects, dopamine only caused a transient reduction (23.8% ± 1.5%; n = 6; p < 0.05, K-S test) of sEPSC amplitude in phase 1, without the delayed increase in phase 2 (5.8% ± 0.8%; n = 6; p > 0.05, K-S test; patient group and normal group comparison: Kruskal-Wallis [K-W] test: 8.9, p = 0.0027) (Figure 2A). For the sEPSC frequency, dopamine induced a delayed increase in iPSC-derived midbrain neurons in both patient and control groups only in phase 2 (parkin patients: 37.2% ± 2.7%; n = 7; p < 0.05, K-S test; normal subjects: 30.8% ± 4.7%; n = 6; p < 0.05, K-S, test; two groups comparison: p > 0.05, K-W test) (Figure 2B). Representative sEPSC recordings in neurons derived from PD patients with parkin mutations (Figure 2C) and normal subjects (Figure 2D) illustrate the delayed increase of sEPSC amplitude in phase 2 only in neurons from parkin patients.

The sEPSCs were recorded in the absence of GABA_A receptor antagonist. The addition of 6,7-dinitroquinoxaline-2,3-dione (DNQX) (50 μM), which blocks non-NMDA ionic glutamate receptors, abolished sEPSCs in neurons from parkin patients (Figure 2E) and normal subjects (Figure 2F). We also examined the effect of dopamine on miniature EPSCs (mEPSCs) by using tetrodotoxin (TTX) (1 μM) to block spontaneous neuronal activities. Subsequent treatment of dopamine (100 μM) had no significant effect on mEPSCs in neurons from parkin patients (Figure 2G) or normal subjects (Figure 2H). There was no significant change in the amplitude (Figure 2I) and frequency (Figure 2J) of mEPSCs. These results suggest that the effects of dopamine on sEPSCs are mediated by a presynaptic mechanism, not through a postsynaptic mechanism, which would affect mEPSCs. Quantal content, which represents the number of vesicles released in response to a presynaptic stimulus and can be calculated by dividing the sEPSC amplitude by the mEPSC amplitude, was significantly increased by dopamine in iPSC-derived neurons from parkin patients (1.69 ± 0.05, n = 5) compared to normal subjects (1.17 ± 0.04, n = 6) (p < 0.001, Student's t test). There was no significant difference between patient neurons (1.12 ± 0.02, n = 5) and control neurons (1.13 ± 0.03, n = 6) in quantal content in the absence of dopamine treatment (p > 0.05, Student's t test) (Figure 2K). When TTX was added to block sEPSCs (Figures S1A and S1B), there was no

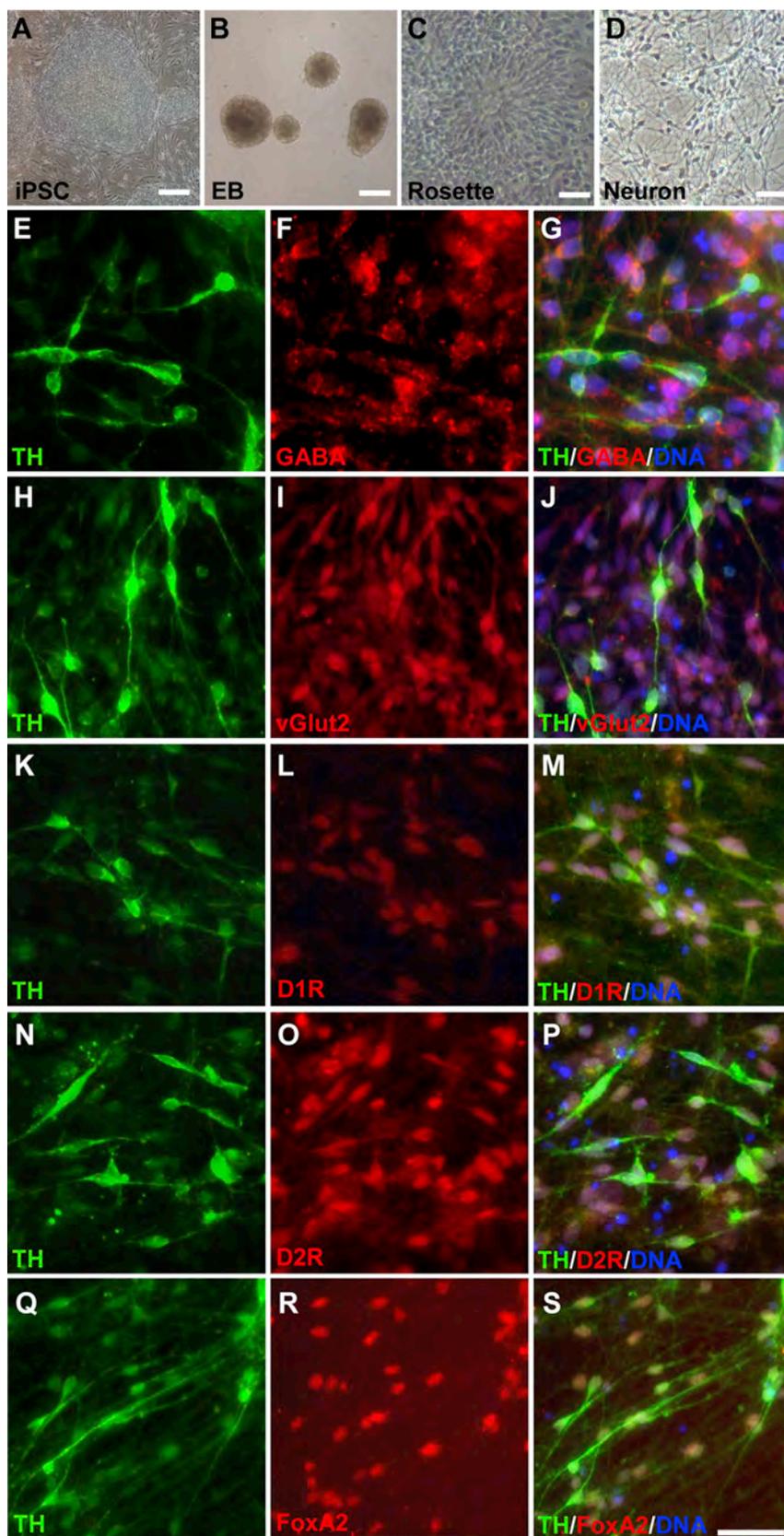
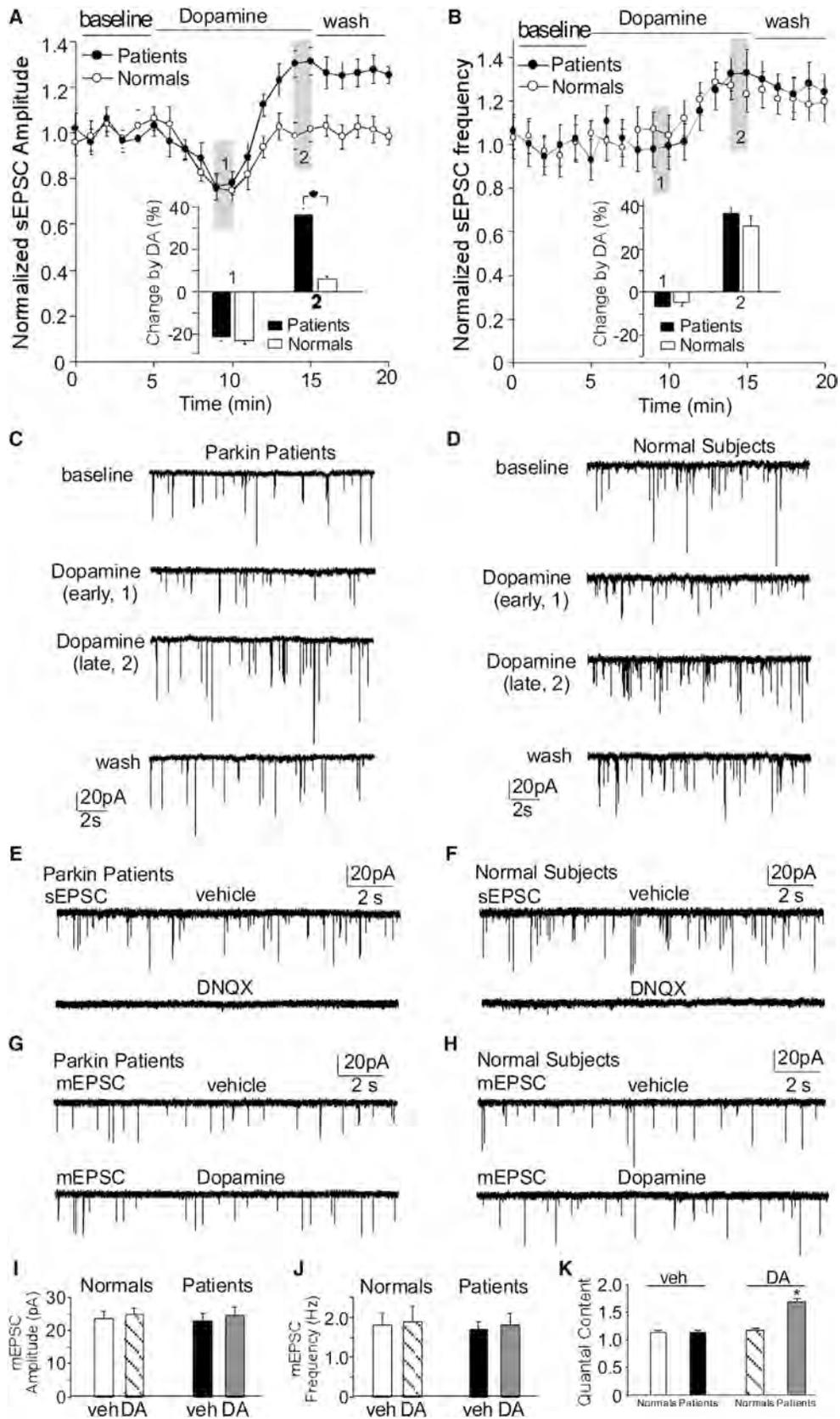


Figure 1. Differentiation of Patient-Specific iPSCs to a Mixed Population of Midbrain Neurons

(A–D) Phase contrast images of iPSCs (A) being differentiated to embryoid bodies (B), neuroepithelial cells (C), and neurons (D). (E–S) Costaining of iPSC-derived neurons for the DA marker tyrosine hydroxylase (TH) (E, H, K, N, and Q), the GABAergic neuronal marker GABA (F), the glutamatergic neuronal marker vGlut2 (I), dopamine D1 receptor (D1R) (L), dopamine D2 receptor (D2R) (O), the midbrain marker FoxA2 (R), and DNA for merged images as indicated (G, J, M, P, and S). Scale bars, 100 μ m.



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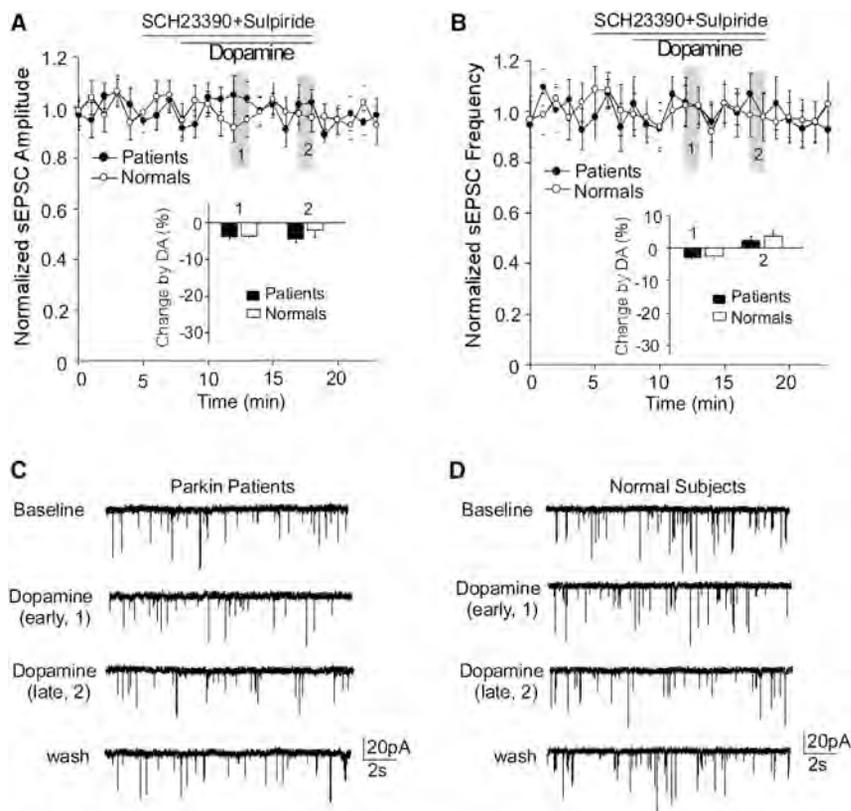


Figure 3. All the Effects of Dopamine on sEPSCs Are Blocked by Co-application of D1-Class and D2-Class Antagonists

(A and B) Normalized plots of sEPSC amplitude (A) and frequency (B), showing the effect of dopamine (100 μ M) in the presence of SCH23390 (10 μ M) and sulpiride (20 μ M) in iPSC-derived neurons from parkin patients versus normal subjects. Inset: bar graph (mean \pm SEM) summary shows the percentage change by dopamine in the presence of SCH23390 and sulpiride in two groups of neurons. (C and D) Representative traces of sEPSCs at different time points (indicated by the numbers in A and B plots) in iPSC-derived neurons from a parkin patient (C) versus a normal subject (D).

SCH23390 (10 μ M) and D2-class antagonist sulpiride (20 μ M). These antagonists completely blocked all the effects of dopamine on sEPSC amplitude (Figure 3A) and frequency (Figure 3B) in iPSC-derived neurons from both groups (parkin patients, $n = 5$; normal subjects, $n = 5$). Representative sEPSC traces for neurons derived from PD patients with parkin mutations (Figure 3C) and normal subjects (Figure 3D) are shown.

Next, we performed experiments to determine which dopamine receptors are

responsible for the differential effects of dopamine on sEPSCs in neurons from parkin patients and normal subjects. As shown in Figure 4A, when D1-class receptors were blocked by SCH23390 (10 μ M), the initial reducing effect of dopamine (100 μ M) on sEPSC amplitude was intact in iPSC-derived neurons from both groups (parkin patients: $24.5\% \pm 1.6\%$; $n = 6$; $p < 0.05$, K-S test; normal subjects: $23.6\% \pm 1.9\%$; $n = 6$; $p < 0.05$, K-S test; two groups comparison: $p > 0.05$, K-W test), suggesting that the reducing effect is not mediated by D1-class dopamine receptors. In the presence of SCH23390, the delayed enhancing effect of dopamine on sEPSC amplitude in parkin patients was eliminated ($-3.1\% \pm 1.1\%$; $n = 6$; $p > 0.05$, K-S test), suggesting that the enhancing effect is mediated by D1-class dopamine receptors. As shown in Figure 4B, the dopamine-induced increase in the sEPSC frequency in both groups was blocked by SCH23390 (parkin patients: $-4.3\% \pm 0.6\%$, $n = 6$; normal subjects: $-3.5\% \pm 1.1\%$, $n = 6$; $p > 0.05$, K-S test), suggesting mediation by

The Delayed Increase of sEPSC Amplitude in Parkin Patients Depends on Dopamine D1 Receptors

To confirm that the effects are dependent on dopamine receptors, we co-applied dopamine (100 μ M) with D1-class antagonist

Figure 2. The Effects of Dopamine on sEPSCs in iPSC-Derived Neurons from Normal Subjects and PD Patients with Parkin Mutations

(A and B) Normalized plots of sEPSC amplitude (A) and frequency (B), showing the effect of dopamine (100 μ M) in iPSC-derived neurons from parkin patients versus normal subjects. Inset: bar graph (mean \pm SEM) summary shows the percentage change by dopamine in two groups of neurons ($*p < 0.05$ versus normals). (C and D) Representative traces of sEPSCs at different time points (indicated by the numbers in A and B plots) in iPSC-derived neurons from a parkin patient (C) versus a normal subject (D).

(E and F) Blocking non-NMDA ionic glutamate receptors with DNQX (50 μ M) abolished sEPSCs in neurons from parkin patients (E) and normal subjects (F).

(G–J) Miniature EPSCs (mEPSCs) in patient (G) or normal (H) neurons treated with vehicle or dopamine (100 μ M) had similar amplitudes (I) and frequencies (J).

(K) Quantal content of sEPSCs as measured by dividing the sEPSC amplitude by mEPSC amplitude in neurons from normal subjects and parkin patients treated with vehicle or dopamine (100 μ M) ($*p < 0.001$ versus normals).

See also Figure S1.

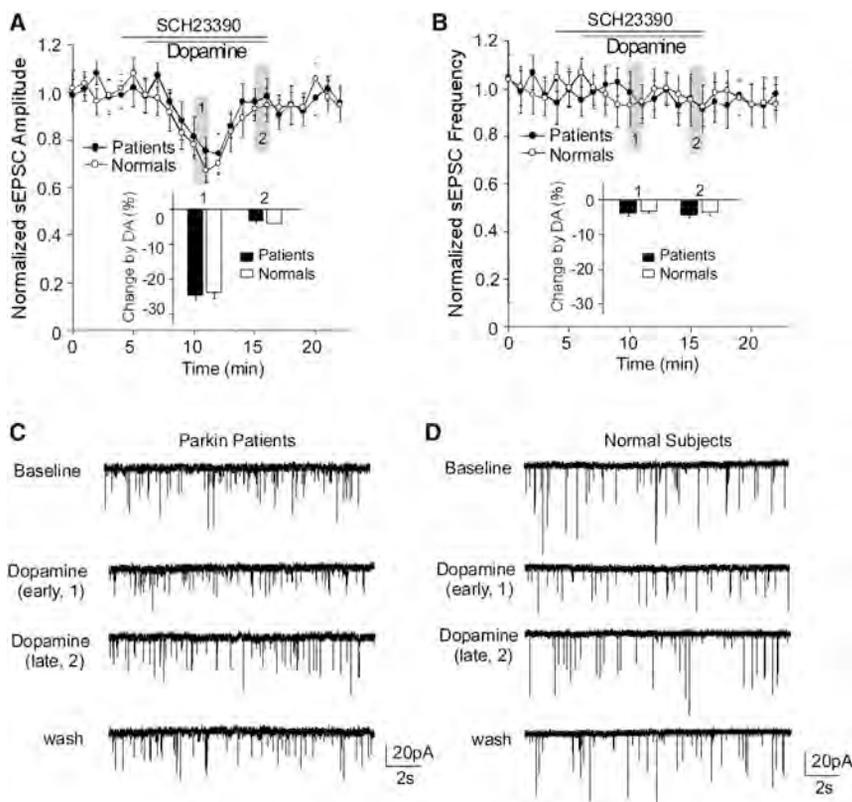


Figure 4. The Effect of Dopamine in the Presence of a D1-Class Antagonist

(A and B) Normalized plots of sEPSC amplitude (A) and frequency (B), showing the effect of dopamine (100 μ M) in the presence of the D1-class antagonist SCH23390 (10 μ M) in iPSC-derived neurons from parkin patients versus normal subjects. Inset: bar graph (mean \pm SEM) summary shows the percentage change by dopamine in the presence of SCH23390 in two groups of neurons. (C and D) Representative traces of sEPSCs at different time points (indicated by the numbers in A and B plots) in iPSC-derived neurons from a parkin patient (C) versus a normal subject (D).

D1-class receptors. Representative sEPSC traces for neurons derived from PD patients with parkin mutations (Figure 4C) and normal subjects (Figure 4D) show that the differential effect of dopamine on the delayed increase of sEPSC amplitude in neurons from parkin patients versus normal subjects was dependent on D1-class dopamine receptors.

Activation of D1 Receptors Induces Oscillatory sEPSC in Midbrain Neurons from Parkin Patients

Since D1-class and D2-class dopamine receptors often exert opposing effects (Gerfen and Surmeier, 2011), we blocked D2-class receptors with sulpiride (20 μ M) to isolate the action of D1-class receptors (Figure 5). Under this condition, dopamine (100 μ M) no longer induced the initial reduction of sEPSC amplitude. Surprisingly, it induced a remarkable rhythmic bursting of sEPSCs in iPSC-derived neurons from parkin patients. The bursting oscillation frequency was 1.1 ± 0.1 events/min ($n = 8$), and each burst lasted for 20.5 ± 1.9 s ($n = 8$). During the bursting, sEPSC amplitude was increased by $36.2\% \pm 2.6\%$ ($n = 8$; $p < 0.05$, K-S test) (Figures 5A and 5C) and sEPSC frequency was increased by $1,703\% \pm 103\%$ ($n = 8$; $p < 0.005$, K-S test) (Figures 5B and 5C). Between bursting, sEPSC amplitude was not significantly changed (Figures 5A and 5C), while sEPSC frequency was increased by $168.6\% \pm 9.8\%$ ($n = 8$; $p < 0.05$, K-S test) (Figures 5B and 5C). In iPSC-derived neurons from normal subjects, dopamine plus sulpiride did not induce rhythmic bursting (Figures 5A, 5B, and 5D) but only a modest increase in sEPSC frequency ($35.3\% \pm 2.3\%$; $n = 6$; $p < 0.05$, K-S test)

(Figures 5B and 5D) and no significant increase in sEPSC amplitude ($3.2\% \pm 0.5\%$; $n = 6$; $p > 0.05$, K-S test) (Figures 5A and 5D).

To confirm that D1-class dopamine receptors mediated the rhythmic bursting of sEPSCs in iPSC-derived neurons from parkin patients, we applied the D1-class agonist SKF81297 (20 μ M). As shown in Figure 6, application of SKF81297 also induced rhythmic bursting of sEPSCs in neurons from PD patients with parkin mutations. The rhythmic bursting frequency was 0.8 ± 0.1 events/min ($n = 6$), and each burst lasted 17.2 ± 1.7 s ($n = 6$)

(Figures 6B and 6C). During bursting, sEPSC amplitude was increased by $33.2\% \pm 2.4\%$ ($n = 6$; $p < 0.05$, K-S test) (Figures 6A and 6C), and sEPSC frequency was increased by $1,442\% \pm 137\%$ ($n = 6$; $p < 0.005$, K-S test) (Figures 6B and 6C). Between the bursting, sEPSC amplitude was not significantly changed (Figures 6A and 6C), and sEPSC frequency was increased by $147.3\% \pm 9.2\%$ ($n = 6$; $p < 0.05$, K-S test) (Figures 6B and 6C). In iPSC-derived neurons from normal subjects, SKF81297 did not induce rhythmic bursting (Figure 6D); it only increased sEPSC frequency modestly ($31.8\% \pm 4.6\%$; $n = 5$; $p < 0.05$, K-S test) (Figures 6B and 6D), and it did not have any significant effect on sEPSC amplitude ($5.1\% \pm 0.8\%$; $n = 5$; $p > 0.05$, K-S test) (Figures 6A and 6D).

Oscillatory sEPSCs in Neurons from Parkin Patients Are Rescued by Overexpression of Parkin

To demonstrate that the oscillatory sEPSCs were caused by parkin mutations, we infected P002 neurons with lentiviruses expressing wild-type parkin (Jiang et al., 2012). Activation of dopamine D1-class receptors by dopamine (100 μ M) and sulpiride (20 μ M) produced no rhythmic bursting of sEPSCs ($n = 10$) (Figures 7A–7C). There was no significant increase in sEPSC amplitude ($6.8\% \pm 0.4\%$; $n = 10$; $p > 0.05$, K-S test) (Figure 7A), but there was a significant increase in sEPSC frequency ($122.6\% \pm 11.7\%$; $n = 10$; $p < 0.05$, K-S test) (Figure 7B). In contrast, P002 neurons transduced with lentivirus expressing the PD-causing T240R mutant parkin (Jiang et al., 2012) exhibited strong rhythmic bursting of sEPSCs (Figures 7A, 7B,

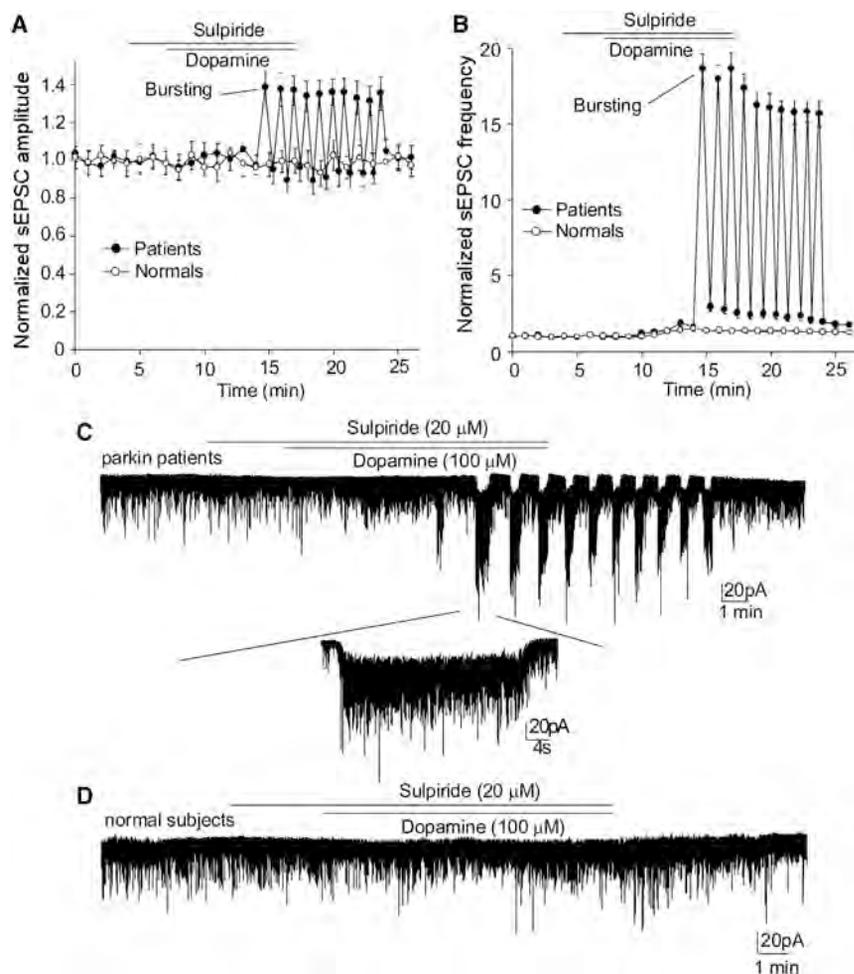


Figure 5. When D2-Class Receptors Are Blocked, Dopamine Induces Rhythmic Bursting of sEPSCs in iPSC-Derived Neurons from Parkin Patients

(A and B) Normalized plots of sEPSC amplitude (A) and frequency (B), showing the effect of dopamine (100 μ M) in the presence of the D2-class antagonist sulpiride (20 μ M) on iPSC-derived neurons from parkin patients versus normal subjects.

(C and D) Representative traces of sEPSCs in iPSC-derived neurons from a parkin patient (C) versus a normal subject (D). Inset in (C) shows the expanded view of sEPSCs during bursting.

DISCUSSION

PD is clinically defined by a core set of movement symptoms that are caused by the dysfunctional basal ganglia neural network consequent of a severe loss of nigral DA neurons. It remains unclear why diminished DA input to striatum leads to movement abnormalities in PD. The discovery of iPSCs (Takahashi and Yamanaka, 2006) makes it possible to generate patient-specific midbrain neurons to study PD (Pu et al., 2012). Our previous study using iPSC-derived midbrain DA neurons from normal subjects and PD patients with parkin mutations has shown that parkin controls the precision of DA transmission by limiting spontaneous DA release and enhancing DA reuptake (Jiang et al., 2012). In the present study, we used the same set of patient-

derived midbrain neurons to study whether the regulation of glutamatergic transmission by dopamine is affected in PD, as neurons in the basal ganglia network receive glutamatergic input from cortex and subthalamic nucleus (STN), as well as DA input from substantia nigra pars compacta (Albin et al., 1989). The balanced modulatory actions of dopamine on glutamatergic inputs on medium spiny neurons (MSNs) in the striatum, through D1-class and D2-class receptors that are expressed on different subsets of MSNs, are disrupted in PD (DeLong, 1990; Gerfen and Surmeier, 2011). Thus, understanding whether PD-linked monogenic mutations, such as those of parkin, affect DA regulation of glutamatergic transmission may reveal the molecular underpinning of PD.

The most significant result of our study is that activation of D1-class dopamine receptors, either by co-application of dopamine with the D2-class receptor antagonist sulpiride (Figure 5) or application of the D1-class receptor agonist SKF81297 alone (Figure 6), induced large rhythmic bursting of sEPSCs in iPSC-derived midbrain neurons from PD patients with parkin mutations, but not from normal subjects. The rhythmic bursting of sEPSCs is reminiscent of oscillatory activities in basal ganglia neurons in PD patients (Wichmann and Dostrovsky, 2011). Recording of local field potentials in PD patients undergoing

and 7D). Oscillation frequency for the bursts was 0.81 ± 0.17 events/min ($n = 8$), and bursts lasted on average for 17.9 ± 1.3 s ($n = 8$). During bursting, sEPSC amplitude was increased by $25.7\% \pm 2.1\%$ ($n = 8$; $p < 0.05$, K-S test) (Figure 7A), and sEPSC frequency was increased by $1,338\% \pm 78\%$ ($n = 8$; $p < 0.005$, K-S test) (Figure 7B). Between bursts, sEPSC amplitude was not significantly increased ($10.9\% \pm 0.7\%$; $n = 8$; $p > 0.05$, K-S test) (Figure 7A), and sEPSC frequency was increased by $95.6\% \pm 6.1\%$ ($n = 8$; $p < 0.05$, K-S test) (Figure 7B). Similarly, P002 neurons transduced with GFP lentivirus (Jiang et al., 2012) also showed robust rhythmic bursting of sEPSCs (Figures 7A, 7B, and 7E). Bursting oscillation frequency was 0.68 ± 0.14 events/min ($n = 6$), and the average duration was 15.1 ± 1.2 s ($n = 6$). During bursting, sEPSC amplitude was increased by $28.3\% \pm 2.4\%$ ($n = 6$; $p < 0.05$, K-S test) (Figure 7A), and sEPSC frequency was increased by $1,402\% \pm 93\%$ ($n = 6$; $p < 0.005$, K-S test) (Figure 7B). Between bursts, sEPSC amplitude was not significantly increased ($6.1\% \pm 0.4\%$; $n = 6$; $p > 0.05$, K-S test) (Figure 7A), and sEPSC frequency was increased by $87.8\% \pm 6.8\%$ ($n = 6$; $p < 0.05$, K-S test) (Figure 7B). Thus, wild-type parkin, but not its PD-linked T240R mutant or an irrelevant protein (GFP), rescued the oscillatory neuronal activities caused by parkin mutations.

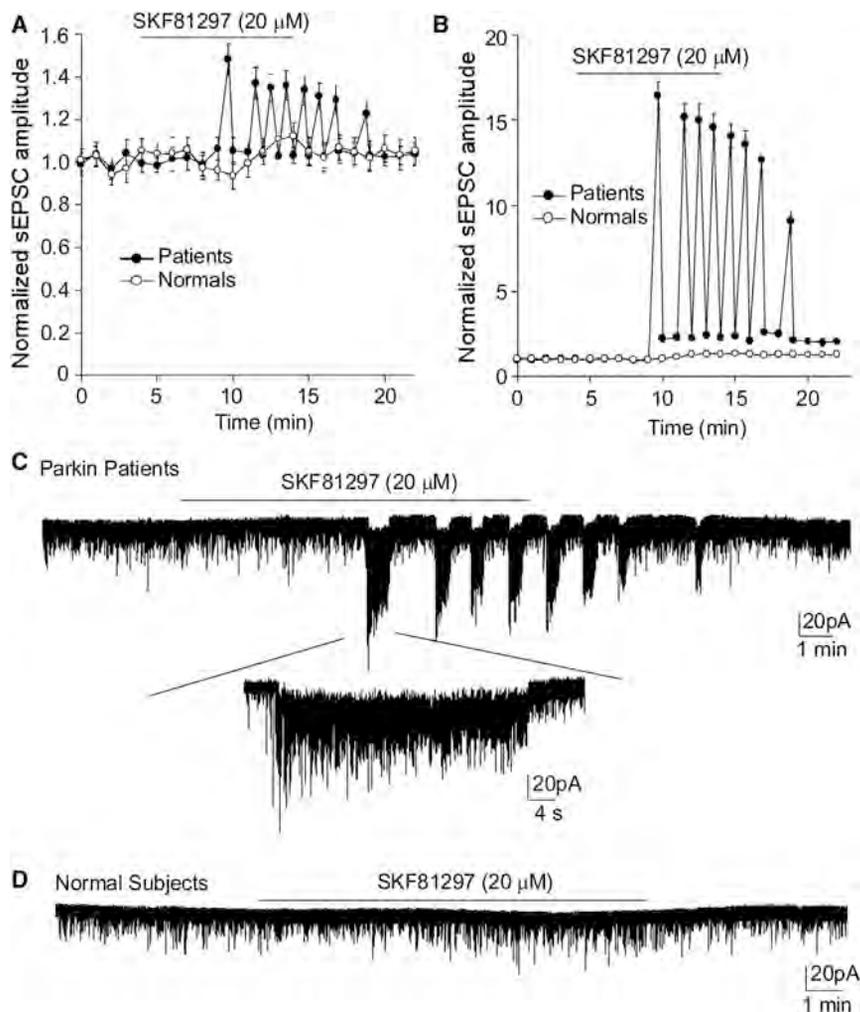


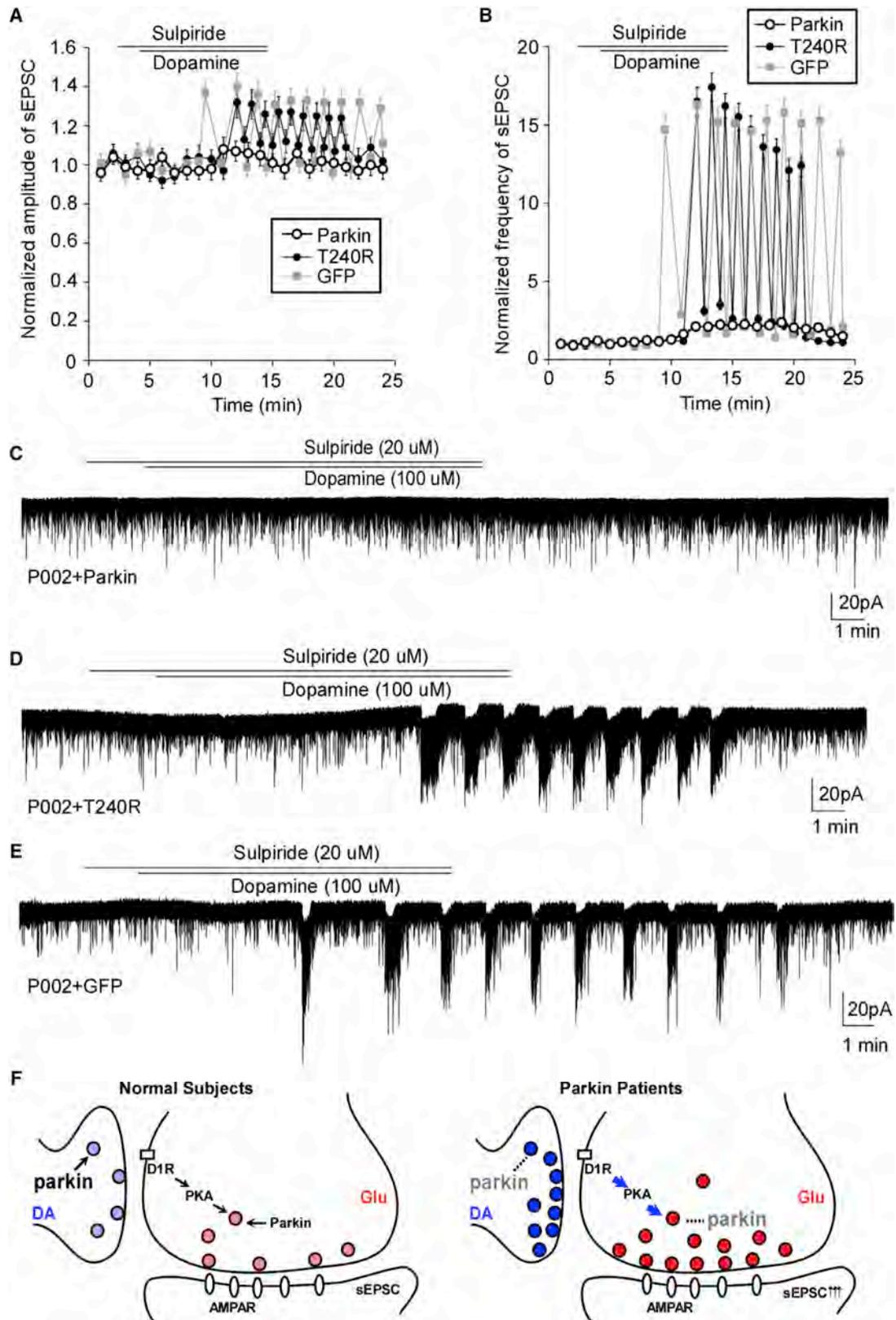
Figure 6. Application of D1-Class Agonist Induces Rhythmic Bursting of sEPSCs in iPSC-Derived Neurons from Parkin Patients (A and B) Normalized plots of sEPSC amplitude (A) and frequency (B), showing the effect of the D1-class agonist SKF81297 (20 μ M) on iPSC-derived neurons from parkin patients versus normal subjects. (C and D) Representative traces of sEPSCs from iPSC-derived neurons from a parkin patient (C) versus a normal subject (D). Inset in (C) shows the expanded view of sEPSCs during bursting.

Consistent with the observations in PD patients, oscillatory activities were found to be similar in different types of neurons in iPSC-derived midbrain neuronal cultures, which contained glutamatergic, GABAergic, and DA neurons (Figure 1). There are, however, important differences between the oscillatory sEPSCs in iPSC-derived neurons and the rhythmic bursting activities in PD patients and animal PD models, which have much higher frequencies and are attenuated by dopamine (Brittain and Brown, 2014). A variety of factors may underlie the discrepancy, including the lack of complex synaptic organizations in neurons differentiated from iPSCs, maturity of the iPSC-derived neurons (around 100 days), lack of inputs from neurons representing other parts of the brain, etc. Nevertheless, the random mixture of midbrain neurons in the dish produced oscillatory sEPSCs when parkin was mutated and when D1-class re-

ceptors were activated. The results suggest an intrinsic problem in parkin-deficient neurons, as manifested in increased quantal content in response to dopamine treatment (Figure 2K). The differences between our observation in this artificial system in vitro and the in vivo situation in PD patients will stimulate further research utilizing an iPSC-based system that can better approximate patient brains, such as organoids (Lancaster et al., 2013). The strong response of parkin-deficient neurons to the activation of D1-class dopamine receptors (Figures 5 and 6) is reminiscent of the dopamine hypersensitivity in PD animal models (Fuxe and Ungerstedt, 1976; Kim et al., 2000). The simultaneous activation of both D1- and D2-class receptors by dopamine only had modest effects on sEPSCs in iPSC-derived midbrain neurons from parkin patients (Figures 2, 3, and 4), suggesting that the opposing effect of D2-class receptors may have masked the strong effect of D1-class receptors. Interestingly, a delayed enhancement of sEPSC amplitude, which was mediated by D1-class receptors, was only observed in parkin patients, but not in normal subjects (Figure 2). This is consistent with the results that only neurons derived from parkin patients exhibited D1-induced oscillation of sEPSCs (Figures 5 and 6). The results

surgeries, such as deep brain stimulation, shows that many parts of basal ganglia, such as striatum, globus pallidus internal segment (GPi), and STN, exhibit oscillatory activities (Wichmann and Dostrovsky, 2011). L-DOPA administration disrupts the oscillatory activities in PD patients (Brown and Williams, 2005). Oscillation of neuronal activities is also seen in STN, globus pallidus external segment (GPe), and GPi of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkey models of PD (Wichmann et al., 2011). In contrast, activities of STN and GPe in normal humans and monkeys do not have any rhythm or obvious pattern (Wilson and Bevan, 2011). Although it is unclear why basal ganglia neurons in the parkinsonian state have synchronized, rhythmic bursting activities, it is remarkable that iPSC-derived midbrain neurons from PD patients with parkin mutations exhibit oscillatory sEPSCs in vitro. The absence of oscillatory activities in iPSC-derived midbrain neurons from normal subjects corroborates that the rhythmic bursting of sEPSCs is associated with PD. The ability of wild-type parkin, but not its PD-linked T240R mutant or GFP, to rescue the oscillatory activities (Figure 7) indicates that parkin mutations indeed cause the oscillation.

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(legend on next page)

suggest that the actions of dopamine on D1-class receptors are markedly altered in PD patients with parkin mutations. Since the initial inhibitory effect of dopamine on sEPSC amplitude (Figure 2A), which was mediated by D2-class receptors (Figure 4A), was similar in iPSC-derived neurons from normal subjects and parkin patients, it suggests that the impact of dopamine on D2 receptors appears to be largely unaffected by parkin mutations. It is widely recognized that an overactive indirect pathway emanated from striatal MSNs expressing D2-class receptors may underlie PD motor symptoms (DeLong, 1990) and the oscillatory activities in vivo (Brittain and Brown, 2014). Further studies are needed to understand why mutations of parkin render human midbrain neurons much more sensitive to dopamine D1 receptor activation and why the increased sensitivity manifests in rhythmic bursting of sEPSCs.

Here we propose a working model (Figure 7F) based on the current study, our earlier finding on increased spontaneous dopamine release in DA neurons from PD patients with parkin mutations (Jiang et al., 2012), and previous studies linking parkin to synaptic vesicles (Trempe et al., 2009; Cao et al., 2014; Huynh et al., 2003; Zhang et al., 2000). In normal neurons, parkin may regulate the function of synaptic vesicles through proteins, such as endophilin A, synaptojanin 1, and dynamin (Trempe et al., 2009; Cao et al., 2014). The lack of this regulation in patient neurons elevates spontaneous DA release (Jiang et al., 2012). The action of dopamine is balanced by D1-class and D2-class receptors. When D2-class receptors were inhibited (Figure 5) or when only D1-class receptors were activated (Figure 6), protein kinase A (PKA) in presynaptic glutamatergic neurons was strongly activated to speed up the recycling of synaptic vesicles (Greengard, 2001). This may synergize with the concomitant increase in the quantal content of sEPSCs (Figure 2K) and cause huge increases in sEPSCs, which are periodically attenuated by temporary exhaustion of recycling vesicles. Thus, we observed oscillation of sEPSCs in parkin-deficient neurons when D1-class receptors were activated.

Our results have demonstrated the utility of patient-specific iPSC-derived neurons in a mechanistic study of PD. This preparation exhibits oscillatory neuronal activities resembling those in PD patients in vivo as well as in animal models of parkinsonism. Since the iPSC-based cell model system captures the intrinsic properties of neurons from PD patients, it would be a useful platform to dissect the molecular mechanism underlying the pathophysiology of PD. Understanding the differences between iPSC-derived neurons from normal subjects and PD patients will reveal important targets for the development of disease-modifying therapies.

EXPERIMENTAL PROCEDURES

Human Subjects

Written informed consent was received from participants prior to inclusion in the study. The study was approved by the Health Sciences Institutional Review

Board of the University at Buffalo, the State University of New York. Two normal subjects (C001 and C002) and two PD patients with parkin mutations (P001, who has compound heterozygous deletions of exon 3 and exon 5 of parkin, and P002, who carries homozygous deletion of exon 3) were described previously (Jiang et al., 2012). Another normal subject C003 and PD patient with parkin mutations (P005) were added to the study. Skin fibroblasts from P005 were purchased from Coriell Cell Repository (ND30171, with parkin exon 3 deletion and R42P mutation). Details of the subjects are listed in Table S1. All normal subjects were the unaffected spouses of idiopathic PD patients. The different age profiles of the control and patient groups had no significant effect on the derivation of iPSCs. There was no evidence that donor age affected any phenotype of iPSC-derived cells.

Generation of iPSC Lines

Skin fibroblasts from normal subjects or PD patients with parkin mutations were reprogrammed to iPSCs according to our previous publication (Jiang et al., 2012). Briefly, 1×10^5 human skin fibroblasts were infected with human Oct4, Sox4, Klf4, c-Myc, Nanog, and M2rTA lentiviruses in the presence of 4 μ g/mL polybrene for 1 day. The infected fibroblasts were plated on mitomycin C-treated mouse embryonic fibroblast (MEF) feeder cells in DMEM containing 10% fetal bovine serum (FBS), 2 mM L-Glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin for another day. Then the media were changed to human embryonic stem cell (hESC) media (DMEM/F12 supplemented with 20% knockout serum replacement, 2 mM L-Glutamine, 0.1 mM nonessential amino acids [NEAAs], 0.1 mM β -ME, penicillin/streptomycin, and 4 ng/mL basic fibroblast growth factor [bFGF]) supplemented with 1 μ g/mL doxycycline and 0.5 mM valproic acid (VPA) (for 7 days). The hESC-like clones normally appeared between day 24 and 40. Then the clones with good morphology were picked manually, expanded on MEF in hESC media, and passaged every 5–7 days by dispase (1 mg/mL) treatment.

Differentiation of iPSCs to Midbrain Neurons

Differentiation of iPSCs was performed according to our previous paper (Jiang et al., 2012). Briefly, iPSCs were treated with 1 mg/mL dispase to detach them from MEF feeder cells. The iPSC clumps were grown as EBs in hESC medium with 10 μ M SB431542 for 4 days. At day 5, EBs were transferred to neural induction media (DMEM/F12 with 1×10^5 N2 supplements, 0.1 mM NEAA, and 2 μ g/mL heparin) containing 20 ng/mL bFGF for 2 more days. Then the EBs were plated on Matrigel-coated six-well plates in neural induction media for 1 week. When some elongated, columnar cells appeared in the center of the differentiated colonies, the culture media were further supplied with FGF8a (20 ng/mL) and sonic hedgehog (SHH) (100 ng/mL) for 1 more week. Then these rosettes were isolated manually and cultured in neural induction media with FGF8a (50 ng/mL), SHH (100 ng/mL), B27 supplements (1 \times), and ascorbic acid (200 μ M) in suspension for 6 days to form neurospheres. The neurospheres were digested with accutase/trypsin (1:1) into single cells. The cells (1×10^5 cells/mL) were plated on polyornithine/laminin/Matrigel-coated coverslips in neural differentiation medium (Neurobasal medium with 1×10^5 N2 supplements, 1×10^5 B27 supplements, and 0.1 mM NEAA) containing FGF8a (50 ng/mL), SHH (100 ng/mL), ascorbic acid (200 μ M), cyclic AMP (cAMP, 1 μ M), laminin (1 μ g/mL), TGF- β 3 (1 ng/mL), brain-derived neurotrophic factor (BDNF) (10 ng/mL), and glial cell line-derived neurotrophic factor (GDNF, 10 ng/mL). Media were half changed every other day. Neuronal cultures were maintained for at least 100 days to obtain mature neurons for electrophysiological recording.

Electrophysiology

Recordings were performed on iPSC-derived neurons (after at least 100 days since the start of differentiation) from three parkin patients and three normal subjects. Standard patch-clamp techniques were used in the sEPSC

Figure 7. Rhythmic Bursting of sEPSCs in Parkin-Deficient Neurons Was Rescued by Wild-Type, but Not Mutant, Parkin

(A and B) Normalized sEPSC amplitude (A) and frequency (B) for P002 neurons transduced with lentiviruses expressing parkin, T240R mutant parkin, or GFP. (C–E) Representative traces of sEPSCs from P002 neurons transduced with lentiviruses expressing parkin (C), T240R mutant parkin (D), or GFP (E). (F) A model for oscillatory activities in neurons from parkin patients in response to D1 receptor activation. Details are in the Discussion.

measurement (Jiang et al., 2015). The iPSC-derived neurons on coverslips were put in a recording chamber, and they were perfused with artificial cerebrospinal fluid (ACSF) (in mM: 130 NaCl, 26 NaHCO₃, 3 KCl, 1 CaCl₂, 5 MgCl₂, 1.25 NaH₂PO₄, 10 glucose [pH 7.4], and 300 mOsm) bubbled with 95% O₂ and 5% CO₂. Neurons were visualized with a 40× water-immersion lens and illuminated with near infrared light. The internal solution contained (in mM): 124 K-gluconate, 1 MgCl₂, 10 KCl, 0.5 CaCl₂, 1 EGTA, 10 HEPES, 3 Na₂ATP, 0.5 Na₂GTP, 12 phosphocreatine (pH 7.2–7.3), and 280 mOsm. Tight seals (2–5 GΩ) were first generated by negative pressure, followed by additional suction to obtain the whole-cell configuration. Neurons were held at –70 mV and recorded in ACSF without GABA_AR antagonist. To record mEPSCs, TTX (1 μM) was added in external solution (ACSF) for at least 10 min to block spontaneous neuronal activities. Dopamine (100 μM) was then added to examine its effect on mEPSCs in different types of neurons. Quantal content of sEPSCs was calculated by dividing the sEPSC amplitude by the mEPSC amplitude. All recordings were performed using a Multiclamp 700A amplifier. Data analysis was performed with Clampfit (Axon Instruments), Mini Analysis Program (Synaptosoft), and Kaleidagraph (Albeck Software).

Statistical Analysis

K-S tests (tailed, unpaired) were used to determine the significance of effects on sEPSC. K-W tests (non-parametric tests) were used for comparison between the patients and the normal groups.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.04.023>.

AUTHOR CONTRIBUTIONS

J.F. and Z.Y. designed research, analyzed data, and wrote the paper. P.Z., Z.H., and H.J. conducted the experiments and analyzed the data.

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