Novel Functional Phenotypes In Induced Pluripotent Stem Cell-derived Neurons From Patients With LRRK2 G2019S Mutation

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Abstract

Virtually all experimental models aimed at mimicking Parkinson's disease (PD) are limited in their ability to faithfully replicate disease phenotypes. A great hope relies on new patient-specific cell culture systems, in which PD-specific phenotypes can be studied and also successfully applied for high-throughput drug discovery platforms. Here, we show that induced pluripotent stem cell (iPSC) derived neurons from patients carrying the most common PD mutation in the leucine-rich repeat kinase 2 (LRRK2) gene, p.G2019S, can be characterized morphologically by using high-content imaging (HCI) system and electrophysiologically by using automated patch clamp system. iPSCs were derived from patient skin fibroblasts using 4-factor retroviral approach. All iPSC lines were characterized for pluripotency, differentiation potential, and were karyotypically normal. Then, we differentiated iPSCs into neural progenitor cells (NPCs) using our published embryoid body approach and dual SMA inhibition. For final neuronal maturation, we differentiated NPCs into dopaminergic (DA) neurons using smoothened agonist (SAG) and fibroblast growth factor 8 (FGF8) for the first 10 days and then further differentiated cells in brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF) and cyclic adenosine monophosphate (dcAMP) for another 25 days. For HCI studies, we examined differentiated cells with microtubule-associated protein 2 and tyrosine hydroxylase to identify the neurons of interest, DA neurons. By utilizing automated imaging and high-content image analysis software, multiple parameters such as the numbers, length and branching of neurites were quantitated and compared between the patient derived neurons and the controls. For the electrophysiological studies, we analyzed the functional profiles of endogenously expressing ion channels, such as voltage-activated inward and outward currents in developing and mature neurons, to evaluate the functional similarity of these cells to primary neurons. By utilizing HCI and electrophysiology, we can gain more insight into neuronal differences in morphology and excitability in the LRRK2 G2019S patient derived neurons compared to the neurons derived from mutation-negative lines. This is crucial towards a better understanding of the LRRK2 function in these novel human in vitro models.

Introduction

- Patient-specific iPSC-derived differentiated cells have become an attractive tool to study disease mechanisms on a human background and are a vanguard into a new era of science and potentially personalized medicine.
- The goal is that patient-derived cell lines are "authentic" in their cellular response compared to any cell culture or animal model to date.
- Autosomal-dominant mutations in leucine rich repeat kinase-2 (LRRK2) were described in familial parkinsonism that mimics the clinical and pathological features of sporadic PD (Paisan-Ruiz et al., 2004; Zimprich et al., 2004).
- Neurons express disease-associated mutant forms of LRRK2 displayed reduced neurite process length and complexity (MacLeod et al, 2006; Winner et al, 2012).
- In this study, we used high content imaging system (HCI) and electrophysiology to demonstrate phenotypic differences between LRRK2 G2019S mutated iPSC derived neurons and wild type controls.
- Using HCI and electrophysiology are the tools to understand neuronal differences in morphology and excitability in the LRRK2 G2019S patient derived neurons compared to those derived from the control line.
- This is very important information to further investigate the function of the LRRK2 in these novel human in vitro models.

Materials & Methods

Generation and maintenance of Neural Progenitor Cells (NPCs) from patient specific iPSCs: LRRK2 mutant and control NPCs were derived from iPSCs following our previously published protocol (Mak et al, 2012).

Differentiation of NPCs into dopaminergic neurons: Dopaminergic differentiation was initiated by culturing NPCs on Geltrex™ coated 24 well culture dishes (BD Bioscience) coated with Poly-L-ornithine (20 µg/ml) (Sigma) and laminin (Sigma) (20 µg/ml). Dopaminergic differentiation in defined media was initiated by culturing ~0.5 x 106 NPCs (in 35 mm culture dish) in DA1 media for 10 days in Neurobasal media supplemented with 1× NEAA, 1× L-Glu, 1x penicillin-streptomycin, 1× B27, FGF8b (100 ng/ml) (R&D Systems) and 0.4 µM SAG (Enzo Life Sciences). Final maturation into dopaminergic neurons was carried out in DA2 media containing Neurobasal media supplemented with 1× NEAA, 1× L-Glutamine, 1× B27 supplement, 1x penicillin-streptomycin, 20 ng/ml BDNF (R&D Systems) and 20 ng/ml GDNF (R&D Systems) and 1 mM dibutyryl cAMP (Sigma) for 25 days.

Immunocytochemistry: Neurons were fixed with 4% paraformaldehyde at room temperature for 10 minutes. Fixed cells were permeablized with 0.3% Triton X-100 for 5 minutes and were blocked in blocking buffer (5% normal goat serum) in 1x Phosphate buffered saline (PBS) and followed by incubation with the primary antibody at 4° C overnight in 5% normal goat serum and PBS. The following primary antibodies were used: microtubule-associated protein 2 (MAP2) (Sigma), 1:500; Tyrosine hydroxylase (TH) (Pel Freeze), 1:300 and secondary antibodies were Alexa Fluor 488 Goat Anti-Mouse IgG and Alexa Fluor 555 Goat Anti-Rabbit IgG (Invitrogen), 1:300 as well as Hoechst 33342 as the nucleus counterstain.

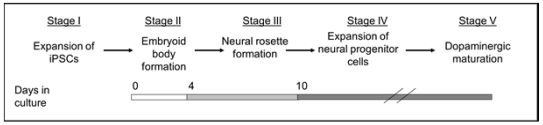
High content imaging analysis: Using ImageXpress® Micro XL High Throughput Imaging System (Molecular Devices, LLC), cells were imaged with 20x Plan Fluor objective, 2-6 sites per well with standard DAPI, FITC, TRITC filter cubes for identifying Hoechst stained nuclei and markers labeled with Alexa Fluor 488 and Alexa Fluor 555. Automated Image Analysis was applied using MetaXpress® Software (Molecular Devices, LLC). Images were processed using either pre-configured software modules or Custom Module Editor to characterize phenotypic changes and allow specific outputs.

Electrophysiology: Cells were dissociated with 0.05% Trypsin (Life Technologies) and, resuspended in the external solution, before they were transferred to PatchXpress 7000A Plus®, Automated Parallel Patch Clamp System. Voltage and current protocols for each set of experiment were included in the figure legends. The internal solution contains : 121 mM K-glu, 10 mM KCl, 3 mM NaCl, 4 mM MgATP, 5 mM EGTA, and 10 mM HEPES, pH 7.3 with KOH. The external solution contains 135 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2 10 mM HEPES, and 10 mM glucose, pH 7.4.

Statistical analysis: Statistical analysis was performed using GraphPad Prism. Data were analyzed by student's t-test ($p<0.05$). Data were presented as the means + standard error of the mean (SEM). All results were pooled from at least three independent experiments.

Differentiation of iPSC into dopaminergic neurons

We have used this five step embryoid body (EB) mediated neuronal differentiation and the differentiated cells were analyzed at day 35 during the maturation process.



Media Name	EB 4 days	NIM 4 days	NPC 2-3 days	DA1 10 days	DA2 25 days
Basal Media	DMEM/F12	DMEM/F12	Neurobasal medium	Neurobasal medium	Neurobasal medium
Media supplement	β-ME Dorsomorphine SB431524	Bottenstein N2 FGF2	B27 FGF2	B27 FGF8 SAG	B27 BDNF GDNF

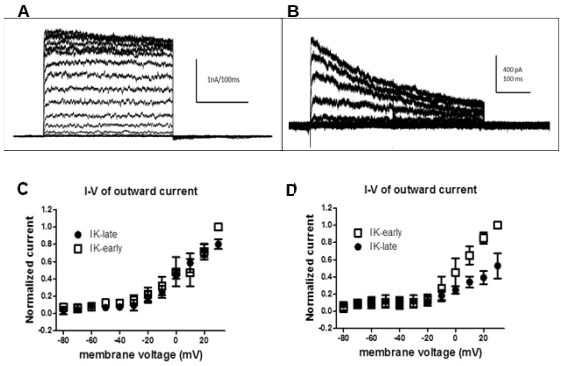


Figure 1, Endogenous currents recorded from the iPSC-derived neurons. **A.** Typical voltage dependent outward currents recorded from a control cell showing no significant inactivation of outward current. **B**. Typical voltage dependent outward currents from a LRRK2 G2019S cell showing significant current inactivation. The difference in current inactivation kinetic was consistent between these two cell lines, thereby suggesting differences in potassium ion channel subunit composition. **C.** I-V relationship of outward currents recded from the control cells. Current amplitudes were measured at the beginning (IK-early) and end (IK-late) of depolarizing voltage steps. **D.** I-V relationship of outward currents recorded from LRRK2 G2019S cells. Current amplitudes were measured at the beginning (IK-early) and end (IK-late) of depolarizing voltage steps.

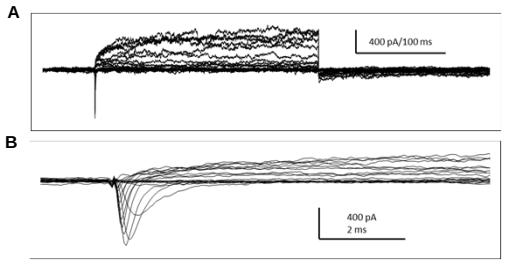
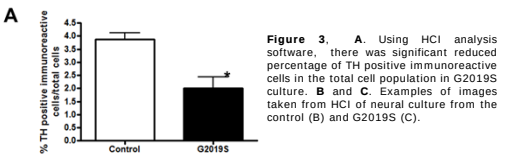


Figure 2, Sample recordings of inward sodium-like currents from the control group (8 out of 10 cells). Similar inward sodium-like currents were rarely observed in the LRRK2 G2019S cells (1 out of 11 cells). **A.** Presence of both inward and outward currents from one control cell. **B.** Expanded view of the inward sodium-like currents from the same cell.



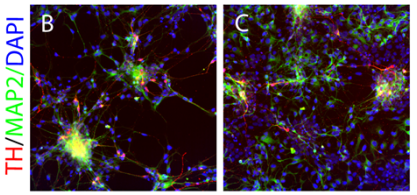


Figure 3, **A**. Using HCI analysis software, there was significant reduced percentage of TH positive immunoreactive cells in the total cell population in G2019S culture. **B** and **C**. Examples of images taken from HCI of neural culture from the control (B) and G2019S (C).

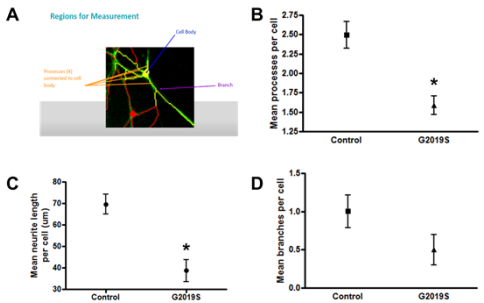


Figure 4, **A**. In the neurite outgrowth module, regions for measurement were defined. **B** and **C**. There were significant decreases in mean processes and mean neurite length (µm) per cell in the G2019S culture compared with the control. **D**. However, there was no significant difference in the mean branches per cell demonstrated in the G2019S culture (panel D). The asterisk,* represents means + SEM (n=3).

Results and Conclusions

- We demonstrated the functional evidences of ion channel expression in LRRK2 G2019S mutated and the control iPSC derived neural cells by whole cell patch clamp technique on PatchXpress® .
- High content imaging (HCI) has been applied to assess the efficiency for dopaminergic neuronal differentiation and to analyze the neurite length, process and branching in the LRRK2 G2019S mutated and mutation-negative iPSC derived neural cultures.
  - Significant difference in TH positive immunoreactive cells in the total cell culture (difference of 1.872% + 0.52, *p<0.01, n=3) implied that in LRRK2 G2019S mutated culture did not generate dopaminergic neurons efficiently or there is a delay in maturation compared to control cells.
  - There are significant reduction in the mean length of neurite per cell and mean processes per cell in the LRRK2 G2019S compared to the control. The difference in the mean length of neurite per cell was 30.98 µm + 6.90, *p<0.011, n=3 and in the mean processes per cell was 0.9027 + 0.21, *p<0.013, n=3.

In summary, LRRK2 G2019S iPSC derived neurons demonstrated significant differences in the morphological and functional phenotypes compared to control cultures. These results establish an in vitro model that can be used for high-throughput analyses of LRRK2 function and lays the foundation for drug screening.

Acknowledgement

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