Progressive degeneration of human neural stem cells caused by pathogenic LRRK2

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Nuclear-architecture defects have been correlated with the manifestation of a number of human diseases as well as ageing1–4. It is therefore plausible that diseases whose manifestations correlate with ageing might be connected to the appearance of nuclear aberrations over time. We decided to evaluate nuclear organization in the context of ageing-associated disorders by focusing on a leucine-rich repeat kinase 2 (LRRK2) dominant mutation (G2019S; glycine-to-serine substitution at amino acid 2019), which is associated with familial and sporadic Parkinson’s disease as well as impairment of adult neurogenesis in mice5. Here we report on the generation of induced pluripotent stem cells (iPSCs) derived from Parkinson’s disease patients and the implications of LRRK2(G2019S) mutation in human neural-stem-cell (NSC) populations. Mutant NSCs showed increased susceptibility to proteasomal stress as well as passage-dependent deficiencies in nuclear-envelope organization, clonal expansion and neuronal differentiation. Disease phenotypes were rescued by targeted correction of the LRRK2(G2019S) mutation with its wild-type counterpart in Parkinson’s disease iPSCs and were recapitulated after targeted knock-in of the LRRK2(G2019S) mutation in human embryonic stem cells. Analysis of human brain tissue showed nuclear-envelope impairment in clinically diagnosed Parkinson’s disease patients. Together, our results identify the nucleus as a previously unknown cellular organelle in Parkinson’s disease pathology and may help to open new avenues for Parkinson’s disease diagnoses as well as for the potential development of therapeutics targeting this fundamental cell structure.

Nuclear-envelope disruption has been correlated recently with ageing as well as a number of pathological manifestations in humans6–9. To study the role that the nuclear envelope might have during ageing-related neurodegenerative processes we chose to focus our attention on Parkinson’s disease, the second most prevalent neurodegenerative disease among ageing individuals. LRRK2 is a large multi-domain protein bearing kinase activity10 whose mutations correlate with inherited and sporadic Parkinson’s disease10,11. Specifically, the G2019S mutation leads to an increase in LRRK2 kinase activity correlating with the manifestation of disease10–13. However, the molecular and cellular implications of LRRK2(G2019S) mutation during Parkinson’s disease manifestation and progression remain elusive.

We decided first to evaluate potential novel interactions. By using a flag-tagged version of LRRK2(G2019S) and MudPIT (multi-dimensional protein identification technology) analysis, we identified unreported nuclear components in LRRK2(G2019S)-containing protein complexes (Supplementary Fig. 2a, b and Supplementary Table 1). Furthermore, LRRK2(G2019S) overexpression resulted in deformed nuclei with a pedal-like structure (Supplementary Fig. 2c, d). The fact that LRRK2(G2019S) leads to impaired adult neurogenesis in mice5 led us to take advantage of iPSC technologies and their potential for directed differentiation into specific human cell lineages (Supplementary Figs 3 and 4). Differentiation of iPSCs into neural progenitors and then serial passaging resembling cellular ‘ageing’14 showed progressive deterioration of nuclear architecture in mutant but not in wild-type NSCs (referred to here as ipsNSCs-LRRK2(G2019S) and ipsNSCs-wt, respectively) (Fig. 1a–f and Supplementary Figs 5, 6 and 7). Nuclear aberrations started to manifest by passage 14 and progressively resulted in compartmentalized pedal-like nuclei. Staining for lamin B1 in late-passage (for example, passage 19) ipsNSCs-LRRK2(G2019S) as well as LRRK2(G2019S)-overexpressing wild-type NSCs showed a markedly enlarged nuclear area, accompanied by a decrease in nuclear circularity (Fig. 1c–e, Supplementary Fig. 5c and Supplementary Fig. 8a). Noticeably, late-passage ipsNSCs-LRRK2(G2019S) showed local loss of lamin B1 and lamin B2 at specific folds of the nuclear envelope (Fig. 1c, f, g and Supplementary Figs 6e and 7), whereas all other examined nuclear components remained correctly localized (Fig. 1f and Supplementary Figs 6e and 7). Co-immunoprecipitation experiments revealed that ectopically expressed LRRK2(G2019S) associated in protein complexes with both lamin B1 and lamin B2 (Fig. 1h). Furthermore, increased phosphorylation of lamin B1 and lamin B2 was seen in late-passage ipsNSCs-LRRK2(G2019S) (Supplementary Fig. 8b), suggesting that excessive LRRK2 kinase activation may directly or indirectly stimulate phosphorylation of B-type lamins in NSCs. Finally, analysis of the mitochondria, an organelle frequently implicated in Parkinson’s disease pathogenesis15, showed the normal expression of the outer mitochondrial membrane receptor TOM20 as well as the mitochondrial heat-shock protein HSP60 in both NSCs-wt and NSCs-LRRK2(G2019S) (Supplementary Fig. 9).

We next examined whether LRRK2(G2019S)-elicited nuclear defects contributed to epigenetic alterations such as those observed during cellular ageing1,2,13. Fluorescence in situ hybridization revealed a dramatic increase of centromeric signals accompanied by reorganization of centromeric, but not telomeric, heterochromatin in passage 15 ipsNSCs-LRRK2(G2019S) (Supplementary Fig. 10a). Genome-wide epigenetic analysis showed passage-dependent differences in global H3K4me3 modifications, an epigenetic mark associated with human neuronal ageing16, at the analysed promoter regions (Fig. 2a and Supplementary Figure 10b). At passage 15, 437 genes in ipsNSCs-LRRK2(G2019S) displayed ≥5-fold enrichment for the epigenetic mark H3K4me3 compared to ipsNSCs-wt, whereas no significant differences were seen when comparing to undifferentiated iPSCs. Interestingly, most of these genes were associated with neurogenesis and neuronal function (Supplementary Table 2).

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These results led us to propose that LRRK2(G2019S) might progressively affect other in vitro NSC functions such as clonal expansion and neuronal differentiation. Newly generated ipsNSCs-wt and ipsNSCs-LRRK2(G2019S) were similarly capable of both clonal expansion and neuronal differentiation, whereas only ipsNSCs-wt retained clonogenic and differentiation capacity after extensive passaging (passage 14 and above) (Fig. 2b–d and Supplementary Fig. 8c). Notably, passage 16 to 17 ipsNSCs-LRRK2(G2019S) failed to give rise to MAP2+ (or Tuj1+) cells and resulted in aberrant neuronal cellular morphologies upon differentiation (Fig. 2c, d and Supplementary Figs 8c and 11a, b). Injection of proteasome inhibitors into animal brains has been shown to recapitulate Parkinson’s disease phenotypes17. Interestingly, treatment of NSCs with the proteasome inhibitor MG132 resulted in increased apoptosis in ipsNSCs-LRRK2(G2019S) in a passage-independent manner, compared to their wild-type counterparts (Fig. 2e and Supplementary Fig. 11c, d).

To validate further that the observed cellular phenotypes were related to NSCs carrying the LRRK2(G2019S) mutation, we evaluated different somatic cell types and also generated two different unbiased cellular systems, an isogenic control iPSC line by targeted correction of the LRRK2(G2019S)-corrected iPSCs to NSCs (c-ipsNSCs-LRRK2(G2019S)) in vitro culture of differentiated somatic cell populations other than NSCs did not result in misshapen nuclei (Supplementary Fig. 15). Differentiation of LRRK2(G2019S)-corrected iPSCs to NSCs (c-ipsNSCs-LRRK2(G2019S)) resulted in the rescue of aberrant cellular phenotypes, as compared to the respective uncorrected controls (Fig. 3d–g). Conversely, LRRK2(G2019S) knock-in in ENSCs resulted in the progressive appearance of disrupted nuclear architecture, compromised clonal expansion, impaired neural differentiation and increased susceptibility to proteasomal stress upon differentiation into NSCs (esNSC-LRRK2(G2019S)).
Interestingly, Ser 935 phosphorylation, an indicator for LRRK2 activation, was increased in passage 15 IPSCs (Fig. 4a and Supplementary Fig. 16a, b). This observation further enhanced the role of LRRK2 in the pathogenesis of Parkinson's disease.

As LRRK2(G2019S) displays enhanced Ser/Thr protein kinase activity that correlates with its pathogenic role in Parkinson's disease, we investigated whether kinase activity correlated with the presence or absence of LRRK2(G2019S) mutation. We found that kinase activity was significantly higher in LRRK2(G2019S) heterozygous mutant IPSCs before (left; LRRK2GS/+ and LRRK2GS/+) relative to total LRRK2 levels (Fig. 4a and Supplementary Fig. 16c).

For LRRK2 downstream targets and marked rescue of the aberrant cellular parameters seen in late-passage IPSCS-LRRK2(G2019S), we found that chemical inhibition has been shown previously to protect against LRRK2-associated toxicity in vitro and in vivo. Accordingly, treating late-passage IPSCS-LRRK2(G2019S) with LRRK2-In-1, a potent and selective LRRK2 inhibitor, reduced in resulting phosphorylation of representative IPSCS-LRRK2GS/+ and IPSCS-C-LRRK2GS/+ at passage 19. Scale bars, 10 µm.

Finally, we investigated nuclear morphology in post-mortem human brain samples. Lamin B1 immunostaining showed that a high proportion of cells displayed altered nuclear morphologies within the human brain samples. Lamin B1/Lamin B2/DNA, Representative of IPSCS-LRRK2GS/+ and their gene-corrected counterparts bearing neo cassette, Schematic representation of HDAdV-based correction of the G2019S mutation in the LRRK2 gene locus. Upwards and downwards arrows indicate the primer sites for polymerase chain reaction (PCR; P1, P2, P3 and P4). The probes for Southern blot analysis are shown as black bars (A, 5’ probe; B, neo probe; C, 3’ probe). Blue crosses, FLPo recognition target (FRT) site. Black crosses, homologous recombination between homology arms. HSV tk probe; B, neo probe; C, homology arm. Scale bars, 10 µm.

Figure 3 | Phenotypic analyses of isogenic iPSC and ESC lines in the presence or absence of the LRRK2(G2019S) mutation. a, Schematic representation of HDAdV-based correction of the G2019S mutation in the LRRK2 gene locus. Upwards and downwards arrows indicate the primer sites for polymerase chain reaction (PCR; P1, P2, P3 and P4). The probes for Southern blot analysis are shown as black bars (A, 5’ probe; B, neo probe; C, 3’ probe). Blue crosses, FLPo recognition target (FRT) site. Black crosses, homologous recombination between homology arms. HSV tk, herpes simplex virus (HSV) thymidine kinase (tk) gene. P, the FRTI restriction enzyme sites. Red cross, mutation site in exon 41. b, Sequencing results of the G2019S mutation site in exon 41 of the LRRK2 gene in LRRK2(G2019S) heterozygous mutant IPSCs before (left; LRRK2GS(+)) and after (right; C-LRRK2GS(+)) gene correction. c, Southern blot analysis of LRRK2(G2019S) heterozygous mutant IPSCs (LRRK2GS/+) and their gene-corrected counterparts bearing neo cassette (C-LRRK2GS neo(+)). d, Subcellular distributions of lamin B1 and lamin B2 in representative IPSCS-LRRK2GS/+ and IPSCS-C-LRRK2GS/+ at passage 19. Scale bars, 10 µm.

*e, Apoptosis assays after MG132 treatment in corrected and uncorrected IPSCs. f, g, Colony formation (f) and neuronal-differentiation (g) assays of IPSCs at passage 19.

Figure 4 | Representative images of lamin B1 and lamin B2. a, Lamin B1/Lamin B2/DNA, Representative of IPSCS-LRRK2GS/+ and their gene-corrected counterparts bearing neo cassette.

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controls (78.8% ± 3.41 and 16.49% ± 2.41, respectively (± s.e.m.)) (Fig. 4h, Supplementary Table 5 and Supplementary Fig. 18). Interestingly, nuclear aberrations were not only present in LRRK2 (G2019S)-bearing individuals and Parkinson’s disease5,28,29. However, these results indicate the possibility that mentioned phenotypes have a 100% penetrance in all Parkinson’s disease patients. Nevertheless, these results indicate the possibility that dysfunctional NSC pools, and/or their respective neuronal derivatives at early stages of maturation, might contribute to the hippocampal and microdomains deficient in lamin B1. Scale bars, 5 μm. d, e, Quantification of nuclear area (d) and nuclear envelope (NE) circularity (e) in ipsNSCs at passage 15 treated and untreated with LRRK2-In-1 for 4 days. Bars show the relative similarities between different groups. f, ipsNSCs-LRRK2(G2019S) at passage 17 were treated with or without LRRK2-In-1 for 8 days, and then with MG132 for 20 h. Data are shown as mean ± s.d., n = 3. **P < 0.01. g, Hierarchical clustering of gene-expression profiles on the brain slices (hippocampal region) of esNSCs-H9, esNSCs-H9-LRRK2GS/GS and esNSCs-H9-LRRK2GS/GS treated with 3 μM LRRK2-In-1 for 5 days at passage 14. Bars show the relative similarities between different groups. h, Immunofluorescence analysis on the brain slices (hippocampal region) of Parkinson’s disease patients bearing the LRRK2(G2019S) mutation (right panel) and age-matched healthy individuals (left panel). Scale bars, 25 μm.

Together, our results show that LRRK2(G2019S)-elicited aberrant phenotypic manifestations seem to be particularly prominent in human NSC populations and are more noticeable at late passages. Of most relevance, our studies further indicate the suitability of iPSC-based models of disease and ageing not only for the in vitro recapitulation of known disease phenotypes, as extensively described, but also as a platform for discovery and study of otherwise novel and elusive cell parameters and populations30 (Supplementary Fig. 1). Furthermore, through the analysis of human brain samples, our study demonstrates that these cellular aberrations can also be found in vivo. The fact that this...
validation was only possible in human samples once a reliable iPSC in vitro system was employed highlights further the potential of patient-specific iPSCs, not only for disease modelling but, most importantly, for the advancement of disease-pathology knowledge.

METHODS SUMMARY

iPSC generation. Parkinson’s disease fibroblasts were obtained from Telethon Genetic Biobank Network. Normal fibroblasts were purchased from the Coriell Cell Repository. Fibroblasts were reprogrammed with retrovirus expressing OCT4, SOX2 and KLF4. The generated iPSC lines were maintained in hESC medium on IMEF feeder cells or in mTeSR medium (StemCell Technologies) on Matrigel1–4.

Targeted gene correction in iPSCs-LRRK2(G2019S) and generation of the isogenic ESC line with an LRRK2(G2019S) mutation. Gene targeting in iPSCs-LRRK2(G2019S) and hiPSCs was carried out using a helper-dependent adenoviral vector (HDAvD) method.5–21

Statistical analysis. Results are presented as mean ± s.d. or mean ± s.e.m. for at least three independent biological replicates. Comparisons were performed using the student’s t-test. Distributions of nuclear area and nuclear envelope circularity were analysed with the Kolmogorov–Smirnov test.

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Supplementary Information is available in the online version of the paper.

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