



Impaired serine metabolism complements LRRK2-G2019S pathogenicity in PD patients



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ABSTRACT

Parkinson's disease (PD) is a multifactorial disorder with complex etiology. The most prevalent PD associated mutation, LRRK2-G2019S is linked to familial and sporadic cases. Based on the multitude of genetic predispositions in PD and the incomplete penetrance of LRRK2-G2019S, we hypothesize that modifiers in the patients' genetic background act as susceptibility factors for developing PD. To assess LRRK2-G2019S modifiers, we used human induced pluripotent stem cell-derived neuroepithelial stem cells (NECs). Isogenic controls distinguish between LRRK2-G2019S dependent and independent cellular phenotypes. LRRK2-G2019S patient and healthy mutagenized lines showed altered NEC self-renewal and viability, as well as impaired serine metabolism. In patient cells, phenotypes were only partly LRRK2-G2019S dependent, suggesting a significant contribution of the genetic background. In this context we identified the gene *serine racemase* (*SRR*) as a novel patient-specific, developmental, genetic modifier contributing to the aberrant phenotypes. Its enzymatic product, D-serine, rescued altered cellular phenotypes. Susceptibility factors in the genetic background, such as *SRR*, could be new targets for early PD diagnosis and treatment.

1. Introduction

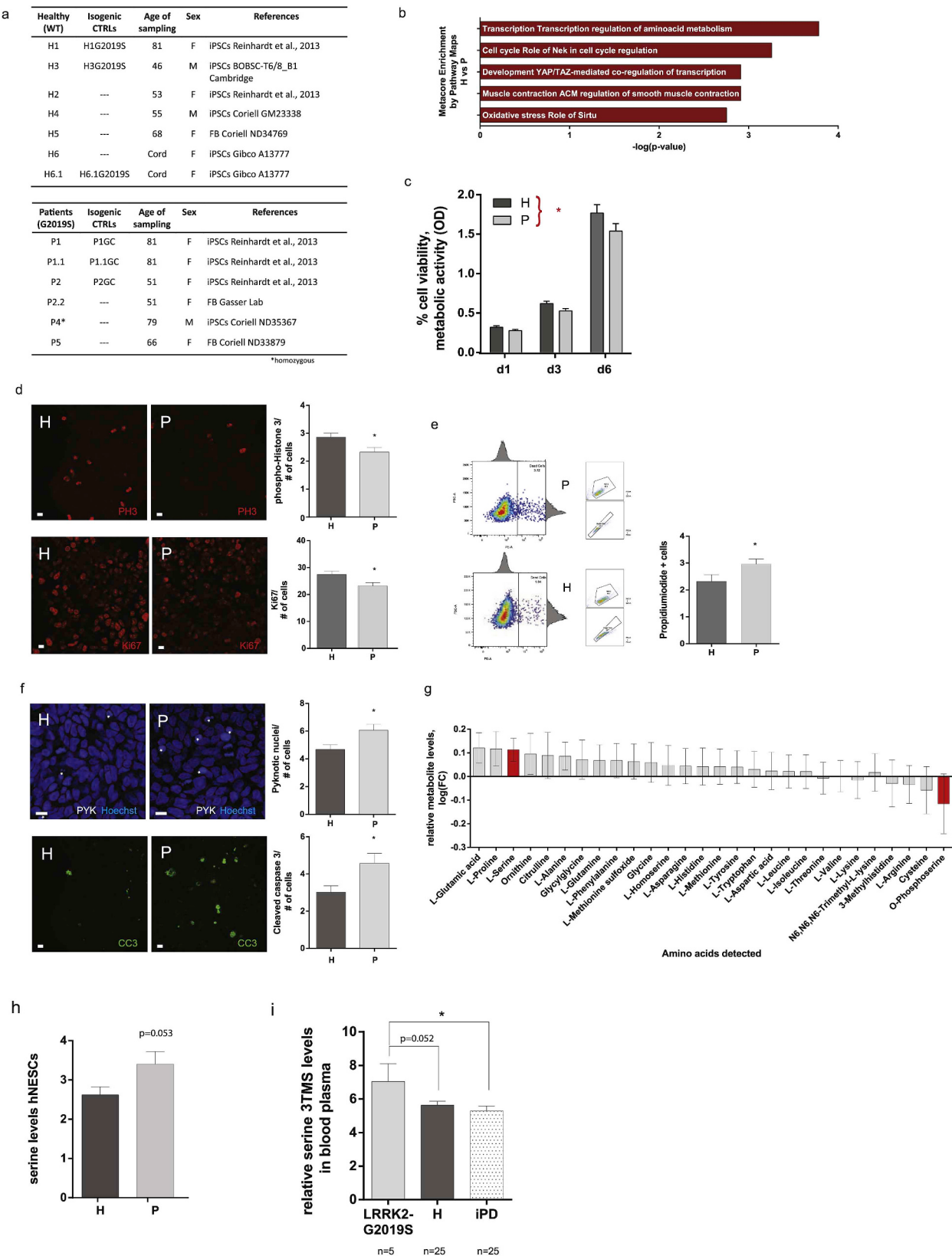
Parkinson's disease (PD) is the second most common progressive neurodegenerative disease. Pathophysiologically, PD is characterized by the loss of midbrain dopaminergic neurons (DN) in the *substantia nigra*. The triggers that lead to the onset of PD and finally to DN degeneration are poorly understood and thus, PD pathogenesis remains largely elusive with no effective protective treatment. PD has been proposed to encompass different multifactorial diseases, with similar phenotypic outcome [1]. Multiple genetic predispositions, monogenic mutations, and risk loci can cause similar parkinsonian phenotypes and symptoms.

The most common mutation that causes autosomal dominant PD is a glycine to serine substitution at position 2019 (G2019S) in the leucine-rich-repeat-kinase-2 (LRRK2) [2,3]. LRRK2 is of special interest as its mutations are the monogenic cause of familial PD and the reason for

1–2% of the sporadic cases [4,5]. Furthermore, a single nucleotide polymorphism (SNP) in the *LRRK2* gene acts as common risk modifier for developing PD [6]. Epidemiologic studies suggest that the age of onset between individuals carrying LRRK2-G2019S is highly variable and that different ethnicities show higher prevalence's [7]. Accordingly genetic variations within the patient-specific genetic background may underlie this variability. Such so-called second hits may explain why asymptomatic carriers exist, why the penetrance of LRRK2-G2019S is age-dependent and why the LRRK2 phenotypic spectrum is practically indistinguishable from that of idiopathic PD (iPD) [4].

Previous studies suggest that LRRK2 has an impact on neural stem cell (NSC) proliferation and integrity and plays a role in neurogenesis [8–12]. Furthermore, *LRRK2* expression is enhanced in the stem cell niches of the developing mouse brain during neurogenesis [13]. These observations imply a possible link between LRRK2, impaired neurogenesis, and the onset or progression of PD and therefore, we

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hypothesize that PD might have a neuro-developmental component that could explain DN vulnerability.

To explore this hypothesis, we investigated human PD patient-specific, iPSC-derived, neuroepithelial stem cells (NEC). To differentiate

between LRRK2-G2019S dependent phenotypes and genetic background-specific changes, isogenic control lines were used [14]. The identification of phenotypes in LRRK2-G2019S PD patient-specific NECs allows to gain new insights into NSC deregulations in PD

Fig. 1. Patient cell lines show impaired stem cell renewal and serine metabolism compared to healthy hNESCs. (A) Summary of cell lines: patients (P) healthy donors (H), insertion of LRRK2-G2019S (HG2019S) or gene correction (PGC). (B) Metacore pathway enrichment analysis for H1-6 vs. P1-5, based on 865 DEGs (RankProduct). (C) Growth curve of hNESCs by MTT at day 1, 3 and 6. Data as Mean $\pm$ SEM. * $p \leq 0.05$, 2-way ANOVA. Sidak's multiple comparison * $p < 0.05$ at day 6. N = 6 (H1-6 vs. P1-5), n = 5. (D) Representative images of phenotypes. P hNESCs show decreased mitosis (pH3) and proliferation (Ki67). Quantifications as Mean $\pm$ SEM. * $p \leq 0.05$, t-test, GRUBB's test $\alpha = 0.1$, no outlier, N = 6 (H1-6 vs. P1-5), n = 3. For Ki67 N = 3 (H1-2, H6 vs. P1-2), n = 3. See Fig. S3F for individual values. (E) Propidium Iodide positive cell count after flow cytometry. Mean $\pm$ SEM. * $p \leq 0.05$, t-test, GRUBB's test $\alpha = 0.1$, 1 outlier, N = 5/6 (H1-2, H4-6 vs. P1-5) n = 3. (F) Representative images of phenotypes. P hNESCs show increased pyknosis (white stars) and apoptosis (CC3). Average percentages of positive cells $\pm$ SEM. * $p \leq 0.05$, t-test, GRUBB's test $\alpha = 0.1$, no outlier for CC3, 1 outlier in H and P for PYK. N = 6 (H1-6 vs. P1-5), n = 3. See Fig. S3F for individual values. (G) Intracellular amino acid metabolite levels of hNESCs show increased serine levels in P. Data is shown as logFC, Mean $\pm$ SEM. (H) Serine metabolite levels in P vs. H individuals. Data is shown as Mean $\pm$ SEM $p \leq 0.053$, t-test. GRUBB's test within 3 technical and 3 biological replicates per line $\alpha = 0.1$. 1 outlier in H2, H3, H5 and P5. N = 6 (H1-6 vs. P1-5), n = 3. See also Fig. S3B for individual values. (I) Serine blood plasma levels are increased in P vs. H and in P vs. iPD cases. Mean $\pm$ SEM. * $p \leq 0.05$, t-test, GRUBB's test $\alpha = 0.1$, no outliers. 1-way ANOVA $p = 0.0555$.

pathogenesis and development. Finally, analysing the patient genetic background enables to discover susceptibility factors that may control disease-related cellular phenotypes and predispose to PD. These novel genetic modifiers could potentially be used as biomarkers for early diagnosis or as personalized therapeutic targets for precision medicine.

2. Results

2.1. Patient cell lines show impaired stem cell self-renewal and serine metabolism

To get an unbiased overview of the pathways that are affected in patients compared to healthy individuals, we assessed the transcriptome of the healthy (H) vs. patients (P) NESCs lines depicted in Fig. 1A. Quality controls regarding characterisation, derivation, gene-editing and individual absolute values of hNESCs phenotypes can be found in the supplements. Microarray analysis was performed and 865 significantly differentially expressed genes (DEGs) were identified between the six healthy and the six patient derived cell lines (H-6 vs. P-5), $FDR \leq 0.05$ [15]. Metacore pathway enrichment analysis revealed that the transcriptional regulation of amino acid metabolism, the cell cycle, cellular development as well as oxidative stress are within the top five networks enriched in the PD patients compared to healthy individuals (Fig. 1B). We decided to focus the further analysis on the deregulated cell cycle and amino acid metabolism by assessing the corresponding phenotypes, such as metabolic activity, proliferation and cell death (Fig. 1C–F). MTT assay derived growth curves revealed reduced cell viability or metabolic activity of the patient cell lines when compared to the healthy lines (H1-6 vs. P1-5) (Fig. 1C). To link this decrease to lower proliferation rates and increased cell death, we stained for the mitosis marker phospho-histone H3 (PH3) (H1-6 vs. P1-5) and proliferation marker Ki67 (H1, H2, H6 vs P1, P2, P1.1). In the patient-derived lines, less cells were undergoing cell division as compared to healthy controls (Fig. 1D and S3E). Moreover, immunostainings for cleaved caspase 3 (CC3) (H1-6 vs. P1-5) and for pyknotic nuclei (PYK) (H1-6 vs. P1-5), as well as flow cytometric counts for propidium iodide (H1-2&4-5 vs. P1-5) revealed increased cell death and apoptosis in patient-derived lines (Fig. 1E and F and S3E).

Next, we used a metabolomics approach to investigate the intracellular levels of amino acids in the NESC cultures from healthy individuals and patients (H1-6 vs. P1-5) (Fig. 1G). From all amino acids measured (H1-6 vs. P1-5), the L-serine increase in patients was the most promising candidate (Fig. 1H). Interestingly, the most decreased amino acid was phospho-serine, the precursor metabolite of serine (Fig. 1G), suggesting that serine metabolism is deregulated in LRRK2-G2019S patients. This hypothesis is further strengthened by the investigation of serine levels in the blood plasma of 25 healthy individuals, 25 iPD patients and 5 PD patients with the LRRK2-G2019S mutation (Fig. 1I). (Fig. 1I), suggesting that impaired serine metabolism is caused by LRRK2-G2019S. Furthermore, this observation supports the concept that findings from a PD patient-specific NSC model can be replicated in patients biofluids.

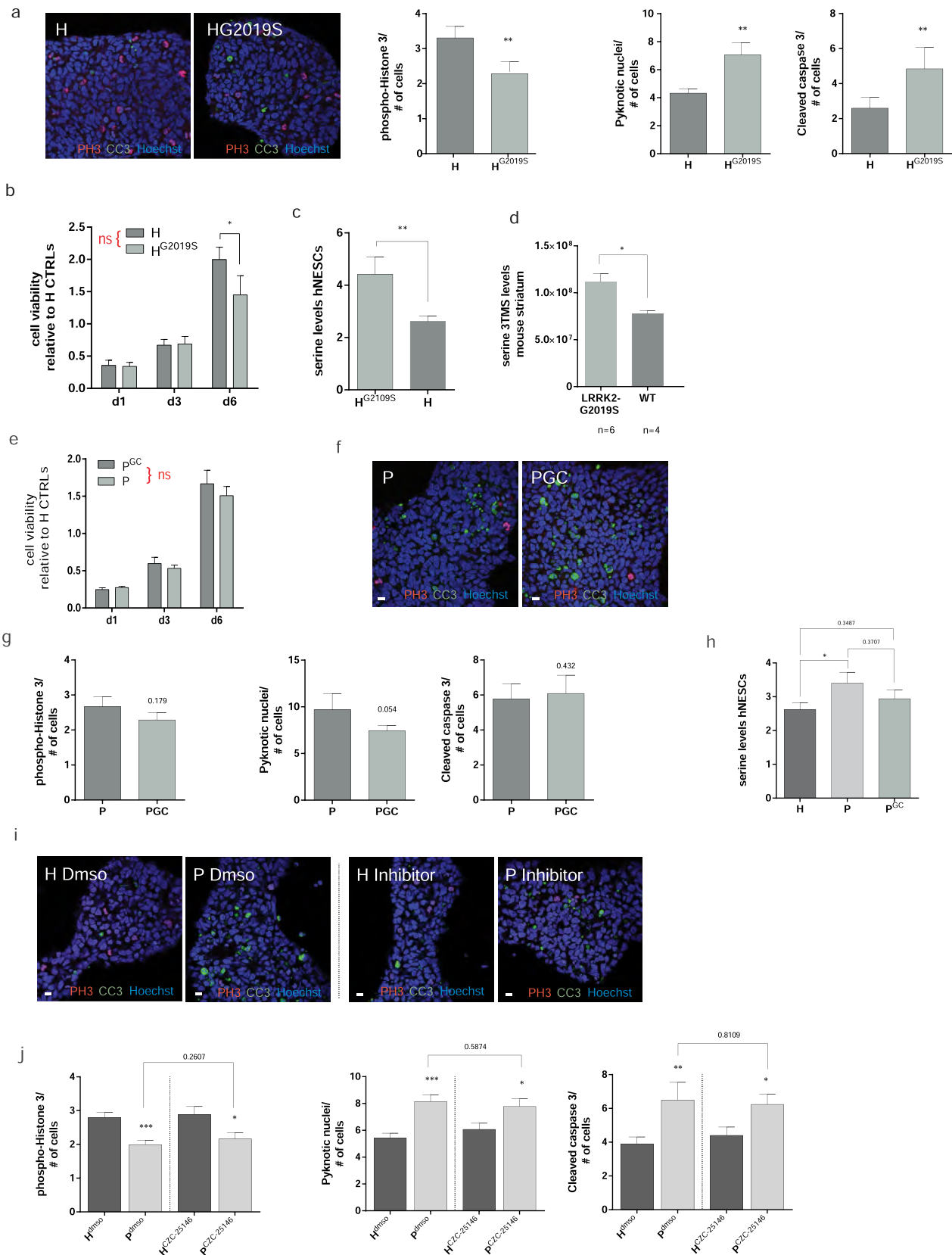
2.2. Stratification of LRRK2-G2019S dependent and independent phenotypes

Next, we investigated whether the phenotypes we observed in patient cells (Fig. 1), were indeed caused by the LRRK2-G2019S mutation (Fig. 2). For this purpose, we made use of the NESCs where the mutation had been introduced into the healthy background (H1, H3, H6.1 vs H1G2019S, H3G2019S, H6.1G2019S). We were able to detect phenotypes similar to the ones observed in patient lines, indicating that the LRRK2-G2019S mutation is sufficient to alter proliferation, cell death and metabolic activity (Fig. 2A and B). Moreover, LRRK2-G2019S dependency of these phenotypes was highlighted by treatment with the LRRK2 inhibitor, CZC-25146, that was able to rescue the mutation-dependent increase in cell death and the decrease in mitosis (Fig. S4A). We further analysed the serine levels upon the insertion of the mutation and could show that LRRK2-G2019S increases serine levels in hNESCs (Fig. 2C). Furthermore, we also detected an increase in the serine levels in mice expressing human LRRK2-G2019S (Fig. 2D). These observations, support the hypothesis that the observed serine deregulations are specific for the LRRK2-G2019S mutation.

Next, we gene-corrected the LRRK2-G2019S mutation in the patient cell lines (P1, P1.1, P2 vs P1GC, P1.1 GC, P2GC). Indeed, correcting the mutation resulted in the rescue of previously published phenotypes [16], such as increased protein levels of alpha-Synuclein and TAU (Fig. S3A). Strikingly, the correction of the mutation was, not able to rescue proliferation and survival phenotypes in patient-specific NESCs (Fig. 2E–G). Moreover, concerning the serine levels only a partial rescue can be observed (Fig. 2H). This observation suggests that in the patient-derived lines, the genetic background is contributing to the phenotypes. To get further insights into the origin of the observed phenotypes and to delineate their dependency on LRRK2-G2019S, we repeated the proliferation and cell death assays in the presence of the LRRK2 kinase inhibitor CZC-25146 [17]. Previously, CZC-25146 was shown to rescue LRRK2-G2019S induced apoptosis in DNs [17]. Within the PD patient-derived NESCs, increased cell death and decreased mitosis were not rescued by inhibiting LRRK2-kinase activity (Fig. 2I and J). This observation suggests that other factors, which are independent of LRRK2-G2019S, strongly contribute to these phenotype. Moreover, inhibiting LRRK2 in the gene-corrected cells had as expected no effect (Fig. S4B). These results emphasise the finding that the gene-correction of LRRK2-G2019S was not sufficient to rescue the phenotypes and highlight the important contribution of the genetic background.

2.3. Identification of potential susceptibility factors in the patient's genetic background

Based on the observation that cellular phenotypes partially depend on the information carried by the patient's genetic background, we aimed at identifying responsible genes. For this purpose, we reanalysed the gene expression via whole transcriptome microarray profiling taking into account also the isogenic controls. Moreover to narrow down, which genes could act as possible genetic modifiers, we performed a stricter analysis using the eBayes method [18]. The data



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confirmed that deregulation of the NESC transcriptome is strongly influenced by the patient's genetic background. The heatmap shows that patient cells cluster with their gene-corrected counterpart, suggesting a limited contribution of LRRK2-G2019S alone to the changes in gene

expression (Fig. 3A). To investigate which genes could act as possible susceptibility factors accounting for the patients' genetic background-dependent part of the phenotypes, we evaluated the DEGs from the Healthy vs. Patient comparison. The top 50 candidates are represented

Fig. 2. LRRK2-G2019S is sufficient to increase cell death, and serine metabolism and to decrease proliferation and metabolic activity. However within patients, LRRK2-G2019S is not necessary to maintain phenotypes. (A) Representative images of HG2019S hNESCs. Introduction of LRRK2-G2019S in H cell lines increases pyknosis (Hoechst) and apoptosis (CC3) and reduces mitosis (PH3). Average percentage of positive cells $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, paired t -test, GRUBB's test $\alpha = 0.1$, no outliers. $N = 3$ (H1,H3,H6 vs. H1G2019S, H3G2019S, H6G2019S), $n = 3$. (B) Growth curve of hNESCs by MTT after 1, 3 and 6 days. Mean $\pm$ SEM. 2-way ANOVA followed by Sidak's multiple comparison * $p < 0.05$ at day 6. $N = 2$ (H1, H3 vs. H1G2019S, H3G2019S), $n = 5$. (C) Serine levels are increased in HG2019S vs. H individuals. Mean $\pm$ SEM. ** $p \leq 0.001$, t -test, GRUBB's test $\alpha = 0.1$, 1 outlier in H2, H3 and H5. $N = 2/6$ (H1G2019S, H3G2019S vs. H1-6), $n = 3$. (D) Serine level in striatum of LRRK2-G2019S carrying mice is increased compared to WT mice from the same strain. Mean $\pm$ SEM. * $p \leq 0.05$, t -test, GRUBB's test $\alpha = 0.1$, 1 outlier in WT and G2019S. $N = 7/5$. (E) Growth curve of hNESCs by MTT after 1, 3 and 6 days. Mean $\pm$ SEM. * $p < 0.05$, 2-way ANOVA followed by Sidak's multiple comparison. $N = 3$ (P1-2 vs. P1-2 GC), $n = 5$. (F) Representative images of PGC hNESCs. Gene correction is not rescuing reduced mitosis (PH3) and increased pyknosis (Hoechst) and apoptosis (CC3). (G) Quantification of B. Percentages as Mean $\pm$ SEM. * $p \leq 0.05$, paired t -test, GRUBB's test $\alpha = 0.1$, 1 outlier for PYK (P). $N = 3$ (P1-2 vs. P1-2 GC), $n = 3$. (H) Serine levels are not significantly decreased by gene-correcting LRRK2-G2019S in patient lines. Mean $\pm$ SEM. * $p \leq 0.053$, t -test, GRUBB's test $\alpha = 0.1$, 1 outlier in P5 and P2GC. $N = 3$ (P1-5 vs. P1-2 GC), $n = 3$. (I) Representative images of hNESCs stained for Hoechst, PH3 and CC3 after treatment with 0.5 μ M LRRK2 inhibitor CZC-25146 in DMSO. (J) Quantification of D. Inhibition of LRRK2-G2019S is not rescuing reduced mitosis or cell death in patient lines. Data as Mean $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, t -test, GRUBB's test $\alpha = 0.1$, 1 outlier in Pdmso and PLRRK2in for CC3 and PYK and HdmsO for PH3, $N = 6$ (H1-6 vs. P1-5), $n = 3$.

in a second heat map (Fig. 3B). These top candidates include genes such as RAB32 and PTGR1 that have implications in LRRK2-G2019S induced PD pathogenesis [19] or were linked to the PD genetic background in DN [20]. To further emphasize the top candidate genes we performed a variable importance ranking based on the significance (Fig. 3C). Interestingly, among the top five genes that were unique to the Healthy vs. Patient comparison we found 2 genes, *ARL17a*, *ARL17b*, in which SNPs have been linked to PD before [21] (Fig. 3D). Other significant DEGs involved in pathways potentially relevant for PD, were serine racemase (*SRR*), neurotensin (*NTS*) and DLEU7 Antisense RNA 1 (*DLEU7-AS1*). The most promising candidates that may act as the here sought-after genetic modifiers were *SRR* and *NTS* because of their involvement in neuromodulation, -transmission and neurological diseases, such as PD [22,23].

2.4. Patient cell lines show impaired *SRR* expression, deregulated serine metabolism and the enzymatic product of *SRR*, D-serine, rescues patient phenotypes

SRR is the enzyme that catalyzes the conversion between L-serine and D-serine (Fig. 4A). Since serine metabolism was already affected by LRRK2-G2019S in hNESCs (Figs. 1 and 2), we decided to focus on *SRR* as a potential genetic modifier for LRRK2-G2019S induced PD. RT-qPCR for *SRR* mRNA validated the reduced expression in patient cell lines (H1-6 vs. P1-5) (Fig. 4B). Consistent with the reduced *SRR* expression, the patient lines showed increased levels of L-serine compared to the healthy lines (H1-6 vs. P1-5) (Fig. 4A). Therefore, we hypothesized that the reduced levels of *SRR* lead to increased levels of serine and a deficiency in the conversion to D-serine (Fig. 4A). Based on the described function of D-serine in NSCs [24,25], the deficiency in D-serine production might contribute to the observed cellular phenotypes, and consequently, the effects may be rescued through treatment with D-serine. Indeed, treatment with 100 μ M D-serine led to the complete rescue of the proliferation and cell death phenotypes in PD patient-derived cells without affecting healthy lines (H1-6 vs P1-5) (Fig. 4C). Strikingly, this rescue is specific for the NSC-related phenotypes. No rescue of the neuronal phenotypes (H1-6 vs. P1-5) was achievable through D-serine treatment (Fig. S5). Furthermore, D-serine was rescuing LRRK2-G2019S dependent phenotypes in the three healthy lines where we inserted the mutation (Fig. S4C). In addition, D-serine rescued proliferation and partially rescued apoptosis in the three patient lines where the mutation had been corrected (Fig. S4D). These rescue effects highlight the contribution of impaired serine metabolism within the phenotypes and emphasizes the role of the LRRK2-G2019S mutation.

3. Discussion

This study supports the concept that PD is not a monogenetic disease, a common interpretation of its diversity follows the multiple-hit hypothesis, which states that different incidences, or genetic

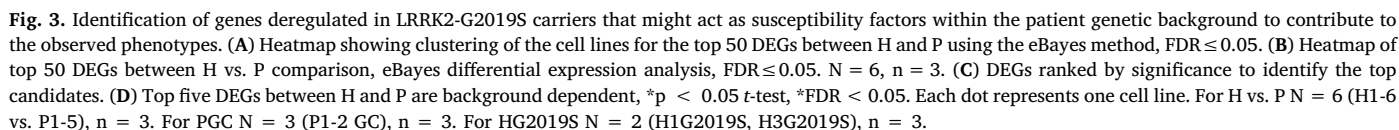
predispositions may to act in parallel or consecutively to cause PD [26].

Our data suggests that both a decrease in *SRR* expression caused by the genetic background as well as an increase of serine levels caused by LRRK2-G2019S influence serine metabolism. In patients both deregulations, the reduction of *SRR* caused by the genetic background and the LRRK2-G2019S mutation, may complement each other (Fig. 4D). Based on these results, we propose an interplay between impaired serine metabolism, the PD genetic background and LRRK2-G2019S. However, we are aware that some of the here presented results are unexpected and have clear constraints, including the facts that the sample sizes is limited and that some of the observed phenotypes are rather subtle or have marginal statistical significance.

Initially, we thought that within patients LRRK2-G2019S alone leads to increased serine levels as observed in NESCs. Increased serine levels were, for instance, previously reported in the striatum of 6-OHda lesioned rats [27] and serine inversely correlates with PD progression, suggesting that it might inhere a compensatory mechanism [28]. This view, however, was challenged in our data by the discrepancy that gene-correcting LRRK2-G2019S within the patient genetic background did not rescue phenotypes, meaning that other susceptibility factors contribute to phenotype manifestation. A screen for potential genes that could act as genetic modifier and represent the driving force behind the phenotypes identified *SRR*, which is directly involved in serine metabolism. We showed that cell lines having the patient background have decreased *SRR* expression, which is in accordance with existing transcriptomic data from the *substantia nigra* of PD patients [29]. Accordingly it seems more likely that two independent pathways exist that both affect serine metabolism and might act together under disease conditions. Mouse studies showed for instance similar phenotypes in *SRR*-KO and LRRK2-G2019S mice, suggesting that LRRK2-G2019S and *SRR* might have opposing modulating capacities [8,30]. The mechanism on how LRRK2-G2019S deregulates serine levels, however, remains an open question and the possibility of a direct interaction between both proteins cannot be excluded (Fig. 4d).

In regards to our hypothesis that PD may have a developmental component, patient NESCs show significant alterations in cell death and proliferation, which are both indicators of functionally relevant developmental impairments and could have significant consequences for *in vivo* neurogenesis. LRRK2 indeed is necessary for maintaining the balance between self-renewal and cell death in NESCs. Moreover, pathway analysis confirmed that developmental genes, independent of LRRK2-G2019S, are altered between healthy and patient cell lines demonstrating that both LRRK2-G2019S and the patient genetic background can influence organism development in the context of PD.

Lastly, the deregulated serine metabolism by LRRK2-G2019S and decreased *SRR* expression levels consolidated the use of D-serine as potential rescue strategy. Moreover, a pilot study described that D-serine treatment alleviates behavioural and motor symptoms in PD [31]. The fact that LRRK2 alone is not responsible for the investigated impairments, could have implications on PD therapeutic strategies in



Detailed experimental procedures are described in the supplements

53

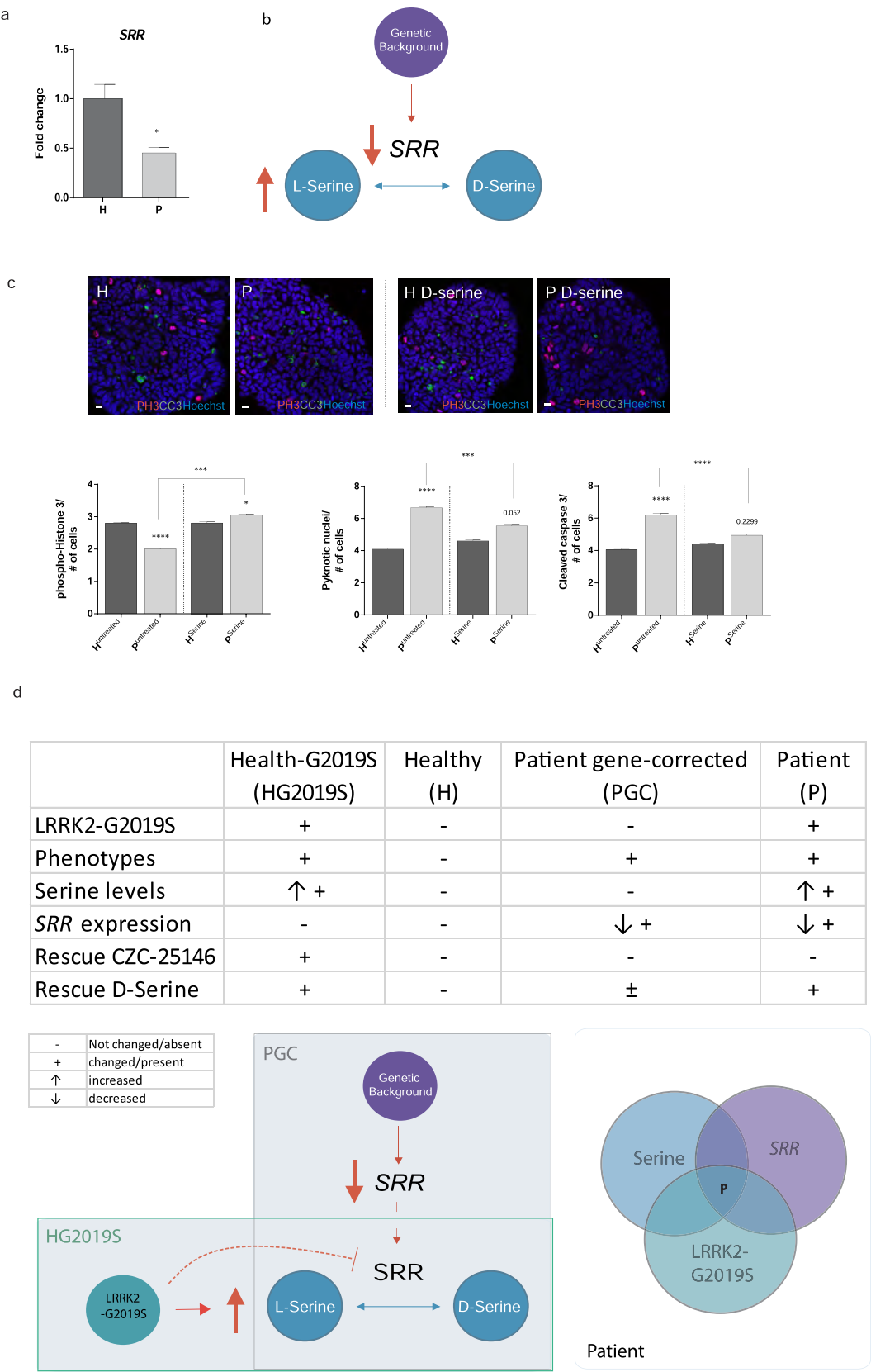


Fig. 4. SRR might contribute to deregulated serine metabolism, and D-serine the metabolic product of SRR rescues phenotypes in patients. (A) Validation of *SRR* expression by RT-qPCR. The H lines were set to 1. Mean $\pm$ SEM. * $p \leq 0.05$, *t*-test. N = 6 (H1-6 vs. P1-5), n = 3. (B) SRR converts L-serine to D-serine and is downregulated in the patient genetic background. (C) Representative images of hNESCs stained with Hoechst, PH3 and CC3 after treatment with 100 μ M D-serine for 6 days. Quantification: D-serine rescues cell death and mitosis in patient cell lines; Mean $\pm$ SEM. * $p < 0.05$, *t*-test, GRUBB's test $\alpha = 0.1$, 1 outlier in Pser for PH3 and in Hser for CC3. N = 6 (H1-6 vs. P1-5), n = 3. (D) SRR acts as genetic modifier for LRRK2-G2019S induced PD. Overview showing the synthesis of previous Figures.

funder line C5713467. Chemical reagents and treatments can be found in Table 1. RNA was extracted using miRNA easy kit. Samples were processed with EMBL Genomics Core Facility using Affymetrix Human Gene 2.0 arrays. GEO accession number [GSE101534](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE101534).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.parkreldis.2019.09.018>.

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