Parkinson disease-related phenotype characterization of A53T alpha-synuclein iPSC-derived dopaminergic cultures

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INTRODUCTION

Parkinson disease (PD) is a progressive neurological disease caused by selective loss of dopaminergic neurons in the substantia nigra. Although the majority of PD cases are sporadic, familial PD mutations provide a valuable tool for understanding and modelling basic pathophysiological mechanisms. We used MyCell® DopaNeurons carrying the A53T mutation in the SNCA gene (A53T DaNs) and healthy isogenic control iCell® DopaNeurons (WT DaNs) to investigate disease-relevant phenotypes including:

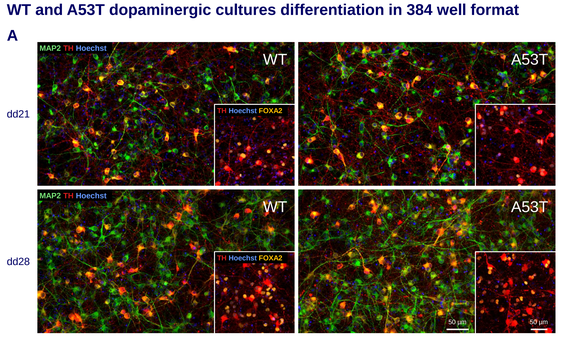
- alpha synuclein (αSyn) accumulation
- mitochondrial dysfunction
- calcium dysregulation

AIM

The aim of the study was to establish PD-related read-out assays to be potentially applied in drug-discovery programs for the identification of neuroprotective compounds for PD.

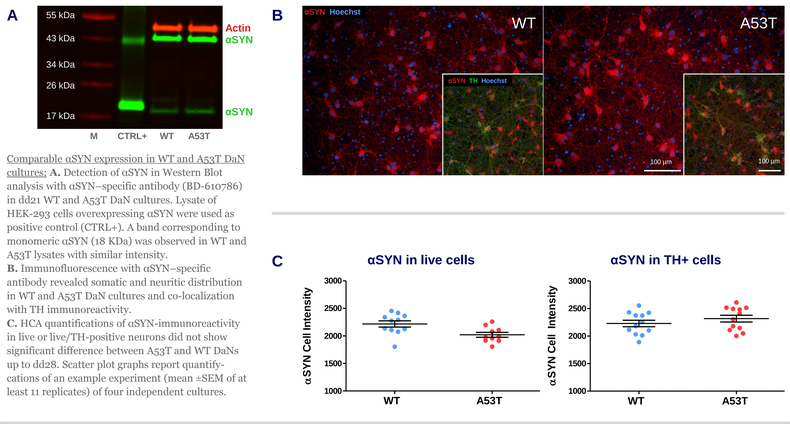
RESULTS

WT and A53T dopaminergic cultures differentiation in 384 well format



Dopaminergic neurons maturation in 384 well format: **A.** iPSC-derived DA-neurons maturation was confirmed by the expression of the neuronal marker MAP2 and DA-specific markers tyrosine-hydroxylase (TH) and FOXA2. WT and A53T cells were seeded at 65,000 cells/well and 50,000 cells/well, respectively, to achieve comparable viability; overall viability estimated for the two cultures is <40%, with WT DaNs displaying lower viability than A53T after seeding. **B.** Bar graph reports the % of TH-positive cells over live cells quantified by high content analysis (HCA). **C.** Viability of the cultures is quantified by HCA at dd21 and 28 by measure of the number of live cells/well. Bar graph represents mean ±SEM of at least 14 replicate wells.

Detection of αSYN in WT and A53T DaN cultures with Western Blot and immunofluorescence

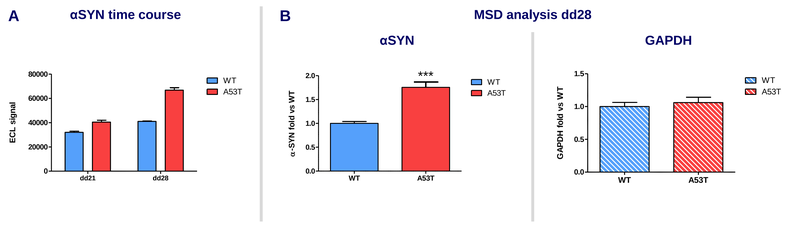


Comparable αSYN expression in WT and A53T DaN cultures: **A.** Detection of αSYN in Western Blot analysis with αSYN–specific antibody (BD-610786) in dd21 WT and A53T DaN cultures. Lysate of HEK-293 cells overexpressing αSYN were used as positive control (CTRL+). A band corresponding to monomeric αSYN (18 KDa) was observed in WT and A53T lysates with similar intensity. **B.** Immunofluorescence with αSYN–specific antibody revealed somatic and neuritic distribution in WT and A53T DaN cultures and co-localization with TH immunoreactivity. **C.** HCA quantifications of αSYN-immunoreactivity in live or live/TH-positive neurons did not show significant difference between A53T and WT DaNs up to dd28. Scatter plot graphs report quantifications of an example experiment (mean ±SEM of at least 11 replicates) of four independent cultures.

Mesoscale assay set up for αSYN quantification from DaN cellular lysates

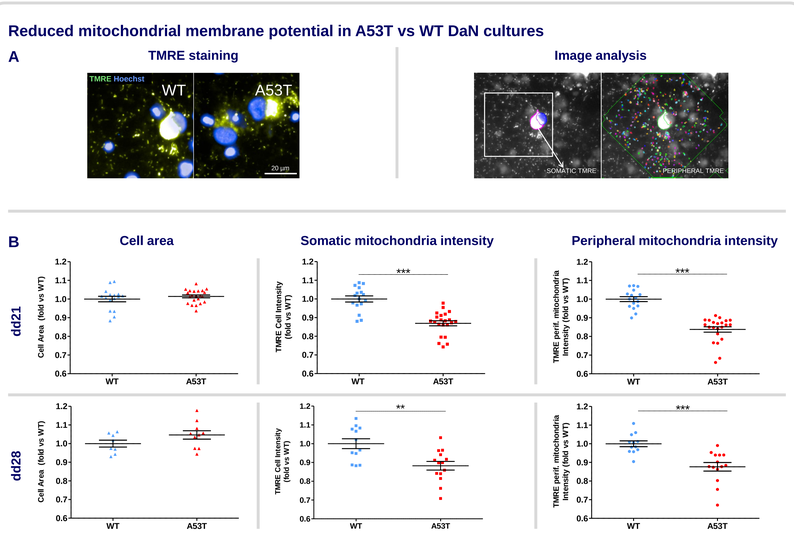
αSYN quantification assay set up: Mesoscale Discovery (MSD) 96-well assay kit (U-PLEX human αSYN K151WKK, for use with human fluid matrices) was employed for quantification of total αSYN from DaNs cell lysates. **A.** Assay set up was performed to adapt the assay kit to αSYN quantification from cell lysates prepared with RIPA buffer. Lysates from HEK-293 over-expressing αSYN and naïve HEK-293 cells were tested in MSD assay. Specific αSYN signal was detected within the linearity range of the assay. **B.** Linearity test of A53T lysates (dd28). 1µg and 0.5 µg of lysates of A53T DaNs were employed in αSYN MSD. ECL signal revealed linear αSYN detection within the linear range of the calibration curve. 0.25 µg/well was the condition selected for the following quantifications in DaN cultures.

αSYN accumulation in A53T DaN cultures



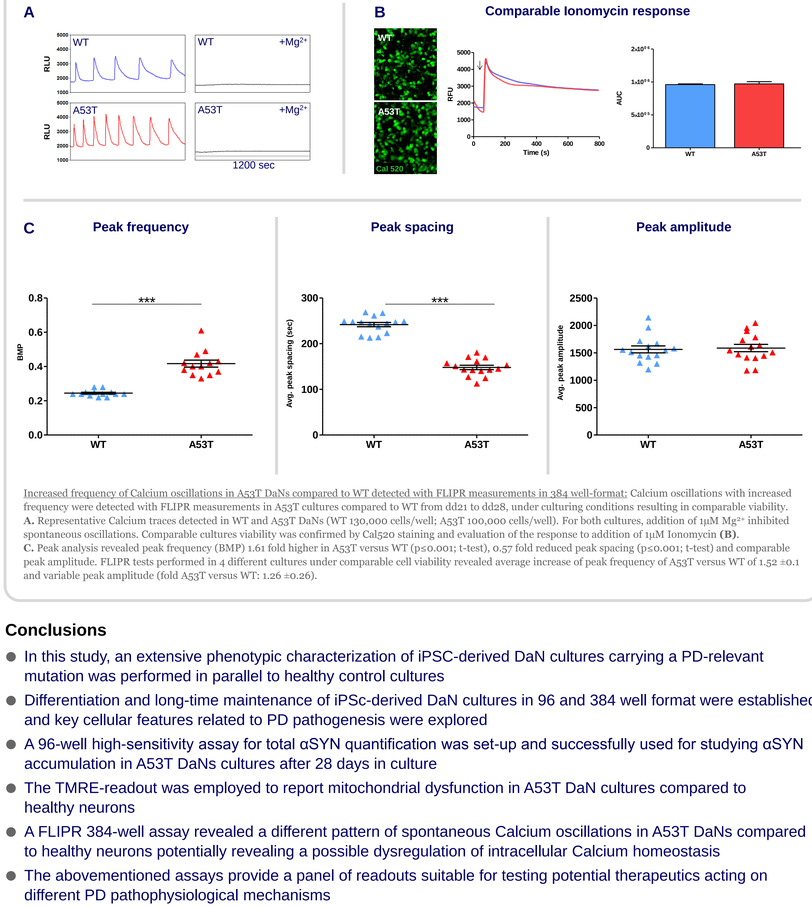
Time dependent αSYN accumulation in A53T DaN cultures: **A.** αSYN MSD quantifications performed at dd21 and 28 revealed time-dependent accumulation of αSYN in A53T versus WT DaN cultures. The graph bars report the fold increase of the αSYN MSD signal of A53T over the signal of WT at each time point. The maximum fold of 1.63 was detected at dd28. **B.** αSYN MSD quantifications in four independent cultures lysates at dd28 confirmed higher αSYN MSD signal in A53T versus WT (fold: 1.76 ±0.11, p≤0.001; t-test). Comparable GAPDH MSD signal in WT and A53T dd28 lysates (subset samples) confirmed uniform sample loading in αSYN MSD studies.

Reduced mitochondrial membrane potential in A53T vs WT DaN cultures



TMRE intensity quantifications performed in the cell soma and in the peripheral mitochondria showed lower signal in A53T cultures versus WT: **A.** TMRE assay was performed on WT and A53T cultures at dd21 and dd28 and HCA was employed for analysis . Nuclear masks were identified based on Hoechst staining and cell masks were segmented on the TMRE signal. For peripheral mitochondria identification, TMRE spot masks were identified in the area surrounding the cells. **B.** Average data for each time-point from two independent cultures of WT and A53T DaNs are reported. Comparable cell area was quantified in live WT and A53 cells. TMRE intensity quantified in the cell soma of A53T displayed significantly lower signal at both time points (fold A53T vs WT: dd21, 0.87±0.01, p≤0.001; dd28 0.88±0.02, p≤0.01; t-test). Additionally, lower TMRE average intensity was detected in peripheral mitochondria (fold A53T vs WT: dd21, 0.84±0.01, p≤0.001; dd28, 0.87±0.02, p≤0.001; t-test).

Different pattern of spontaneous Calcium oscillations in WT and A53T DaN cultures



Increased frequency of Calcium oscillations in A53T DaNs compared to WT detected with FLIPR measurements in 384 well-format: Calcium oscillations with increased frequency were detected with FLIPR measurements in A53T cultures compared to WT from dd21 to dd28, under culturing conditions resulting in comparable viability. **A.** Representative Calcium traces detected in WT and A53T DaNs (WT 130,000 cells/well; A53T 100,000 cells/well). For both cultures, addition of 1µM Mg^{2+} inhibited spontaneous oscillations. Comparable cultures viability was confirmed by Cal520 staining and evaluation of the response to addition of 1µM Ionomycin **(B)**. **C.** Peak analysis revealed peak frequency (BMP) 1.61 fold higher in A53T versus WT (p≤0.001; t-test), 0.57 fold reduced peak spacing (p≤0.001; t-test) and comparable peak amplitude. FLIPR tests performed in 4 different cultures under comparable cell viability revealed average increase of peak frequency of A53T versus WT of 1.52 ±0.1 and variable peak amplitude (fold A53T versus WT: 1.26 ±0.26).

Conclusions

- In this study, an extensive phenotypic characterization of iPSC-derived DaN cultures carrying a PD-relevant mutation was performed in parallel to healthy control cultures
- Differentiation and long-time maintenance of iPSc-derived DaN cultures in 96 and 384 well format were established and key cellular features related to PD pathogenesis were explored
- A 96-well high-sensitivity assay for total αSYN quantification was set-up and successfully used for studying αSYN accumulation in A53T DaNs cultures after 28 days in culture
- The TMRE-readout was employed to report mitochondrial dysfunction in A53T DaN cultures compared to healthy neurons
- A FLIPR 384-well assay revealed a different pattern of spontaneous Calcium oscillations in A53T DaNs compared to healthy neurons potentially revealing a possible dysregulation of intracellular Calcium homeostasis
- The abovementioned assays provide a panel of readouts suitable for testing potential therapeutics acting on different PD pathophysiological mechanisms

Methods

DaN cultures: iCell® DopaNeurons (WT) and MyCell® DopaNeurons (A53T) were seeded according to vendor's instruction in ICell Neural medium (Fujifilm Cellular Dynamics) on PDL/PLO/Laminin coated 384 or 96-well plates. Cells were seeded at the density of 6.5 x 10^5/cm^2 (WT) and 5 x 10^5/cm^2 (A53T) for imaging studies; for FLIPR studies cells were seeded from 0.7 x 10^6 to 1.8 x 10^6/cm^2. Culture medium was replaced with BrainPhys™ Neuronal Medium (StemCell Technology) after 5 days in culture and maintained for up to dd21 or dd28 with half medium volume exchange twice/week.

Western blot: cell lysates were prepared in RIPA buffer and separated on 4-12% Bis-Tris gels in MES-SDS running buffer. After transfer to Nitrocellulose membrane, an incubation of 30 min in 0.4% paraformaldehyde solution was performed prior to proceeding to blocking, primary antibody staining (Ms anti-αSYN: BD-610786; Rb anti-actin: CellSignaling 13E5) and incubation with secondary antibodies (anti-Rb IRDye 680 e anti-Ms IRDye 800). Western blot signal was revealed using Odyssey CLx.

Immunofluorescence: cells were fixed in 4% paraformaldehyde solution, permeabilized with 0.2% Triton X-100 and blocked with 0.5% BSA-PBS before incubation with primary antibody. The following primary antibodies were used for immunostaining: Ms-anti-αSYN BD-610786; Ch-anti-TH (Abcam ab76442), Rb-anti-FOXA2 (Cell Signalling 8186), Ms-anti-MAP2 (Abcam ab32454). The secondary antibodies anti-Ms-AlexaFluor 488, Anti-Chicken-AlexaFluor 647, Anti-Rb AlexaFluor 546 were used. Images were captured with InCell Analyzer 2200.

Mesoscale assays: U-PLEX human αSYN -K151WKK assay and GAPDH-K151PWD were employed according to supplier's protocols. 0.25µg/well were used in αSYN – MSD assay and 0.125 µg/well were used in GAPDH-MSD assay. MSD assays were performed in technical duplicates.

TMRE assay: cells were incubated with TMRE (Invitrogen T669) for 30 minutes in culture media without phenol-red, supplemented with a solution containing Hoechst and imaged with InCell Analyzer 2200.

FLIPR assay was performed by using 10µM Cal520 calcium indicator (Abcam, AB171868) in Tyrode's buffer without Magnesium. Measurements were carried out for 20 minutes. Final addition of 1 µM Ionomycin was performed to evaluate total Calcium increase. FLIPR Screenworks® Peak Pro™ Software was used for Peak analysis.

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