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Original Research

Characterisation of Oxidative Stress, DNA Damage and Inflammation in a Cellular Model of Parkinson's Disease

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Abstract

***Background*:** Parkinson's disease (PD) is the second most common neurodegenerative disease and is a synucleinopathy due to the critical role of α-synuclein (α-Syn) in its pathology. α-Syn is able to translocate from the cytoplasm to the nucleus and cause DNA damage.

***Methods*:** SH-SY5Y cells were stably transfected with plasmids containing wild type (WT) α-Syn and A53T mutant α-Syn as fusion proteins with EGFP and an EGFP only control vector. The cells were differentiated using retinoid acid (RA) and treated with hydrogen peroxide (H_2O_2) and analysed in a differentiated state for the effects of oxidative stress using flow cytometry, DNA damage using γH2A.X staining and inflammatory and senescence markers using qPCR.

***Results*:** Cells expressing the A53T mutation exhibited a higher sensitivity to treatment with H_2O_2 with significantly higher amounts of DNA damage. Intriguingly, WT α-Syn seemed to have a protective effect in differentiated cells under increased stress with no increase in DNA damage after H_2O_2 treatment. In line with these results, inflammatory markers of COX-2, IL-6 and TNF-α as well as the stress-and senescence marker p21 were all significantly increased in differentiated treated cells expressing mutant α-Syn compared to cells harbouring WT α-Syn or EGFP controls only. Interestingly, both forms of α-Syn equally increased the levels of mitochondrial ROS already in untreated condition.

***Conclusions*:** Our data demonstrate a higher stress sensitivity of SH-SY5Y neuroblastoma cells harbouring an A53T mutant α-Syn for DNA damage and the expression of selected inflammatory marker. Intriguingly, WT α-Syn seemed to provide a protection against oxidative stress in our model which was most pronounced for the absence of increased DNA damage after hydrogen peroxide treatment. Although the mechanism of protection is not clear yet, we suggest that the prevention of DNA damage correlates to less neuro-inflammation, an area of intense research in PD pathology.

Keywords

SH-SY5Y neuroblastoma cells; DNA damage; oxidative stress; inflammation; Parkinson's disease; cellular model α-synuclein

1. Introduction

Parkinson's disease (PD) is prevalent in 1-2% of the over 65 years old population [1] and is the second most commonly occurring neurodegenerative disease [2]. An increase of 2-5 times in mortality rate is seen in older patients suffering from PD compared to age-matched controls [1]. Characteristic clinical symptoms include a resting tremor, bradykinesia, rigidity and postural instability [3]. In addition, known co-morbidities can constitute non-motor features such as gastrointestinal complications, insomnia and depression, among many others, which all contribute to the debilitating nature of the disease [3]. The two main characteristics of PD are α-synuclein (α-Syn) inclusions- Lewy bodies (LBs) and the loss of dopaminergic neurons in the *substantia nigra* [4, 5]. Aggregation of truncated, full-length and partially insoluble α-Syn is found in LBs [4], characterising PD as a synucleinopathy [2].

α-Syn consists of 140 amino acids and had been described as a 'natively unfolded' protein previously, however, Bartels and co-authors described α-Syn from natural sources such as brain tissue as to a large extent folded, 58 kDa tetramer, which is proposed to destabilise and become susceptible to misfolding and aggregation in PD [6]. Interaction with lipids require the α-helical conformation of α-Syn, in contrast to an unfolded state found in the cytoplasm, which implies various roles for the protein depending on its cellular localisation [7]. α-Syn is mainly found at presynaptic vesicles which resulted in the hypothesis that it might be involved in vesicle availability and turnover [8]. *In vitro* studies have shown that α-Syn can be secreted into the culture medium and this increases with enhanced stress. Consequently, exocytosis of α-Syn may explain the spread of PD pathology within the brain [9]. Not only can α-Syn spread from cell to cell

under stressful conditions, but it can also translocate from the cytoplasm to the nucleus [10] as well as to mitochondria compromising the organelle's vital functions [11].

Familial PD frequently involves the A53T substitution of alanine to threonine as a missense mutation in *SNCA,* the α-Syn encoding gene [2]. This and other mutations cause α-Syn to be more susceptible to aggregation through gaining a toxic function, which is the main reason for its neurotoxicity [11]. Overexpression of the human α-Syn with the A53T mutation, which is more likely to form oligomers and aggregates, has been shown to induce serious motor impairment in mice and a particular inhibition of mitochondrial motility, as well as a disturbance in mitochondrial respiration and membrane potential [2, 12]. Additionally, it contributes to the inhibition of Complex I of the electron transport chain and an increase in levels of reactive oxygen species (ROS) [2, 13]. Similarly, wildtype (WT) α-Syn also causes Complex I inhibition and higher ROS production, but these effects occur later in the disease in comparison to A53T α-Syn [14]. α-Syn is found in the cytoplasm, nucleus and mitochondria [2]. It's migration into mitochondria is physiological since it contains a mitochondrial localisation signal and it specifically accumulates in the striatal and nigral mitochondria as part of PD pathology [14]. A study by Devi and colleagues found a significant association between the accumulation of α-Syn in mitochondria and subsequent decrease in the activity of Complex I, which is in accordance with studies utilising Complex I inhibitors and their findings of mitochondrial dysfunction and neuronal death [14].

ROS and consequent oxidative stress are thought to have a significant contribution to the pathology of PD [13, 15]. Patients suffering from neurodegenerative diseases including PD, exhibit higher than normal levels of ROS in their post-mortem brain tissues [15]. As well as causing mitochondrial dysfunction, ROS is a by-product of this dysfunction, causing a pathological cycle [15]. ROS include hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and superoxide ($O_2^{\bullet-}$) [14]. Activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an inflammatory enzyme that produces ROS, is present in PD in addition to other inflammatory enzymes including cyclooxygenase-2 (COX-2) [15]. Knockouts of these enzymes are able to reduce dopaminergic cell death, further supporting the pathological role of ROS in PD [15]. Inflammation is a hallmark of Parkinson's disease pathophysiology [16] as synucleinopathies are known to activate inflammatory pathways [17]. Pro-inflammatory responses were found after α-Syn was injected into mouse *substantia nigra*, including a significant increase in TNF-α mRNA, and enhanced levels of interleukin-6 (IL-6) have also been reported [17]. Inflammation has been suggested to play a major role in response to exogenous stress [18], providing tentative evidence for the involvement of environmental factors in PD.

In this study, the commonly used SH-SY5Y neuroblastoma cell line was differentiated and stably transfected with wild type and A53T mutated human α-Syn (both fused to EGFP), as well as a control vector containing EGFP only (Figure 1). Oxidative stress was induced in these cells by administering hydrogen peroxide (H_2O_2) in order to investigate the influence of the two α-Syn forms onto DNA damage under increased oxidative stress, and to determine whether the A53T mutation increases the susceptibility of the neuron-like cells to stress, in comparison to the effects of WT α-Syn. Additionally, COX-2, IL-6, TNF-α and p21 gene expression was analysed in order to better understand the role of α-Syn in PD-related inflammation and senescence. Finally, oxidative stress was measured as mitochondrial ROS generation using dihydroethidium (DHE) staining and flow cytometry.

2. Materials and Methods

2.1 *SH-SY5Y Cell Culture*

2.1.1 Culture of Undifferentiated SH-SY5Y Cells Culture and G418 Selection

Undifferentiated SH-SY5Y cells around population doubling (PD) number 21 (*ECACC* cell repository, London, UK) were maintained in DMEM/F12 medium (GIBCO) supplemented with 10% Fetal Bovine Serum (FBS, SIGMA), 2mM L-Glutamine (GIBCO), Penicillin/Streptomycin (100 IU/ml / 100µg/ml, (GIBCO), and 1% non-essential amino acids (SIGMA) under normoxic condition (37°C, 21% O_2 and 5% CO_2). 100 µg/ml concentration of G418 were found to kill untransfected cell within 2 weeks of selection and thus used for selection of stable cells transfected with plasmids containing the Neo gene (see Figure 1).

2.1.2 Transfection of SH-SY5Y Cells with pEGFP-*SNCA*-WT, pEGFP-*SNCA*-A53T and pEGFP-N1 (Control) Plasmids

Undifferentiated SH-SY5Y cells were seeded with a density of 50,000 cells/well in 12-well plates overnight. Plasmids were transfected with Lipofectamine 2000 (Invitrogen). Medium containing 100 µg/ml G418 concentration was used to get stably transfected cell lines. Stably expressing cells were maintained in 75 ml flasks with SY5Y medium containing 50 µg/ml G418. The maps of the 3 plasmids are shown in Figure 1.

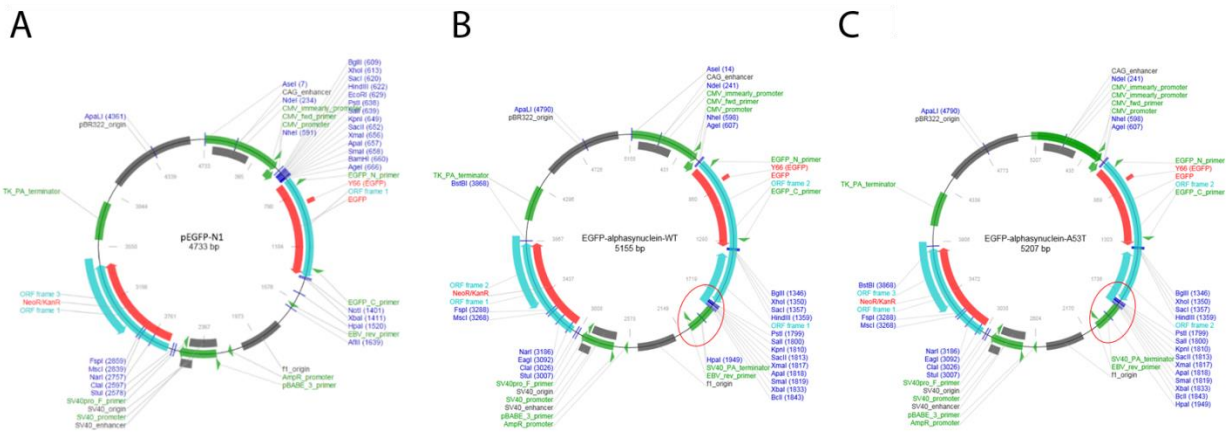

Figure 1 Maps of plasmids used for transfection. **A:** pEGFP-N1 plasmid (Clontech) containing the EGFP gene only used as vehicle control; **B:** plasmid containing the human WT *SNCA* gene fused to EGFP (Addgene); **C:** plasmid containing the human WT A53T mutated *SNCA* gene fused to EGFP (Addgene). For B and C the target sequences are shown in red circles. All three plasmids contain the Neomycin resistance gene (NeoR), which provides the resistance to G418.

2.1.3 Differentiation of the SH-SY5Y Cells into Neuron-Like Cells

Stably transfected cell lines were differentiated into more neuron-like cells. For this purpose, surfaces were coated with 0.005% calf-skin collagen. Cells were seeded initially into DMEM/F12

medium containing 1.5% FBS, 2 mM L-Glutamine, Penicillin/Streptomycin (100 IU/ml / 100 µg/ml), 1% non-essential amino acids (SIGMA) and 10 µM retinoic acid (RA) for 2 days. From then on medium was changed every other day with DMEM/F12 supplemented with 2 mM L-Glutamine, Penicillin/ Streptomycin (100 IU/ml / 100 µg/ml), 1% non-essential amino acid, 10 µM RA (SIGMA) and 2% B27 growth supplement (GIBCO) for 10 days to complete the differentiation. After the differentiation, cells in the 6-well plates were harvested for RNA isolation and cells in the 12-well plates with cover slips were used for H_2O_2 treatment.

2.1.4 H_2O_2 Treatment on Differentiated SY5Y Cells

Differentiated transfected SY5Y cells were treated with 100 µM and 200 µM H_2O_2 in serum-free DMEM/F12 medium for 2.5 hours. Afterwards, treatment medium was changed to serum containing medium for 3 minutes to neutralise H_2O_2. Afterwards, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 10 minutes, air dried and stored in PBS for immunofluorescent staining at 4°C.

2.2 Immunofluorescent Staining

Immunofluorescent staining was performed with rabbit anti-γH2A.X primary antibody (Cell Signaling), and AlexaFluor® Goat anti-rabbit594 (Invitrogen) secondary antibody to investigate the percentage of DNA damage positive cells. Briefly, coverslips were blocked with PBS containing 5% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.1% Triton X-100 for one hour. Afterwards the coverslips were incubated with the primary antibody (1:400 diluted in PBS containing 2% NGA and 1% BSA) for 1 hour at room temperature. After washing with PBS containing 0.005% Tween 20 and they were incubated with the secondary antibody (1:1000 diluted in PBS with 2% NGS and 1% BSA) for 30 minutes. After washing DAPI staining (CyStain® UV Ploidy, Sysmex) was applied for 10 minutes and coverslips mounted on glass slides using Vectashield mounting medium (Vector Laboratories) Images (at least 5 per coverslip and condition) were taken with the Leica fluorescent microscope DM5500 and data were analysed with ImageJ software. All experiments were repeated at least twice to ensure reproducibility.

2.3 Polymerase Chain Reaction for Gene Expression

2.3.1 RNA Isolation and Reverse Transcription

Cells from 6-well plates were trypsinised and collected as cell pellets by centrifugation. RNA from each pellet was isolated by the RNeasy® kit (QIAGEN) according to the manufacturer's protocol. The concentration of isolated RNA was measured by a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Inc.). For the reverse transcriptase reaction, 1 µg RNA was mixed with 1 µl of random primers (Promega) and RNase/DNase-free water (QIAGEN) for a total volume of 11 µl and denatured at 75°C for 7 min followed by immediate chilling on ice. Afterwards, the denatured RNA was mixed with 4 µl 5X First Strand Buffer (Invitrogen), 2 µl 0.1 M DTT (Invitrogen), 1 µl 10 mM dNTP (Thermo Scientific), 1 µl RNase Inhibitor (Applied Biosystems) and 1µl Superscript III Reverse Transcriptase (Invitrogen). The reverse transcriptase reaction was

performed in a thermal cycler (Hybaid) with a programme consisting of a 90 min 42°C reaction step and a 5 min 95°C enzyme inactivation step. The RT product (cDNA) was then stored at -80°C.

2.3.2 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was applied to analyse the expression of inflammation and senescence markers in SH-SY5Y cells. Primers sequences are described in Table 1 and the amplification efficiency for each primer pair was confirmed by standard curve analysis. For the qPCR analysis, 1 µl cDNA was mixed with 5 µl mastermix of the SensiFAST™ SYBR® Hi-ROX Kit (Bioline), 3 µl RNase/DNase-free water, 0.5 µl forward primer (10 µM), and 0.5 µl reverse primer (10 µM) in each reaction well of an optical 96-well reaction plate or 8-well reaction strip. Every sample was prepared in triplicates to reduce the handling error and the qPCR was implemented on a Step One Plus instrument (Applied Biosystems). The programme contained a 2 min heating at 95 °C for enzyme activation, 40 cycles consisting of a 5s 95 °C step for denaturation and 20 s 60 °C step for annealing and extension in each cycle, as well as a melt curve analysis step, which increased the temperature gradually from 68 °C to 95 °C to record the melting temperature of the products, for the determination of product purity.

The expression of target genes (for primer sequences please see Table 1) was normalised to the expression of a housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the results were analysed in the format of comparative threshold cycle ($2^{-\Delta\Delta CT}$). All PCR analysis were repeated twice to ensure reproducibility.

Table 1 Primer sequences for gene expression analysis.

Gene	Primer Sequence (5' -> 3')
GAPDH	Fwd: TGCACCACCAACTGCTTAGC Rev: GGCATGGACTGTGGTCATGGAG
COX-2	Fwd: GCTCAAACATGATGTTTGCATTC Rev: GCTGGCCCTCGCTTATGA
IL-6	Fwd: GTAGCCGCCCCACACAGA Rev: CATGTCTCCTTTCTCAGGGCTG
TNF-α	Fwd: GGAGAAGGGTGACCGACTCA Rev: CTGCCCAGACTCGGCAA
P21	Fwd: GCAGACCAGCATGACAGATTT Rev: GGATTAGGGCTTCCTCTTGGA
SNCA	Fwd: GTGGCTGCTGCTGAGAAAAC Rev: CACCACTGCTCCTCCAACAT

2.4 Flow Cytometry Analysis (FACS)

Cells were trypsinised and stained with DHE (Dihydroethidium, Molecular Probes, Invitrogen) at a concentration of 10 µg/ml for 30 minutes in the dark. Cells were then analysed at FL3 (red fluorescence) in a Partec Flow cytometer (Partec, Germany). The instrument was calibrated with multifluorescent particles (Polysciences) and values for unstained samples were subtracted from the FL3 values to correct for autofluorescence.

2.5 Statistical Analysis

One or Two Way ANOVA together with Holm-Sidak post-hoc test were used in *SigmaPlot* v12.5 software. Statistical significance was set at $p < 0.05$.

3. Results

3.1 SH-SY5Y Cells Decrease Proliferation and Show Neuron-Like Characteristics Following Differentiation

All three cell lines differentiated during the 12 days under treatment with retinoid acid (RA) without any obvious morphological differences. Differentiated cells acquired neuronal-like processes as demonstrated by immunofluorescence staining with the neuronal marker beta III-tubulin (Figure 2 B and C) while undifferentiated cells (Figure 2A) have a rather diffuse pattern of βIII-tubulin. As expected during cellular differentiation, proliferation decreased gradually from over 90% in undifferentiated cells to less than 20% on day 10 of differentiation (Figure 2D). Maintenance of differentiation seems to depend heavily on retinoid acid since its removal reverses differentiation and increases proliferation (data not shown).

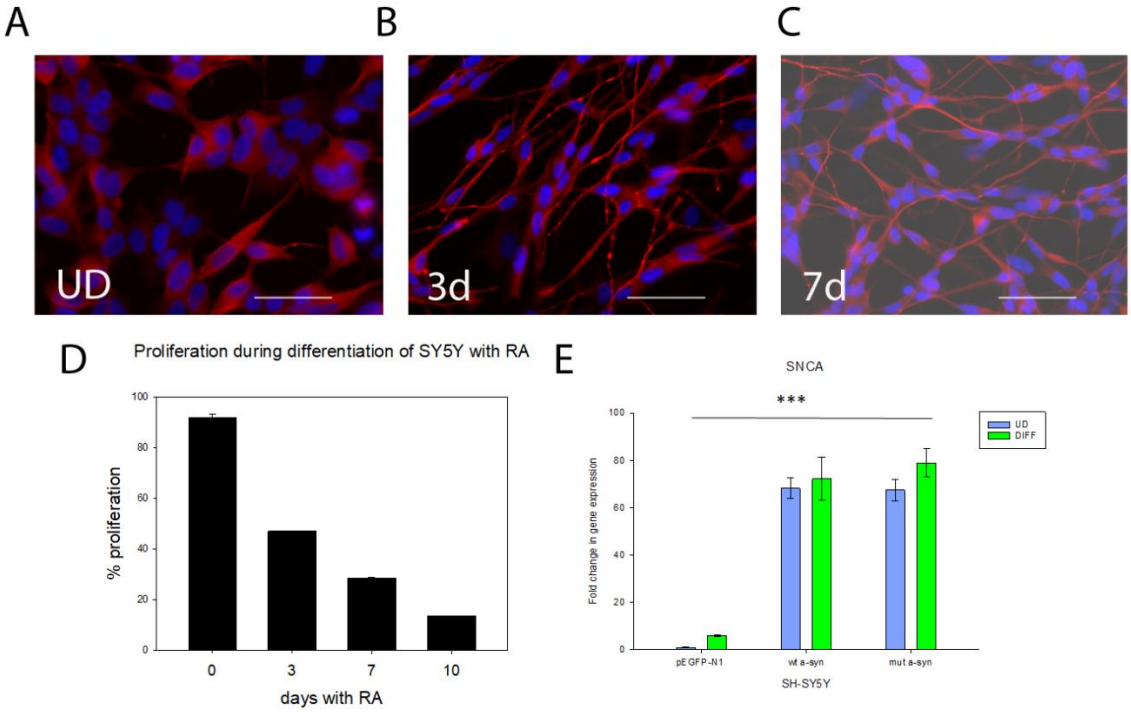

Figure 2 Characterisation of differentiation stages of SH-SY5Y cells into neuron-like cells and expression analysis of SNCA. **A-C:** βIII tubulin staining (red) and DAPI (blue) on undifferentiated (UD, A) and 3 (B) and 7 (C) days differentiated SH-SY5Y cells. **D:** Ki67 staining to characterise the proliferation ability of differentiating SH-SY5Y cells on indicated days of differentiation. **E:** qPCR analysis of SNCA expression in undifferentiated (UD, blue bars) and differentiated (DIFF, green bars) SY5Y cells transfected with either EGFP only or wt *SNCA*-EGFP and mut *SNCA* -EGFP. Statistics for E has been performed using a Two Way ANOVA where no difference was found due to differentiation, but of transfection with α-synuclein containing plasmids. All data were compared to the undifferentiated EGFP control. *** $p < 0.001$.

Additionally, we characterised the expression level of α-Syn in undifferentiated and differentiated cells using qPCR. While we found a high expression level of overexpressed α-Syn there was no difference between cells expressing WT or mutated α-Syn. There was also no change during differentiation in these two cell lines (Figure 2E). These results suggest that differences we found between the two α-Syn containing cell lines are not due to *a priory* different expression levels in our cell model. However, we did not analyse whether the expression levels might change during acute stress treatment with H_2O_2.

3.2 Differentiated A53T SNCA Expressing SH-SY5Y Show Higher Sensitivity to Oxidative Stress While WT SNCA Expression Seems to have a Protective Effect

There was no difference in basal DNA damage level in all 3 cell lines (Figure 3A) there was a trend for increased DNA damage in A53T containing cells for 100 µM H_2O_2 treatment (Figure 3B). However, the control and A53T *SNCA* expressing cells showed an increase in DNA damage under 200 µM H_2O_2 treatment compared to the WT *SNCA* expressing cell line as visualised by nuclear signals from the yH2A.X antibody (Figure 3 D-F). Importantly, the level of cells containing DNA damage in WT *SNCA* expressing cells stayed at the same level under all conditions rendering these cells apparently resistant to the oxidative stress suggesting a potentially protective effects of WT α-Syn in the differentiated SH-SY5Y cells to oxidative stress.

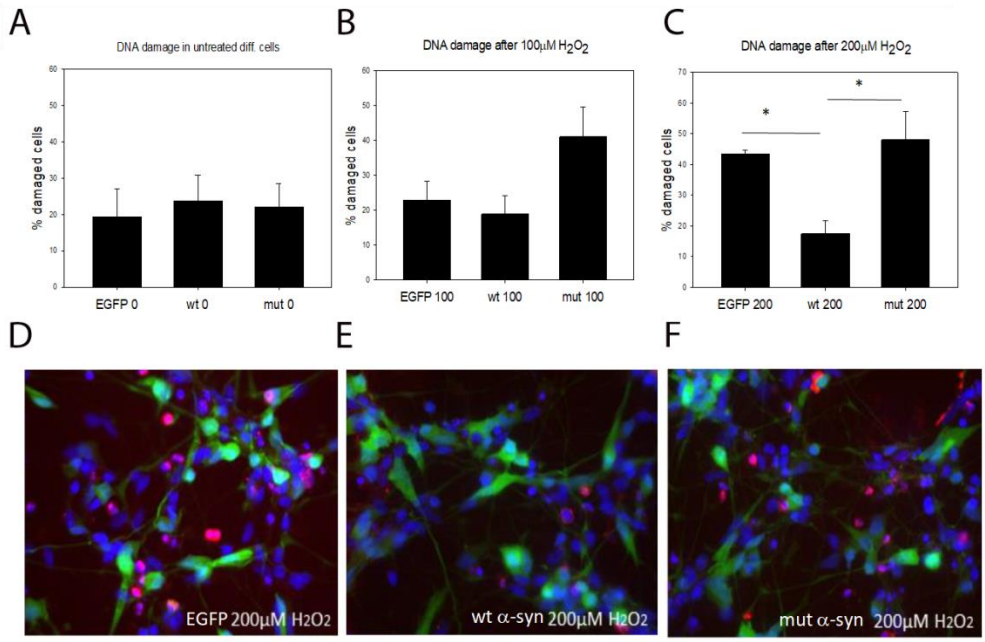

Figure 3 No increase in DNA damage in in differentiated SH-SY5Y cells with wild type α-synuclein under H_2O_2 treatment while A53T containing and control cells are more sensitive to oxidative stress. **A-C:** The amount of damaged was quantified by counting damage positive cells using fluorescence microscopy images and expressed as a percentage of total cell numbers after stress induction with 0 µM, 100 µM and 200 µM of H_2O_2 respectively. Bars represent mean and SEM from 3-4 biological repeats. One way ANOVA with Holm-Sidak test. * $p < 0.05$. **D-F:** Representative images of cells transfected with EGFP, wt or A53T α-Syn at 200 µM H_2O_2. Green signal: EGFP, red: H2A.X/DNA damage foci, blue: DAPI nuclear stain.

3.3 A53T SNCA Expression Increased Inflammatory and Senescence Markers in Differentiated SH-SY5Y Cells under Oxidative Stress

To determine whether *SNCA* expression in differentiated SH-SY5Y cells resulted in increased inflammation and cellular senescence, the expression of different inflammatory markers (COX-2, IL-6 and TNF-α) and a stress- and senescence marker (p21) were analysed by RT-qPCR. There was a similar tendency for the three inflammatory markers and p21 as for the analysed DNA damage: the expression of A53T *SNCA* dramatically increased cellular inflammation after treatment with 200 µM H_2O_2 compared to cells with either WT α-Syn or EGFP only controls (Figure 4 A-C). Corresponding to the lack of increase in DNA damage the WT SNCA expressing cell line did not show any increase in expression of any inflammatory marker after treatment with 200 µM H_2O_2 and even significantly decreased it. For the stress marker p21 there was a slight increase in WT *SNCA* expressing cells while A53T expressing cells were higher than controls and WT *SNCA* under increased stress.

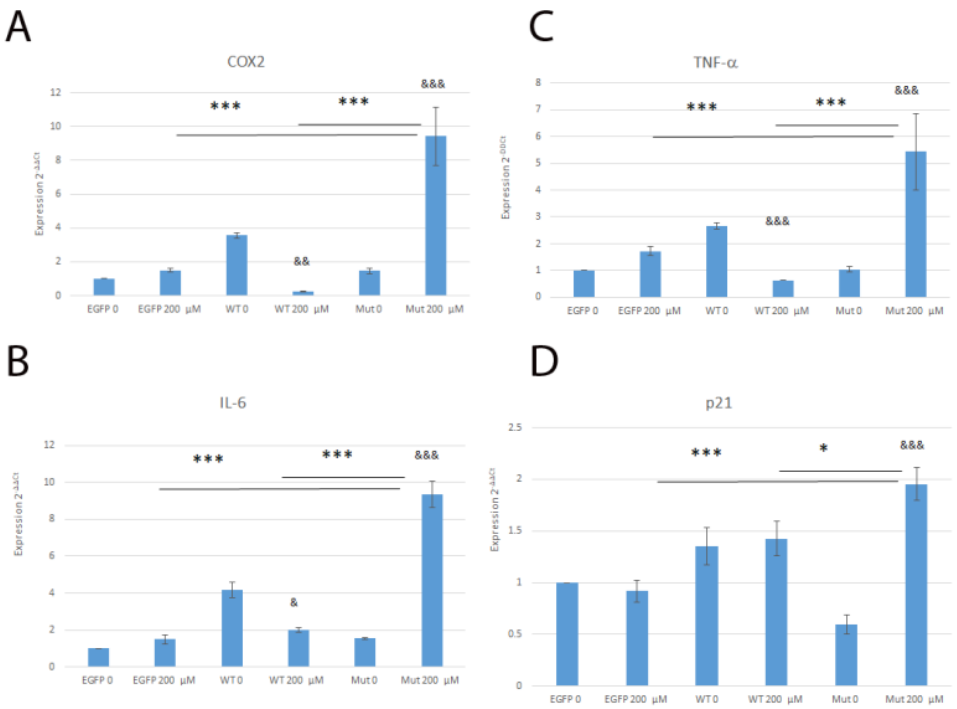

Figure 4 Expression of inflammatory and senescence markers of treated and untreated differentiated cells. Expression of COX-2, IL-6, TNF-α and p21, was analysed by RT-qPCR. Results are shown as the $2^{-\Delta\Delta Ct}$ value averages of three technical repeats and error bars represent SEM. **A-C:** Inflammatory marker (COX-2, IL-6 and TNF-α) expression in differentiated cells, respectively. **D:** Stress-and senescence marker p21. Two Way ANOVA was performed for the influence of the genotype and the treatment with Holm-Sidak post-hoc test. ***/$^{\&\&\&}$p<0.001, **/$^{\&\&}$p<0.01, */$^{\&}$p<0.05. &: comparison between treatments within the same genotype, *: comparison between genotypes within the same treatment (H_2O_2).

3.4 Alpha-Synuclein Expression Causes Higher Mitochondrial ROS Generation in Undifferentiated and Differentiated SH-SY5Y Cells

In order to analyse whether the expression of α-Syn changes superoxide levels, we stained cells with the dye DHE and measured red fluorescence using flow cytometry. As shown in Figure 5 A, B there was an increase in mitochondrial ROS levels in both, undifferentiated and differentiated cells harbouring α-Syn with the latter having in general higher ROS levels than the former. However, there was no difference between cells expressing wild type and A53T mutated α-Syn. This data suggest that there is no initial difference in ROS prior to treatment with H_2O_2 in the two α-Syn expressing cell lines.

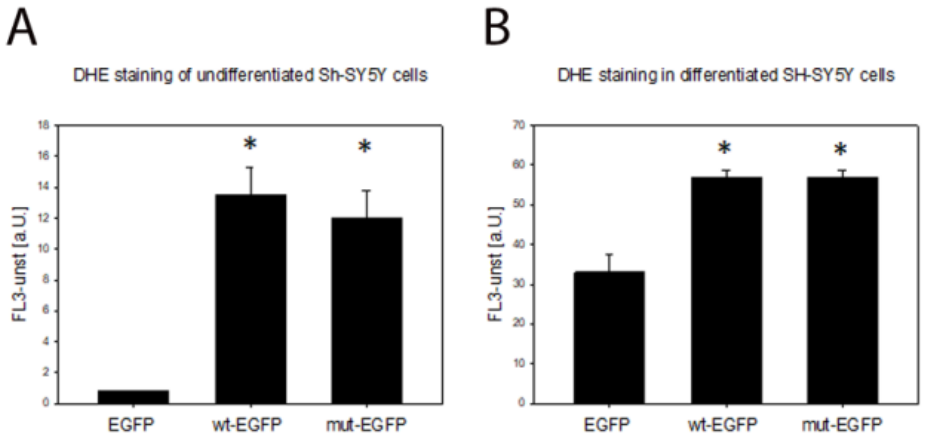

Figure 5 Cellular superoxide levels in undifferentiated **(A)** and differentiated **(B)** SH-SY5Y cells. DHE staining was performed and red fluorescence (FL3) measured in a Flow Cytometer. Values for unstained cells (autofluorescence) were subtracted. One way ANOVA was used to assess significance and α-synuclein containing cells were compared against control (EGFP only) * $p < 0.05$.

4. Discussion

In this study, SH-SY5Y human neuroblastoma cells transfected with wild type or A53T mutated *SNCA* in a differentiated state were used as a cellular model of Parkinson's disease to quantify the levels of DNA damage, inflammation and senescence present after induction of stress.

Unexpectedly, there were no differences in *SNCA* gene expression neither between differentiated and undifferentiated cells nor between WT and mutated α-Syn in either differentiated or undifferentiated state. It is well accepted that both ageing and the increase in oxidative stress are important promoting factors for the development of PD and other age-related neurodegenerative diseases in addition to the presence of α-synuclein. We focussed on the use of differentiated cells since we did not find any major differences between cell lines and treatments in undifferentiated cells (data not shown). It is possible that α-Syn as a synaptic protein [8] requires a certain differentiated state with neuronal processes which are not present in undifferentiated neuroblastoma cells which are cancer cells. Morphological similarities between human neurons and differentiated neuroblastoma cells have been described recently together with a marked increase in gene expression of synaptic components and dopamine processes in differentiated cells [19].

Our results show that during differentiation there is seems to be a higher sensitivity to oxidative stress in the A53T mutated *SNCA* expressing cells compared to EGFP only controls as well as WT *SNCA* expressing cells which included a higher DNA damage level as well as a consistently higher expression of inflammatory and senescence markers.

The A53T mutation is causally involved in early-onset familial PD [20], while WT α-Syn is predominantly associated with spontaneous PD [21]. Surprisingly, we found that WT α-Syn might even have a protective effect under oxidative stress, as shown by the lower levels of DNA damage compared to the mutated α-Syn, under increased oxidative stress. This could be connected to its physiological function at synaptic vesicles, their transport and regeneration. However, we were not successful in determining differences in synaptic proteins using immuno-staining in these cell lines (data not shown).

Possibly, the resilience of WT α-Syn to increased oxidative stress may also explain the later onset of sporadic PD, in which case higher levels of or prolonged, chronic exposure to stress could trigger the effects required to drive this WT α-Syn into a PD-causing state. The role of increased oxidative stress in the pathology of neurodegenerative disease including PD is well established [22]. In addition, it is known that complete knock-out of WT α-synuclein has a detrimental phenotype in mice [23]. Previous studies using primary cortical neurons from α-synuclein knock-out and wild-type mice have also demonstrated that endogenous levels of α-synuclein provide resistance to oxidative stress and apoptosis through the inhibition of the MAPK signalling pathway [24].

Neuro-inflammatory processes are strongly implicated in PD pathology, with aggregating α-Syn driving initiation and acting as a mediator [25]. We found that differentiated cells with A53T α-Syn, displayed the highest levels of inflammation. This seems in good accordance with previous studies on mouse models. A study using transgenic mice with overexpression of A53T α-Syn implicated this mutation in chronic neuro-inflammation, with consequential α-Syn aggregation, formation of Lewy body (LB)-like inclusions and death of dopaminergic neurons [26]. Another study that used mice with transgenic A53T α-Syn, found that oligomers used in the formation of LBs were recognised by antibodies that bind to α-Syn oligomers after they have undergone oxidation [27]. α-Syn accumulation accelerated as a result of oxidative stress and there was significant neurodegeneration, as opposed to the oligomers from non-LB regions, which showed no neurotoxicity and later progression to fibril formation [27]. Oxidative stress can be generated by different cellular systems: NADPH oxidase over-activation and consequential ROS formation is able to promote oxidation of α-Syn, followed by its aggregation [26]. Furthermore, mitochondrial dysfunction and an ineffective ubiquitin-proteasome system can arise from accumulation of aggregated α-Syn [26]. This detrimental α-Syn can also be released from the cell, thereby enabling it to activate microglia that release more pro-inflammatory cytokines also resulting in more ROS, leading to neuronal death and inducing a vicious cycle of chronic inflammation characteristically seen in PD [25].

Interestingly, cytoplasmic α-Syn preferentially displays the A53T mutation, which also increases the likelihood of its aggregation [28]. Additionally, the speed of nuclear shuttling of α-Syn increases with the presence of the A53T mutation, suggesting an alteration to its normal physiology [9]. The nuclear functions of α-Syn are unknown, but may encompass inhibition of acetylation, histone interactions and chromatin binding alterations, causing DNA damage and ultimately neurotoxicity [28]. Recently, mechanistic links have been uncovered between DNA

damage and the immediate occurrence of inflammation, well before any senescence can develop. This is due to the recognition of nuclear and mitochondrial DNA fragments after occurring DNA damage which activate the cGas-STING pathway, NF-κB and downstream inflammation [29]. Thus, our results regarding increased DNA damage and inflammation provide some novel data in confirming this connection in a neuronal model setting. Moreover, the protective effect of WT α-Syn in our system consistently correlates to less DNA damage under increased oxidative stress together with no increase and even a decrease in acute inflammation after H_2O_2 treatment in this genotype. However, we do not yet understand the mechanism for the higher resistance of WT α-Syn compared to cells with no α-Syn overexpression or overexpression of the A53T mutant.

p21 is an acute stress-related marker downstream of p53 that also plays an important role in the establishment of senescence, even in postmitotic neurons [30]. While short exposure to H_2O_2 can induce an acute stress response, we were also interested to examine whether the two α-Syn forms can induce senescence-associated gene expression.

Senescence can be triggered by a DNA damage response, enabling p21 activation [31]. In addition, ROS, through various mechanisms, are likely to mediate senescence as a result of stress and cause mitochondrial dysfunction, producing even more ROS, and eventually lead to cellular senescence [31]. During senescence, an increase in p21 occurs initially, followed by increase in p16 levels that are thought to maintain the senescent state [31]. DNA damage due to ROS and an abundance of unrepaired double-stranded breaks upregulate p21 downstream of p53 [32]. Similar to DNA damage and inflammatory markers, we found an increase in p21 expression predominantly in the A53T *SNCA* expressing cells but there was also a moderate increase in p21 in cells expressing WT α-Syn which could be a more general feature of α-synuclein overexpression. Postmitotic cells such as neurons have been shown to display a senescent phenotype with increased inflammation and DNA damage [30]. We found an increase in mitochondrial ROS generation in differentiated cells harbouring the two α-Syn types compared to undifferentiated cells. Increased oxidative stress due to wild type α-Syn expression in SH-SY5Y cells has been described recently [13]. However, we did not find any differences between wild type and A53T mutated α-synuclein regarding superoxide levels without any additional oxidative stress. The identical increase in ROS parallels the similar expression levels of both α-synuclein forms as well as the lack of differences in DNA damage and expression of inflammatory genes without any additional oxidative stress. However, we cannot exclude that ROS levels are different between the two α-Syn types after increased oxidative stress. Other, more suitable treatment methods for ROS measurement such as paraquat should generate a better understanding of this topic in the future.

Together, our results suggest that a differential sensitivity to increased oxidative stress of wild type or A53T α-synuclein-bearing neuronal cells might be responsible for the accumulation of DNA damage in line with inflammation and senescence in the mutated form. This could be potentially due to different regulation of antioxidant enzymes or other, yet unknown factors such as different subcellular localisation of different α-Syn forms [28] or the potential differences in binding of A53T α-Syn to phospholipids compared to WT α-Syn [21].

5. Conclusions

Our results reveal increased DNA damage, inflammation and senescence in differentiated neuroblastoma cells harbouring the A53T mutation of α-Syn compared to control cells or those

expressing wild type SNCA. Importantly, we found that wild type α-Syn seems even to provide a protective effect regarding the accumulation of DNA damage during oxidative stress correlating to a decrease in the acute expression of selected inflammatory markers. Although the underlying mechanism remains elusive at this stage, our results correlate well to a later onset of sporadic PD compared to its mutated forms such as A53T which demonstrated higher DNA damage, inflammation and senescence induction in our cellular model.

Author Contributions

TW generated the cell lines, performed experiments and helped writing the manuscript, AK, CH, PS, MH, SN and JM performed experiments and analysis, AI performed data analysis and GS designed the study and wrote the manuscript.

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Competing Interests

The authors declare that no competing interests exist.

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