SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The role of Drp1-a mitochondrial fission protein- in mitochondrial morphology rearrangement during neuronal differentiation

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1. Introduction

Mitochondria divide during cell division to be distributed to the daughter cells. Even quiescent cells show mitochondrial division during cellular differentiation and cell growth or in response to external signals. The processes of mitochondrial division and fusion must be closely regulated, as the survival of a cell relies on maintaining a sufficient number of mitochondria within it. The mechanisms behind these processes are probably quite intricate due to the double membranes of mitochondria, which pose unique topological and energetic challenges (E. Smirnova, L. Griparic, D. L. Shurland, & A. M. van der Bliek, 2001). The proteins that play a vital role in the process of mitochondrial fusion are three GTPases: MFN1, MFN2, and OPA1 (H. Chen, Chomyn, & Chan, 2005). Conversely, dynamin-related protein 1 (Drp1) and Fis1 have been recognized as part of the fission mechanism. (E. Smirnova et al., 2001). Drp1 is part of the dynamin superfamily (DSP). Dynamins are a family of large GTPases. Dynamin, the classical member of this family, coils around the endocytic vesicles and aids in separating them from the cell membrane (Hinshaw, 2000).

Drp1 contains four distinct regions. At the protein's N-terminus, there is a large GTPase domain essential for the binding and hydrolysis of GTP. After the GTPase domain is the middle domain necessary for the self-assembly of the Drp1 protein. Next is a variable domain with a B insert specific to Drp1 (Michalska, Duszyński, & Szymański, 2016). At the protein's C-terminus end, an effector domain (GED) is associated with GTP hydrolysis (Sesaki, Adachi, Kageyama, Itoh, & Iijima, 2014). Human fibroblast 5756-Ti cells modified to express a dominant negative form, Drp1 (DLPS39N), exhibited large, networked peroxisomes due to decreased GTP affinity (X. Li & Gould, 2003). X-ray structural analysis established that the Ser39 amino acid plays a crucial role in Drp1's interaction with the α-phosphate of GTP. This residue is essential for GTP function as it is situated within the phosphate binding loop, also known as the P-loop. The sequence pattern (GSQSSGKSS) containing the underlined Ser39 residue is widely preserved. Ser39 plays a role in binding to the α-phosphate through hydrogen bond interaction via its backbone. Still, it does not interact with the β-phosphate, unlike the corresponding Ser residues in dynamin-1 at that position (Wenger et al., 2013).

Endogenous Drp1 is located in cell cytosol and, upon stimulus, moves around the mitochondria and is crucial in mitochondria division. Time-lapse imaging of cells transfected with Drp1 fused

with green fluorescent protein showed assembly at specific sites of mitochondrial division. Like the dynamin family, Drp1 forms a ring-like structure around the mitochondria (E. Smirnova et al., 2001). Any imbalance in the fusion and fission cycle of mitochondria leads to various abnormalities, contributing to different neurodegenerative diseases such as Huntington's disease (HD), Alzheimer's disease (AD), and Parkinson's disease (PD), as well as some diseases such as cancer, cardiomyopathies, and several metabolic disorders (Oliver & Reddy, 2019; Reddy et al., 2011). In Huntington's disease, the mutant huntingtin protein (Htt) binds with Drp1, boosting its enzymatic activity, which leads to excessive fragmentation and abnormal distribution of mitochondria in HD neurons. The abnormal interaction of mutant Htt and Drp1 causes mitochondrial damage and synaptic degeneration (Shirendeb et al., 2012). In Alzheimer's disease, Drp1 interacts with monomers and oligomers, and these atypical interactions advance as the disease progresses. Loss of neuronal branches and degeneration were observed in neurons containing Aβ oligomer aggregates, suggesting a potential link between oligomeric Aβ and neuronal degeneration (Manczak, Calkins, & Reddy, 2011). In Parkinson's Disease (PD), mutated α-synuclein interacts with spectrin, a cytoskeleton protein causing alteration in F-actin dynamic. This altered dynamic causes dysfunctional mitochondria by relocating Drp1 (Ordonez, Lee, & Feany, 2018).

Drp1 is crucial for the growth, function, and survival of neurons. Mice with a specific deletion of $Drp1^{-/-}$ died soon after birth due to nervous system development issues (Ishihara et al., 2009). Drp1 perturbations displayed brain development defects, indicating the necessity of Drp1-mediated mitochondrial fission for neuronal development. In developing chick motor neurons, mitochondria became shorter over time alongside an increase in Drp1 expression (S. Y. Choi et al., 2013). Blocking Drp1 activity led to longer mitochondria, suggesting that increased Drp1 expression contributed to adjusting mitochondrial length during development. Conversely, cultured mouse cortical neurons are known to experience gradual elongation of mitochondria. A significant change occurs in mitochondrial structure during the conversion from somatic cells to induced pluripotent stem cells (iPSC). During reprogramming, the pro-fission factor Drp1 undergoes phosphorylation, and both its knockdown and inhibition hinder mitochondrial fragmentation and iPSC cell formation (Prieto et al., 2016). Drp1 plays a crucial role in developing human iPSCs into cardiac mesodermal cells. Blocking Drp1 activity enhances the differentiation of induced iPSCs in cardiac

cells, shifting mitochondrial behavior towards fusion and altering energy production from glycolysis to oxidative phosphorylation (Hoque et al., 2018).

Our findings highlight that shDrp1 displays a fused mitochondrial structure. Drp1 knockdown leads to an increase in genes related to the development of the nervous system. High-Content Analysis showed enhanced growth and formation of nerve cell projections, segments, and extremities in differentiated shDrp1 cells. This study sheds light on the process of neuronal differentiation linked to a decrease in ERK1/2 phosphorylation independent of dual specificity phosphatases 1 and 6 (DUSP1/6) in shDrp1 cells. Our research demonstrates that control cells undergo changes in mitochondrial shapes while shDrp1 cells maintain highly fused mitochondria during differentiation. We also mention that control and shDrp1 cells exhibit similar responses to specific triggers for programmed cell death. We also show that reducing Drp1 leads to a decrease in huntingtin protein aggregate formation under experimental conditions. Our findings indicate that Drp1 plays a crucial role in neuronal differentiation. Knockdown of Drp1 leads to extensive changes in gene expression. It enhances RA-BDNF-induced neuronal differentiation in SH-SY5Y human neuroblastoma cells by enhancing longer neurites and more roots, neurite segments, and extremities by increasing cellular bioenergetics during differentiation. We also highlight the importance of the Drp1 GTPase domain and its role in maintaining mitochondrial morphology.

2. Aims and objectives

This study investigates the role of Dynamin-related protein 1 (Drp1) on neuronal development and mitochondrial remodeling during differentiation. This study seeks to analyze the effect of Drp1 knockdown on neuronal differentiation and morphology using SH-SY5Y human neuroblastoma cells. This study explores the signaling pathways activated during neuronal differentiation and changes in other mitochondrial proteins related to the fusion/fission cycle Drp1 is stably depleted. This research will employ different methods and techniques, including High-Content analysis, transmission electron microscopy, RT-qPCR, and RNA sequencing, to assess the changes in differentiated and undifferentiated neuroblastoma cells. The research hopes to lay the groundwork for future studies on mitochondrial dynamics, cellular ATP production, and neuronal death related to Drp1 by addressing these objectives. Therefore, Drp1 could provide future insight for understanding the therapeutics for treating neurodegenerative disorders.

3. Materials and Methods

3.1. Cell culture

SH-SY5Y human neuroblastoma cells (European Tissue Culture), modified for DNM1L knockdown and control (unmodified), were cultured in high-glucose DMEM supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, and 1x (V/V) antibiotic-antimycotic solution (Gibco), at 37 ºC in a 5% CO_2 incubator. HEK293T cells were grown in DMEM with conditions and supplements similar to SH-SY5Y cells.

3.2. *hDNM1L*/hDrp1 knockdown

We used lentiviral technology to reduce the expression of h*DNM1L*/hDrp1 as described by (Abdennour Douida et al., 2020).

3.3. Titration of antibiotic selection

The concentration of the selected antibiotic was adjusted to identify the most effective dosage for selecting transduced target cells to establish a stable cell line effectively. 1.25 µg/ml concentration initiated significant cell death within 7 days, leading to complete cell death by day 10. This specific concentration was chosen for subsequent selection and maintenance.

3.4. Quick change mutagenesis

The mCherry-Drp1 S39A mutant was created using the manufacturer's quick-change site-directed mutagenesis protocol (cat. 200523, Agilent Technologies). The mCherry-Drp1 WT plasmid utilized in this research was a generous gift from Gia Voeltz (Addgene plasmid # 49152; http://n2t.net/addgene:49152; RRID: Addgene_49152) (J. R. Friedman et al., 2011). The mCherry control vector was a gift from Dr. György Vámosi (Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, Hungary).

3.5. Transfection and generation of stable cell lines

Cells were transfected with the mCherry control, mCherry-Drp1 WT, and mCherry-Drp1 S39A plasmids using Lipofectamine 3000 (cat. L3000001, Thermofishers Sci.) as per the manufacturer's guidelines. After 72 hrs of transfection, cells underwent geneticin antibiotic selection at a concentration of 800 µg/ml (cat. 11811023, Thermofisher Sci.) to establish stable cells expressing.

3.6. Neuronal Differentiation

The SH-SY5Y cells underwent differentiation following the method (Forster et al., 2016) described, with a slight modification of using 3% heat-inactivated FBS in phase 1 media.

3.7. Live cell imaging

To stain the cells with neuron-specific ß-tubulin III, 5000 cells per well were seeded in a 96-well plate (Corning, Merck) coated with 5 ug/cm^2 laminin at 37 ºC for two hours before seeding cells. On Day 6, cells were treated with 1 µM Tubulin Tracker Deep Red (Thermofisher Sci.) to observe neurite outgrowth and with 10 µM Hoechst 333642 for nuclei staining for 30 minutes at 37 ºC and 5% CO_2. After staining, the cells were washed twice with HBBS (Thermofisher Sci). Live cell images were captured at 37 ºC with a 5% CO_2 Opera Phoenix High Content Screening system (Perkin Elmer).

3.8. Neurite Analysis

Automated confocal microscopy was performed on an Opera Phenix High Content Analysis system. Image-acquisition settings were 40x water objective (NA = 1.1), and appropriate lasers and filters for Hoechst, eGFP, and Tubulin Tracker Deep Red were used sequentially to exclude spectra overlap. Neurites were detected with a 16-bit camera under nonsaturating conditions. Quantitative image analysis was performed with the built-in software (Harmony 4.9, PerkinElmer). Cell segmentation was based on Hoechst and eGFP staining to detect the nuclei and cytoplasm, respectively. The neurite analysis utilized the “CSIRO neurite Analysis 2” method of the Harmony 4.9 software as described by (D. Wang et al., 2010).

3.10. RNA Extraction

According to the manufacturer's instructions, total RNA was isolated using a TRI reagent (Molecular Research Center, lnc). Before reverse transcription with random primers, samples were treated with DNase I in a DNA digestion buffer for 15 minutes. The cDNA synthesis was carried out by reverse transcribing 1 µg of total RNA using a High-capacity cDNA Reverse Transcription Kit

(Applied Biosystems). A quality check of cDNA was conducted by loading a 1 µl sample onto a 1% agarose gel.

3.11. Quantitative Real-Time PCR

Real-time PCR was carried out using a Lightcycler 480 Thermocycler (Roche) and Xceed qPCR Probe 2x Mix Hi-ROX (Institute of Applied Biotechnologies, IAB) per the manufacturer's instructions. CT values (threshold values) were normalized to GAPDH and/or PPIA, with each biological replicate including at least three technical replicates per gene in every sample. Gene expression fold change relative to undifferentiated cells was determined using the ΔΔCT method (Livak & Schmittgen, 2001).

3.12. Western Blotting

Cells were initially washed with 1x phosphate buffer saline (PBS) and then lysed in RIPA buffer containing 50 mM Tris-HCL Ph 7.4, 150 mM NaCl, 0.5 % Na-deoxycholate, 2 mM EDTA, 1% NP-40, and 50 mM NaF. The lysis buffer was supplemented with a protease inhibitor cocktail consisting of 1 mM benzamidine, 1 mM PMSF, and a cOmplete Mini-EDTA-free protease inhibitor cocktail. Following this step, wells containing 20 µg of protein were prepared and resolved on SDS-PAGE. The resolved proteins were then transferred to the nitrocellulose membrane using the wet transfer method from GE Healthcare Life Sciences. Samples that received primary antibodies underwent overnight incubation at 4 ºC and afterward were incubated with secondary antibodies for two hours at room temperature. The blots were developed using an enhanced chemiluminescence western blotting detection system from Santa Cruz Biotechnologies.

3.13. Mitochondrial Staining for High Content Screening Live Cell Confocal Imaging

To label the mitochondria, laminin was applied to 96 well-plate at a concentration of 5 µg/cm^2 and incubated at 37 ºC for two hours before seeding 5000 cells in each well. The control and shDrp1 neuroblastoma cells underwent neuronal differentiation using the designated protocol. On Day 6 of the differentiation process, the cells were washed with 1x PBS and then stained with a solution containing 100 nM Mitotracker Red CMX ROS and 10 µM Hoechst 333242 in serum-free media for 20 minutes at an optimal temperature of 37 ºC, inside a 5% CO_2 incubator. Subsequently, fresh complete DMEM was added as replacement media prior to conducting live cell imaging using an Opera Phoenix High-Content Screening system by Perkin Elmer operating under consistent conditions of 37 ºC and 5% CO_2. The image-acquisition procedure entailed employing specific settings: usage of water objective lens rated at 63x magnification power with NA = 1.15; appropriate lasers along with filters were used for Hoechst staining, eGFP, and a detection signal

from Mitotracker Red conducted sequentially to exclude any potential interference arising due to shared spectra overlaps. Image acquisition occurred via the employment of non-saturating conditions-driven operation facilitated using a 16-bit camera setup.

3.14. Mitochondrial Morphology Analysis

Opera Phenix HCA system was used for live cell imaging. The system configuration included a water objective (63x, NA=1.15), and filters were adjusted for mCherry, Hoechst 33342, and Mitotacker Green for mCherry, mCherry Drp1, and mCherry Drp1 mutant S39A cell to prevent spectral overlap. However, for control and shDrp1 cells, eGFP, Hoechst 33342, and Mitotracker Red channels were selected. A 16-bit camera under nonsaturating conditions was used for imaging detection after determining the most suitable z-frame position. Image analysis used Harmony 4.9 software with different fluorescence staining for cell segmentation.

3.15. Seahorse Assay

Oxygen consumption rate was assessed in undifferentiated cells at Day 0 and during phases 1 (Day 3) and 2 (Day 6), comparing the control and shDrp1 cells. For mCherry control, mCherry Drp1, and mCherry Drp1 S39A cells, the OCR was measured after 24 hours of cell seeding. Cells were plated in XF96 cell culture microplates with appropriate background correction wells, then incubated overnight at 37 ºC in a 5% CO_2 incubator. Concurrently, the sensor cartridge was prepared a day before the assay by adding Seahorse Bioscience XF96 calibrant solution to each Seahorse Bioscience utility plate well in a non-CO2 incubator overnight. Measurement was conducted using the Seahorse XF96 Analyzer. For XF cell Mito stress analysis on measurement day, the media was replaced with assay media supplemented with L-glutamine and glucose before an hour of incubation without CO_2. Mitochondrial inhibitors Oligomycin, FCCP, and Antimycin A/Rotenone were introduced sequentially with a final concentration of 1 µM, 1 µM, and 1 µM. The OCR values underwent normalization based on total protein concentration determined through quick Bradford protein assays using software such as Wave Agilent Seahorse. Statistical analysis relied upon GraphPad Prism.

3.16. Sulphorhodamine B assay

The cell viability assay using the sulphorhodamine B method was assessed after 24 hours of cell seeding in mCherry, mCherry Drp1, and mCherry Drp1 S39A cells. For control and shDrp1, the

cells were further treated with all-trans-retinoic acid and mitochondrial inhibitors such as Oligomycin, Antimycin A, and Rotenone in control and shDrp1 cells. Suphorhodamine B assay evaluates the cell viability by measuring the cellular protein content, and the calculation for cell viability was performed according to the formula % cell viability = Absorbance sample/ Absorbance negative control or untreated sample x 100, as described by (Vichai & Kirtikara, 2006).

3.17. Propidium Iodide Staining to Assess Cell Viability

Cell viability was evaluated for undifferentiated, Phase 1 and 2 differentiated control and shDrp1 cells by using propidium iodide (PI) at a concentration of 500 ng/ml, along with counterstaining with Hoechst at a concentration of 10 μM. In short, the respective differentiation media was removed, and complete DMEM containing PI at 500 ng/ml and Hoechst at 10 uM was added to the cells. The cells were then incubated for 15 minutes at 37 ºC in a CO_2 incubator with a level of 5%. Live cell imaging was conducted utilizing an Opera Phenix High Content Screening confocal microscopy system from PerkinElmer set to operate at an ambient temperature of 37 ºC and 5% CO_2. Image acquisition settings included using a 10x objective (NA=0.3), appropriate lasers and filters for Hoechst, and Alexa 568 specifically used for PI detection in sequential mode to eliminate any spectral overlap. The analysis stage involved utilization of the “Live/Dead cells” built-in method found within Harmony 4.9 software developed by Perkin Elmer.

3.18. Immunofluorescence Staining

1.5 x 10^4 control and shDrp1 cells were plated in a 12-well plate and subjected to differentiation as outlined in the neuronal differentiation section of the material and methods. On days 3 and 6, both differentiated control and shDrp1 cells underwent standard fixation, permeabilization, blocking, and stained with Tuj1 antibody for neuron-specific Tubulin beta-III before being mounted on slides using Moviol 4-88: Dabco 33-LV (1:50) for imaging with a Leica SP8 confocal laser scanning microscope.

Approximately 7000 control and shDrp1 cells were seeded onto laminin-coated Perkin Elmer microplates for transient transfection experiments. The following day, transient transfection was carried out with pHM6-Q23 expressing wild-type N-terminal Huntington fragment and pHM6-Q74 expressing mutant N-htt Huntington fragment using lipofectamine 3000 as per the protocol

by (Aladdin et al., 2020). After a 72-hour incubation period, cells were fixed, permeabilized, and processed for antibody staining before analysis using an Opera Phenix High Content Screening System from Perkin Elmer.

3.19. Transmission Electron Microscopy (TEM)

The Aclar thermoplastic film (EMS-Electron Microscopy Sciences) was used to grow control and shDrp1 monolayers. Cell pellets were fixed for one hour at room temperature. After washing, the samples were subsequently fixed in 2% osmium tetroxide dissolved in 0.1 M cacodylate buffer (Ph: 7.4) for an hour at room temperature. After further dehydration and embedding. The sections were examined using a JEOL 1010 transmission electron microscope and captured at a magnification ranging from 5000-30000x with an Olympus Veleta CCD camera. Cell and mitochondria outlines were manually delineated. Only mitochondria with observable and intact internal membranes were included for subsequent analysis. Amira 3D (version 2022; Thermofisher Scientific) imaging software was utilized to assess the numerical characteristics of the outlined structures.

3.20. Human phospho-Kinase Array

Relative protein expression was obtained by analysis of 43 specific phosphorylation sites using the proteome profiler human phospho kinase assay (cat. ary003b R & D systems) according to the manufacturer’s protocol.

3.21. RNA-Seq method

A high-throughput mRNA sequencing analysis was conducted using an Illumina sequencing platform to acquire comprehensive transcriptomic data. Total RNA was isolated from control and shDrp1 fllowing the protocol provided by Zymo Research. The quality of total RNA was assessed using the Eukaryotic Total RNA Nano Kit on Agilent Bioanalyzer. Samples with an RIN value exceeding 7 were deemed suitable for library preparation. The generation of RNA-Seq libraries from total RNA involved utilizing an Ultra II RNA samples prep kit by the manufacturer's guidelines. In brief, poly-A RNAs were captured using Oligo-Dt conjugated magnetic beads, followed by elution and fragmentation of mRNAs at 94 ºC. Subsequently, first-strand cDNA synthesis occurred through random priming reverse transcription; this was succeeded by double-stranded cDNA production after seconds-strand synthesis.

3.22. RNA-Seq data analysis

Raw sequencing data in fastq format were aligned to the human reference genome GRCh38 using the HISAT2 algorithm, leading to the generation of BAM files. Subsequent analysis was conducted utilizing Strand NGS software (www.strand-ngs.com). The BAM files were imported into DESeq for normalization, and differential gene expression was determined via moderated T-test with Benjamini-Hochberg FDR for multiple testing corrections. A significance level of $p < 0.05$ was applied to identify significant changes. Gene ontology analysis of differentially expressed genes was performed using CytoScape 3.4.0 with ClueGo application.

3.23. In silico molecular modeling

Protein data for molecular modeling was sourced from UniProt (000429; DNM1L_HUMAN), and post-translational modifications were obtained from the PhosphoSitePlus database. (Hornbeck et al., 2015). Furthermore, ligand-bound structures of human Drp1 protein (PDBID: 3W6N) (Kishida & Sugio, 2014) and (PDBID: 4H1V) (Wenger et al., 2013) as well as the structure of the oligomeric protein (PDBID: 4BEJ) (Fröhlich et al., 2013) were downloaded from the RCSB protein Data Bank (Berman et al., 2000). The NetPhos-3.1 online tool was utilized to predict phosphorylation sites (Blom, Sicheritz-Pontén, Gupta, Gammeltoft, & Brunak, 2004)

3.24. Statistical analysis

Data from 3 or more than 3 (≥) independent experiments were presented as mean values ±SD. Statistical analysis was done using an unpaired t-test, Mann-Whitney test, One-Way ANOVA, or Two-Way ANOVA followed by Sidak or Tukey's multiple comparisons t-test after passing the normality test of the Shapiro-Wilk test. The samples with negative normality tests were analyzed using a non-parametric analysis of Kruskal-Wallis's test following the DUNN's multiple comparison t-test. GraphPad Prism v9.5.1 software was used where statistical significance was defined as * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

4. Results

4.1. Drp1 knockdown generates long filamentous mitochondrial ultrastructure and induces changes in global transcriptomics of SH-SY5Y cells

The protocol described previously (Abdennour Douida et al., 2020) was used to generate a depleted Drp1/DNML1 cell line using SH-SY5Y human neuroblastoma cells. qRT-PCR and western blotting were used to confirm the efficiency of knockdown. Two primer pairs were used to verify the efficient relative change in the gene expression of DNM1L (Drp1 gene name). The cells stably expressing pGIPZ-GFP and those stably depleted of Drp1 will be referred to hereinafter as control and shDrp1, respectively.

Performed transmission electron microscopy (TEM) to assess the characteristics and physiological effects of Drp1 depletion on the mitochondrial ultrastructure of both control and shDrp1 cells. The shDrp1 cells showed a high number of long, hyperfused and compact mitochondrial shapes as compared to control cells in TEM images. Using the Amira 3D software, we determined the mitochondrial inside length, area, and the formation of clusters of mitochondria. shDrp1 cells showed a significant enhancement in the average inside length of mitochondria, area, and total sum mitochondrial area compared to the control group. However, we could not detect any difference in the total inside length of mitochondria in shDrp1 cells. The results proved that the average distance between two mitochondria was shorter in shDrp1 cells compared to control cells.

We performed RNA Seq on both control and shDrp1 cells and analyzed GO term enrichment analysis on significantly up and downregulated genes to determine the significant biological relevance of DEGs. The stably depleted Drp1 cells showed significant downregulated DEGs of heart and circulatory system development processes, cellular response to chemical and growth factor stimuli, and regulation of signaling and MAPK cascade. The enriched GO terms analysis showed the genes associated with the regulation of neurogenesis, neuron projection development, synapse assembly, and axon development were significantly increased in shDrp1 cells compared to the control.

4.2. RA-BDNF-induced neuronal differentiation improves in Drp1 depleted cells *in vitro*

As our transcriptomic data indicated, GO terms related to neuronal differentiation, synapse formation, and axon formation, we differentiated shDrp1 and control cells. The differentiated cells were monitored after phase 1 (Day 3) and phase 2 (Day 6) by bright field and fluorescence

microscope. Neuronal differentiation was confirmed by the neuron-specific β-III tubulin (Tuj1) staining on fixed cells and tubulin tracker staining on live cells. Undifferentiated cells showed a characteristic clumping structure. The differentiated cells showed neurofilament outgrowth and interconnected neuronal networks. The imaging results demonstrate that the shDrp1 cells showed longer neurite outgrowth and enhanced neuronal networks than the control cells.

SYN1 and SNAP25 showed an increased (not significant) expression in differentiated control cells compared to undifferentiated cells. However, the expression level of NES showed no significant difference between differentiated and undifferentiated control cells. SOX2 showed significant downregulation in differentiated control, indicating initiation of differentiation. On the other hand, differentiated shDrp1 compared to undifferentiated cells showed a significant increase in various genes such as SYN1, SNAP25, and TUBβ3, SOX2 showed a substantial decrease in shDrp1 differentiated cells compared to shDrp1 undifferentiated cells. While comparing the differentiated shDrp1 cells to the differentiated control cells, SYN1, SNAP25, NES, TUBβ3, and DDC were significantly upregulated.

Multiple parameters for the neurite analysis were assessed in live cells to evaluate the nervous system's development critically. We found that maximum and total neurite length parameters were significantly higher in both control and shDrp1 compared to their undifferentiated cells. However, differentiated shDrp1 cells have substantially higher neurite lengths than differentiated control cells to connect to other neuron-like cells synaptically. We found a significantly higher number of segments, number of extremities, and number of roots in both control and shDrp1 differentiated cells vs. their undifferentiated cells. Differentiated shDrp1 cells showed a significant increase in all these parameters compared to the undifferentiated control and shDrp1 and differentiated control groups. The number of nodes type 1 & 2 were enhanced in differentiated cells compared to undifferentiated cells, but no significant difference was detected. In conclusion, these results indicate that RA-BDNF-induced neuronal differentiation enhances the expression of neuronal markers genes and neurite outgrowth parameters in Drp1-depleted SH-SY5Y human neuroblastoma cells *in vitro*.

4.3. RA-BDNF induced neuronal differentiated shDrp1 cells exhibited low level of phospho-ERK1/2

To check the effect of drp1 depletion on pathways triggered upon RA and BDNF treatment, we did a western blotting analysis of all major proteins, including MAP kinases and their phosphorylation status. According to our western blotting analysis, JNK, AKT, P38, and ERK1/2 showed the same trend in undifferentiated Day3 and Day6 differentiated cells of both control and shDrp1 cells. JNK and AKT phosphorylation increased on Day 3, remaining the same until Day 6 in both cell lines. BDNF differentiation induced the phosphorylation of JNK and AKT induced by RA on Day 3. However, P38, MEK1/2, and ERK1/2 phosphorylation were activated on RA treatment but abolished on BDNF treatment. shDrp1 cells compared to control showed a reduction in ERK1/2 phosphorylation on Day 3 of differentiation. These results demonstrate that Drp1 knockdown impacts the ERK1/2 phosphorylation during phase 1 of neuronal differentiation.

4.4. DUSP1 and DUSP6 has no impact on ERK1/2 dephosphorylation in shDrp1 cells during neuronal differentiation

To investigate the role of dual specificity phosphatases on ERK1/2, we treated control and shDrp1 cells with BCI (a specific DUSP1/6 inhibitor) for 24 hrs. 1μM treatment of BCI induced a strong level of ERK1/2 phosphorylation in control cells but not in shDrp1 cells. We compared the ERK1/2 and ERK1/2 phosphorylation levels in undifferentiated and differentiated control and shDrp1 cells with or without BCI treatment. ERK1/2 remained steady in treated or untreated control or shDrp1 cells. In undifferentiated control and shDrp1 cells, BCI treatment did not cause any increase in phospho-ERK1/2 level. However, in phase 1 differentiation, control cells showed significant upregulation in phospho-ERK1/2 level compared to undifferentiated control cells. BCI treatment induced substantial levels of ERK1/2 phosphorylation in control cells on Day 3 differentiation. Still, no significant impact was shown on shDrp1 cells. We measured the DUSP2 gene analysis by RT-qPCR in undifferentiated control and shDrp1 and in differentiated control and shDrp1 cells. We found a significant upregulation of DUSP2 in differentiated shDrp1 cells compared to undifferentiated shDrp1 cells and differentiated control cells. However, a significant decrease was shown in differentiated control cells compared to corresponding undifferentiated cells, indicating that DUSP2 is more active in differentiated shDrp1 cells. These results suggest that ERK1/2 dephosphorylation by DUSP1/6 might depend on Drp1 and DUSP2 upregulation might play a part in the dephosphorylation of ERK1/2 in shDrp1 cells.

4.5. During neuronal differentiation control and shDrp1 cells rearrange the mitochondrial morphology and adapt to metabolic changes

To check the effects of Drp1 depletion on mitochondrial morphology during the neuronal differentiation, we performed live staining in High Content Analysis imaging system. Mitotracker Red CMXRos was used to stain the mitochondria, and Hoechst was used to stain nuclei. Undifferentiated control cells exhibited dispersed tubular mitochondrial structure. However, differentiated control cells on day 6 contained densely connected elongated tubular and compact mitochondrial structures. These changes suggest that mitochondria in control cells underwent remodeling during differentiation. Undifferentiated and differentiated shDrp1 cells showed elongated tubular, compact, and densely packed mitochondrial morphology, implying that the mitochondria in shDrp1 cells do not experience remodeling during differentiation. The quantification confirmed a notable increase in both cell lines' long, elongated, and round compact tubular mitochondrial populations during differentiation. extended, elongated, and round compact tubular mitochondria than differentiated control cells.

we also checked the Drp1 levels but found no significant changes in our control cells. shDrp1 cells maintained its essential depletion of Drp1 throughout the RA-BDNF-induced differentiation. We also checked these phosphorylation levels in phase 1 and phase 2 of differentiation in control cells. The Ser616 phosphorylation gradually increased with RA and BDNF treatments. However, Ser637 phosphorylation was triggered upon RA treatment and abolished upon BDNF treatment.

Mitofusins (MFN1 and MFN2) and OPA1 are mitochondrial fusion proteins. Interestingly, reduced levels of Drp1 did not lead to any significant changes in the expression level of OPA1 in both undifferentiated and differentiated control and shDrp1 cells. Amid differentiation, the MFN1 levels increased significantly in control and not in shDrp1 cells. However, the levels of MFN2 did not change in either of the cell lines. The level of MFN1 increases during the differentiation. In comparing shDrp1 cells to the respective control cells in all phases, the levels of both MFN1 and MFN2 decrease significantly. These results indicate that mitochondrial protein reduction is a coping mechanism to ensure the proper functioning of the cells with reduced mitochondrial proteins.

The cellular bioenergetics of undifferentiated control and shDrp1 were not significantly different. However, differentiated shDrp1 cells indicated an increasing trend in maximal respiration compared to undifferentiated shDrp1 cells and differentiated control cells. Other parameters such

as basal respiration, ATP-linked respiration, proton leak, and spare respiratory capacity also showed an increase but not significant change in differentiated cells. These results suggest the RA-BDNF-induced neuronal differentiation in SH-SY5Y cells *in vitro* causes functional and morphological reprogramming of mitochondria in control cells and shDrp1 cells, and Drp1 knockdown alter cellular bioenergetics during differentiation.

4.6. RA and mitochondrial inhibitors exposure to Drp1 depleted cells is dispensable for cell death

To check the cell viability of Drp1-depleted cells, we treated both control and shDrp1 cells with 10 μM of RA for three days. Sulphorhodamine B assay (SRB), which measures cellular protein content, was used to check the cellular viability. As anticipated, the RA caused a significant increase in cell death in both control and shDrp1 cells. However, there were no differences between the two cell lines. Propidium iodide stains, specifically necrotic and late apoptotic cells, excluding live and apoptotic cells. Propidium iodide staining revealed a slight increase in cell death at Day 3 and 6 in both control and shDrp1 cells. A significant increase was found in shDrp1 cells on Day 6, but there was no significant difference between control and shDrp1 cells. The treatment of 100 nM Antimycin does not cause any decrease in cell viability of both cell lines. The 3 μM oligomycin and 10 μM rotenone treatment decreased cell viability equally in both cell lines but not significantly. These results suggest that Drp1 does not play a role in the modulation of cell death induced either during RA-induced neuronal differentiation or during mitochondrial inhibitor treatment in undifferentiated SH-SY5Y neuroblastoma cells in vitro.

4.7. Drp1 depletion provides protective effects upon overexpression of mutant Huntington protein in SH-SY5Y neuroblastoma cells

We investigated the role of Drp1 knockdown in the formation of toxic mutant huntingtin protein aggregates. 23 polyQ N-Htt represents the normal length of the protein, and 74 polyQ N-Htt represents the mutant form of the huntingtin protein. Normal and mutant forms of huntingtin or Htt proteins (fused with HA tag) were transiently transfected in the undifferentiated control and shDrp1 cells for 72 hrs. After 72 hrs post-transfection, the fixed cells were monitored using High Content Analysis. The 23 polyQ N-Htt fragments showed cytoplasmic distribution in control and shDrp1 cells. However, 74 polyQ N-Htt fragments depicted a combination of cytoplasmic and aggregated distribution in control and Drp1-depleted cells. The aggregated distribution varied in

size, number, and shape. An algorithm was established to assess the mutant huntingtin protein formation quantitatively. The fluorescent intensity of N-Htt/mHtt was normalized to true nuclei using batch analysis in untransfected cells, cells expressing normal N-Htt, and cells expressing mutant N-Htt fragments. There was an increase in the number of aggregates in both control and Drp1 knockdown cells expressing mutant huntingtin proteins compared to their respective untransfected cells or cells expressing 23 polyQ N-Htt fragments. However, Drp1 knockdown cells showed a significant reduction in mutant N-Htt aggregate formation compared to control cells expressing mutant N-Htt. In conclusion, these results suggest that the Drp1 knockdown causes a reduction in the pathogenic formation of mutant huntingtin proteins in SH-SY5Y cells.

4.8. Mutation at Ser39 of Drp1 results in cells lacking mitochondrial fission, leading to hyperfused mitochondria

According to the PhosphoSite database, the Ser39 residue is not a site for PTM and does not undergo phosphorylation. However, neighboring residues Ser40 and Ser44 are documented as PTM sites. These residues can be targeted for phosphorylation by glycogen synthase 3β, leading to mitochondrial fragmentation. The positioning of the Ser39 residue within the protein structure indicates that it is mainly concealed and, therefore, less accessible for phosphorylation.

Consequently, we created a mutant by introducing the S39A substitution via quick change mutagenesis into the mCherry-Drp1 backbone plasmid. Western blot analysis confirmed overexpression of both mCherry-Drp1 S39A and wild-type mCherry-Drp1 in human neuroblastoma SH-SY5Y cells. Confocal microscopy revealed cytosolic localization with punctate structures in Drp1 S39A mutant when transfected into SH-SY5Y cells.

Following this, we established stable SH-SY5Y neuroblastoma cell lines that expressed the mCherry fusion proteins. These included the mCherry control, mCherry-Drp1 WT, and mCherry-Drp1 S39A variants. We examined the phosphorylation status of Drp1 at Ser-616 and Ser-637 to determine whether the loss of function mutant impacted these two sites. Both endogenous Drp1 and the recombinant proteins were present in both the mCherry-Drp1 WT and mCherry-Drp1 S39A cell line. The overexpression of the loss of function S39A mutant did not alter the phosphorylation status of Drp1 at Ser-637 and Ser-616 compared to control. However, it is noteworthy that overexpressing Drp1 WT increased the phosphorylation of S637 compared to both controls and Drp1 mutant S39A.

4.9. Transmission electron microscopy confirms the presence of elongated mitochondrial morphology in cells that express S39A mutant stably

We visualized the effects of this mutation on mitochondrial morphology by staining the cells with a TOMM20 (Translocase of outer mitochondrial membrane 20) antibody. This mutation was reported to cause elongated peroxisomes. Overexpression of wild-type Drp1 protein did not alter the mitochondrial. To assess cellular mitochondrial structures in cells overexpressing Drp1 S39A, we established SH-SY5Y neuroblastoma cell lines with stable overexpression of the mCherry fusion protein (referred to as control), mCherry-Drp1, and mCherry-Drp1 S39A. TEM imaging revealed that stable expression of mCherry-Drp1 S39A led to a significant increase in highly elongated giant mitochondria throughout the cells indicating that serine 39 amino acid residue plays a crucial role in initiating GTP hydrolysis for promoting mitochondrial fission. Conversely, cells overexpressing mCherry-Drp1 exhibited more round-shaped, smaller-sized perinuclear-localized mitochondria suggesting a shift toward increased fission dynamics. The ultrastructure analysis confirmed that control cells displayed a mixed population of perinuclear mitochondria undergoing continuous balanced fusion-fission processes without affecting morphology significantly whereas Ser39 mutation had a substantial effect on promoting mitochondrial fusion.

We conducted measurements of the mitochondria, including mitochondrial number, area, and internal length, using Amira 3D analysis software (Ghani et al., 2024). The number of mitochondria per cell was notably lower in cells expressing the mCherry-S39A mutant compared to the control. Furthermore, both the average mitochondrial internal length per cell and the average mitochondrial area per cell were significantly higher in cells expressing the mCherry-Drp1 S39A mutant compared to both control and mCherry-Drp1 WT. On another note, the total mitochondrial internal length was significantly reduced in the mCherry-Drp1 S39A cell line compared to the control; however, no significant difference was observed in the total mitochondrial area. In mCherry-Drp1 mutant S39A cells, the total mitochondrial area does not change, but a decrease in the number of mitochondria and an increase in the average inside length due to fusion in cells was observed. These findings indicate that the Ser39 mutation substantially impacts the mitochondrial morphology as it blocks fission.

4.10. Computational analysis of mitochondrial morphology classification unveils compact hyperfused and compact tubular mitochondria in cells stably expressing Drp1 mutant S39A

Cells with stable expression of mCherry control, mCherry-Drp1, and mCherry-Drp1 S39A were stained with MitoTracker Green and Hoechst for live cell imaging. Both the control and mCherry-Drp1 cells demonstrated an interconnected mitochondrial network primarily located in the perinuclear region, consistent with our observation in TEM. Overexpression of Drp1 led to a shorter tubular rearrangement compared to the control; however, statistical analysis of High Content Analysis measurement. In contrast, cells overexpressing mCherry-Drp1 S39A exhibited a collapsed mitochondrial network, forming large perinuclear blebby aggregates with the same long retained tubules.

Five mitochondrial classes were defined by using morphology and intensity properties. These classes are referred to as hyperfused, round/ compact tubular, long tubular, short tubular, and fragmented. Measures were computed for the morphology and intensity of mitochondrial structures across all wells. The quantitative analysis revealed that mCherry-Drp1 S39A cells exhibited a significantly higher proportion of hyperfused and compact tubular mitochondria than mCherry control. Fragmented mitochondria were lower than mCherry control and mCherry-Drp1. Furthermore, we detected no difference in mitochondria's short and long tubular structure in three cell lines.

Here, we combined the hyperfused, round/compact and long tubular mitochondria into one class as fused mitochondria. The areas of mitochondria in cells expressing the mCherry, mCherry-Drp1, or mCherry mutant S39A were not significantly different. Additionally, both the mean mitochondrial width and mean mitochondrial length were considerably higher in cells expressing the mCherry-Drp1 S39A mutant compared to both mCherry control and mCherry-Drp1. These results indicate that while there is a decrease in the number of mitochondria and an increase in average inside length due to fusion in cells expressing the mCherry-Drp1 mutants, the overall mitochondrial area remains unchanged. In conclusion, the Ser 39 mutation significantly impacts the mitochondrial morphology due to neglected fission activity.

4.11. Drp1 S39A mutation reinstates cellular energy production and cell survival to the same level as control compared to overexpressing Drp1 cells

Our data suggests that the overexpression of Drp1 triggers a stress response in which cells try to cope with increased spare reserve, showing an increasing but insignificant trend of ATP production. Intriguingly, we found that overexpressing the Drp1 S39A mutant reversed the effect

of Drp1 overexpression and reduced both maximal respiration and spare capacity to the control level.

We did an SRB assay to check the effects of Drp1 WT and Drp1 S39A mutation on cell death. Our results indicate that cells overexpressing Drp1 showed excessive cell death compared to mCherry control and Drp1 S39A cells. However, overexpressing Drp1 mutant S39A cells showed no significant cell death compared to the control. These results demonstrate that Drp1 S39A overexpressing cells are more viable or restore viability than overexpressing Drp1 WT cells.

5. Summary

The study explored the impact of Drp1, an essential protein in spitting mitochondria, on differentiating SH-SY5Y cells into neurons. Decreasing the Drp1 levels led to a reorganization of the mitochondrial network and an increase in the expression of genes related to neuronal development, such as synapse formation, neurogenesis, differentiation, and morphogenesis. The reduction of Drp1 enhanced neuronal differentiation, resulting in longer neurite outgrowth, improved number of segments, and more branch points. Neuronal differentiation induced by RA-BDNF was accompanied by a notable decrease in ERK1/2 phosphorylation, indicating an ERK1/2 independent pathway for neuronal differentiation in shDrp1 cells. Additionally, there was no influence from dual specificity phosphatases DUSP1/6 on the phosphorylation status of ERK1/2 in shDrp1 cells, which maintained highly fused and elongated mitochondrial structures during differentiation while displaying significantly elevated maximal respiration. These findings suggest that lowering Drp1 promotes neuronal maturation through genomic and mitochondria rearrangement in undifferentiated cells. Regarding apoptosis or cell death, the shDrp1 reacted similarly to control when examined in vitro. Several crucially toxic aggregates associated with mutant huntingtin protein (mHtt) were notably reduced following the overexpression within these shDrp1 cells. This implies that lowering Drp1 in a regulated manner can increase several parameters, such as cell viability, neurotoxicity, and cellular bioenergetics. In conclusion, the research findings have shed light on the multifaceted roles of Drp1 in neuronal function. Further research is needed to investigate the mechanisms that can be very helpful in identifying therapeutic targets to treat neurodegenerative diseases.

Drp1 and its function in the GTPase domain are essential for morphology and cellular respiration. S39A mutation of Drp1 caused elongated, fused, and clustered tubular mitochondria compared to normal cells. Despite these changes, the energy remained unchanged, and cell death was reduced in the S39A mutant compared to those with the wild-type Drp1. These findings suggest that Serine 39 plays a crucial role in distributing mitochondria within a cell by regulating GTPase activity and that mutations at this site cause structural abnormalities in the mitochondrial network. This study contributes new insight into understanding how Drp1 functions.

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Registry number: DEENK/323/2024.PL
Subject: PhD Publication List

Candidate: Marvi Ghani
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Ghani, M.**, Szabó, B., Alkhatibe, M., Amsalu, H., Zohar, P., Janka, E. A., Mótyán, J. A., Tar, K.: Serine 39 in the GTP-binding domain of Drp1 is involved in shaping mitochondrial morphology.
   FEBS Open Bio. [Epub ahead of print], 2024.
   DOI: http://dx.doi.org/10.1002/2211-5463.13820
   IF: 2.6 (2022)

2. **Ghani, M.**, Zohar, P., Ujlaki, G., Tóth, M., Amsalu, H., Póliska, S., Tar, K.: Stable knockdown of Drp1 improves retinoic acid-BDNF-induced neuronal differentiation through global transcriptomic changes and results in reduced phosphorylation of ERK1/ 2 independently of DUSP1 and 6.
   Front. Cell. Dev. Biol. 12, 1-25, 2024.
   DOI: http://dx.doi.org/10.3389/fcell.2024.1342741
   IF: 5.5 (2022)

List of other publications

3. Ullah, H., Ihsan, J., Mohamed, R. M. K., Khan, M. A., **Ghani, M.**, Rauf, N., Ullah, S., Javed, A., Farooq, M.: Bionanocomposite scaffolds based on MnS-nanorods loaded acacia-Senegal-gum hydrogels: Fabrication, characterization and biological evaluation.
   Bioactive Carbohydrates and Dietary Fibre. 30, 1-9, 2023.
   DOI: http://dx.doi.org/10.1016/j.bcdf.2023.100368

4. Khalil, I., **Ghani, M.**, Khan, M. R., Akbar, F.: Evaluation of biological activities and in vivo amelioration of CCl4 induced toxicity in lung and kidney with Abutilon pannosum (G.Forst.) Schltdl. in rat.
   J. Ethnopharmacol. 249, 1-14, 2020.
   DOI: http://dx.doi.org/10.1016/j.jep.2019.112395
   IF: 4.36

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5. Aladdin, A., Yao, Y., Yang, C., Kahlert, G., **Ghani, M.**, Király, N., Boratkó, A., Uray, K., Dittmar, G., Tar, K.: The Proteasome Activators Blm10/PA200 Enhance the Proteasomal Degradation of N-Terminal Huntingtin.
Biomolecules. 10 (11), 1-33, 2020.
DOI: http://dx.doi.org/10.3390/biom10111581
IF: 4.879

6. Khalil, I., Khan, M. R., **Ghani, M.**, Akbar, F.: Abutilon pannosum stem bark enhances the aphrodisiac activities and spermatogenesis in rat.
Andrologia. 51 (10), 1-11, 2019.
DOI: http://dx.doi.org/10.1111/and.13404
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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