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MAILIS LIIV

Role of mitochondrial dynamics in Wolfram syndrome

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Role of mitochondrial dynamics in Wolfram syndrome

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Press

Department of Pharmacology, Institute of Biomedicine and Translational Medicine, Faculty of Medicine, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to by the Roman numerals I–III.

I Cagalinec, M., Safiulina, D., **Liiv, M.**, Liiv, J., Choubey, V., Wareski, P., Veksler, V., & Kaasik, A. (2013). Principles of the mitochondrial fusion and fission cycle in neurons. *Journal of Cell Science*, *126*(10), 2187–2197. https://doi.org/10.1242/jcs.118844

II Cagalinec, M.*, **Liiv, M.***, Hodurova, Z., Hickey, M. A., Vaarmann, A., Mandel, M., Zeb, A., Choubey, V., Kuum, M., Safiulina, D., Vasar, E., Veksler, V., & Kaasik, A. (2016). Role of Mitochondrial Dynamics in Neuronal Development: Mechanism for Wolfram Syndrome. *PLoS Biology*, *14*(7), e1002511. https://doi.org/10.1371/journal.pbio.1002511

III **Liiv, M.***, Vaarmann, A.*, Safiulina, D., Choubey, V., Gupta, R., Kuum, M., Janickova, L., Hodurova, Z., Cagalinec, M., Zeb, A., Hickey, M. A., Huang, Y.-L., Gogichaishvili, N., Mandel, M., Plaas, M., Vasar, E., Loncke, J., Vervliet, T., Tsai, T.-F., … Kaasik, A. (2024). ER calcium depletion as a key driver for impaired ER-to-mitochondria calcium transfer and mitochondrial dysfunction in Wolfram syndrome. *Nature Communications*, *15*(1), 6143. https://doi.org/10.1038/s41467-024-50502-x

* Contributed equally to this work

The author's contribution to each article was as follows:

I The author participated in designing and conducting immunohistochemistry experiments and participated in analyzing mitochondrial velocity, density, and length experiments.

II The author contributed to the conception and design of the project, including defining research objectives and experimental approaches. The author developed analytical tools for the estimation of mitochondrial motility and was involved in the design, execution, and analysis of experiments related to mitochondrial density and length, mitochondrial membrane potential, mitophagy assays, luciferase reporter assays, neuronal viability, neuronal maturation, and axonal growth. Additionally, the author contributed to the preparation of the manuscript, ensuring accurate presentation and interpretation of the results.

III The author was responsible for the conception and design of the project, including the formulation of research objectives and experimental methodologies. The author contributed to the design, execution, and analysis of experiments investigating mitochondrial density and length, mitochondrial

membrane potential, mitophagy, immunoprecipitation, and axonal growth. Furthermore, the author participated in the preparation of the manuscript, ensuring accurate presentation and interpretation of the experimental findings.

ABBREVIATIONS

ADP	adenosine diphosphate
ALS	amyotrophic lateral sclerosis
ATF6α	activating transcription factor 6α
ATG	autophagy-related protein
ATP	adenosine triphosphate
BECN1	beclin 1
NIX/BNIP3L	NIP-like protein X/ BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
CDGSH2	cysteine desulfurase iron-sulfur (Fe-S) cluster binding homolog 2 domain
cDNA	complementary DNA
CFP	cyan fluorescent protein
CISD2	CDGSH iron-sulfur domain 2
CMT2A	Charcot-Marie-Tooth disease 2A
GABARAP	GABA (gamma-aminobutyric acid) type A receptor-associated protein
GDAP1	ganglioside-induced differentiation-associated protein 1
GLP1	glucagon-like peptide 1
DHPG	dihydroxyphenylglycine, a metabotropic glutamate receptor agonist
DIV	day *in vitro*
DNM1L	dynamin-1-like gene
DOA	dominant optic atrophy
DRP1	dynamin-related protein 1
EF-hand	is a helix–loop–helix structural domain or motif found in a large family of calcium-binding proteins
ER	endoplasmic reticulum
ERIS	endoplasmic reticulum IFN (interferon) stimulator
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, a mitochondrial oxidative phosphorylation uncoupler
Fe	iron
FIS1	mitochondrial fission 1
FRET	fluorescence resonance energy transfer
HEK293	human embryonic kidney cells
HeLa	a human cell line derived from cervical cancer cells of a patient named Henrietta Lacks in 1951
HTT	huntingtin
IMM	inner mitochondrial membrane
iPSC	induced pluripotent stem cells
IP3R	inositol 1,4,5-trisphosphate receptor

KIF5	the kinesin-1 family
LC3	microtubule-associated protein 1A/1B-light chain 3
LRRK2	leucine-rich repeat kinase 2
MAM	mitochondria-associated endoplasmic reticulum (ER) membranes
MCU	mitochondrial Ca^{2+} uniporter
MEF	mouse embryonic fibroblasts
MiD49	mitochondrial dynamics protein of 49 kDa
MiD51	mitochondrial dynamics protein of 51 kDa
Miner1	mitoNEET-related 1
MIRO 1 and 2	mitochondrial Rho (Ras homologous) GTPase 1 and 2
Mff	mitochondrial fission factor
MFN1 and 2	mitofusin 1 and 2
MRI	Magnetic Resonance Imaging
MUL1	mitochondrial E3 ubiquitin protein ligase 1
Na^{+}	sodium ion
NAF-1	nutrient-deprivation autophagy factor-1
NCS1	neuronal calcium sensor 1
NDP52	nuclear dot protein 52 kDa, also known as CALCOCO2 (calcium binding and coiled-coil domain 2)
NCLX	mitochondrial Na^{+}/Ca^{2+} exchanger
OMM	outer mitochondrial membrane
OPA1	optic atrophy 1
OPTN	optineurin
PARK2	parkin gene
PC12	rat pheochromocytoma cells
PDH	pyruvate dehydrogenase
PINK1	PTEN-induced kinase 1
P724L WFS1	a mutant of wolframin found in Wolfram syndrome 1
ROS	reactive oxygen species
RYR	ryanodine receptor
S	sulfur
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SIR	sigma-1 receptor
SLC25A46	solute carrier family 25 member 46
SOD1	superoxide dismutase 1
TAU	tubulin associated unit
TMRE	tetramethylrhodamine, ethyl ester
TRAK 1 and 2	trafficking kinesin-binding protein 1 and 2
UPR	unfolded protein response
VDAC	voltage-dependent anion channel
WFS1	wolframin
WS1 and 2	Wolfram syndrome type 1 and 2
wt	wild-type
$\Delta\Psi_m$	mitochondrial membrane potential

INTRODUCTION

According to the World Health Organization, there is a substantial global burden associated with neurological and neurodevelopmental conditions, with brain diseases emerging as the leading cause of disability-adjusted life years (DALYs).

In the last 20 years, it has become increasingly clear that dysfunctional mitochondria are important contributors to brain diseases, as well as to the pathogenesis of various other human pathological conditions, including cardiovascular disease, diabetes, cancer, and obesity (Clemente-Suárez et al., 2023; Kargaran et al., 2021). Mitochondria play a crucial role as eukaryote organelles, participating in cellular metabolism, Ca^{2+} homeostasis, regulating apoptosis, and serving an essential function in cellular energy production. Moreover, it has been uncovered that the dynamic nature of mitochondria, namely constantly fusing, splitting apart, moving around, and being degraded by mitophagy, is of immense importance in maintaining mitochondrial functioning and neuronal health (H. Chen & Chan, 2009). Thus, restoring mitochondrial dysfunction could offer novel therapeutic approaches for treating brain diseases in the future.

Much of our understanding of the molecular basis of common and burdensome diseases comes from studying rare familial forms or even rarer monogenic diseases that share similarities with common conditions (Peltonen et al., 2006). Wolfram syndrome (WS) type 1 and type 2 are rare multi-systemic monogenetic diseases characterized by progressive endocrinological and neurological symptoms, serving as valuable models to study molecular and cellular pathology behind neuronal damage and axonal loss. WS was initially proposed to be a mitochondriopathy (Bu & Rotter, 1993) and was later found to involve two causative genes, *WFS1* and *CISD2*, which belong to different protein families with distinct properties (Amr et al., 2007; Osman et al., 2003; Takeda et al., 2001). Interestingly, despite differences, loss-of-function mutations in *WFS1* and *CISD2* result in comparable disease phenotypes.

So far, the exact cellular functions of wolframin (WFS1) and CISD2 remain unclear. Previous studies have primarily emphasized the significance of endoplasmic reticulum (ER) stress and regulating intracellular Ca^{2+} levels as relevant functions of WFS1. The mitochondrial involvement in the cellular pathogenesis of WS has more support in WS type 2 involving a mutated *CISD2* gene (Y.-F. Chen et al., 2010; Kanki & Klionsky, 2009). However, it remains to be elucidated what impact WFS1 has on mitochondrial functioning in WS type 1 and what cellular mechanisms could be involved in the possible alterations of mitochondrial dysfunction.

The general aim of this thesis was to study the role of mitochondria in the disease pathogenesis of WS using an *in vitro* model. First, the general principles governing fusion and fission balance were described in living neurons. Next, we investigated the involvement of mitochondrial dynamics in the cellular pathogenesis of WS. Finally, we specified the cellular mechanisms underlying mitochondrial dysfunction in WS and explored pharmacological interventions to alleviate the impaired mitochondrial phenotype and delayed neuronal development.

1. REVIEW OF LITERATURE

1.1. The dynamic nature of mitochondria

Mitochondria are intracellular organelles consisting of an outer and an inner membrane that generate two separate compartments – an intermembrane space and a matrix. They are found in the cytoplasm of almost all eukaryotic cells and are responsible for generating most of the energy the cell needs via oxidative phosphorylation. Mitochondria also participate in numerous other cell functions such as cell proliferation and differentiation, intermediary metabolism (production of fatty acids, phospholipids, amino acids, nucleotides, and heme groups), reactive oxygen species (ROS) production, innate immunity, calcium signaling, and apoptosis (Osellame et al., 2012). The number, size, and shape of mitochondria in a cell are constantly changing. The term "mitochondrial dynamics" encompasses processes such as mitochondrial fusion, fission, mitochondrial transport, also referred to as trafficking, and removal of excessive or damaged mitochondria, known as mitophagy (Figure 1).

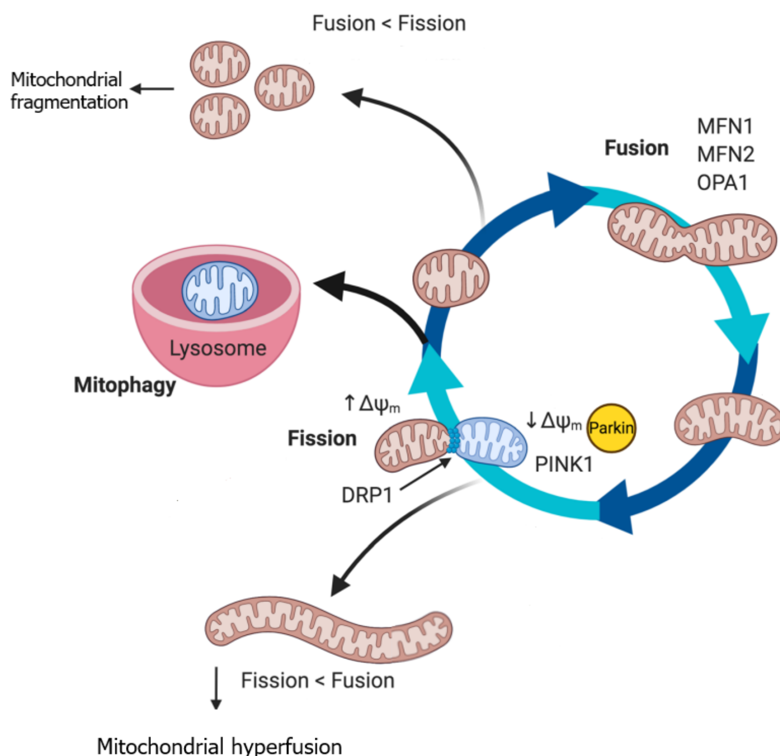


Figure 1. Mitochondrial dynamics. Mitochondria undergo tightly interconnected dynamic processes of fusion, fission, and mitophagy. Balance of fission and fusion processes and selective degradation of defective mitochondria are needed to maintain normal mitochondrial function (modified from Garbern & Lee, 2021).

1.1.1. Fusion and fission

Mitochondrial fusion involves the physical merging of outer and inner mitochondrial membranes and the matrix from two originally distinct mitochondria to mix and unify mitochondrial membranes and contents (Chan, 2006). Mitochondrial fusion promotes homogenization of the mitochondrial population, facilitated by proteins such as mitofusin 1 and 2 (MFN1 and 2) and optic atrophy 1 (OPA1). Mitofusins merge outer mitochondrial membranes, while OPA1 fuses inner mitochondrial membranes (Song et al., 2009).

On the other hand, mitochondrial fission is the separation of one mitochondrial body into two or more smaller daughter organelles. It plays a crucial role in regulating cell death and removing damaged mitochondria via mitophagy (Gomes et al., 2011; Suen et al., 2008). The central proteins in mitochondrial fission include mitochondrial fission 1 (FIS1) and dynamin-related protein 1 (DRP1). FIS1, mainly located on the outer mitochondrial membrane, interacts with DRP1, which translocates from the cytosol to mitochondria (Y. Wang et al., 2019; Westermann, 2008). Recent findings suggest that outer membrane proteins like Mff, MiD49, and MiD51 are essential in recruiting DRP1 to mitochondria (Chan, 2020). Depletion of these proteins results in elongation of mitochondria and a fission defect similar to DRP1 depletion (Losón et al., 2013; Osellame et al., 2016; Otera et al., 2016).

There is a growing amount of evidence on the interdependent nature of fusion and fission, meaning the fission events occurring shortly after fusion events, suggesting a feedback loop between the two processes (Twig et al., 2008; S. Wang et al., 2012).

The equilibrium between fusion and fission is pivotal in organelle distribution and bioenergetics, especially in neurons with specialized, compartmentalized energy requirements. Mitochondrial fission is involved in quality control, promoting the elimination of damaged mitochondria through mitophagy (Gomes et al., 2011) and aiding in the initiation of apoptosis during cellular stress (Suen et al., 2008).

1.1.2. Mitochondrial movement

Neuronal mitochondria exhibit high mobility, moving bidirectionally in cellular processes, pausing briefly, and changing direction repeatedly. Long-distance transport within neuronal processes relies on microtubule-based motors. Dynein motors are responsible for retrograde movement in axons, and kinesin motors mediate anterograde transport (Hirokawa et al., 2010). Additionally, actin motors facilitate short-distance movement along filaments (Frederick & Shaw, 2007; Hatch et al., 2014).

Mitochondrial anterograde transport in neurons involves the kinesin-1 family (KIF5) motors (Hurd & Saxton, 1996; Pilling et al., 2006). KIF5 motors attach to mitochondria with the assistance of trafficking kinesin-binding proteins TRAK1 and TRAK2 (Milton in Drosophila) and the MIRO 1/2, a Ca^{2+} -sensing mito-

chondrial outer membrane protein (Glater et al., 2006; Koutsopoulos et al., 2010; MacAskill et al., 2009; Stowers et al., 2002). TRAK proteins are required for the KIF5- and dynein-mediated two-way transportation of axonal mitochondria.

Mitochondrial axonal retrograde transport is driven by the dynein/dynactin complex, composed of multiple chains, which is suggested to interact with MIRO (Guo et al., 2005; Pilling et al., 2006; Russo et al., 2009).

Mitochondrial transport along the microtubule cytoskeleton is relevant for organelle intracellular distribution and bioenergetics, especially in complex and energy-intensive cells such as neurons. Neurons heavily rely on targeted delivery and regulated removal of organelles along the axon, ensuring mitochondria are distributed where energy demand is high, like at synapses, active growth cones, and axonal branches.

The main mechanism immobilizing mitochondria to necessary cellular sites involves the mitochondrial-anchoring protein syntaphilin (Y.-M. Chen et al., 2009; Kang et al., 2008). Deletion of syntaphilin increases motile mitochondria, while overexpression abolishes axonal mitochondrial transport. Recent studies suggest a causal link between immobilized mitochondria induced with syntaphilin overexpression and increased axonal branching (Courchet et al., 2013; Tao et al., 2014). Increased intracellular $[ADP]_i$ slows down mitochondrial movement, recruiting mitochondria to subcellular regions and sustaining local $[ATP]_i$ depletion (Mironov, 2007). Additionally, mitochondria may sense energy needs through increased local cytosolic Ca^{2+} levels using the EF-hands of MIRO as a Ca^{2+} sensor, leading to the inactivation or disassembling of the KIF5-MIRO-TRAK transport machinery (MacAskill et al., 2009; X. Wang & Schwarz, 2009).

1.1.3. Mitophagy

Mitophagy, an essential aspect of mitochondrial dynamics, involves the removal of aged or irreversibly damaged mitochondria through autophagy, also known as mitophagy – a major mechanism for mitochondrial quality control. This process is crucial for maintaining a functional mitochondrial population, adjusting mitochondrial numbers to changing cellular metabolic needs, and during specific cellular developmental stages. The elimination of dysfunctional mitochondria follows a sequence of steps, including marking them for degradation, subsequent sequestration by an autophagosome, and degradation through lysosomal fusion. To facilitate the degradation of dysfunctional mitochondria, prevention of their fusion with others and/or promotion of fission is necessary (Duvezin-Caubet et al., 2006; Ishihara et al., 2006; Mao et al., 2013; Twig et al., 2008). Mitophagy can occur in the form of bulk/non-selective autophagy or selective removal of mitochondria (for review, see ref. (Evans & Holzbaur, 2020)).

In non-selective autophagy, also known as macroautophagy, cytoplasmic components, including mitochondria, are engulfed into a double-membraned vesicle or autophagosome. This structure fuses with late endosomes or lysosomes to form autolysosomes, resulting in cargo degradation. The protein machinery driving this pathway is well-established and incorporates autophagy-related pro-

teins (ATGs) (for reviews, see Refs. (Klionsky et al., 2011; Yin et al., 2016)). In cultured neurons, LC3-positive organelles form in the distal part of the axon and are retrogradely transported to the soma (Cheng et al., 2015; Hollenbeck, 1993; Maday et al., 2012; Maday & Holzbaur, 2014).

The best-characterized pathway of selective mitophagy is the PINK1/Parkin-dependent pathway. Under cellular stress, mitochondrial depolarization stabilizes PINK1 on the outer mitochondrial membrane (OMM) (D. P. Narendra et al., 2010; Vives-Bauza et al., 2010). The autophosphorylation of PINK1 leads to the recruitment, phosphorylation, and activation of an E3 ubiquitin ligase Parkin (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012; Vives-Bauza et al., 2010). Together, ubiquitin binding and PINK1-phosphorylation of Parkin induce full activation of the latter, thus leading to the conjugation of ubiquitin chains to OMM substrates (Kane et al., 2014; Ordureau et al., 2014). In the end, phospho-ubiquitination of OMM proteins (voltage-dependent anion channel VDAC, MFN2, MIRO1/2, etc.) by PINK1 and Parkin generates a specific signal and a platform for the recruitment of autophagy receptors (e.g., OPTN and NDP52) for organelle degradation. Consequently, removing MFN and MIRO proteins prevents mitochondrial fusion and transport and connects mitophagy to mitochondrial motility. It is noteworthy that although mitochondrial fragmentation is permissive for mitophagy, it is not a sufficient signal for mitophagy (Ashrafi & Schwarz, 2013).

Recent studies conducted *in vivo* have suggested that the PINK1/Parkin-dependent pathway is not functionally dominant, and other selective mitophagy pathways may play an equally important role in the mitochondrial quality control system (Villa et al., 2018). For example, an E3 ligase MUL1 is functionally independent of Parkin but has similar substrates to Parkin and can directly bind to GABARAP and regulate mitophagy (Ambivero et al., 2014; Yun et al., 2014). Moreover, upon phosphorylation of a mitochondrial OMM protein NIX/BNIP3L, mitophagy is initiated by binding to LC3 or GABARAP on the phagophore (Novak et al., 2010). Also, the externalization of cardiolipin from IMM to the OMM leads to lipid-mediated mitophagy, as cardiolipin can directly bind to LC3 (Chu et al., 2013).

1.2. Mitochondrial dynamics in brain disorders

Studies about the connections between mitochondrial dynamics and various brain disorders are underway and much remains to be elucidated. Mitochondrial morphology, dynamics, and distribution appear to play crucial roles in the development of neurons due to their high demand for ATP and the need for timely calcium buffering (Iwata et al., 2023; Son & Han, 2018). There is extensive evidence that mutations and knockouts introduced in mitochondrial fusion, fission, and transport proteins lead to issues in mammalian embryonic and tissue development (D. T. W. Chang et al., 2006; H. Chen et al., 2003; H. Chen & Chan, 2004; Fang et al., 2016; Z. Li et al., 2004; Nemani et al., 2018; van Spronsen et al.,

2013). For example, in humans, mutations in the *DNM1L* gene, encoding DRP1, lead to a condition known as encephalopathy due to defective mitochondrial and peroxisomal fission-1 (EMPF1; OMIM 614388) (Robertson et al., 2023). Individuals affected by EMPF1 exhibit diverse symptoms, including neurodevelopmental delay, refractory seizures, muscle abnormalities (hypotonia), and ataxia (Robertson et al., 2023). Moreover, in mouse models, global DRP1, as well as MFN knockout, are embryonically lethal (H. Chen et al., 2003; Ishihara et al., 2009; Wakabayashi et al., 2009).

Neurodegenerative diseases are a heterogeneous group of disorders characterized by the selective and progressive loss of the structure and function of neurons in the central and/or peripheral nervous system. Most neurodegenerative diseases are characterized as proteopathies as they are frequently associated with the aggregation of one or more misfolded proteins. Interestingly, mitochondrial abnormalities are regularly described as a common prominent early pathological feature in Alzheimer's disease, sporadic Parkinson's disease, amyotrophic lateral sclerosis (ALS), and Huntington's disease (Johri & Beal, 2012; Lin & Beal, 2006). Disease-associated proteins such as phosphorylated TAU, amyloid beta (Aβ), LRRK2 G2019S, SOD1 G93A, and mutant HTT have been demonstrated to impair mitochondrial dynamics, including fusion, fission, and transport, resulting in mitochondrial dysfunction (for review, see ref. Gao et al., 2017). However, in cases of Huntington's disease, Alzheimer's disease, and sporadic Parkinson's disease, it remains unclear whether the observed mitochondrial deficits primarily contribute to pathogenesis or if they are secondary effects of another pathogenic process.

Furthermore, the best examples of direct involvement of mitochondrial proteins affecting mitochondrial dynamics in neurodegeneration include human diseases such as autosomal dominant optic atrophy (ADOA), Charcot–Marie–Tooth type 2A (CMT2A), and hereditary early-onset Parkinson's disease (mutations in *PINK1* or *PARK2* genes). *OPA1* is the causative gene for ADOA, the predominant form of inherited childhood blindness. Individuals affected by ADOA experience a gradual vision decline due to the degeneration of retinal ganglion cells, whose axons constitute the optic nerve (Westermann, 2010). CMT2A is attributed to mutations in MFN2, resulting in progressive impairments in distal sensory and motor neurons from the degeneration of long peripheral nerves (Züchner et al., 2004). Moreover, it has been discovered that mutations in the genes encoding PINK1 and Parkin are accountable for 50% of cases of autosomal recessive juvenile parkinsonism (Quinn et al., 2020). These mutations manifest a deficiency in mitochondrial quality control, resulting in the accumulation of dysfunctional mitochondria in the dopaminergic neurons, prominently affected by the disease. Furthermore, there have been reports of rare mutations in the mitochondrial fission protein MFF and the SLC25A46 protein. The latter is a mitochondrial carrier protein that interacts with fusion machinery. In patients with mutated MFF, the clinical features emerged within the first year of life, including microcephaly, hypotonia, epileptic seizures, spasticity, and optic neuropathy (Chan, 2020). Recessive mutations in SLC25A46 result in various diseases,

such as optic atrophy with axonal CMT features, Leigh syndrome, pontocerebellar hypoplasia, and progressive myoclonic ataxia (Chan, 2020).

1.3. Wolfram syndrome

1.3.1. The clinical picture of Wolfram syndrome

Wolfram syndrome (WS) is a rare, progressive, and severe autosomal recessive neuroendocrine disease characterized by diabetes mellitus, optic atrophy, diabetes insipidus, and deafness (also referred to as DIDMOAD; Online Mendelian Inheritance in Man (OMIM) #222300) (Rigoli et al., 2018; Toppings et al., 2018; Urano, 2016). The estimated prevalence of WS has been suggested to be 1 in 770,000 in the United Kingdom and 1 in 100,000 in North America (Barrett et al., 1995; Toppings et al., 2018). The prognosis of WS is poor and typically terminal between 30 and 40 years of age. The death results mainly from respiratory failure because of brain stem atrophy and neurodegeneration (Urano, 2016). The condition was first described in 1938 by Wolfram and Wagener, who found 4 of 8 siblings with juvenile diabetes mellitus and optic nerve atrophy, which has been considered the minimal criteria for the diagnosis of WS (Dj, 1938; Pallotta et al., 2019; Toppings et al., 2018; Urano, 2016). The genetic basis of WS involves mutations in two specific genes. The mutation in the wolframin gene causes Wolfram syndrome type 1 (WS1) (WS1; 606201), first described in 1998 (Inoue et al., 1998; Strom, 1998), and Wolfram syndrome type 2 (WS2) is caused by a mutation in the *CISD2* gene (WS2; 604928) (Amr et al., 2007).

The clinical picture of WS is diverse (Figure 2). The additional presentations include neurologic abnormalities such as nystagmus, mental retardation, seizures, myoclonus, urinary tract abnormalities, ataxia, peripheral neuropathy, and dementia, as well as hypogonadism, infertility, and hypopituitarism (Chaussenot et al., 2011; Medlej et al., 2004; Swift et al., 1990; Urano, 2016). Furthermore, WS2 can present with profound upper gastrointestinal ulceration, impaired platelet aggregation, and bleeding while diabetes insipidus is absent (El-Shanti et al., 2000).

Alongside the neuroendocrine manifestations, WS1 frequently presents with psychiatric symptoms (Strom, 1998). A review of 68 case findings in the USA identified that 60% of WS1 patients had severe depression, psychosis, organic brain syndrome, suicide attempts, as well as impulsive verbal and physical aggression (Swift et al., 1990). Also, according to Swift et al., 1991 heterozygous carriers of the mutant wolframin gene, who are estimated to be 1% of the general population, are predisposed to significant psychiatric illness (Swift et al., 1991) and are 26 times more likely to require psychiatric hospitalization than non-carriers (Swift et al., 1998). These observations have been validated in subsequent studies (Munshani et al., 2021; Sequeira et al., 2003; Zalsman et al., 2009). Although certain reports have not identified an association (Furlong et al., 1999; Ohtsuki et al., 2000; Torres et al., 2001). This inconsistency is likely attributed to variations in patient cohorts and warrants additional investigation.

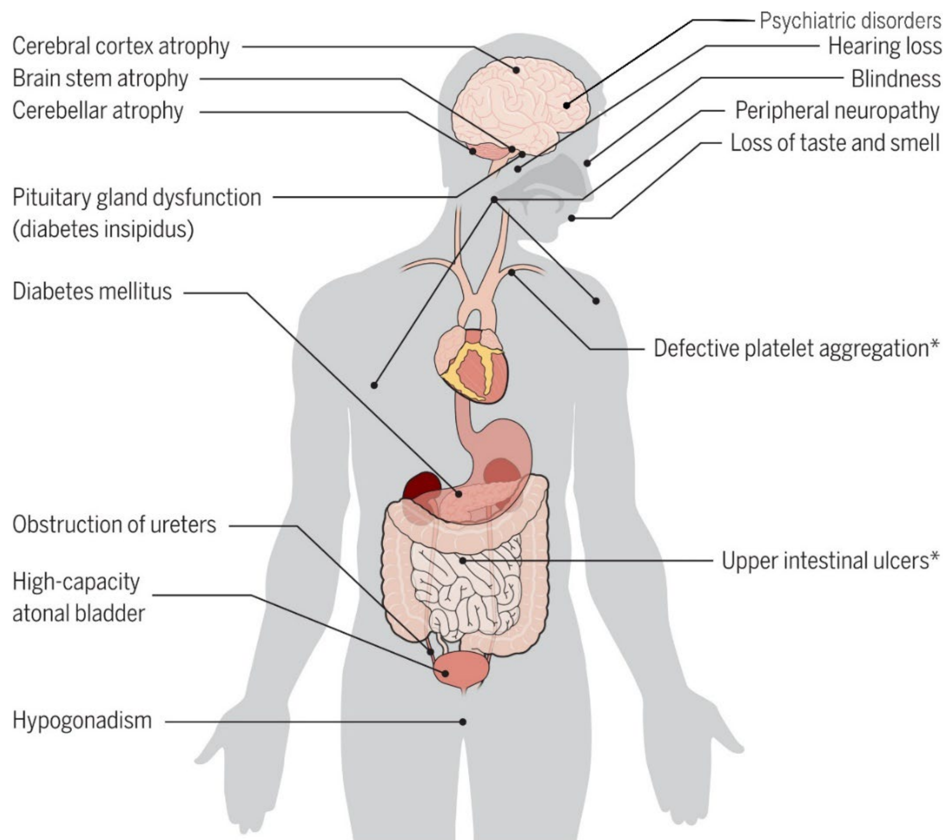


Figure 2. The clinical manifestations of Wolfram syndrome. The implicated systems comprise the central nervous system, sensory and peripheral nervous systems, digestive system, endocrine system, urinary system, reproductive system, and vascular system. Symptoms marked with an asterisk are linked to WS2 (modified from Loncke et al., 2021).

1.3.2. The *WFS1* and *CISD2* genes and the corresponding proteins

The linkage of the gene implicated in WS1 was reported to markers on chromosome 4p (Collier et al., 1996; Polymeropoulos et al., 1994). The gene itself was first described in 1998 by two separate groups (Inoue et al., 1998; Strom, 1998). The *WFS1* gene was found to produce a tetrameric endoplasmic reticulum transmembrane glycoprotein with a molecular weight of 100 kDa (890 amino acids) known as wolframin (Hofmann et al., 2003). The protein consists of 9 transmembrane segments and a large hydrophilic region at both ends, with C-terminus localizing to ER lumen and N-terminus to cytosol (Hofmann et al., 2003). Initial findings suggested that WFS1 predominantly localizes within ER membranes (Osman et al., 2003; Takeda et al., 2001). Subsequent investigations revealed a specific enrichment of wolframin at mitochondria-associated ER membranes (MAMs) (Angebault et al., 2018; Delprat et al., 2019). Wolframin is an abundant

protein in pancreatic β-cells, brain, heart, and muscle and is also found in the liver, kidneys and spleen (Hofmann et al., 2003; Inoue et al., 1998; Strom, 1998).

Over 200 different mutations (insertions, deletions, frameshift mutations, nonsense, and missense mutations) have been recognized in the *WFS1* gene, which represents 99% of WS cases (Crouzier, Richard, et al., 2022; Rigoli et al., 2022). Most of these mutations are situated in exon 8, the largest exon of the *WFS1* gene, and encodes the transmembrane region and the C-terminus of the wolframin protein. Mutations in WFS1 are commonly inactivating, and the majority follow an autosomal recessive inheritance pattern. Less disruptive missense mutations are presumed to be linked to milder phenotypes of WS1 compared to mutations that entirely inhibit *WFS1* expression (Loncke et al., 2021). Supporting this, a study has demonstrated an inverse relationship between the residual expression of WFS1 protein and the severity of optic atrophy (Hu et al., 2022). Furthermore, autosomal dominant mutations of WFS1 also have clinical relevance and lead to non-syndromic low-frequency hearing loss (OMIM #600965), Wolfram-like syndrome (OMIM #614296), non-syndromic autosomal-dominant diabetes, non-syndromic optic atrophy, and hearing loss (Delprat et al., 2018; Niu et al., 2017; Rendtorff et al., 2011).

WS2 is caused by mutations in the CDGSH iron-sulfur domain-containing protein 2 (*CISD2*) gene, located on chromosome 4q22-q23 and comprising three exons (Amr et al., 2007). *CISD2* encodes a 15 kDa, single-pass, transmembrane ER protein called CISD2, also known as ERIS, Miner1, NAF-1, and WS2 (Amr et al., 2007), enriched explicitly at MAMs (Wiley et al., 2013). The protein has a redox-active 2Fe-2S cluster domain within its cytosolic CDGSH domain and forms homodimers (Conlan et al., 2009). CISD2 is highly expressed in the pancreas and brain (Y.-F. Chen et al., 2010) but can be found in all cell types.

To date, four WS-associated disease mutations in CISD2 have been described, leading to a truncated or a nonfunctional protein (Loncke et al., 2021). CISD2 is involved in ER stress, the unfolded protein response (UPR), and the regulation of Ca^{2+} homeostasis between the ER and cytosol (Wiley et al., 2013). Additionally, it facilitates iron transfer to the mitochondria, playing a crucial role in regulating iron and reactive oxygen species (ROS) and redox homeostasis (Tamir et al., 2015). Also, CISD2 deficiency shortens lifespan and accelerates aging in mice (Y.-F. Chen et al., 2009), while an excess of CISD2 seems to be implicated in cancer pathogenesis (Sohn et al., 2013). Furthermore, CISD2 modulates autophagy by influencing BECN1 activity and regulates apoptosis through modulating calpain 2 activity (Shen et al., 2021).

1.3.3. The pathogenesis of Wolfram syndrome

WS has been considered both a neurodegenerative and a neurodevelopmental disorder (Hershey et al., 2012; Rando et al., 1992). In post-mortem and MRI studies, the atrophy of the specific areas of the brain, e.g. hypothalamic nuclei, optic nerves, chiasm and tracts, olfactory bulbs and tracts, cochlear nerve and nuclei, medulla, pons, cerebellum, alongside demyelination of the pyramidal

tracts, have been demonstrated repeatedly (Barrett et al., 1995; Carson et al., 1977; Genís et al., 1997; Hilson et al., 2009; Rando et al., 1992; Shannon et al., 1999). Neurodegeneration has also been established *in vivo* animal models. Knockdown of WFS1 in the fruit fly *Drosophila* nervous system resulted in age-dependent behavioral deficits and premature neuronal death (Sakakibara et al., 2018). In rats, the loss of WFS1 results in optic nerve atrophy and medullary degeneration (Plaas et al., 2017).

The Washington University Wolfram Study Group conducted research intending to characterize regional patterns of neuroanatomical abnormalities in WS patients across a range of disease duration. The study revealed a diminished intracranial volume, primarily attributed to reduced volumes of the brainstem, cerebellum, and optic radiation, manifesting from the initial stages of the disease in Wolfram patients when compared to both healthy controls and type 1 diabetic patients (Hershey et al., 2012). The authors concluded that the observed changes in neuroimaging and deficits in white matter myelination detected in further studies resulted from a neurodevelopmental disruption dating back to the early stages of the central nervous system development (Hershey et al., 2012; Lugar et al., 2016, 2019). A recent case study involving a neonatal patient with WS also suggests the involvement of the *WFS1* gene in the development of specific brain structures during the fetal period (Ghirardello et al., 2014). Furthermore, an *in vitro* study involving neurons derived from induced pluripotent stem cells (iPSCs) carrying mutations associated with WS displayed impaired neurite outgrowth and abnormally extensive fasciculation (Pourtoy-Brasselet et al., 2021).

The mitochondrial hypothesis of WS emerged from observations that the clinical presentation of WS is varied, with several symptoms being consistent with an ATP supply defect and resembling those of a mitochondrial disease, e.g., deafness, optic atrophy, ataxia, and psychiatric disorders. Mitochondrial aberrant morphology and dysfunction were found in a muscle biopsy of a WS patient in 1992 (Bundey et al., 1992). Still, another group did not confirm the findings (Jackson et al., 1994). The pattern of axonal degeneration observed in post-mortem optic nerves from a 25-year-old male patient with WS closely resembled that of mitochondrial optic neuropathies, supporting the hypothesis that mitochondrial dysfunction is a fundamental factor in the pathogenesis of WS (Ross-Cisneros et al., 2013). The mitochondrial involvement in the cellular pathogenesis of WS has mainly been suggested in WS2 involving CISD2 mutations (Y.-F. Chen et al., 2010; Kanki & Klionsky, 2009), while WS1 (mutations in wolframin) is mainly considered an ER disease (Urano, 2014).

1.3.4. Molecular mechanisms of Wolfram syndrome 1

The exact cellular functions of the WFS1 protein remain unclear. Wolframin has been found to participate in ER stress regulation and influence intracellular Ca^{2+} levels. Osman et al. were the first to demonstrate that the changes in intracellular Ca^{2+} levels were dependent on the expression level of wolframin and suggested that wolframin activates endogenous ER channels or could act as a Ca^{2+} channel

(Osman et al., 2003). The significance of calcium was further affirmed by Takei et al., who characterized the WFS1 protein as a positive regulator of Ca^{2+} uptake in the ER of HEK293 cells overexpressing WFS1 (Takei et al., 2006). However, the exact mechanisms behind the changes in Ca^{2+} homeostasis in WFS1 deficiency were not elucidated.

Notably, the ER functions as a primary reservoir for intracellular calcium ions, playing a pivotal role in regulating both the uptake and release of $Ca^{2+,}$ and is responsible for normal cellular Ca^{2+} signaling. Calcium ions are sequestered within the ER through the activity of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump and are released into the cytosol in response to various physiological triggers (Wuytack et al., 2002). Specifically, Ca^{2+} channels known as ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP3Rs) are resident in the ER membrane, mediating Ca^{2+} efflux from the ER (Figure 3) (Loncke et al., 2021; Zhang et al., 2020). The interactions between mitochondria and the ER, referred to as MAMs, actively participate in intracellular Ca^{2+} regulation and the transfer of Ca^{2+} from the ER to mitochondria (Islinger et al., 2015). Earlier work indicates that IP3Rs primarily localize to MAMs, forming complexes with the voltage-dependent anion-selective channel (VDAC) at the mitochondrial outer membrane and the mitochondrial Ca^{2+} uniporter (MCU) at the mitochondrial inner membrane (Lee et al., 2018). ER-mitochondrial Ca^{2+} transport is critical for Ca^{2+} buffering and cellular metabolism and has important connections with ER stress and apoptosis (Kaufman & Malhotra, 2014).

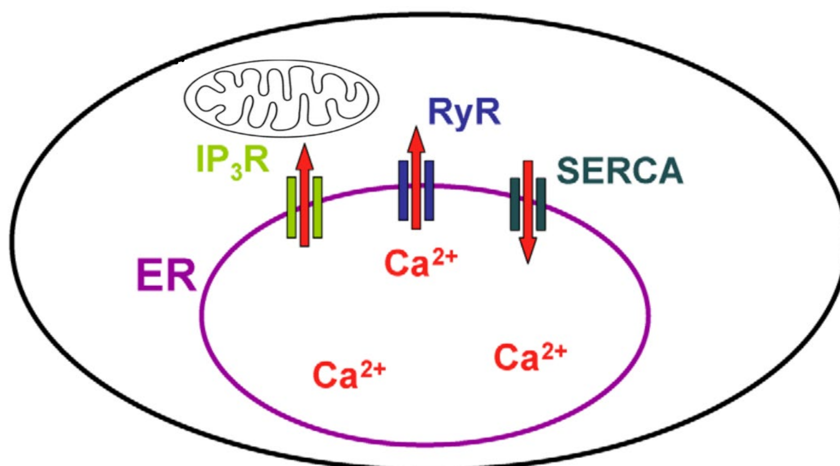


Figure 3. ER calcium homeostasis. The ER Ca^{2+} levels are regulated by SERCA, an ATP-dependent pump that transports free Ca^{2+} from the cytosol to the ER lumen. Additionally, Ca^{2+} efflux channels, namely ryanodine (RyRs) and inositol 1,4,5-trisphosphate receptors (IP3Rs), release Ca^{2+} into the cytosol. It's noteworthy to mention that the release of Ca^{2+} to the mitochondria from the ER primarily occurs through IP3Rs (modified from Schrödl et al., 2009).

Further studies discovered the implication of wolframin in regulating ER stress signaling (Fonseca et al., 2005). Besides Ca^{2+} regulation, the ER also functions as a quality control system, identifying misfolded proteins and directing them for degradation. In the accumulation of misfolded proteins, the ER triggers a stress response known as the unfolded protein response (UPR) (Delprat et al., 2019). It was found that WFS1 interacts with one of the regulators of UPR, the transcription factor ATF6α, and protects pancreatic β cells from dysfunction and premature cell death induced by the excessive activation of ER stress signaling (Abreu et al., 2020; Fonseca et al., 2010). Additionally, by preventing ER stress and subsequent UPR signaling, WFS1 may indirectly influence the expression of various genes (Loncke et al., 2021). Induction of UPR pathways during ER stress diminishes both transcriptional and translational processes, inhibiting gene expression (Hetz et al., 2020). This was suggested as a mechanism explaining the decreased presence of plasmalemmal Na^{+}/Ca^{2+} exchanger (NCX) mRNA and protein detected in cardiac myocytes derived from a rat model of WS, though ER stress in WFS1 deficiency has been described in the heart inconsistently (Kureková et al., 2020; Yamada et al., 2006).

The Ca^{2+} involvement in WS disease mechanisms was again emphasized in a study by Lu et al., 2014. The authors observed Ca^{2+}-dependent calpain activation induced by high cytosolic calcium mediated by the loss of function of wolframin in WFS1 knockout mice and neural progenitor cells derived from iPS cells of WS patients (Lu et al., 2014). Moreover, the hypothesis of ryanodine receptor leakage in the progression of WS has been proposed, supported by the observation that treating WFS1-knockdown cells with dantrolene and ryanodine – agents that inhibit ryanodine receptors and decrease calcium leakage from the ER to the cytosol, thus reducing cytosolic calcium levels – could prevent cell death mediated by WFS1 knockdown (Lu et al., 2014).

Moreover, similar changes with calpain hyperactivation and ER calcium efflux were also observed in WFS1-deficient β cells (Hara et al., 2014). Interestingly, the functional and physical interaction of WFS1 with SERCA was demonstrated in different cell models, but contrary to earlier, reduced and delayed rise in cytosolic free Ca^{2+} concentration in *WFS1* KO MIN6 cells was found (Zatyka et al., 2015). A study by Morikawa et al. showed again the involvement of SERCA in the disturbances of Ca^{2+} homeostasis in a cell line transfected with mutant *WFS1* cDNA as opposed to those transfected with wt *WFS1* cDNA, but to the contrary, suggested that the *WFS1* mutant elevated cytosolic Ca^{2+} by diminishing the expression of SERCA (Morikawa et al., 2017).

Delprat et al. suggested that because SERCA is present in MAMs and serves as a recognized mediator of ER Ca^{2+} uptake, WFS1 could represent a novel physiological effector within MAMs being crucial for maintaining Ca^{2+} homeostasis (Delprat et al., 2018). Supporting the prior idea, an essential role of WFS1 could be its involvement in regulating the transfer of calcium to mitochondria through the MAMs (Crouzier, Richard, et al., 2022). Indeed, Angebault et al., 2018 demonstrated that WFS1 interacts with the neuronal Ca^{2+} sensor NCS1 in MAMs, forming a complex with IP3R to activate ER-mitochondria Ca^{2+} transfer.

This triplet complex ensures effective Ca^{2+} transfer to mitochondria and proper activation of mitochondrial oxidative respiration (Angebault et al., 2018). Interestingly, the notion that WFS1 might either elevate IP3R abundance or enhance the sensitivity of IP3Rs to IP3 was initially suggested by Osman et al. in 2003, as mentioned earlier in this chapter. The discoveries of Angebault et al., 2018 were validated in a rat insulinoma β cell model lacking WFS1. The absence of WFS1 was identified to elevate basal cytosolic Ca^{2+} concentrations, leading to heightened calpain activity and reduced IP3R-mediated Ca^{2+} release, subsequently impacting Ca^{2+} transfer to the mitochondria (Nguyen et al., 2020). Furthermore, the overexpression of NCS1 restored cytosolic Ca^{2+} homeostasis. Finally, a recent study affirmed the involvement of WFS1 in the transfer of Ca^{2+} between the ER and mitochondria in WS patient-derived fibroblasts, although no subsequent mitochondrial defects were documented (La Morgia et al., 2020). Altogether, the existing understanding of intracellular calcium regulation in the presence or absence of wolframin remains controversial and requires further clarification (Figure 4).

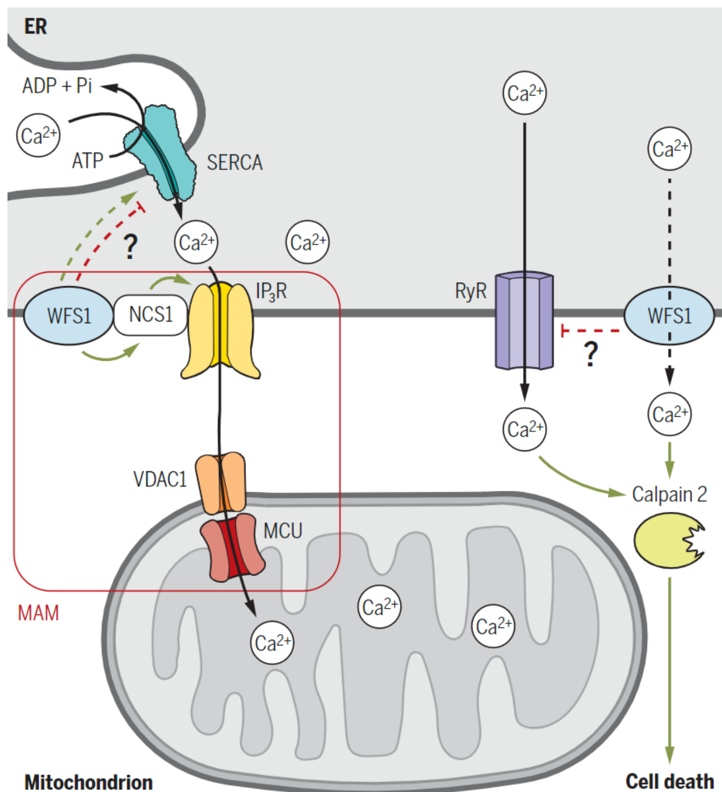


Figure 4. The involvement of WFS1 in Ca^{2+} homeostasis. WFS1 is proposed to act as a Ca^{2+} leak channel and regulate Sarco/endoplasmic reticulum Ca^{2+}-ATPase's (SERCA) degradation, activity, or both. WFS1 interacts with Neuronal calcium sensor 1 (NCS1) to

enhance the Ca^{2+}-release activity of Inositol 1,4,5-trisphosphate receptors (IP3Rs). Additionally, WFS1 is believed to inhibit Ryanodine (RyR) -mediated Ca^{2+} flux, preventing hyperactivation of calpain 2 and thereby blocking cell death. Green arrows denote stimulatory interactions, red arrows indicate inhibitory interactions, dashed arrows represent uncertain interactions, and black arrows signify Ca^{2+} fluxes. MCU, mitochondrial Ca^{2+} uniporter; VDAC1, voltage-dependent anion-selective channel 1. Modified from Loncke et al., 2021.

1.3.5. The treatment perspectives for Wolfram syndrome

Beyond symptomatic approaches like insulin replacement, there are presently no interventions available to modify the progression of Wolfram syndrome. Nevertheless, various investigations in the cellular pathology of WS have opened novel avenues for treatment. Some treatments are under clinical investigation, as most are in preclinical testing.

The first completed uncontrolled open-label clinical trial in WS patients was published in 2021 (Abreu et al., 2021). The study enrolled adult and pediatric Wolfram syndrome patients. It aimed to determine the safety and tolerability of dantrolene sodium – a drug known for inhibiting ryanodine receptors on the ER and stabilizing ER Ca^{2+} (Abreu et al., 2021). The study incorporated 22 individuals with a genetically confirmed diagnosis of WS and lasted 6 months. Overall, dantrolene was very well tolerated. The small study population complicated the evaluation of dantrolene's effectiveness. Nevertheless, in a subgroup analysis, a minority of subjects appeared to experience improvements in the β cell functioning.

The second repurposing drug for WS is sodium valproate, which is currently under a phase II, placebo-controlled clinical trial with 70 pediatric and adult WS patients (trial number: NCT03717909) (Shah et al., 2021). In a preclinical WS study, sodium valproate increased WFS1 mRNA expression in neuronal cells by activating its promoter. It also suggested potential ER stress-modulating effects by enhancing the dissociation of WFS1 from a molecular chaperone GRP94 (Kakiuchi et al., 2009). A recent study revealed that sodium valproate reduces ER stress and cell apoptosis in WS1 models with dominant WFS1 mutations (Batjargal et al., 2020). Moreover, valproate is recognized for enhancing the expression of $p21^{cip}$, a cyclin-dependent kinase inhibitor with an anti-apoptotic effect. Consequently, valproate can potentially protect WS1 cells from cell death, as cell cycle assays in WFS1-depleted cells demonstrated a decrease in $p21^{cip}$ levels along with increased apoptosis (Gharanei et al., 2013).

Glucagon-like peptide-1 receptor (GLP-1R) agonists are proposed as potential therapeutics for WS1 due to their demonstrated antidiabetic effects in both animal models and WS2 patients (Rigoli et al., 2022). Considering the widespread presence of GLP-1R in neuronal cells, liraglutide proved to be neuroprotective in a WS rat model as it protected retinal ganglion cells from apoptosis and the optic nerve from degeneration (Seppa et al., 2019). Furthermore, the administration of exenatide (GLP-1R agonist) in a patient with WS type 2 led to a 70% reduction

in daily insulin requirements, enhanced glycemic control, and a 7-fold increase in maximal insulin secretion (Danielpur et al., 2016). Moreover, recently the dual-incretin agonist DA-CH5 exhibited therapeutic efficacy in a WS rat model. The treatment reversed glucose intolerance and intra-islet alterations in knockout rats, and visual acuity and retinal ganglion cell density were better preserved (Jagomäe et al., 2023). Accordingly, a clinical trial involving exenatide for WS type 2 (trial number: NCT010302327) was initiated. Also, a second trial using liraglutide (Victoza®) in patients with WS type 1 has recently been announced but not officially registered by Washington University in collaboration with the Snow Foundation (Shah et al., 2021).

Other potential treatment strategies for WS under preclinical investigations encompass the use of chemical chaperones to enhance the structure of mutant WFS1 proteins, ER calcium stabilizers targeting ER calcium transporters, compounds regulating ER-stress-mediated cellular apoptosis and activating cell survival mechanisms, modulators of mitochondrial function, as well as gene replacement therapy and gene editing to correct pathogenic *WFS1* variants (Mishra et al., 2021).

1.4. Summary of literature review

Neurodevelopmental and neurodegenerative disorders are both growing major public health concerns decreasing life quality and causing substantial financial burdens (Magnin, 2021). Interestingly, mitochondrial abnormalities are regularly described as a common prominent early pathological feature in neurodegenerative diseases, e.g., Alzheimer's disease, sporadic Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease (Johri & Beal, 2012; Lin & Beal, 2006). Moreover, intact mitochondrial functioning (e.g., mitochondrial dynamics) is crucial for neuronal development (Son & Han, 2018; Iwata et al., 2023). Despite the growing recognition of the role of mitochondria in various brain disorders, it is still unclear how exactly mitochondria contribute to the pathology of neurodegenerative/neurodevelopmental diseases and whether the observed mitochondrial deficits are primary or secondary contributors. Therefore, while the primary proteins and principles governing mitochondrial dynamics, specifically fusion and fission, in neurons, have been previously studied, there is still a lack of clarity regarding the interactions between mitochondrial fusion and fission events at the neuronal level (Chan, 2020; Twig et al., 2008; Wang et al., 2012).

The value of studying rarer monogenic diseases that share similarities with common conditions comes from broadening our understanding of the molecular basis of general burdensome diseases (Peltonen et al., 2006). In this context, we investigated an *in vitro* model of Wolfram syndrome, a progressive autosomal recessive neuroendocrine disorder characterized by diabetes mellitus, optic atrophy, diabetes insipidus, and deafness. Interestingly, WS was initially proposed as a mitochondriopathy (Bu & Rotter, 1993). Subsequent research has predominantly suggested mitochondrial involvement in the cellular pathogenesis of WS

type 2, characterized by loss-of-function mutations of CISD2 protein (Chen et al., 2010; Kanki & Klionsky, 2009). Conversely, WS type 1, associated with mutations in the WFS1 protein, is primarily considered an ER disease affecting Ca^{2+} homeostasis and ER stress regulation (Urano, 2014). However, sparse findings of mitochondrial involvement in the disease pathology of WS type 1 have also been reported (La Morgia et al., 2017). Possible mechanisms explaining how an ER resident protein like WFS1 could influence mitochondrial functioning suggest a role in both direct ER-mitochondrial connections and deviations in SERCA, IP3R, and RyR receptor functioning as crucial players in maintaining ER, mitochondrial, and cytosolic Ca^{2+} homeostasis.

Based on previous suggestions of mitochondrial involvement in WS disease mechanisms, we sought to explore the role of mitochondrial dynamics in the cellular pathogenesis of WS1. Additionally, we aimed to clarify the controversial aspects of intracellular Ca^{2+} regulation and its associations with mitochondrial dynamics in the disease pathogenesis of Wolfram syndrome and suggest possible pharmacological interventions.

2. AIMS OF THE STUDY

This study aimed to investigate the role of mitochondria in the pathogenesis of Wolfram syndrome.

The specific objectives were:

1) To elucidate the main principles governing mitochondrial fusion-fission balance in cultured cortical neurons
2) To investigate in an *in vitro* model the importance of mitochondrial dynamics in the cellular pathogenesis of Wolfram syndrome
3) To specify the cellular mechanisms of Wolfram syndrome and find possible pharmacological interventions to alleviate the impaired mitochondrial phenotype and delayed neuronal development

3. MATERIAL AND METHODS

3.1. Cell culture and transfection

Primary neuronal cultures of rat cortical cells were prepared from less than 1-day-old neonatal Wistar rats. Approximately >60% of the cells in the culture had neuronal phenotype. The cells were plated onto 35 mm glass-bottom dishes (MatTek, MA, USA) pre-coated with poly-L-lysine at a density of 10^6 cells per dish in 2 ml of cell suspension. Neurons were cultured in Neurobasal™-A medium with or without phenol red containing B-27 supplement, 2 mM GlutaMAX-I, and 100 µg/ml gentamicin.

Next, neuronal cultures were transiently transfected 1 to 3 days after plating using Lipofectamine™ 2000 (Invitrogen) according to the provided guidelines. Shortly, the conditioned medium was replaced with 100 µl Opti-MEM I medium containing 2% Lipofectamine™ 2000 and 1 to 2 µg of total DNA with an equal amount of each plasmid. The dishes were incubated for 3 to 4 h followed by adding fresh Neurobasal™-A medium. Finally, neurons were cultivated for 3 to 8 days to allow for the expression of the transfected DNA. During microscopy, the cells were kept in a Krebs–Ringer solution supplemented with 1 mM Ca^{2+} and 15 mM glucose or, alternatively, in a colorless Neurobasal™-A medium.

Our research didn't involve procedures that require ethical authorization under the Estonian 'Animal Protection Act,' Chapter 8. The project involved only the preparation of primary neuronal cultures, and no experiments were performed on live animals. We also certify that the animals used in the project were bred and kept at the Laboratory Animal Centre according to the Estonian Animal Protection Act and under the activity license given by the Estonian Veterinary and Food Board.

3.2. Plasmid and chemical list

Reagent or resource	Source	Identifier
Plasmids		
Fluorescent sensors		
CEPIA3mt	Addgene	Cat# 58219
ER-GCamp6-210	Addgene	Cat# 86919
ATeam	Addgene	Cat# 51958
Syn-jGCaMP7b	Addgene	Cat# 104489
Camk2-Ace-8aa-mScarlet	Addgene	Cat# 129702
GW1-PercevalHR	Addgene	Cat# 49082
mito-KikGR1	Generated in our lab (Cagalinec et al., 2013)	
mito-CFP	Evrogen (Moscow, Russia)	
mtKeima	Amalgaam	AM-V0251
DsRed2-Mito	Clontech	Cat# 632421

Reagent or resource	Source	Identifier
EGFP	Clontech	6085–1
hSyn-DsRed1	Addgene	Cat#22907
mKate2-mito	Evrogen	FP187
EGFP-LC3	Addgene	Cat#24920
Overexpression plasmids		
IP_3R1 wt and D2550A	Gift from Dr. David Yule	
IP_3R1 active fragment	Plasmid ID	MmCD00312368
SERCA2b	Addgene	Cat# 75188
Mfn1	Gift from Dr. S. Hirose	
wt Mfn2	Gift from Dr. S. Hirose	
dn Mfn2ΔTM	Gift from Dr. S. Hirose	
Drp1	Gift from Dr. G. Szabadkai	
Drp1 K38A (dominant negative)	Gift from Dr. G. Szabadkai	
Fis1	Gift from Dr. J.-C. Martinou	
WFS1 P724L	Addgene	Cat# 13012
WFS1-Flag	Addgene	Cat# 13011
shRNA-s		
Wfs1 shRNA	SABiosciences	KR46208N
Cisd2 shRNA	SABiosciences	KR47927N
Wfs1 siRNA	Sigma-Aldrich	SASI_Rn02_00265296 NM_031823
Drp1 shRNA	SABiosciences	KRxxxxxN
Parkin shRNA	SABiosciences	KR50238N
Pink1 shRNA	SABiosciences	KR55105N
Cell Culture		
B-27™ Plus supplement	ThermoFisher Scientific	Cat# A3582801
B-27™ supplement	ThermoFisher Scientific	Cat#17504044
Basal Medium Eagle (BME)	ThermoFisher Scientific	Cat# 41010109
Lipofectamine™ 2000	ThermoFisher Scientific	Cat# 11668019
Neurobasal™-A with or without fenol red	ThermoFisher Scientific	Cat# 10888022 or 12349015 (without fenol red)
N-TER Nanoparticle siRNA Transfection System	Sigma-Aldrich	Cat#N2913
Opti-MEM I Reduced Serum Medium	ThermoFisher Scientific	Cat# 11058021
Poly-L-lysine hydrobromide	Sigma-Aldrich	P6282
Chemicals		
(RS)-3,5-DHPG	Tocris	Cat# 0342
deoxyglycose	Sigma-Aldrich	D8375
oligomycin	Sigma-Aldrich	75351
glutamate	Sigma-Aldrich	G2128
azymolene sodium salt	Cayman Chemical	Cat# 16462
CDN1163	Tocris	Cat# 5869

Reagent or resource	Source	Identifier
CGP 37157	Tocris	Cat# 1114
liraglutide	Tocris	Cat# 6517
RYR-Calstabin Interaction Stabilizer (RyCal) S107	Millipore, Sigma-Aldrich	Cat# 500469
FCCP	Tocris	Cat# 0453
JC-10 [Enhanced JC-1], Ultra-Pure	Enzo	ENZ-52305
Mag-fluo-4 AM	ThermoFisher Scientific	Cat# M14206

3.3. Mitochondrial fusion rate

To analyze mitochondrial fusion and fission events, cortical neuronal cultures were transfected with the mito-KikGR1 plasmid and the plasmids of interest. A laser scanning confocal microscope (LSM 510 Duo, Carl Zeiss Microscopy GmbH) equipped with an LCI Plan-Neofluar 63×/1.3 water immersion DIC M27 objective was used. The temperature was maintained at 37°C during scanning using a climate chamber. Mito-KikGR1 green signal was elicited with a 488-nm argon laser line and selected axonal mitochondria were subsequently photoconverted to red using a 405-nm diode laser, and red mitochondrial signal was illuminated with a 561 nm DPSS laser. To compare the fusion rate difference of various transfection conditions, images were taken at 10 s intervals for 10 min, and the progression of all activated (red) mitochondria was followed throughout the time-lapse. To analyze the fusion-fission cycle, images were captured every 10 s for 2 hours.

3.4. Mitochondrial density and length

To measure mitochondrial length and density in axons, the neuronal cultures were transfected with mitochondrial pDsRed2, EGFP, scrambled shRNA, or *Wfs1* shRNA (*Wfs1* shRNA suppressed 84±4% of endogenous WFS1 protein expression in PC12 cells as estimated by Western blotting, $n = 5$) under vehicle or treatment conditions. Imaging was carried out 6 to 7 days after transfection. Fluorescence images of 10 randomly chosen axons from each culture dish were acquired using an Olympus IX70 inverted microscope equipped with a WLSM PlanApo ×40/0.90 water immersion objective and an Olympus DP70 CCD camera. Morphometric analysis was conducted using MicroImage software (Media Cybernetics, Bethesda, MD). For mitochondrial length and density assessments between different transfection conditions, a minimum of 40 axons were examined for each group.

3.5. Mitophagy assays

Mitochondrial degradation through autophagy was assessed by employing mitochondria-targeted pH-sensitive fluorescent protein Keima, whose excitation spectrum changes from 440 to 586 nm when mitochondria enter acidic lysosomes. Briefly, primary cortical neurons were incubated for 5 to 6 days with scrambled shRNA or *Wfs1* shRNA under vehicle or treatment conditions. Images were captured with a laser scanning confocal microscope (LSM 510 Duo, Carl Zeiss Microscopy GmbH equipped with an LCI Plan-Neofluar 63×/1.3 water immersion DIC M27 objective) using the 458 nm (green, indicating mitochondria at a neutral pH) and 561 nm (red, indicating mitochondria at an acidic pH) laser lines. Subsequently, the red dots per neuronal body were counted blindly.

In a different series of mitophagy experiments, neurons were transfected with pEGFP-LC3, mKate2-mito, and either scrambled shRNA or *Wfs1* shRNA. The analysis of co-localization between EGFP-LC3 dots and mitochondrial mKate2-mito was performed using ImageJ software (Schneider et al., 2012).

3.6. Mitochondrial membrane potential

Mitochondrial membrane potential was measured using JC-10 dye loading. JC-10 selectively enters mitochondria and reversibly changes its color from green to orange-red dependent on the membrane potential (emission changes from 520 nm to 570nm).

First, primary cortical neurons (seeded at lower density, 2.5×10^5 cells/ml) were transfected with 20 nM validated siRNA against Wfs1 (*Wfs1* siRNA suppressed 80 ± 1% of endogenous Wfs1 expression in primary cortical cells as estimated by RT-PCR, n = 3) using the N-TER Nanoparticle siRNA Transfection System according to the provided guidelines. Prior microscopy, the transfected cells were incubated for 72 to 96 h in a humidified 5% CO2/95% air incubator at 37°C. The N-TER Nanoparticle siRNA Transfection System displayed a relatively low level of toxicity (neuronal survival rate of 82% ± 4% (n=5 per group) determined using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Invitrogen)) 16 hours after transfection.

For JC-10 loading, siRNA-transfected dishes were kept in 10 µM JC-10 dissolved in culture media and incubated at 37°C for 20 min. During microscopy, the cells were maintained in Krebs-Ringer solution supplemented with 1 mM Ca^{2+} and visualized using a laser scanning confocal microscope equipped with an LCI Plan-Neofluar 63×/1.3 water immersion DIC M27 objective. Finally, the dishes were treated with 5 µM FCCP to establish background values.

To visualize mitochondria with intact membrane potential in autophagosomes, the neurons were co-transfected with LC3-EGFP and Wfs1 shRNA and incubated for 4 days. Next, the cells were treated with a 50 nM solution of the mitochondrial membrane potential-sensitive dye, tetramethylrhodamine ethyl ester (TMRE), in completed Neurobasal™-A medium at 37°C for 30 minutes.

Subsequently, neurons were visualized in Krebs-Ringer solution supplemented with 1 mM Ca^{2+} using a confocal microscope with a 100×/oil objective.

3.7. Axonal growth and synaptic density

For axonal growth analysis, at DIV1, cortical neurons were transfected with plasmids coding neuron-specific hSyn-DsRed1 and either scrambled shRNA or *Wfs1* shRNA under vehicle or treatment conditions. Images of randomly selected neuronal axons were captured at different time points (DIV2 to DIV6) employing an Olympus IX70 inverted microscope with a 20x objective. The length of the axonal tree, the length of the longest axon, and the number of axonal endings were measured, and neuronal reconstructions were established using Neurolucida (MBF Bioscience) or Fiji software (Meijering et al., 2004).

To visualize synapses, neurons were co-transfected with GFP and *Wfs1* shRNA on DIV2. Next, neurons were fixed and permeabilized at DIV4, DIV6, or DIV18 using the Image-iT™ Fixation/Permeabilization Kit (Life Technologies) as indicated in the manufacturer's protocol. The cells were then incubated first with the primary antibody, mouse anti-PSD95 (1:1000, ab2723, Abcam), in the presence of 3% normal goat serum at 4°C for 24 hours, and afterward with a secondary antibody, goat anti-mouse DyLight 594 (1:1000, ab96873, Abcam), at room temperature for 1 hour. The fluorescence signal was visualized using an LSM 510 confocal microscope with a 63×/1.3 water immersion objective. The red immunofluorescent puncta that closely neighbored the GFP-labeled soma or colocalized with neurites were manually quantified.

3.8. Neuronal viability

Cortical neurons were co-transfected with neuron-specific hSyn-DsRed1 and scrambled or *Wfs1* shRNA. Fluorescent living cells with visible neurites were manually counted in 8 dishes per group (50 fields per dish) under an Olympus IX70 inverted microscope equipped with a WLSM PlanApo 40x/0.90 water immersion objective.

3.9. Ca^{2+} measurements

Basal or treatment-induced live-cell Ca^{2+} measurements were conducted using genetically encoded GCaMP-based probes targeted to different subcellular compartments (Nakai et al., 2001). Specifically, neurons were transfected on DIV 2 to 3 with organelle-specific Ca^{2+} sensors targeted to mitochondria (Cepia 3mt), endoplasmic reticulum (ER-GCamp6-210), or cytosol (jGCaMP7b). Ratiometric Ca^{2+} imaging was conducted 6 to 7 days later by recording the green fluorescence emission of GCaMP from 495 to 555 nm following excitation with both 405 and

488 nm lasers. Time-lapse Ca^{2+} imaging experiments using 100 µM glutamate (100 µM glutamate and 10 µM glycine) or DHPG treatment were performed on DIV 9 to 10.

ER Ca^{2+} uptake was measured in permeabilized cells loaded with Mag-Fluo-4 AM. Firstly, neurons were transfected with scrambled, *Wfs1* shRNA or *Cisd2* shRNA along with the mitochondrial marker mito2Kate at DIV 2 to 3. At DIV 9 to 10, the growth media was removed, and cells were incubated in Krebs-Ringer solution containing 1 mM Ca^{2+} and 5 µM Mag-Fluo-4 AM for 1 hour at 37°C. Subsequently, cells were permeabilized in a basic solution containing ethylene glycol-bis(β-aminoethyl ether)N, N, N′, N′-tetra-acetic acid 10 mM (EGTA), N, N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid 30 mM (BES, pH 7.1), free Mg^{2+} 1 mM, taurine 20 mM, glutamic acid 5,56 mM, malic acid 1,5 mM, K_2HPO_4 3,9 mM, dithiothreitol 0.5 mM, NaATP 3,16 mM; (ionic strength was adjusted to 160 mM with potassium methanesulfonate, pH 7.1) with saponin (50 µg/ml) for 16 minutes at 4°C and washing with the same solution containing additionally sodium azide 3 mM. Imaging was conducted using an LSM 780 confocal microscope (Plan Apochromat 20X/0.5 objective) at room temperature. A random field of 424 X 424 µm was selected from each dish and imaged at 3s intervals over 10 min. After 20 frames, $CaCl_2$ was added to achieve pCa 6.5.

3.10. ATP measurements

The intracellular ATP levels were determined using a ratiometric fluorescence resonance energy transfer (FRET)-based ATP sensor ATeam or a genetically encoded fluorescent ratiometric probe PercevalHR (Tantama et al., 2013).

In the experiments using ATeam, previously transfected cortical neurons were illuminated with 458 nm line (10%) of an argon laser. Next, the CFP emission was acquired at 465 to 500 nm and the FRET signal at 520 to 570 nm. The FRET/CFP fluorescence intensity ratio was then determined based on the signal coming from the cytosol.

PercevalHR has been previously validated for our experimental conditions (Vaarmann et al., 2016). Firstly, neurons were transfected with ATP/ADP ratio sensor PercevalHR and scrambled or *Wfs1* shRNA followed by vehicle or treatment conditions. Next, the fluorescence signal was visualized in the neuronal endings using a 405 nm diode laser and the 488 nm line from an Argon or diode laser. Emission was collected within a 495 to 555 nm emission window. The data is presented as the ratio of fluorescence emission from axons evoked by 488 nm excitation divided by 405 nm excitation (F488 nm/F405 nm).

3.11. Statistics

Data are presented as the mean ± SEM. The D'Agostino-Pearson omnibus test was used to test the normality of distribution. To test the equality of variances, we used the F test for two conditions or the Brown and Forsythe test for more than two conditions. In the Ca^{2+} experiments, the outliers were removed (ROUT, Q 1%) to exclude bursting neurons.

Student's t-tests, one-way ANOVA followed by Bonferroni post-hoc test (selected pairs) or Newman-Keul's post-hoc tests or Sidak's multiple comparison test or Brown-Forsythe ANOVA followed by Dunnett's T3 multiple comparisons test or Welch's ANOVA and Dunnett's T3 multiple comparisons test were used to compare the parametric data. Mann-Whitney U-tests or Kruskal–Wallis tests followed by the Dunn test were used to analyze the non-parametric data. Two-way ANOVA was used to obtain the interaction between the two treatments. The χ^2-test was used to determine whether the observed distribution differed significantly from the expected distribution. P-values of less than 0.05 were considered statistically significant.

4. RESULTS

4.1. Mitochondrial fusion-fission balance in cortical neurons (Paper I)

Mitochondrial length is determined by two processes: mitochondrial fusion and mitochondrial fission. Two mitochondria merging and unifying their contents results in mitochondrial fusion, which subsequently leads to mitochondrial elongation (Westermann, 2008). On the other hand, mitochondrial fission, the splitting apart of mitochondria, leads to mitochondrial shortening and the formation of morphologically and functionally distinct organelles (Westermann, 2008). The balance between mitochondrial fission and fusion is finely tuned within cells (Adebayo et al., 2021). Nevertheless, although the proteins involved and the potential interplay between these reciprocal events are relatively well known, the practical principles governing fission-fusion processes in neurons have not been systematically elucidated (Abrisch et al., 2020; Pozo Devoto et al., 2022; Twig et al., 2008; S. Wang et al., 2012).

4.1.1. Fusions and fissions are balanced and sequential events

We used confocal microscopy and the KikGR1 protein, which changes color from green to red upon laser irradiation, to understand the fundamental principles of fusion-fission balance in rat primary cortical neurons. The mitochondrial localization of KikGR1 was confirmed by observing its co-localization with the established mitochondrial marker mito-CFP (Figure 5A and 5B).

Firstly, we found that the fusion rate was the same as the fission rate in cortical neurons (0.023±0.003 fusions or fissions/mitochondria/min). Secondly, we identified, similar to Twig et al., that a fusion or a fission event was likely followed by corresponding fission or fusion forming a cyclic pattern (Twig et al., 2008). In cortical neurons, a fusion was followed by fission in 86.4% of instances and a second fusion in only 13.6% of cases (Figure 5C). Similarly, fission was followed by fusion in 83.5% of cases and by a second fission in 16.5% of cases (Figure 5C). Thus, non-cyclic events occur in cortical neurons in 15% of the cases. Similar observations were reported by Wang et al., where 35% of events in HeLa cells and 40% in MEF cells deviated from the cyclic pattern (S. Wang et al., 2012).

Subsequently, we assessed the durations of the two phases within the fusion-fission cycle in cortical neurons. We found that the average time interval between fusion and fission was 4.7±0.6 minutes, while the interval between fission and the subsequent fusion was 15.3±2.0 minutes. The complete duration of the cycle was approximately 20 minutes. It is important to highlight that this analysis specifically targeted the active subpopulation of mitochondria and could explain the shorter cycle duration compared to calculations based on the fusion or fission rates of the entire mitochondrial population (approximately 43 minutes). These findings indicate that neuronal mitochondria primarily spend most of the time in

the post-fission state, similarly found earlier by Twig et al. in different cell lines (Twig et al., 2008).

Next, we aimed to determine the practical principles regulating fusion-fission balance in neurons.

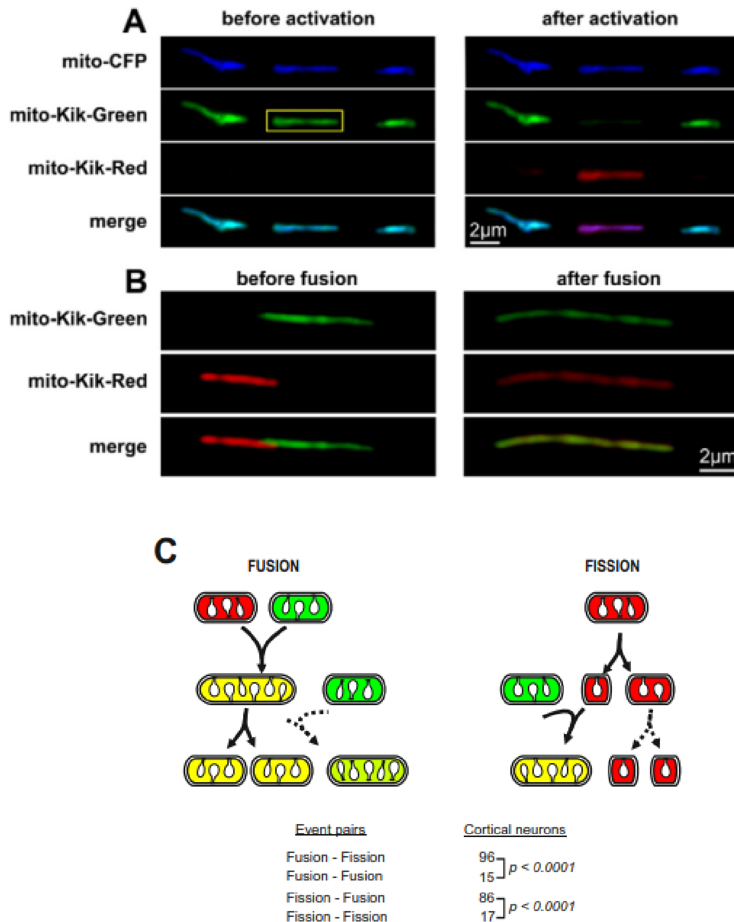


Figure 5. Mitochondrial fusion-fission events in cortical neurons. (A) For fusion-fission rate analysis randomly chosen mitochondria, designated with a yellow rectangle, were irradiated using a 405-nm laser line, causing mito-Kikume-Green to convert into mito-Kikume-Red (right panels). **(B)** An example of a fusion event between mito-Kikume-Green and the photoactivated mito-Kikume-Red mitochondria resulting in a yellow descendant due to the blending of contents from the red and green mitochondrial matrices. **(C)** Each fusion event (left) was succeeded by either a fission or a secondary fusion, and each fission event (right) was followed by a fusion or a secondary fission. To determine the frequency of sequential fusion and fission events in cortical neurons, we analyzed 111 event pairs starting with fusion and 103 pairs starting with fission, and the count for each type of event is displayed. A χ^2-test was conducted to assess whether the observed distribution significantly deviated from the expected distribution.

4.1.2. Fission rate is determined by mitochondrial length

Given the intermittent nature of fusion and fission, it would be logical to assume that the feedback mechanisms regulating those processes depend on mitochondrial length. Thus, we conducted a so-called 'twin study' comparing daughter mitochondria originating from the same parent mitochondrion.

For that purpose, we monitored 30 pairs of the same parent mitochondria (so-called twins) until one of the daughters underwent a second fission. Similarly, we followed 42 pairs of the same parent mitochondria until one of the daughters fused. We found that the average length of the daughter undergoing second fission exceeded twice that of its non-splitting twin. Interestingly, the average length of the fusing daughter precisely matched the length of the non-fusing daughter. Hence, mitochondrial length determines the fission rate but does not influence the fusion rate (Figure 6A). Therefore, shorter mitochondrial length reduces the likelihood of the next event being fission and, correspondingly, heightens the probability of the next event being a fusion.

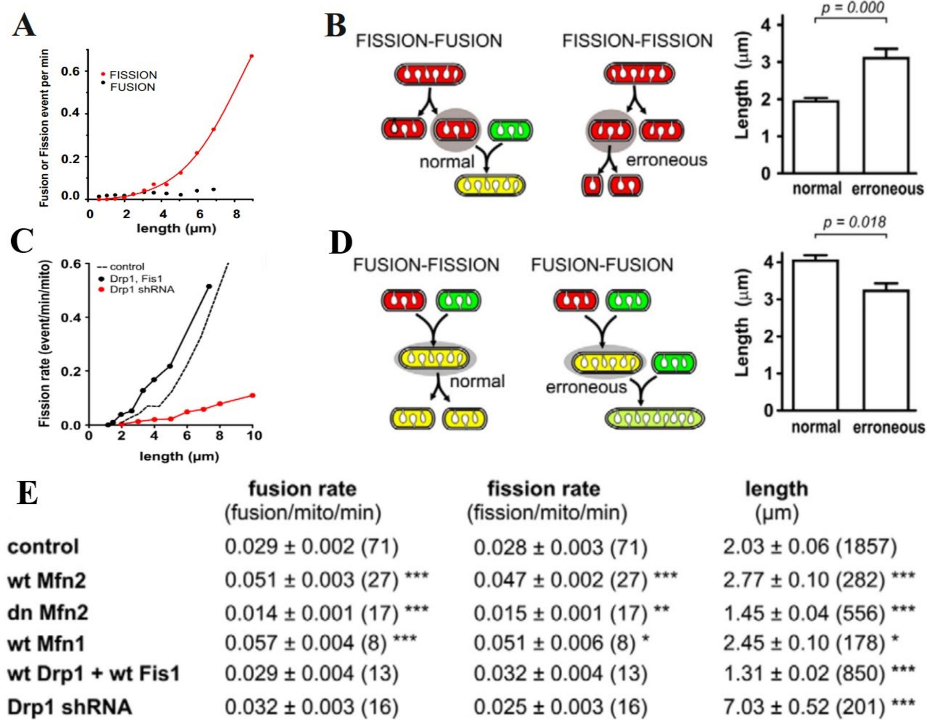
This finding suggests that the longer mitochondria following fission are more prone to experience subsequent 'corrective' fission. Hypothetically, post-fusion mitochondria that are too short are more likely to undergo secondary fusion. We next examined specific cases to ascertain whether fusion-fission cycle errors were associated with mitochondrial length. Indeed, we found that mitochondria undergoing second fission and interrupting their regular cycle were notably longer than those entering fusion (Figure 6B). Furthermore, mitochondria experiencing a second fusion event after fusion were notably shorter than those undergoing fission (Figure 6D). This suggests that such a feedback mechanism may function as a quality control mechanism to maintain cellular average mitochondrial length and avoid extremes.

Next, we investigated the role of major fusion-fission proteins Drp1, Fis1, and Mfn1/2 levels in regulating fission rate and mitochondrial length. First, Drp1 and Fis1 expression levels were manipulated in cortical neurons. As anticipated, overexpression of the fission proteins Drp1 and Fis1 resulted in shortened mitochondria, whereas silencing of Drp1 led to significant mitochondrial elongation. However, neither Drp1 silencing nor Drp1 and Fis1 overexpression led to consistent changes in fission or fusion rates (Figure 6E). An additional analysis of the fission rate–length relationship showed that overexpression of Drp1 and Fis1 increased the sensitivity of the fission rate to mitochondrial length. In contrast, silencing Drp1 with shRNA dramatically decreased this sensitivity (Figure 6C). In contrast, no such fission-mitochondrial length relationship was observed following the overexpression of wild-type (wt) Mfn2 or dominant negative Mfn2 (data not shown). These results indicate that the length–fission feedback loop is Drp1 dependent.

Therefore, we concluded that the length–fission dependency controls mitochondrial length in neurons and is the primary feedback tool that enables neurons to sense and correct mitochondrial length. This feedback mechanism also explains the well-tuned balance between fission and fusion rates. We observed no

significant change in the fusion rate to fission rate ratio upon altering fusion-fission protein levels. The overexpression of wt Mfn2 doubled both fusion and fission rates, with only a minor increase in mitochondrial length (Figure 6E). It is plausible that an increase in fusion activity caused a slight elongation of mitochondria, which triggered the fission machinery to prevent further elongation.

Moreover, these findings explain the ineffectiveness of manipulating the fission machinery to alter the fission rate. For instance, while upregulating Drp1 did induce mitochondrial shortening, it did not elevate the fusion rate. Attempting to increase the fission rate to shorten mitochondria is likely counteracted by inhibiting the fission machinery resulting from the same mitochondrial shortening. Conversely, suppressing the fission rate by inhibiting Drp1 had the opposite effect, leading to mitochondrial elongation, which, in turn, activated the fission machinery. Thus, mitochondrial length is self-regulated through Drp1-dependent fission and determines the fission rate. Relevantly, mitochondrial length does not influence the fusion rate.



	fusion rate (fusion/mito/min)	fission rate (fission/mito/min)	length (μm)
control	0.029 ± 0.002 (71)	0.028 ± 0.003 (71)	2.03 ± 0.06 (1857)
wt Mfn2	0.051 ± 0.003 (27) ***	0.047 ± 0.002 (27) ***	2.77 ± 0.10 (282) ***
dn Mfn2	0.014 ± 0.001 (17) ***	0.015 ± 0.001 (17) **	1.45 ± 0.04 (556) ***
wt Mfn1	0.057 ± 0.004 (8) ***	0.051 ± 0.006 (8) *	2.45 ± 0.10 (178) *
wt Drp1 + wt Fis1	0.029 ± 0.004 (13)	0.032 ± 0.004 (13)	1.31 ± 0.02 (850) ***
Drp1 shRNA	0.032 ± 0.003 (16)	0.025 ± 0.003 (16)	7.03 ± 0.52 (201) ***

Figure 6. Mitochondrial fission rate, contrary to fusion rate, is length dependent. (A) Mitochondria from cortical neurons (1277) were subgrouped based on their lengths, and the fission (red circles) and fusion rates (black circles) were determined for each subgroup. **(B)** The length of post-fission mitochondria entering either fusion (normal cycle, n=586) or fission (erroneous, cycle-breaking, n=517) and **(D)** the length of post-fusion mitochondria entering either a fission (normal cycle, n=596) or fusion event (erroneous,

cycle-breaking, n=515) are presented. **(C)** The length dependency of mitochondrial fission in neurons expressing Drp1 shRNA (red line, n=513), Drp1 and Fis1 (black line, n=531), and control (dashed line). **(E)** Additionally, fusion-fission rates and mitochondrial lengths were examined in control, wild-type (wt) Mfn2-, dominant negative (dn) Mfn2-, wt Mfn1-, wt Drp1+ wt Fis1-overexpressing, and Drp1-suppressed neurons. The data is represented by means ± S.E.M., with significance denoted as $*p<0.05$, $**p<0.01$, and $***p<0.001$ versus control. The number of dishes analyzed (for fusion and fission rate) or the number of mitochondria analyzed (for length) is indicated in brackets.

4.2. Role of mitochondrial dynamics in the disease mechanisms of Wolfram syndrome 1 (Paper II)

WS1 is a rare neurodevelopmental and neurodegenerative disease in which mitochondrial dysfunction has been suggested. The hypothesis relies on the observation that the tissues and organs impacted in WS exhibit a heightened metabolic demand. Moreover, most WS clinical manifestations align with a defect in energy metabolism (e.g., deafness, optic atrophy, psychiatric disorders). The idea finds further support from discoveries indicating that another causative gene, *CISD2*, found in individuals with WS type 2, is linked to mitochondrial abnormalities and the initiation of mitophagy (Y.-F. Chen et al., 2009; Tsai et al., 2015; Wiley et al., 2013).

Besides the plausible mitochondrial involvement, several studies have highlighted the role of WFS1 in regulating Ca^{2+} homeostasis and managing ER stress (Fonseca et al., 2005; Osman et al., 2003; Takei et al., 2006). There is a suggestion that ER stress contributes causatively to WS. Importantly, these two hypotheses are not mutually exclusive, as ER stress can also compromise mitochondrial function (Malhotra & Kaufman, 2011; Vannuvel et al., 2013; Win et al., 2014).

4.2.1. Mitochondrial dynamics is impaired in WFS1 deficiency

WS1 was modeled in primary neuronal cultures using *Wfs1* shRNA treatment (the efficiency of *Wfs1* shRNA estimated by Western blotting was 83.8% ± 1.7%). We observed a significant decrease in the number of fusion events compared with scrambled shRNA-treated controls (from 0.029 ± 0.001 to 0.010 ± 0.001 fusion/mito/min, respectively, n = 80 neurons, $p < 0.0001$) (Figure 7A). Additionally, the mitochondrial fission rate was decreased in *Wfs1* shRNA-treated neurons (from 0.027 ± 0.001 to 0.010 ± 0.001 fission/mito/min, n = 80 neurons, $p < 0.0001$), indicating that WFS1 deficiency extends the fusion-fission cycle. Also, the changes in the fusion-fission cycle led to a 20% decrease in mitochondrial length (Figure 7B), which means mitochondrial fragmentation and suggests that mitophagy may be activated.

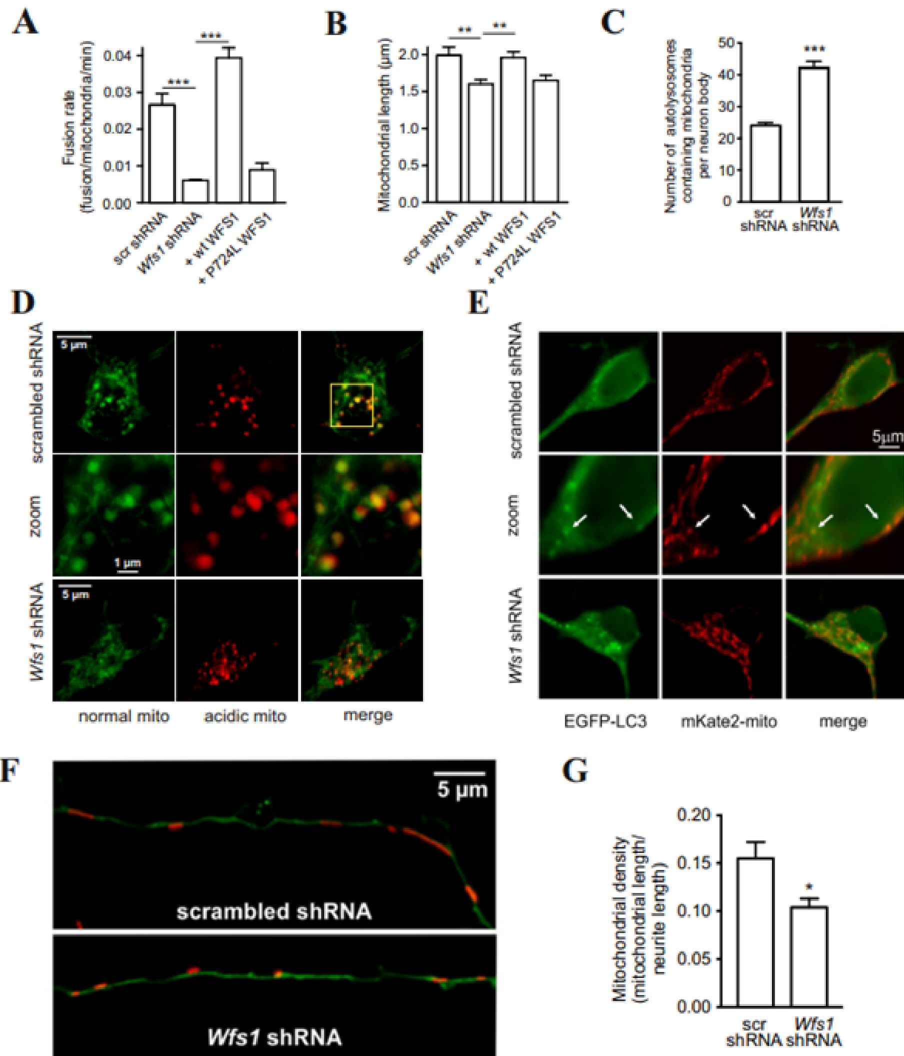


Figure 7. WFS1 deficiency disrupts mitochondrial dynamics in primary cortical neurons. Wfs1-deficient neurons show reduced fusion rate **(A)** and mitochondrial length **(B)**. These effects are restored by the overexpression of wild-type (wt) WFS1, but not by a WS1 mutant P724L WFS1. **(C)** The number of autolysosomes containing mitochondria was increased in Wfs1-silenced neurons measured with mitochondria-targeted pH-sensitive fluorescent protein mtKeima. **(D)** Representative images of mtKeima in scrambled- and *Wfs1*-shRNA transfected neuronal bodies. **(E)** In different mitophagy experiments, neurons were transfected with the autophagosome marker EGFP-LC3, the mitochondrial marker mKate2-mito, and either scrambled shRNA or *Wfs1* shRNA. Four days later, the number of co-localizations was analyzed. **(F)** Representative images of mitochondrial morphology and density in the axons of scrambled- and *Wfs1*-shRNA transfected neurons are presented, indicating a reduction in axonal mitochondrial density in *Wfs1*-silenced neurons **(G)**. Statistical significance is denoted as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared to the control groups.

To assess mitophagy, we introduced the mitochondrially targeted pH-sensitive protein Keima, which exhibits a shift in its excitation spectrum (from green to red) when mitochondria are transported to acidic lysosomes (Figure 7D). The count of autolysosomes containing mitochondria notably increased in wt neurons transfected with *Wfs1* shRNA (Figure 7C). Furthermore, the number of autophagosomes containing mitochondria (identified by the co-localization of LC3-positive dots with a mitochondrial marker) significantly increased within the neuronal bodies of *Wfs1* shRNA-treated neurons (13.8 ± 1.1 compared to 8.8 ± 0.8 in neurons transfected with scrambled shRNA, n = 50 neurons, p = 0.0004) (Figure 7E).

The LC3 and Keima assays reveal that WFS1 deficiency enhances the elimination of mitochondria rather than impeding the formation of autolysosomes containing mitochondria. As an expected result of augmented mitophagy within WFS1-deficient axons of cortical neurons, an approximate 30% reduction in mitochondrial mass was observed (Figure 7F and 7G).

Altogether, in neurons lacking WFS1, mitochondria exhibit a reduced frequency of fusion and fission compared to their wild-type counterparts and a higher incidence of mitophagy, resulting in decreased mitochondrial length and reduced axonal mitochondrial density. It can be assumed that besides dysregulated Ca^{2+} homeostasis in WFS1-deficient cells described earlier in the literature, the changes in mitochondrial dynamics could also be among the factors influencing the maintenance of mitochondrial membrane potential and compromising cellular energy production (Oliveira, 2012).

4.2.2. Mitochondrial membrane potential and ATP production decrease in WFS1-deficient cells

Impaired mitochondrial dynamics is frequently associated with abnormalities in mitochondrial function, such as insufficient ATP production and difficulties maintaining an adequate membrane potential (Van Laar and Berman, 2013).

Hence, we used the N-TER nanoparticle siRNA transfection system to introduce *Wfs1* siRNA into neurons (achieving transfection efficiency of over 70%), and conducted a quantitative evaluation of mitochondrial membrane potential with a ratiometric fluorescent probe JC-10. The results revealed a 10% reduction in the red-to-green fluorescence ratio, thus a slight depolarization in the *Wfs1* siRNA group was found (Figure 8A). Moreover, an increased quantity of polarized mitochondria, identified as positive for TMRE, was noted within autophagosomes in WFS1-deficient neurons (Figure 8D and 8E). Our findings suggest that WFS1 deficiency may trigger mitophagy, potentially targeting active and polarized mitochondria.

To better understand the cellular energy status in WFS1 deficiency, we assessed cellular ATP levels utilizing the fluorescence resonance energy transfer (FRET)-based ATP sensor ATeam. The validity of ATeam in our experimental settings was confirmed through control experiments involving deoxyglucose/

oligomycin or glutamate. Under these conditions, a reduction in the FRET signal was observed, affirming the anticipated decline in neuronal ATP levels (Figure 8B). Overall, as illustrated in Figure 8C, WFS1 deficiency resulted in reduced cytosolic ATP levels in WFS1-deficient neurons.

Though the disrupted mitochondrial dynamics and bioenergetics in WS are well-evident in our cell model and have also been shown by Zatyka et al., the causal link between WFS1 deficiency and mitochondrial dysfunction needs further elucidation (Zatyka et al., 2023). La Morgia et al. have suggested the possibility of a secondary mitochondrial dysfunction, potentially mediated by Ca^{2+} mishandling between the ER and mitochondria, mainly manifested as defective Ca^{2+} efflux from the ER (La Morgia et al., 2020).

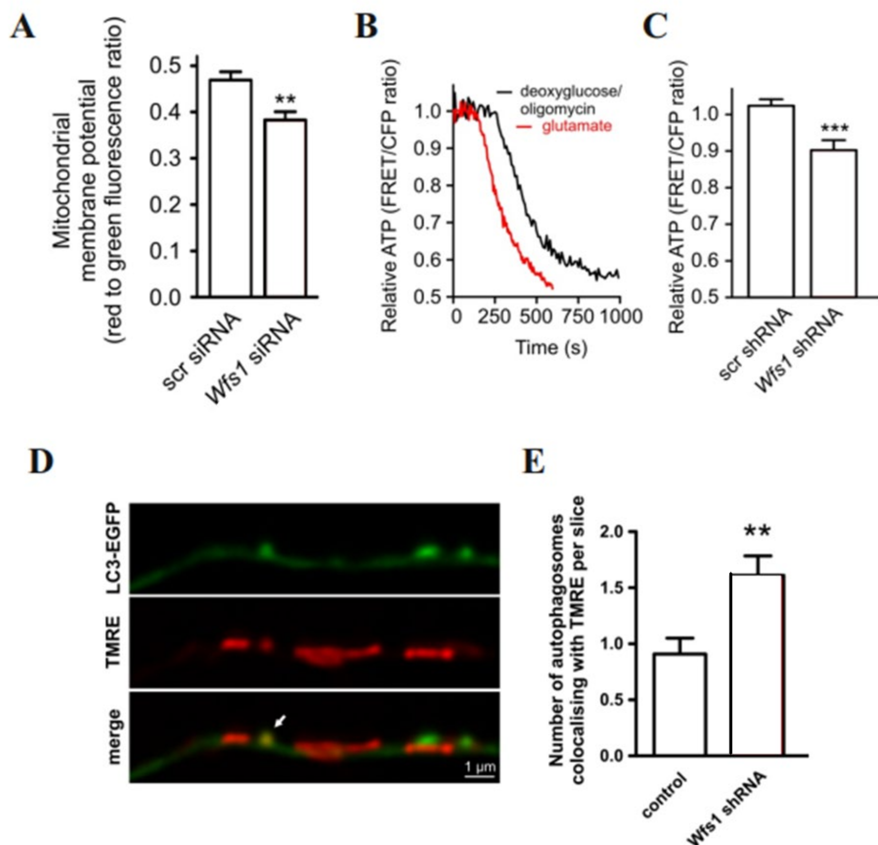


Figure 8. WFS1 deficiency reduces mitochondrial membrane potential and diminishes cytosolic ATP levels in neurons. (A) Wfs1-silenced neurons show a modest yet significant decrease in the red-to-green ratio of JC-10 fluorescence, indicating reduced mitochondrial membrane potential. The presented values are adjusted by subtracting the values acquired during FCCP incubation (5 μM). **(B)** Neurons transfected with the ATP sensor ATeam and subjected to 2-deoxyglucose (12 mM)/oligomycin (2.5 μM) or glutamate (2 mM) treatments (both used as positive controls), exhibit a decline in relative

cytosolic ATP levels. **(C)** Wfs1-deficient neurons show lower cytosolic ATP levels than the control group estimated with an ATP sensor ATeam. **(D)** An illustration of a TMRE-positive mitochondrion surrounded by an EGFP-LC3-positive autophagosome (indicated by a white arrow) in a *Wfs1* shRNA transfected neuron. **(E)** Quantification of autophagosomes colocalizing with TMRE-positive mitochondria per optical slice in neuronal bodies. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with a respective control group.

4.3. Alterations in intracellular Ca^{2+} homeostasis in Wolfram syndrome (Paper III)

In the existing literature, the disease mechanisms of WS are mainly attributed to the ER involving ER stress and impaired ER Ca^{2+} regulation. Yet, the precise mechanisms of how these disruptions contribute to the disease progression have remained elusive (Loncke et al., 2021). One possible hypothesis is that perturbations in ER Ca^{2+} levels may impact mitochondrial function, including fusion-fission balance, mitophagy, and suppressed mitochondrial ATP production. Remarkably, in cells derived from individuals with WS that lack either functional WFS1 or CISD2, mitochondria receive less calcium from the ER (Angebault et al., 2018; Crouzier, Danese, et al., 2022; Rouzier et al., 2017). Therefore, we hypothesized that intracellular basal calcium levels are altered in WFS1-deficient neurons and aimed to specify the connections behind the disturbances of ER-mitochondria Ca^{2+} fluxes.

4.3.1. Intracellular compartmental Ca^{2+} levels are altered in Wolfram syndrome

First, to investigate the basal Ca^{2+} homeostasis in distinct cellular compartments and assess intracellular Ca^{2+} levels in the axons of cortical neurons lacking WFS1 or CISD2, we measured baseline free Ca^{2+} concentrations within the ER and cytosol, specifically in the axoplasm (i.e., cytosol in axons). For this purpose, we used ratiometric Ca^{2+} sensors, such as the GCaMP family of fluorophores with dual excitation peaks at 410 and 474 nm. The ratio between the 488 nm and 405 nm wavelengths facilitated the measurement of fluorescence signals directly proportional to Ca^{2+} concentration while minimizing the impact of variations in probe expression levels (Figure 9A and 9B). Notably, the affinity of the ER Ca^{2+} sensor ER-GCaMP6-210 matches the resting axonal $[Ca^{2+}]_{ER,}$ and the cytosolic Ca^{2+} sensor jGCaMP7b has a relatively high baseline fluorescence at low resting axonal $[Ca^{2+}]_{cyto}$. Our observations revealed that in axons deficient in WFS1 or CISD2, the diminished basal ER Ca^{2+} levels (Figure 9C) correlated with elevated resting axoplasmic Ca^{2+} levels (Figure 9D). This suggests that ER Ca^{2+} regulation is compromised in the axons of neurons lacking WFS1 or CISD2, leading to elevated cytosolic $[Ca^{2+}]_{cyto}$.

To confirm that lower ER Ca^{2+} is unrelated to other factors, such as an altered cytosolic environment, we estimated ER Ca^{2+} levels in permeabilized cells under a constant "cytosolic" Ca^{2+} concentration. The ER Ca^{2+} levels remained lower in WFS1-deficient neurons, suggesting it is a primary intrinsic deficit in ER Ca^{2+} handling and not an increase in cytosolic Ca^{2+} that induces the loss of ER Ca^{2+} (Figures 9E and 9F).

Previously, it was demonstrated that the depletion of ER Ca^{2+} hinders the direct Ca^{2+} flux from the ER to mitochondria at the ER-mitochondria contact sites, mediated via IP3R (Bartok et al., 2019; Cárdenas et al., 2010; Szabadkai et al., 2006). Indeed, our findings reveal that neurons deficient in WFS1 or CISD2 released significantly lower levels of Ca^{2+} into the axoplasm when stimulated by DHPG, a metabotropic glutamate receptor agonist known to induce IP3 production in glutamatergic neurons (Figure 9G). Thus, the deficiency of WFS1 and CISD2 is linked to lower IP3R-mediated ER to mitochondria Ca^{2+} transfer, potentially leading to decreased mitochondrial Ca^{2+} content. Additional ratiometric assessments of mitochondrial Ca^{2+} levels, utilizing the mitochondrially targeted G-Cepia3mt (as shown in Figure 9H), revealed that decreased IP3R-mediated ER to mitochondria Ca^{2+} flux was linked to reduced mitochondrial calcium uptake (as depicted in Figure 9I) and a decline in basal Ca^{2+} levels within the mitochondrial matrix (as indicated in Figure 9J). It is worth noting that, like GCamp6 and 7, GCepia3 probe features two excitation peaks, allowing for ratiometric measurements independent of probe expression levels.

Altogether, under physiological conditions, the ER serves as a primary intracellular Ca^{2+} reservoir that actively participates in orchestrating the dynamic control of both cytosolic and mitochondrial Ca^{2+} levels (Stutzmann & Mattson, 2011). Our findings indicate that a deficiency in either WFS1 or CISD2 reduces ER Ca^{2+} store content. This compromised ER Ca^{2+} storage affects axoplasmic Ca^{2+}, leading to an accumulation of Ca^{2+} in the axoplasm and an elevation of basal cytosolic Ca^{2+} levels, as previously suggested in WS (Angebault et al., 2018; Lu et al., 2014). Moreover, in the absence of WFS1 and CISD2, the depletion of ER Ca^{2+} also results in mitochondrial Ca^{2+} depletion related to hindered IP3R-mediated Ca^{2+} delivery from the ER to mitochondria.

To further elucidate the possible connections between disrupted intracellular Ca^{2+} levels (decreased $[Ca^{2+}]_{ER}$, $[Ca^{2+}]_{mito}$, and increased $[Ca^{2+}]_{cyto}$) and mitochondrial functioning in WFS1 deficiency we asked how we can correct the alterations in the observed Ca^{2+} levels and whether it also restores mitochondrial health.

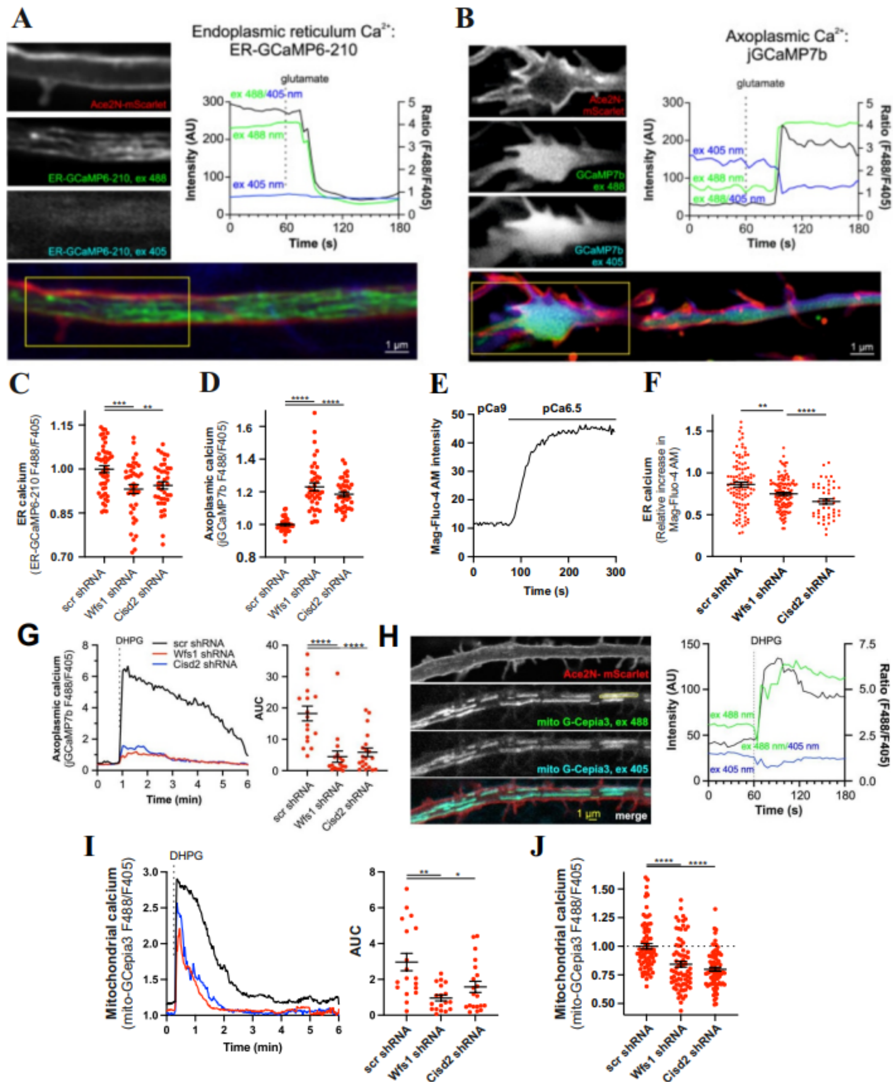


Figure 9. Decreased ER Ca^{2+} uptake and reduced ER basal Ca^{2+} levels are associated with increased cytosolic Ca^{2+} levels and suppressed mitochondrial Ca^{2+} uptake together with decreased basal mitochondrial Ca^{2+} levels in the axons of WFS1- and CISD2-deficient neurons. (A) To visualize axonal ER basal Ca^{2+} levels, neurons were transfected with ER-GCamp6-210 and Ace2N-mScarlet at DIV 2 to 3. Ratiometric imaging of the axonal shaft was performed 6 to 7 days later. **(B)** For imaging cytosolic Ca^{2+} levels in axonal endings, neurons were transfected at DIV 2 to 3 with GCaMP7b and Ace2N-mScarlet, and ratiometric imaging of the axons was conducted 6 to 7 days later. **(C)** WFS1- and CISD2-deficient neurons demonstrated reduced basal ER Ca^{2+} levels in axons. **(D)** WFS1- and CISD2-deficient neurons exhibited elevated basal cytosolic Ca^{2+} levels. **(E-F)** ER Ca^{2+} uptake was measured in permeabilized neurons loaded with Mag-Fluo-4 AM. Results are shown as the relative Ca^{2+} uptake of the transfected cell compared to the Ca^{2+} uptake in non-transfected neurons within the same field. **(G)** DHPG-induced

Ca^{2+} release from ER to cytosol is lower in WFS1- and CISD2-deficient neurons. The left panel illustrates the averaged Ca^{2+} transients following treatment with 100 μM DHPG, while the right panel displays the area under the curve (AUC) of these transients. **(H)** Visualization of mitochondrial Ca^{2+} levels in axons was conducted by transfecting neurons with mitochondrially targeted Cepia3mt and Ace2N-mScarlet at DIV 2 to 3. Ratiometric imaging of the axonal shaft was performed 5 to 6 days later. **(I)** DHPG-induced Ca^{2+} uptake to axonal mitochondria is diminished in WFS1- and CISD2-deficient neurons. The left panel illustrates the averaged Ca^{2+} transients following treatment with 100 μM DHPG, while the right panel shows the area under the curve of these transients. **(J)** Basal levels of mitochondrial Ca^{2+} are reduced in the axons of WFS1- and CISD2-deficient neurons. The statistical significance is indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

4.3.2. Pharmacological manipulation of intracellular Ca^{2+} homeostasis improves mitochondrial health in WFS1-deficient neurons

As previously described, our results indicate that changes in mitochondrial Ca^{2+} homeostasis, dependent on the proper functioning of ER, are an important factor in the pathological mechanisms linked to WFS1 deficiency. In theory, pharmacological interventions could restore this balance by enhancing ER calcium uptake and release and improving Ca^{2+} uptake by mitochondria. Consequently, we tested whether mitochondrial health (mitochondrial density, length, and mitophagy) and neuronal bioenergetics (ATP/ADP ratio) could be restored by pharmacological compounds known for their capacity to safeguard against calcium depletion in both the ER and mitochondria. Furthermore, we also included liraglutide (500 nM) in our experiments, considering its status as a primary therapeutic option for WS patients and its known neuroprotective effects in animal studies (Frontino et al., 2021; Jagomäe et al., 2021; Seppa et al., 2019).

We found that treatment of WFS1-deficient neurons with a SERCA activator CDN1163 (0.5 μM), an inhibitor of Ca^{2+} release via RYR azumolene (20 μM), an inhibitor of Ca^{2+} leak through the RyR Rycal-S107 (5 μM), a selective inhibitor of Ca^{2+} efflux through the mitochondrial Na^{+}/Ca^{2+} exchanger CGP37157 (10-20 μM) and GLP1 agonist liraglutide restored mitochondrial density and length as well as mitophagy intensity along with ATP levels in most settings, with CPG37157 being effective in all settings (Figure 10).

These findings strongly suggest that adjusting the balance of ER/mitochondrial calcium homeostasis could be an effective approach to restoring mitochondrial dynamics and cellular bioenergetics in individuals with WS.

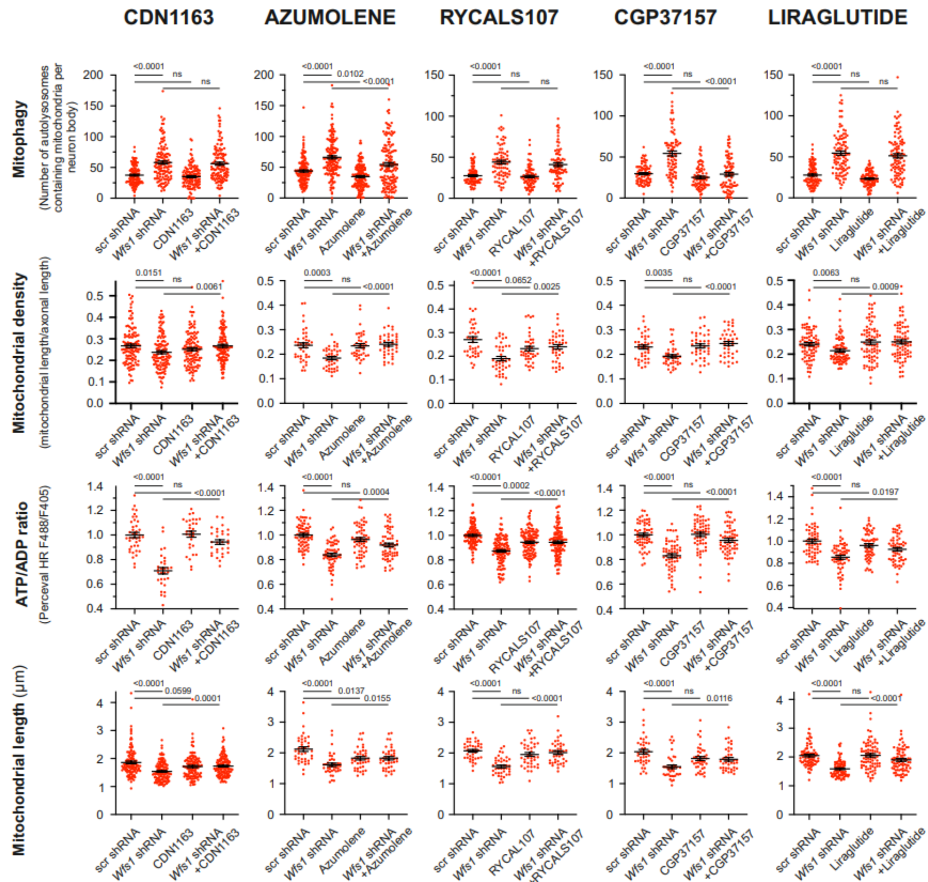


Figure 10. Effects of different chemical compounds on mitophagy, mitochondrial density, mitochondrial length, and neuronal ATP levels in Wfs1-deficient neurons. Pharmacological agents used: a SERCA activator CDN1163 (0.5 μM), an inhibitor of Ca^{2+} release via RYR azumolene (20 μM), an inhibitor of Ca^{2+} leak through the RyR Rycal-S107 (5 μM), a selective inhibitor of Ca^{2+} efflux through the mitochondrial Na^{+}/Ca^{2+} exchanger CGP37157 (10-20 μM), and GLP1 (glycogen-like peptide-1) agonist liraglutide (500 nM).

4.4. WFS1 deficiency leads to delayed neuronal development which can be rescued by normalizing mitochondrial dynamics and/or restoring intracellular Ca^{2+} balance (Paper II and III)

4.4.1. WFS1-deficient neurons show delayed neuronal development and impaired neuronal survival *in vitro*

Earlier observations in WS patients have found both neurodegeneration and deviations in early brain development (Hershey et al., 2012)Therefore, we decided to investigate whether disrupted mitochondrial dynamics and Ca^{2+} homeostasis in WFS1-deficient neurons also impact neuronal development and/or survival *in vitro*.

We found that in WFS1 deficiency, the development of cortical neurons was markedly delayed (Figure 11A). During the early stages of development (DIV2-DIV4), *Wfs1* shRNA-transfected neurons displayed notably shorter axons and reduced axonal branching, resulting in fewer axonal tips (Figure 11B-D). However, the differences disappeared at DIV6, as the axonal length and branching in relatively mature neurons appeared comparable to the control group. Furthermore, as illustrated in Figure 11E, the survival of WFS1-deficient neurons was impaired, as the loss of neurons was small but significant in the *Wfs1* shRNA-treated group. Moreover, WFS1 suppression led to a considerable reduction in synapse density when measured at DIV19, but this effect was not evident at earlier developmental stages (Figure 11F and 11G).

Hence, these findings indicate that WFS1 is essential for proper neuronal development, morphology, and neuronal survival *in vitro*.

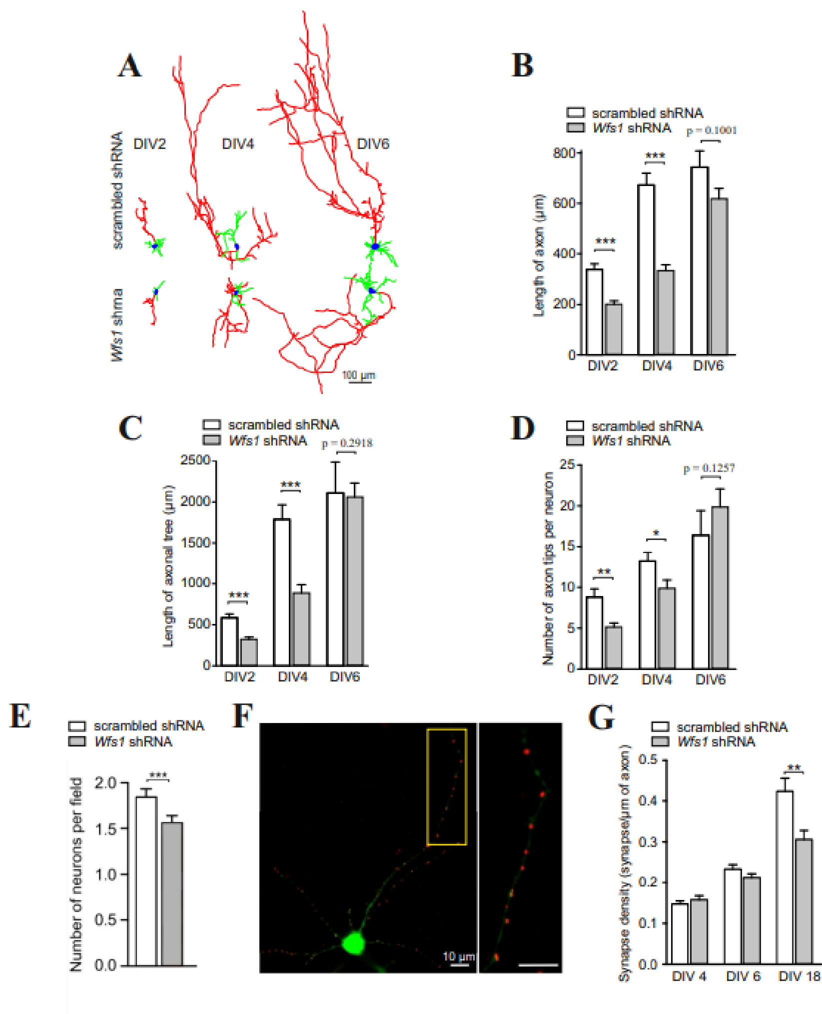


Figure 11. WFS1 deficiency leads to impaired neuronal development. (A) Examples of reconstructed control and WFS1-deficient neurons at DIV (day *in vitro*) 2, DIV4, and DIV6 transfected with the neuronal marker hSyn-DsRed1 and either scrambled shRNA or *Wfs1* shRNA. **(B–D)** WFS1 deficiency impairs the growth of the longest axon **(B)** and the development of the axonal tree **(C)**, along with reducing the number of axon tips **(D)** in early developmental phases. **(E)** The survival of WFS1-deficient neurons is diminished compared to control neurons. **(F)** Visualization of synapses (red) using an antibody against the post-synaptic marker PSD-95 in neurons transfected with GFP (green). The right panel displays a magnified image. **(G)** WFS1 deficiency leads to a decrease in synaptic density at later developmental stages. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ in comparison to the corresponding control groups.

4.4.2. Delayed neuronal development in WS is rescued by correction of mitochondrial dynamics and/or restoring intracellular Ca2+ balance

Altogether our findings demonstrate significant impairment in mitochondrial dynamics, bioenergetics, and neuronal development in WFS1 deficiency. We have shown that restoring proper ER Ca^{2+} regulation and enhancing mitochondrial Ca^{2+} uptake can ameliorate disrupted mitochondrial dynamics (Figure 10).

Next, we intended to specify whether restoring Ca^{2+} homeostasis could positively impact the delayed neuronal development seen in WFS1 deficiency. Indeed, our investigations revealed that the calcium-modifying chemical agents (except for azumolene) and the overexpression of SERCA and IP3R in the context of WFS1 deficiency effectively restored axonal length (Figure 12A–C).

Furthermore, we demonstrate that mitigation of increased mitophagy and correction of fusion-fission imbalance can prevent the delayed neuronal development observed in WFS1 deficiency. *Pink1* and *Parkin* shRNAs restored the fusion rate, mitophagy, mitochondrial density, and neuronal development (Figure 12D–M). Also, inhibiting mitochondrial fission proteins could protect mitochondria in WFS1-deficient neurons and rescue the neurons from developmental delay. Treatment with negative dominant DRP1 (nd DRP1) reversed the adverse effects of WFS1 deficiency and restored fusion rate, mitochondrial density, and neuronal development (Figure 12N-P).

We conclude that impaired neural development and survival in Wfs1-deficiency are the ultimate result of upstream pathological events incorporating ER Ca^{2+} dysregulation, negatively impacting mitochondrial functioning. Correcting Ca^{2+} homeostasis, mitochondrial fusion rate, and mitophagy in WFS1-deficient neurons can prevent these negative consequences.

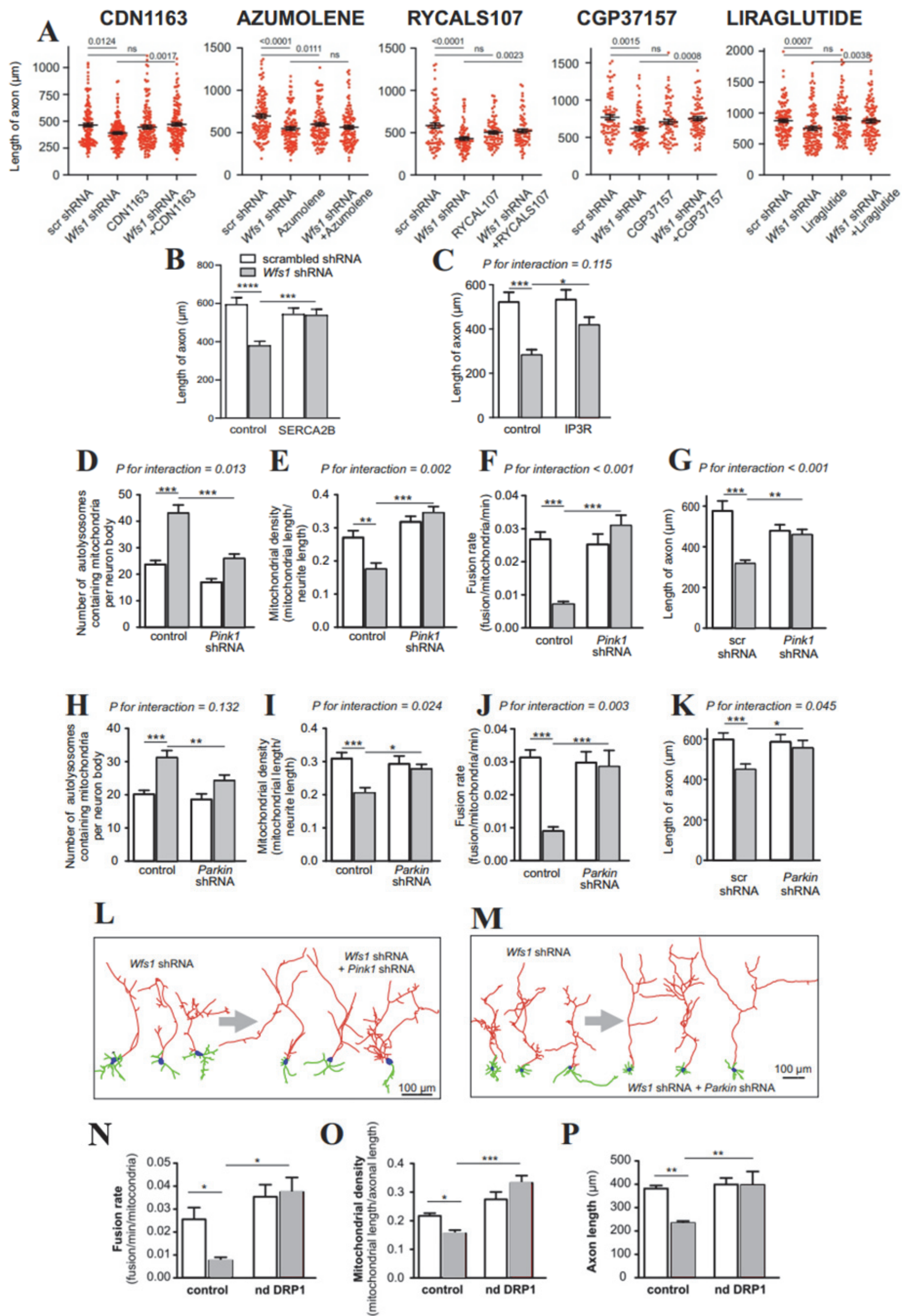


Figure 12. Correction of Ca^{2+} homeostasis with pharmacological agents or over-expression of IP3R or SERCA2b, and blocking mitophagy, and restoring fusion rate rescues impaired neuronal development in WFS1 deficiency. (A-C) The length of the

longest axon in WFS1-deficient neurons is restored when incubated with **(A)** SERCA activator CDN1163 (0.5 μM), an inhibitor of Ca^{2+} release via RYR azumolene (20 μM), an inhibitor of Ca^{2+} leak through the RyR Rycal-S107 (5 μM), a selective inhibitor of Ca^{2+} efflux through the mitochondrial Na^{+}/Ca^{2+} exchanger CGP37157 (10–20 μM), and GLP1 (glycogen-like peptide-1) agonist liraglutide (500 nM), and overexpressing **(B)** SERCA2b and **(C)** IP3R. **(D-G)** PINK1 silencing normalizes mitophagy **(D)**, mitochondrial density **(E)**, fusion rate **(F)**, and axonal growth **(G)**. **(H–K)** Parkin silencing produces comparable effects on all these parameters. **(L-M)** Examples of reconstructed WFS1-deficient neurons co-transfected with **(L)** *PINK1* shRNA and **(M)** *Parkin* shRNA. **(N-P)** nd DRP1 expression in WFS1-deficient neurons restores mitochondrial fusion rate **(N)**, mitochondrial density **(O)**, and axon lengths **(P)**. White bars = scrambled shRNA, grey bars = *Wfs1* shRNA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared with an indicated group. *P*-values for interactions are given in the figure.

5. DISCUSSION

5.1. The practical principles of mitochondrial fusion-fission balance in cortical neurons (Paper I)

Fusion and fission are fundamental processes of mitochondrial dynamics alongside mitochondrial biogenesis, motility, and mitophagy. The well-tuned balance of fusion and fission is vital for neuronal function (Bertholet et al., 2016; Westermann, 2010). Moreover, maintaining a proper balance between mitochondrial fusion and fission appears to be of greater significance than the absolute level of each process (Adebayo et al., 2021; Chan, 2020). It is suggested that specifically, the fusion-fission imbalance is related to human disease, including neurodegenerative diseases, cancer, cardiac and metabolic dysfunction (Y. J. Liu et al., 2020).

Our data confirms the findings from earlier reports that fusion and fission rates are approximately equal in neurons, and the fusion and fission events are forming a cycle. Also, neuronal mitochondria primarily spend most of the time in the post-fission state, similarly found earlier by Twig et al. in different cell lines (Twig et al., 2008). Further elucidation of the feedback mechanisms of the fusion-fission cycle suggested that mitochondrial length is self-regulated through Drp1-dependent fission and determines the fission rate. In contrast, mitochondrial length does not influence fusion rate.

Importantly, the dependency of the fission rate on length offers a straightforward explanation for the alternating nature of mitochondrial fusion and fission, as well as the non-cyclic occurrence of these events. Following each fusion, the mitochondrial length doubles, significantly increasing the likelihood of fission. This doubling predicts a brief lifespan for the post-fusion mitochondrion. Conversely, fission reduces the mitochondrial length by half, decreasing the probability of secondary fission and increasing the likelihood of the next event being fusion. This mechanism facilitates the alternation of fusion and fission events, elucidating why mitochondria spend less time in a post-fusion state and most of their time in a post-fission state.

If fusion occasionally yields a mitochondrion that is too short or a fission product that is too long, the cycle becomes compromised. Deviations from the cycle, such as two consecutive fusions or two consecutive fissions, function as quality control mechanisms to regulate mitochondrial length and aid mitochondria from becoming excessively short or oversized. This mechanism holds potential physiological relevance in maintaining optimal mitochondrial size, as excessively long mitochondria have been demonstrated to exhibit compromised bioenergetic capacity (Benard et al., 2007; Galloway et al., 2012).

Altogether, maintaining a balance between mitochondrial fusion and fission is essential for regulating mitochondrial length and regulating selective degradation of dysfunctional mitochondria, also referred to as mitophagy. Cells can use mitochondrial length as a quality control mechanism to identify mitochondria that need repair or removal. Healthy mitochondria are typically elongated, while

damaged or dysfunctional ones are shorter and fragmented. Shortened and damaged mitochondria are more likely to undergo mitophagy to maintain the overall integrity and functionality of the mitochondrial network within the cell (Gomes & Scorrano, 2013; Twig & Shirihai, 2011).

5.2. Role of mitochondrial dynamics in the disease mechanisms of Wolfram syndrome 1 (Paper II)

Although WS was initially thought to be a mitochondrial disorder (Bu & Rotter, 1993), later research shifted attention away from mitochondria when the subcellular localization of the causative protein wolframin revealed a stronger connection with the endoplasmic reticulum (Inoue et al., 1998; Strom, 1998; Takeda et al., 2001). Investigations into WS mechanisms took a different path with identifying another causative gene, *CISD2* (also known as *ZCD2, Noxp70, NAF-1, ERIS*, and *Miner1*) (Amr et al., 2007). A mouse model of WS2 showed disruptions in mitochondrial integrity and increased autophagic activity (Y.-F. Chen et al., 2009).

Therefore, our investigations led us to be the first to describe perturbations in mitochondrial dynamics in WS1. In WFS1 deficiency, the mitochondrial fusion-fission rate decreases, and there is a higher incidence of mitophagy, resulting in mitochondrial fragmentation and reduced axonal mitochondrial density. Relevantly, the impaired mitochondrial dynamics is accompanied by a slight decline in mitochondrial membrane potential and decreased ATP production. The mitochondrial fragmentation and depolarization could lead to the activation of mitophagy pathways, e.g. PINK1 accumulation in the mitochondrial outer membrane and Parkin translocation to mitochondria, inhibiting further mitochondrial fusion and inducing mitophagy (Choubey et al., 2014; Gegg et al., 2010; Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2008, 2010; Ziviani et al., 2010). Additionally, our observation of polarized mitochondria inside autophagosomes in WFS1-deficient neurons suggests another possibility: the overactivation of the PINK1-Parkin pathway independent of mitochondrial membrane potential, leading to the removal of healthy mitochondria (Jin & Youle, 2013; Michaelis et al., 2022). A comparable phenomenon has been observed earlier in neurons expressing mutant alpha-synuclein, in which our lab's prior study documented the initiation of PINK1-Parkin-dependent mitophagy to eliminate polarized mitochondria (Choubey et al., 2011). It is conceivable that both explanations remain applicable to neurons deficient in WFS1. A marginal depolarization of mitochondria might enhance the pace of their elimination, and PINK1-Parkin-dependent mitophagy could be initiated to clear out functional or partially functional mitochondria. This heightened mitochondrial clearance would consequently result in reduced mitochondrial density and ATP production, as observed in WFS1-deficient neurons, potentially compromising the bioenergetic status of the cells.

Moreover, a multitude of studies investigating mitophagy induction (mainly using mitochondrial membrane potential uncouplers) have established mitochondrial fragmentation as the prerequisite for mitophagy initiation (Gomes et al., 2011; Ikeda et al., 2015; Kageyama et al., 2014; Kanki et al., 2009; Rambold et al., 2011; Twig et al., 2008). Additionally, previous work has demonstrated, in the context of mitochondrial depolarization, that Parkin ubiquitinated MFN1 and MFN2, leading to their degradation (Gegg et al., 2010; Ziviani et al., 2010). Eliminating functional MFN hampers fusions, fosters mitochondrial fragmentation, and facilitates mitophagy. Furthermore, in a study of mouse cardiac myocytes, PINK1 phosphorylated MFN2 to recruit Parkin to impaired mitochondria during depolarization-induced mitophagy (Chen & Dorn, 2013). Therefore, it could be hypothesized that targeting impaired mitochondrial fusion-fission balance-mitophagy axis provides promising therapeutic choices to benefit cellular energy status in WFS1 deficiency.

5.3. Alterations in intracellular Ca^{2+} homeostasis in Wolfram syndrome influencing mitochondrial function (Paper III)

The data in this thesis demonstrates that a deficiency in either WFS1 or CISD2 reduces ER Ca^{2+} store content. This compromised ER Ca^{2+} storage affects axoplasmic Ca^{2+}, leading to an accumulation of Ca^{2+} in the axoplasm and an elevation of basal cytosolic Ca^{2+} levels, as previously suggested in WS (Angebault et al., 2018; Lu et al., 2014). Moreover, in the absence of WFS1 and CISD2, the depletion of ER Ca^{2+} also results in mitochondrial Ca^{2+} depletion related to hindered IP3R-mediated Ca^{2+} delivery from the ER to mitochondria.

Our findings of disrupted ER, cytosolic, and mitochondrial Ca^{2+} content find support from other studies. Decreases in releasable ER Ca^{2+} content have been observed in WFS1 knockout HEK293 cells, pancreatic β cells deficient in WFS1, rat insulinoma cells, and fibroblasts from individuals with WS (Angebault et al., 2018; Hara et al., 2014; Nguyen et al., 2020; Takei et al., 2006). Reductions of ER Ca^{2+} levels have also been shown in MEFs and cardiomyocytes of CISD2 KO mice (Wiley et al., 2013; Yeh et al., 2019), although there are also reports of no change or increase in ER Ca^{2+} content (Amr et al., 2007; N. C. Chang et al., 2010, 2012; Rouzier et al., 2017; C.-H. Wang et al., 2014). The decrease in ER Ca^{2+} content has earlier been linked to reduced SERCA activity and Ca^{2+} leakage through the RyR (Loncke et al., 2021; Lu et al., 2014; Shen et al., 2017; Yeh et al., 2019; Zatyka et al., 2015). This was also our conclusion based on successful rescue experiments in WFS1 and CISD2 deficiency with SERCA overexpression/activation or RyR suppression (data not shown, Paper III). However, recent reports suggest that the main alteration in WFS1 deficiency may be a decline in IP3R-mediated Ca^{2+} efflux (Angebault et al., 2018; Crouzier, Danese, et al., 2022; Delprat et al., 2020). Nevertheless, the latter explanation does not clarify

why ER Ca^{2+} content decreases, as it should increase in that case, and it leaves unclear the increase in basal axoplasmic Ca^{2+} concentrations observed in many earlier reports of WS models (Angebault et al., 2018; Lu et al., 2014).

We have also demonstrated that treatment of WFS1-deficient neurons with a GLP1 agonist liraglutide, which activates adenylate cyclase and the following signal cascade, can promote Ca^{2+} release by activating IP3, or pharmacological agents activating SERCA (CDN1163), inhibiting Ca^{2+} release/leak via RYR (azumolene and Rycal-S107), or selectively inhibiting Ca^{2+} efflux through the mitochondrial Na^{+}/Ca^{2+} exchanger (CGP37157) restore mitochondrial density and length, mitophagy intensity, and ATP levels in most settings.

Hypothetically, compromised ER Ca^{2+} release, increased cytosolic Ca^{2+} levels, and reduced mitochondrial Ca^{2+} uptake could impact mitochondrial function in various ways. Lowered ER Ca^{2+} release and elevated cytosolic Ca^{2+} levels can directly impact mitochondrial and cytoskeleton proteins related to mitochondrial dynamics, e.g. Drp1 recruitment to mitochondria is regulated by the Ca^{2+}-dependent phosphatase calcineurin, also EF-hand containing Ras GTPase Miro1/2 senses cytosolic Ca^{2+} levels and modulates mitochondrial movement (Carvalho et al., 2020). WFS1 has been found to interact with calcium/calmodulin kinase signaling cascade, which may influence mitochondrial and cytoskeleton proteins (Yurimoto et al., 2009). Also, elevated cytoplasmic Ca^{2+} levels could potentially contribute to calpain hyperactivation observed in WS (Lu et al., 2014). In addition, a study has found that mitochondrial motility in axons is actively regulated by mitochondrial matrix Ca^{2+} (K. T. Chang et al., 2011). Moreover, the mitochondrial matrix hosts Ca^{2+}-sensitive enzymes like pyruvate dehydrogenase (PDH) and Krebs cycle dehydrogenases, essential for maintaining mitochondrial electrochemical potential and ATP generation (Gray et al., 2014; Park et al., 2018; Tarasov et al., 2012). Supportively, previous findings in skeletal muscle cells lacking mitochondrial calcium uniporter (MCU) have described impaired oxidative phosphorylation due to suppressed mitochondrial Ca^{2+} influx (Gherardi et al., 2019). These observations imply that reduced mitochondrial Ca^{2+} uptake significantly diminishes pyruvate utilization, converting it into lactate. Our results align with this, showing a slight mitochondrial depolarization and notably lower ATP levels in the axons of WFS1-deficient neurons. Notably, disruption in ER-mitochondrial Ca^{2+} flux causing compromised mitochondrial function has been observed in Charcot-Marie-Tooth disease 4A. In this condition, GDAP1 silencing disrupted ER-mitochondria contact sites, resulting in lower mitochondrial Ca^{2+} levels, inhibition of pyruvate dehydrogenase, and compromised bioenergetics (Wolf et al., 2022).

5.4. WFS1 deficiency leads to delayed neuronal development (Paper II and III)

The disruptions of mitochondrial functioning in WFS1-deficient neurons are accompanied by impaired neuronal development and compromised neuronal survival *in vitro*. Indeed, our *in vitro* observations align with prior clinical investigations reporting brain stem atrophy and fatalities linked to respiratory failure (Barrett et al., 1997). Additionally, compared with both healthy individuals and those with type 1 diabetes, a group of young individuals with WS in the early stages of the disease exhibited reduced intracranial volumes, and preferentially affected grey matter volume and white matter microstructural integrity, suggesting impaired early brain development (Hershey et al., 2012).

The findings demonstrated in this thesis do not provide a definitive explanation for how precisely disrupted mitochondrial dynamics and compromised Ca^{2+} homeostasis hinder neuronal health. The hypothetical assumptions about the negative impact of disruptions in Ca^{2+} homeostasis were discussed in the previous chapter. Besides that, several potential mechanisms regarding impaired mitochondrial dynamics can also be suggested. The altered mitochondrial dynamics in WFS1-deficient neurons may interfere with effectively delivering energy-producing mitochondria to regions with heightened energy demands. For instance, inhibiting mitochondrial transport can lead to mitochondria depletion from peripheral nerve terminals, potentially diminishing local ATP supply and impacting ATP-dependent processes. Additionally, severe disruptions in mitochondrial fusion-fission dynamics may compromise the maintenance of mitochondrial function, thereby jeopardizing neuronal energy requirements (Westermann, 2012). Moreover, excessive mitophagy, which reduces mitochondrial mass, may impede the overall energy production capacity in neurons. Altogether, these energy deficits could impede the increased energy requirements linked to neuronal growth.

5.5. Clinical relevance of mitochondrial dysfunction and intracellular Ca^{2+} dysbalance in WS (Paper II and III)

WS is a multisystemic genetic disorder with no curable treatment to date. Only symptomatic treatment is available in everyday practice for specific symptoms arising during the course of WS, e.g., insulin for diabetes mellitus and vasopressin for diabetes insipidus. Ideally, new therapeutic options should consider relevant molecular disease mechanisms and target disease progression in all affected tissues. According to our experimental data, several pharmacological ways could be explored to restore mitochondrial dynamics and enhance neuronal health in WFS1 deficiency.

Starting with the possibility of improving the Ca^{2+}-pump function of SERCA, which has a fundamental role in maintaining cellular Ca^{2+} homeostasis across

various cell types (J. Chen et al., 2020). SERCA dysfunction is linked to diabetes, heart failure, and neurodegenerative diseases, and restoring SERCA expression or activity is recognized as a potential strategy for disease prevention (Gianni et al., 2005; Kulkarni et al., 2004; Zarain-Herzberg et al., 2014). The recently developed small-molecule allosteric SERCA activator CDN1163 has also demonstrated promising therapeutic effects in models of Parkinson's, Alzheimer's, and metabolic diseases, supporting potential protective benefits in WS (Dahl, 2017; Rakovskaya et al., 2023). Notably, CDN1163 has also shown improvements in glucose intolerance and insulin resistance in obesity (S. W. Park et al., 2010).

We have also demonstrated that drugs suppressing ER Ca^{2+} leakage (dantrolene analog azumolene and Rycal S107) restore normal mitochondrial and neuronal functions. This leakage, extensively studied in neurodegenerative diseases like Alzheimer's, Huntington's, and spinocerebellar ataxia type 2, is associated with posttranslational modifications leading to the dissociation of the stabilizing protein calstabin2 from the RyR channel (Chami & Checler, 2020; Lacampagne et al., 2017; Vervliet, 2018). Experimental drugs of Rycals have shown efficacy in stabilizing the interaction between calstabins and RyRs, particularly in conditions with elevated ER Ca^{2+} leakage. Additionally, dantrolene, known to reduce RyR sensitivity to Ca^{2+}, inhibits this leakage and has demonstrated neuroprotective effects in various neurodegenerative disease models (X. Chen et al., 2011; Fruen et al., 1997; J. Liu et al., 2009; Wei & Perry, 1996). However, a recent phase Ib/IIa clinical trial of dantrolene in WS patients showed no improvement in visual acuity or neurological functions (trial number: NCT02829268) (Abreu et al., 2021), but some important study limitations must be considered. A small sample size, clinical heterogeneity, and a relatively short 6-month dantrolene treatment may have interfered with detecting differences between groups.

Our results also align with recent research findings suggesting that WS could benefit from activating Ca^{2+} flux from the ER through the activation of the sigma-1 receptor (S1R), which is an ER-resident protein involved in Ca^{2+} efflux from the ER via IP3R (Crouzier, Danese, et al., 2022). The S1R agonist PRE-084 demonstrated restoration of Ca^{2+} ion transfer and mitochondrial respiration *in vitro*. It also corrected the associated increase in autophagy and mitophagy while alleviating behavioral symptoms observed in zebrafish and mouse models of WS. Our results complement this, suggesting that it is primarily the ER Ca^{2+} handling capacity, rather than the integrity of mitochondria-associated membranes, compromised in WS. Activating IP3R may more effectively channel Ca^{2+} from the ER to mitochondria, aiding cells in coping with this situation.

Additionally, mitochondrial mishandling of Ca^{2+} in neurons, observed in neurodegenerative diseases and brain aging, suggests the potential significance of maintaining an appropriate matrix Ca^{2+} concentration (Jung et al., 2020). Theoretical approaches include inhibiting the Na^+/Ca^{2+} exchanger, which exports Ca^{2+} to the extramitochondrial space, or activating the MCU and/or its binding subunits. We have shown that inhibiting Ca^{2+} loss from the mitochondrial matrix via the Na^+/Ca^{2+} exchanger is protective and normalizes all impacted mito-

chondrial parameters in WS, including mitochondrial density, length, mitophagy, and ATP levels, as well as neuronal development. This strongly implies that mitochondrial Ca^{2+} is one of the most important factors leading to bioenergetic deficits.

Also, the GLP1 agonist liraglutide has improved Ca^{2+} homeostasis and mitochondrial health in WFS1-deficient neurons. Liraglutide, known for its neuroprotective effects in animal experiments, has emerged as a leading treatment for WS (Jagomäe et al., 2021; Seppa et al., 2019, 2021; Toots et al., 2018). As an analog of GLP1 agonists, liraglutide binds to the Gs-coupled GLP-1 receptor present in both β-cells and neurons, stimulating adenylate cyclase to generate cAMP (Doyle & Egan, 2007; Harkavyi & Whitton, 2010). This, in turn, directly potentiates IP3-evoked Ca^{2+} release (Konieczny et al., 2017; Tovey & Taylor, 2013), thereby enhancing mitochondrial function in WFS1-deficient neurons. Importantly, tirzepatide, a dual glucose-dependent insulinotropic polypeptide and GLP1 receptor, is currently in phase 1 clinical trial in Milan, Italy (EudraCT number 2022-003853-70).

It could be hypothesized that targeting impaired mitochondrial fusion-fission balance-mitophagy axis provides promising therapeutic choices to benefit cellular energy status in WFS1 deficiency. Thus, another strategy to improve mitochondrial health in WFS1 deficiency could be the prevention of mitochondrial fragmentation. We successfully rescued decreased fusion rate and mitochondrial density in axons alongside impaired neuronal development with the overexpression of dominant-negative Drp1. The Mdivi-1, a derivative of quinazolinone, inhibits DRP1-dependent mitochondrial fragmentation and has been shown to give protection from neurotoxicity in mouse models of Parkinson's and Alzheimer's Disease and ALS (Baek et al., 2017; Luo et al., 2013; Rappold et al., 2014). Similar effects have been observed with P110, a specific inhibitor of the Drp1-Fis1 interaction, in cellular and mouse models of Alzheimer's Disease, Parkinson's disease, Huntington's disease, and ALS (Filichia et al., 2016; Guo et al., 2013; Joshi et al., 2017, 2018). Therefore, we hypothesize that the investigations of Drp1 inhibitors could yield favorable results in WS.

The failure to remove aggregate protein molecules and mitophagy defects are the hallmarks of the most prevalent neurodegenerative diseases, including Parkinson's disease, ALS, Alzheimer's disease, and Huntington's disease. A group of novel therapeutics, such as an USP30 inhibitor and a PINK1 activator, in clinical testing, aim to enhance mitophagy pathways (Antico et al., 2025). Our data supports the contrary idea that suppressing mitophagy could be beneficial. It can be proposed that neurons may not be able to afford a high rate of mitochondrial loss resulting probably in energy deficits, as the turnover of mitochondria in neurons is relatively low. Rather than degrading them through mitophagy, it may be energetically more favorable for neurons to retain "partially defective" mitochondria. Accordingly, excessive and undesired mitochondrial clearance could lead to detrimental bioenergetic deficits for neurons in pathological conditions associated with elevated autophagy and mitophagy. In our case, this manifests as an increased presence of partially defective mitochondria and a heightened re-

moval of these partially defective mitochondria, resulting in reduced mitochondrial mass.

Besides suggesting therapeutic options for WS, our results may add to the understanding of molecular pathology in neuropsychiatric conditions. The applicability of WFS1 deficiency-elicited perturbations in Ca^{2+} homeostasis leading to disturbed mitochondrial dynamics and impaired neuronal development could go beyond WS, as alterations in the gene WFS1 occur in different neurologic and psychiatric disorders. Based on limited evidence in the literature, WFS1 is associated with Parkinson's pathways. Shadrina et al. found that the synonymous polymorphism C1645T in the WFS1 gene increases the risk of Parkinson's disease in Russian patients (Shadrina et al., 2006). Kõks et al. demonstrated that WFS1 silencing in HEK cells primarily affected gene expression in the Parkinson's signaling ingenuity canonical pathway (Kõks et al., 2013). Furthermore, WFS1-deficient mice demonstrate impaired dopaminergic system function (Visnapuu et al., 2013). WS has also been associated with psychiatric pathology. The increased risk for mood disorders may apply to the 1% of the general population estimated to be carrying heterozygous mutants of WFS1 (Swift et al., 1998). In general, our data is consistent with a growing body of evidence suggesting that mitochondrial dysfunction (including mitochondrial dynamics) may lead to a disruption of normal neural plasticity and reduced cellular resilience, which may contribute to the development of neuropsychiatric disorders.

In conclusion, our data has identified pharmacological targets that could be adjusted to normalize various Ca^{2+}-dependent mitochondrial processes and enhance neuronal health in WS. In addition, the mitochondrial involvement in the disease mechanisms of WS may broaden our understanding of neurodegenerative and neuropsychiatric conditions.

5.6. Methodological limitations

The main limitation of our work comes from the (disease) model used in elaborating the cellular pathologies in WS. *In vitro* models lack important elements of *in vivo* and multicellular 3D tissue models, like cell diversity and the overall structural and molecular complexity of the central nervous system (CNS) environment, as well as the brain-blood barrier which connects the CNS to both the peripheral circulation and the immune system (Slanzi et al., 2020). However, studying mitochondrial dynamics in primary neuronal cultures is closer to human neurons and more disease-specific than immortalized cell lines, often used in earlier studies of WS disease mechanisms. Furthermore, primary neurons provide a unique opportunity to estimate mitochondrial dynamics and Ca2+ levels in relevant subcellular locations, such as the axon and axonal endings. It needs to be considered that the data obtained from our studies is derived from an *in vitro* model and may not be totally applicable to real-life patients. Also, the data is limited to neurons and not to other cell types possibly impacted by WFS1 deficiency, for example, pancreatic cells etc.

A relevant consideration in interpreting our experimental data is using transitory plasmid transfection to model WS pathology in neurons. Transiently transfected primary neurons limit the generalization of the findings to patient-derived cells as the time frame of introducing the insult and applying the treatment differs. In our *in vitro* model, the neurons had a normal developmental period, which was lacking in *in vivo* models and WS patients-derived cells. Also, we were able to provide rescue at approximately the same time the molecular stress arising from WFS1 deficiency started, possibly giving an advantage to favorable treatment outcomes. A good alternative addressing the limitations arising from transiently transfected neurons would be the usage of KO mice or rats to model WFS1 deficiency. However, it is remarkably more time- and work-consuming.

Furthermore, our knockout model established with Wfs1shRNA corresponds to the more severe disease phenotypes seen in human WS mutations and leaves uncertainties about the treatment effects outweighing toxicity in less affected cellular phenotypes.

Despite the aforementioned limitations, transiently transfected primary cortical neurons used to model WS are sufficiently reliable and applicable to human conditions. Their results align with data obtained from clinical samples, and they offer more flexibility and material to manipulate and test relevant hypotheses and treatments.

5.7. Concluding remarks and future directions

Overall, mitochondria are considered integral to the orchestration of cellular processes during neuronal development, as they provide energy, regulate intracellular Ca^{2+} levels, and participate in the maintenance of cellular homeostasis (Son & Han, 2018). Dysregulation of mitochondrial function can have profound effects on neurodevelopment and may contribute to neurological disorders, e.g. CMT2A, CMT4A, and DOA (Khacho et al., 2016; S. Park et al., 2018). There is also increasing evidence suggesting that mitochondrial dysfunction plays an important role in the etiopathogenesis and progression of neurodegenerative illnesses, such as Alzheimer's Disease, Parkinson's disease, Huntington's disease, and ALS (Klemmensen et al., 2024).

Here, we show that neuronal fusion-fission processes are cyclic, and fissions are regulated by mitochondrial length. We propose that delayed neuronal development in a cellular model of WFS1 deficiency is related to reduced mitochondrial Ca^{2+} uptake, resulting in abnormalities in mitochondrial dynamics and bioenergetics. In turn, the diminished Ca^{2+} uptake by mitochondria in WFS1 deficiency is connected to reduced ER Ca^{2+} content (Figure 13), as the restoration of neuronal development by ER Ca^{2+} homeostasis correction also helped to alleviate the mitochondrial phenotype. This additional understanding expands upon the ER hypothesis of Wolfram syndrome, explaining the energy deficiency symptoms observed in patients with WS and suggesting that WS is at least partly a mitochondria-mediated disease.

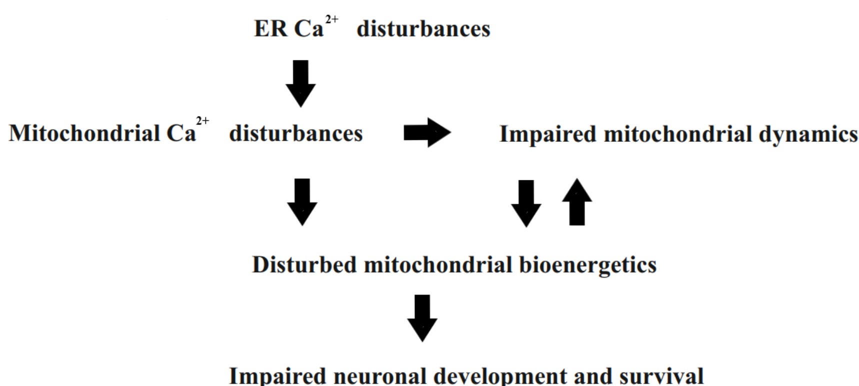


Figure 13. Neuronal health in WFS1-deficiency is compromised by ER-mitochondria Ca^{2+} dysregulation, impaired mitochondrial dynamics, and disturbed mitochondrial bioenergetics.

Although the connections between impaired ER Ca^{2+} homeostasis and mitochondrial dynamics seem to have a significant role in WS, additional mechanisms should be explored to elucidate the complex molecular pathway behind impaired neuronal development and neurodegeneration. It is known that the physical interaction of mitochondria with the ER is established through MAMs. The MAMs function as close contact sites on ER and mitochondria, facilitating Ca^{2+} transfer via IP3R interacting with an OMM protein, VDAC1. WFS1 deficiency is known to disorganize the MAMs, probably disrupting mitochondrial Ca^{2+} uptake (Angebault et al., 2018; Zatyka et al., 2023). Also, WFS1 is implicated in ER stress (Fonseca et al., 2005, 2010). CISD2, having an iron-sulfur cluster, additionally appears to respond to redox stress (Conlan et al., 2009; S.-M. Li et al., 2017). Therefore, it is reasonable to speculate that WFS1 and CISD2, acting as sensors for ER and redox stress, could synergize to integrate these converging signals and regulate cellular ER Ca^{2+} handling. Furthermore, WFS1 deficiency increases cytosolic Ca^{2+} to trigger apoptosis through the hyperactivation of calcium-dependent cysteine proteases, the calpains (Lu et al., 2014). Interestingly, CISD2 is a negative regulator of calpain 2 that limits the cleavage of caspase-3 (Lu et al., 2014).

Our findings indicate a connection between disruptions in ER and mitochondrial Ca^{2+} homeostasis, compromised mitochondrial dynamics, and the subsequent delay in neuronal development in WFS1-deficient neurons. These insights shed new light on the pathogenesis of neuronal abnormalities in Wolfram syndrome, presenting it as a valuable model for identifying drugs and molecules that influence restoring ER Ca^{2+} levels, ER-to-mitochondrial Ca^{2+} flux, mitochondrial matrix Ca^{2+} levels, and normalization of mitochondrial dynamics. Our results offer a significant perspective for addressing neuronal diseases characterized by impaired ER-mitochondria contact sites, such as Parkinson's disease, ALS, and

other rare neurodegenerative or neurodevelopmental conditions. Furthermore, our study reveals an unexpected link with implications beyond Wolfram syndrome. The impact of impaired mitochondrial dynamics on neuronal development suggests the critical role of proper mitochondrial dynamics in neurodevelopment. Finally, given the involvement of WFS1 function alterations in various neuropsychiatric disorders, our work may have broad implications for understanding the role of mitochondrial dynamics in neuropsychiatric diseases (Flint & Kendler, 2014; L. Li et al., 2020; Swift et al., 1998).

CONCLUSIONS

The conclusions of this study were the following:

1. Within cultured cortical neurons, there is an equilibrium between mitochondrial fusion and fission processes. The cycle of fusion and fission is feedbacked by alterations in mitochondrial length – an increase in length post-fusion heightens the likelihood of subsequent fission. In contrast, a reduction in length post-fission diminishes this probability. Variations from the fusion and fission cycle in neurons function as a corrective mechanism, preventing the occurrence of excessively elongated or fragmented mitochondria.
2. The fusion-fission balance is altered in several brain diseases and compromises the functioning of mitochondria and neuronal health. In an *in vitro* model of Wolfram syndrome, mitochondrial fusion rate was decreased, resulting in mitochondrial fragmentation. Furthermore, mitochondrial elimination by mitophagy was increased, leading to reduced mitochondrial density in axons. The disruption of mitochondrial dynamics adversely affected cellular bioenergetics, resulting in a reduction of axonal ATP levels, and negatively impacting neuronal development.
3. The perturbations in mitochondrial dynamics and bioenergetics in an *in vitro* model of Wolfram syndrome were connected to the impaired ER Ca^{2+} homeostasis and diminished ER-mitochondria Ca^{2+} flux. In WFS1 deficiency basal endoplasmic Ca^{2+} levels were decreased and mitochondrial Ca^{2+} uptake was reduced. Additionally, the cytosolic Ca^{2+} levels were increased due to compromised ER Ca^{2+} uptake. The chemical compounds activating ER and mitochondrial Ca^{2+} uptake restored mitochondrial dynamics and rescued neuronal development. Treatment of WFS1-deficient neurons with a SERCA activator CDN1163, an inhibitor of Ca^{2+} release via RyR azumolene, an inhibitor of Ca^{2+} leak through the RyR Rycal-S107, and a selective inhibitor of Ca^{2+} efflux through the mitochondrial Na^+/Ca^{2+} exchanger CGP37157 restored mitochondrial density and length as well as mitophagy intensity and axonal length in most settings, with CPG37157 being effective in all settings.

The significant role of mitochondrial dysfunction in the neuronal pathogenesis of Wolfram syndrome 1 broadens our understanding of the disease's mechanisms and provides new targets for pharmacological interventions.

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SUMMARY IN ESTONIAN

Mitokondriaalse dünaamika roll Wolframi sündroomi korral

Maailma Tervishoiu Organisatsiooni andmetel on tänapäeva üks peamisi tervena elatud eluaastate vähendajaid närvisüsteemi arengulised ja närvirakkude surmaga seotud (neurodegeneratiivsed) ajuhaigused. Viimase 20 aasta jooksul on saanud selgemaks, et ajuhaiguste, aga ka teiste haiguslike seisundite, nagu vähk, suhkruhaigus, südame-veresoonkonna haigused ja rasvumine, väljakujunemises on oluline osa mitokondrite ehituse ja/või talitluse kõrvalekalletel (Clemente-Suárez et al., 2023; Kargaran et al., 2021). Mitokondreid leidub peaaegu kõikides eukarüootsetes rakkudes ja nad osalevad arvukates rakusisestes protsessides. Nende peamised ülesanded on seotud energia tootmise ja rakusisese kaltsiumi tasakaalu säilitamisega. Dünaamiliste organellidena liiguvad nad aktiivselt mööda närvirakkude jätkeid ringi, ühinevad (fusioon) ja lahknevad (fission) omavahel ning vigased/üleliigsed mitokondrid suunatakse lagundamisele (mitofaagia) (H. Chen & Chan, 2009). Fusiooni-fissiooni tasakaal ning mitofaagia on mitokondrite ehituse ja talitluse terviklikkuse hoidmise ning närvirakkude arengu ja elulemuse tagamiseks väga olulised. Eelnevast järeldub, et mitokondrite funktsiooni parandamine ravimite ja muude sekkumiste abil võib osutuda tulevikus üheks võimaluseks, kuidas leevendada ajuhaigustega seotud vaevuseid, invaliidsust ja suremust.

Laialt levinud ja mitmete tegurite koosmõjus avalduvate haigusseisundite rakusiseste mehhanismide kohta uute teadmiste saamisel on abistavaks osutunud harvaesinevate monogeensete haiguste loom- ja katseklaasimudelid (Peltonen et al., 2006). Wolframi sündroomi (WS) on peetud heaks mudeliks, mille abil uurida närvirakkude arengu ja suremise ning aksonite kahjustuse mehhanisme. WS on harvikhaigus, mis avaldub ajas süvenevate neuroendokriinsete kõrvalekalletena, nagu magediabeet, diabeet, optiline atroofia ja kurtus. Haiguse avastamise ja kirjeldamise järel peeti seda kauaaegselt mitokondriaalseks haiguseks. Hiljem avastati, et WS põhjustavad mutatsioonid kahes erinevas geenis – *WFS1* (wolframiin) ja *CISD2*, mille poolt kodeeritud valgud paiknevad endoplasmaatilise retiikulumi (ER) ja mitokondrite ühenduskohtades ehk MAM-des (*mitochondria-associated ER membranes*). Kuigi wolframiini ja CISD2 valkude ehitus ja omadused erinevad märkimisväärselt, siis nende mutatsioonid põhjustavad inimestel sarnaseid sümptomeid. Senini ei ole täpselt teada, millised on WFS1 ja CISD2 täpsed ülesanded rakkudes. WS tüüp 2 varasemad uuringud on kirjeldanud CISD2 defektsuse/puuduse korral avalduvaid kõrvalekaldeid nii mitokondrite ehituses ja talitluses kui ka paljudes teistes olulistes rakufunktsioonides, nagu vananemine, autofaagia, ER stress, raua ainevahetus, kaltsiumi- ja redokstasakaal ja programmeeritud rakusurm. Seevastu WS tüüp 1 on peetud peamiselt ER haiguseks ning WFS1 osaleb ER stressi ja rakusisese kaltsiumi tasakaalu regulatsioonis. Käesolevalt ei ole teada, milline on wolframiini roll mitokondrite dünaamika mõjutamisel. Samuti pole piisavalt teadmisi mehhanismide kohta, kas

ja kuidas saab WS korral närvirakkudes häirunud ER talitlus mõjutada mitokondrite talitlust.

Käesoleva uurimistöö eesmärgid:

1) Kirjeldada seaduspärasusi, mis valitsevad närvirakkude kultuuris mitokondrite fusiooni ja fissiooni tasakaalu hoidmisel
2) Uurida katseklaasimudelis mitokondriaalse dünaamika olulisust Wolframi sündroomi rakulistes kõrvalekalletes
3) Täpsustada Wolframi sündroomi rakulisi mehhanisme ja otsida võimalikke farmakoloogilisi sekkumisi, et leevendada kõrvalekaldeid mitokondrite talitluses ja närvirakkude arengus

Antud doktoritöös kasutati mitokondrite dünaamika ja funktsiooni, kaltsiumi tasemete/voolude ning närvirakkude arengu/suremise kirjeldamiseks roti primaarset närvirakkude kultuuri. Antud katseklaasimudelis muudeti valkude ekspressiooni vastavalt uurimisküsimustele ning vastavalt katse eesmärkidele lisati rakkudele ka raviaineid. Wolframi sündroomi mudeldamiseks transfekteeriti primaarset närvirakkude kultuuri *Wfs1* shRNA-ga, et suruda alla WFS1 valku. Kontrollgrupi ja katsegruppide tulemusi võrreldi raku funktsioone ja ehitust hindavate erinevate keemiliste markerainete abil ning kasutati fluorestents- ja konfokaalmikroskoopia võimalusi.

Uurimistöö tulemused ja järeldused:

Kooskõlas varasemate tulemustega leiti, et roti närvirakkudes valitseb mitokondrite fusioonide ja fissioonide vahel tasakaal ning tsüklilisus – fusioonile järgneb fissioon ja vastupidi. Kahe vastandliku protsessi oluliseks regulaatoriks on mitokondrite pikkus – pikemad mitokondrid lahknevad tõenäolisemalt kui lühemad. Lühiajalised kõrvalekalded fusiooni ja fissiooni tsüklilisuses ehk kaks või enam järjestikkust fusiooni või fissiooni on rakkudele vajalikud, et vältida mittefunktsionaalsete ja liigselt pikenenud või lühenenud mitokondrite tekkimist.

Järgnevalt kirjeldati mitokondrite dünaamika muutuseid Wolframi sündroomi katseklaasimudelis. WFS1 puuduse korral leiti fusioonide sageduse vähenemist ja mitokondrite fragmentatsiooni. Lisaks oli suurenenud mitokondrite lagundamine ja seeläbi vähenenud närvirakkude aksonites ka mitokondrite tihedus. Mitokondrite dünaamika muutustega käis kaasas nende membraanipotentsiaali langus ja ATP tootmise vähenemine, mida seostati omakorda närvirakkude arengu aeglustumisega.

Seejärel leiti, et kõrvalekalded mitokondrite dünaamikas ja bioenergeetikas olid seotud häirunud Ca^{2+} tasakaaluga ER-s ja vähenenud Ca^{2+} vooludega ER-st mitokondritesse. Täpsemalt, WFS1 puuduse korral oli alanenud ER Ca^{2+} basaalne tase ja Ca^{2+} transport tsütoplasmast ER-i koos suurenenud tsütoplasma Ca^{2+} tasemega. Lisaks ER Ca^{2+} tasakaalu häirumisele oli vähenenud ka Ca^{2+} transport ER-st mitokondritesse.

WFS1 puuduse puhuseid kõrvalekaldeid mitokondrite dünaamikas ja närvirakkude arengus aitasid leevendada keemilised ained, mis taastasid Ca^{2+} tasa-

kaalu ER-s ja mitokondrites. Ca^{2+}-pumba SERCA aktivaator CDN1163, RYR Ca^{2+}-väljavoolu kanalite inhibiitor asumoleen, RYR Ca^{2+} kanalite lekke inhibiitor Rycal-S107 ja mitokondrite Na^{+}/Ca^{2+} vahetaja selektiivne inhibiitor CGP37157 aitasid taastada WFS1 puuduse korral mitokondrite pikkuse, tiheduse ja/või vähendasid mitofaagiat ja/või leevendasid närvirakkude arenguhäiret. Seejuures Na^{+}/Ca^{2+} vahetaja suutis seda teha kõigi eelnimetatud parameetrite osas.

Kokkuvõttes laiendavad antud doktoritöö tulemused praeguseid teadmisi WS rakulistest mehhanismidest ning võivad olla abiks tulevikus uute ravimite väljatöötamisel. Esmakordselt on kirjeldatud WS korral seost ER ja mitokondrite Ca^{2+} tasakaalu muutuste ning mitokondrite dünaamika vahel. Antud doktoritöö tulemustest järeldub, et WS neuroloogiliste sümptomite ilmnemisel võib olla oluline osa vähenenud energia tootmisel närvirakkudes. Energiadefitsiidi võimalikeks põhjusteks võivad omakorda olla nii mitokondrite dünaamikahäired kui ka ER-mitokondrite Ca^{2+} voolude vähenemine. Lisaks võivad antud doktoritöö tulemused omada tähtsust mõistmaks ka teiste sagedamini esinevate neurodegeneratiivsete haiguste (Parkinsoni tõbi, Alzheimeri tõbi, amüotroofiline lateraalskleroos) mehhanisme, kus on samuti leitud muutuseid ER ja mitokondrite omavahelises ühendatuses.

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PUBLICATIONS

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List of publications:

Liiv, M.*, Vaarmann, A.*, Safiulina, D., Choubey, V., Gupta, R., Kuum, M., Janickova, L., Hodurova, Z., Cagalinec, M., Zeb, A., Hickey, M. A., Huang, Y.-L., Gogichaishvili, N., Mandel, M., Plaas, M., Vasar, E., Loncke, J., Vervliet, T., Tsai, T.-F., … Kaasik, A. (2024). ER calcium depletion as a key driver for impaired ER-to-mitochondria calcium transfer and mitochondrial dysfunction in Wolfram syndrome. *Nature Communications*, *15*(1), 6143. https://doi.org/10.1038/s41467-024-50502-x

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Teadustöö:

Teadustöö põhisuunaks on olnud uurida mitokondrite biogeneesi, dünaamika ja mitofaagia rolli neurodegeneratiivsete haiguste katseklaasimudelites.

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