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LIST OF ORIGINAL PUBLICATIONS

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1. Terasmaa, A., Soomets, U., Oflijan, J., **Punapart, M**. *et al.* (2011). Wfs1 mutation makes mice sensitive to insulin-like effect of acute valproic acid and resistant to streptozocin. *J Physiol Biochem*, 67 (3), 381–390. DOI: 10.1007/s13105-011-0088-0. (**Paper I**)
2. **Punapart, M.**, Eltermaa, M., Oflijan, J., Sütt, S. *et al*. (2014). Effect of Chronic Valproic Acid Treatment on Hepatic Gene Expression Profile in Wfs1 Knockout Mouse. *PPAR Research*, 2014, 349525. DOI: 10.1155/2014/349525. (**Paper II**)
3. **Punapart, M.**, Seppa, K., Jagomäe, T., Liiv, M. *et al*. (2021). The Expression of RAAS Key Receptors, *Agtr2* and *Bdkrb1*, Is Downregulated at an Early Stage in a Rat Model of Wolfram Syndrome. *Genes*, 12 (11). DOI: 10.3390/genes12111717. (**Paper III**)
4. **Punapart M.**, Reimets R., Seppa K., Kirillov S. *et al*. (2023). Chronic Stress Alters Hippocampal Renin-Angiotensin-Aldosterone System Component Expression in an Aged Rat Model of Wolfram Syndrome. *Genes (Basel)*, 14(4), 827. DOI: 10.3390/genes14040827. (**Paper IV**)

Author contributions:

I. The author performed the tolerance tests (jointly with J. Oflijan) and participated in analysing the data.
II. The author participated in designing the study and writing the animal study application, performed the animal experiments (jointly with A. Terasmaa et al.), performed quantitative real-time PCR, analysed/interpreted the data (jointly with A. Terasmaa) and cowrote the article.
III. The author extracted RNA from tissues, performed quantitative real-time PCR and ELISA (jointly with K. Seppa and R. Reimets), performed data visualization (jointly with K. Seppa) and interpretation (jointly with M. Plaas and K. Seppa), cowrote the manuscript and cohandled the correspondence.
IV. The author participated in the study design (jointly with M. Plaas), data analysis (jointly with M. Plaas) and visualization (jointly with K. Seppa); cowrote the manuscript; and cohandled the correspondence.

ABBREVIATIONS

7,8-DHF	7,8-dihydroxyflavone
Ace	Angiotensin I converting enzyme
Ace2	Angiotensin I converting enzyme 2
Actb	Beta-actin
Agtr1a	Angiotensin II receptor type 1a
Agtr1b	Angiotensin II receptor type 1b
Agtr2	Angiotensin II receptor type 2
Akt	Serine/threonine protein kinase
ALS	Amyotrophic lateral sclerosis
AngI	Angiotensin I
AngII	Angiotensin II
Ang-(1-7)	Angiotensin-(1-7)
Ang-(1-9)	Angiotensin-(1-9)
ANOVA	Analysis of variance
ARRIVE	Animal Research: Reporting of In Vivo Experiments
Bdkrb1	Bradykinin receptor B1
Bdkrb2	Bradykinin receptor B2
BDNF	Brain-derived neurotrophic factor
BK	Bradykinin
CHO	Chinese hamster ovary (cells)
CNS	Central nervous system
cDNA/cRNA	Complementary DNA/RNA
CYP	Cytochrome polypeptide
DIDMOAD	*Diabetes Insipidus*, *Diabetes Mellitus*, Optic Atrophy, Deafness
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
F2	Second filial generation
F9	Mouse embryocarcinoma (cells)
GABA	Gamma-aminobutyric acid
GIP	Glucose-dependent insulinotrophic polypeptide
GLP-1R	Glucagon-like peptide 1 receptor
GLUT2	Glucose transporter 2
GSK3	Glycogen synthase kinase-3
GTT	Glucose tolerance test
Hprt1	Hypoxanthine-guanine phosphoribosyltransferase
HSD	Honestly significant difference
HZ	Heterozygous
i.p.	Intraperitoneal
IP3R	Inositol triphosphate receptor
IPGTT	Intraperitoneal glucose tolerance test
IR	Insulin receptor

KKS	Kallikrein-kinin system
KO	Knock-out
lacZ	Beta-galactosidase
LIR	Liraglutide
LiCl	Lithium chloride
LSD	Least significant difference
MAM	Mitochondria-associated ER membrane
Mas1	MAS1 proto-oncogene, G protein-coupled receptor
NaCl	Sodium chloride
NCX1	Sodium-calcium exchanger
OMIM	Online Mendelian Inheritance in Man
PBS	Phosphate-buffered saline
PEG-300	Polyethylene glycol-300
PI3K	Phosphoinositide 3-kinase
Ppard, *-g*	Peroxisome proliferator-activated receptor beta/delta, -gamma
qPCR	Quantitative polymerase chain reaction
RAAS	Renin-angiotensin-aldosterone system
SAL	Saline
s.c.	Subcutaneous
SEM	Standard error of the mean
SGLT2	Sodium-glucose transport protein 2
siRNA	Small interfering RNA
SMURF1	SMAD specific E3 ubiquitin protein ligase 1
STZ	Streptozotocin
Tbp	TATA box binding protein
TRKB	Tropomyosin receptor kinase B
TSPO	Mitochondrial translocator protein
UPR	Unfolded protein response
V1aR	Vasopressin receptor 1A
VPA	Valproate
VEH	Vehicle
WFS1	Wolframin/Wolfram Syndrome 1
WS	Wolfram Syndrome
WT	Wild-type

*A complete list of gene name abbreviations can be found in Tables 2–4.

INTRODUCTION

Wolfram syndrome (WS) is a severe disease leading to generalized neurodegeneration inherited in an autosomal recessive manner, although autosomal dominant forms have been reported. The disease was first described by Wolfram and Wagener in 1938 as a combination of early-onset *diabetes mellitus*, progressive optic atrophy, *diabetes insipidus* and sensorineural deafness (Wolfram and Wagener, 1938). To date, many other comorbidities have been described, and heterozygous carriers have been found to be at greater risk of developing psychiatric diseases, sensorineural deafness or diabetes. WS patients usually die in their thirties, mainly due to damage to the respiratory centre in the brain stem (Barrett et al., 1995; Barrett and Bundey, 1997; Medlej et al., 2004; Rigoli et al., 2018; R. G. Swift et al., 1998).

WS is caused by homozygous or complex heterozygous mutations in the *WFS1* gene, which encodes the wolframin protein. Wolframin is a transmembrane glycoprotein located mostly in the endoplasmic reticulum (ER) membrane (Inoue et al., 1998; Strom et al., 1998; Takeda et al., 2001); however, its exact molecular function is still unknown. The most likely function of wolframin is regulation of the calcium content in the ER, modulation of the ER stress response and cell survival (Fonseca et al., 2005, 2010; Kakiuchi et al., 2006; Takei et al., 2006). Wolframin is also strongly associated with facilitating communication between the ER and mitochondria via structures called mitochondria-associated ER membranes (MAMs). Importantly, wolframin has been shown to affect mitochondrial dynamics and function (Angebault et al., 2018; Cagalinec et al., 2016).

WS is a rare disease with an estimated prevalence of 1:100 000–1:770 000, which is highly variable depending on the population (Barrett et al., 1995; Barrett and Bundey, 1997; Medlej et al., 2004). However, recent data suggest that recessive aetiologies, such as mutations in *WFS1*, may be more common in childhood-onset monogenic diabetes than previously thought: in Turkish paediatric patients, *WFS1* mutations contribute to ~20% of monogenic diabetes cases; in Han Chinese individuals, the proportion is ~22%; and in Indian children, the proportion is ~26% of antibody-negative type 1 diabetes cases (M. Li et al., 2020; Menon et al., 2023; Patel et al., 2022).

Despite active research, to date, there is no specific treatment for WS. A few promising drug candidates to slow the progression of WS are being studied more extensively, including the endoplasmic reticulum stress modulator valproate (VPA) and the antidiabetic glucagon-like peptide-1 receptor agonist liraglutide (LIR).

The studies herein aimed to describe the actions of VPA and LIR in animal models of WS from a transcriptomic point of view. Specifically, the effect of VPA on gene expression was investigated in the liver, a metabolically relevant tissue. The studies also examined the significance of WFS1 and the modulatory effects of VPA and LIR on the renin-angiotensin-aldosterone system (RAAS). In

a mouse model of WS, VPA was found to have contradictory effects on blood glucose levels depending on the length of treatment; a single administration of VPA improved glycaemic control, but chronic treatment with VPA had no effect. Prolonged treatment with VPA impacted hepatic gene expression, which is mainly related to oxidative processes, lipid metabolism and circadian rhythm. In a rat model of WS, changes in the RAAS were observed early in disease progression. The most prominent finding was the significant downregulation of the key RAAS receptors angiotensin II receptor type 2 and bradykinin receptor B1, specifically in the heart, lungs and hippocampus, in a rat model of WS. Furthermore, in WS rats, a substantial decrease in aldosterone and an increase in bradykinin serum levels were observed. Notably, low aldosterone levels have recently been directly linked to wolframin deficiency (Ma et al., 2023). Although VPA and LIR exhibited potential modulatory effects on the RAAS, they failed to normalize the gene expression levels in WS rats. However, at the peptide level, both drugs increased aldosterone and decreased bradykinin.

Although physiological manifestations suggestive of RAAS disturbance are not apparent in WS rats, the RAAS is evidently dysregulated at the transcriptional level. Moreover, its regulation seems to be strongly affected by stress. Since the RAAS either directly or indirectly regulates a wide variety of processes, from sodium and water balance to inflammatory responses, and has been implicated in the pathophysiology of diabetes and neurodegeneration, among other conditions (Ghadhanfar et al., 2017; Labandeira-Garcia et al., 2017; Ribeiro-Oliveira et al., 2008), its importance in the pathophysiology of WS cannot be underestimated. It is plausible that in WS, the compensatory axis of the RAAS is disturbed, possibly under stressful conditions; thus, the development of WS pathologies may be accelerated, including accelerated ageing. Although neither of the drugs used was able to normalize the RAAS at the transcriptional level, it cannot be excluded that they positively modulate alternative pathways that compensate for the altered RAAS. In addition, the clinical guidelines and criteria for monogenic diabetes as well as WS may need to be reevaluated, thereby providing better treatment options.

1. REVIEW OF THE LITERATURE

1.1 Wolfram syndrome

Wolfram syndrome 1 (WS) (OMIM #222300) is a severe monogenic neurodegenerative disorder with autosomal recessive inheritance (Wolfram and Wagener, 1938) caused by homozygous or compound heterozygous mutations in the *WFS1* gene encoding wolframin (OMIM 606201). WS is rare, with an estimated prevalence ranging from 1:770,000 in the United Kingdom (Barrett et al., 1995; Barrett and Bundey, 1997) to 1:68,000 in Lebanon (Medlej et al., 2004), varying substantially by population.

WS is mostly known by the acronym DIDMOAD due to its characteristic clinical features of disease progression: *diabetes insipidus*, *diabetes mellitus*, optic atrophy, and deafness (Barrett et al., 1995; Barrett and Bundey, 1997; Inoue et al., 1998; Strom et al., 1998; Wolfram and Wagener, 1938). The major criteria required for WS diagnosis are the development of juvenile nonautoimmune *diabetes mellitus* and optic atrophy before 15 years of age, which are often followed by *diabetes insipidus* and sensorineural deafness in the second decade of life (Barrett et al., 1995). Due to the mutational heterogeneity of WS, many other less frequent comorbidities exist, but the clinical picture and severity of the disease depend on the nature of the mutations in each individual (Cryns et al., 2003).

Juvenile nonautoimmune insulin-dependent *diabetes mellitus* develops at a median age of 6 years (Barrett et al., 1995; Barrett and Bundey, 1997). Blood sugar levels in WS patients are controlled by daily injections of insulin (Barrett et al., 1997; Rigoli et al., 2011). Compared to type 1 diabetic patients, WS patients have residual insulin secretion and therefore lower glycaemic variability and a decreased prevalence of microvascular complications (Cano et al., 2007; Zmyslowska et al., 2015). WS and other monogenic forms of diabetes are often misdiagnosed as type 1 diabetes (Zmyslowska et al., 2014). A recent study among Indian children with idiopathic type 1 *diabetes mellitus* revealed that 26% had mutations in *WFS1*, while other WS manifestations were not present (Menon et al., 2023).

Primary optic atrophy causes progressive loss of vision at a median age of 11 years, eventually resulting in blindness in the majority of WS patients (Barrett et al., 1995, 1997; Barrett and Bundey, 1997; Rigoli et al., 2011). The deterioration of visual acuity and colour vision is caused by atrophy of the optic nerve via demyelination of the optic tracts, thinning of the retinal nerve fibre layer and secondary retinal vascular impairment (Battista et al., 2022; Carson et al., 1977; Hoekel et al., 2014). In addition, other ophthalmologic pathologies, such as horizontal nystagmus, central scotomas, and cataracts, may occur (Barrett et al., 1997; Hoekel et al., 2014). WS patients eventually develop diabetic retinopathy, although the progression is not as fast as that in type 1 diabetes patients (Barrett et al., 1997). Notably, in some cases, optic atrophy can present before the onset of *diabetes mellitus* (Ustaoglu et al., 2020).

Approximately three-quarters of WS patients have *diabetes insipidus* in addition to *diabetes mellitus* due to insufficient vasopressin (Barrett et al., 1995; Barrett and Bundey, 1997), which causes a constant urge to urinate and frequent thirst. WFS1 deficiency causes neuronal loss and defective vasopressin precursor processing in the paraventricular hypothalamic nuclei (Gabreëls et al., 1998; Kawano et al., 2009). *Diabetes insipidus* occurs at a median age of 14 years and responds well to desmopressin treatment (Rigoli et al., 2011).

Sensorineural hearing loss affects more than half of patients; it may be partial or complete and can occur at any stage of life, with a median age of onset of 16 years (Barrett et al., 1995; Barrett and Bundey, 1997; Rigoli et al., 2011). Hearing loss can also appear much earlier: in a study by Karzon, the mean age at diagnosis was approximately 8 years (Karzon et al., 2018). The condition is likely induced by a reduction in fibres in the cochlear nerves and dysfunctional cells in the auditory system (Cryns et al., 2003; Genís et al., 1997). Hearing loss in WS patients is most often in the high frequency range, and the progression of hearing loss seems to be rather slow. Due to the subjectivity of reporting by the patient (or their carers), periodic audiologic evaluation is advised (Karzon et al., 2018).

In addition to the main symptoms described above, other clinical manifestations can occur, usually early in the third or fourth decade of life. These include urinary/renal tract abnormalities and neuropathic bladder; gastrointestinal disorders; and neurological complications, such as impaired balance, reduced reflexes, dysarthria, central sleep apnoea, myoclonus, and cerebellar ataxia. In males, hypogonadism, primary gonadal atrophy and other infertility problems may occur (Barrett et al., 1995, 1997; Barrett and Bundey, 1997; Medlej et al., 2004; Rigoli et al., 2018).

In addition, more than half of WS patients suffer from several psychiatric disorders, including depression, increased suicidality, psychosis and impulsivity (Sequeira et al., 2003; R. G. Swift et al., 1990, 1991, 1998). Moreover, *WFS1* heterozygous mutation carriers have a substantially greater likelihood of developing psychiatric diseases (M. Swift and Swift, 2000, 2005; R. G. Swift et al., 1991, 1998). The carrier frequency of *WFS1* heterozygous mutations is reported to be 1 in 354, which is remarkably high (Barrett et al., 1995). Heterozygous mutations increase not only the risk of psychiatric illnesses but also the probability of developing hearing loss and *diabetes mellitus* (Ohata et al., 1998).

The main symptoms usually develop by the age of 15, but in extreme forms of WS, the symptoms can evolve during the first 10 years of life (Khanim et al., 2001; Lynch, 2020). Most of these complications can be attributed to underlying neurodegeneration, which eventually leads to generalized brain atrophy, with the cerebellum, pons and medulla being the most affected regions (Barrett and Bundey, 1997). Brain stem atrophy can lead to central respiratory failure, which is the leading cause of death in WS patients around the age of 30–40 (Barrett et al., 1995; Barrett and Bundey, 1997; Kinsley et al., 1995).

Due to its phenotypic resemblance to other rare disorders, including mitochondrial diseases, a diagnosis of WS is confirmed using molecular analyses such as DNA sequencing (Annamneedi et al., 2023).

1.2 Wolframin and its physiological importance

The wolframin (WFS1)-encoding gene *WFS1* has a total size of 33.4 kb and consists of 8 exons, the first of which is noncoding. Exon 8, which is the largest exon in the gene (2.8 kb), encodes approximately 60% of wolframin (Cryns et al., 2003). In humans, *WFS1* is located on the short arm of chromosome 4 (4p16.1) (Polymeropoulos et al., 1994; Strom et al., 1998). Patients suffering from complications due to WFS1 deficiency harbour approximately 200 different point mutations and several dozen different insertions and deletions (indels), most of which are mapped to exon 8 of *WFS1* (Cárcamo Fonfría et al., 2018; Cryns et al., 2003; Inoue et al., 1998; Rigoli et al., 2018).

Wolframin is an 890 amino acid transmembrane glycoprotein localized in the endoplasmic reticulum (ER) membrane and has an estimated molecular weight of 100 kDa (Hofmann et al., 2003; Inoue et al., 1998; Strom et al., 1998; Takeda et al., 2001). The aminoterminal domain of this protein is located in the cytoplasm, and the carboxyterminal domain is located in the ER lumen (Hofmann et al., 2003).

WFS1 protein is found in multiple tissues, with higher levels in the brain, heart, lungs, retina and neuroendocrine tissues such as the pancreas (insulin-secreting beta cells). In the brain, WFS1 levels are greatest in regions related to anxiety, adaptation and cognitive functions, including the amygdala, hippocampus, hypothalamus and olfactory tubercles. In addition, lower levels of WFS1 have been observed in the liver, muscles, kidney and spleen (Hofmann et al., 2003; Ishihara et al., 2004; Kato et al., 2008; Luuk et al., 2008; Schmidt-Kastner et al., 2009; Strom et al., 1998; Takeda et al., 2001).

Knowledge of the exact function of WFS1 is limited, but it has been primarily associated with the ER stress response and calcium homeostasis in the ER (Fonseca et al., 2005, 2010; Kakiuchi et al., 2006; Takei et al., 2006). ER stress is an accumulation of mis- or unfolded proteins initiated in response to perturbations in ER processes (Bhattarai et al., 2020; Rigoli et al., 2011). WFS1 dysfunction leads to ER stress in mice (Kakiuchi et al., 2009) and eventually to cell apoptosis (Ishihara et al., 2004; Ueda et al., 2005); conversely, increased WFS1 can alleviate ER stress and protect against cell death, including that of pancreatic beta cells (Fonseca et al., 2010; Kakiuchi et al., 2009; Yamada et al., 2006). The proposed mechanism of action involves modulation of the unfolded protein response (UPR) and free calcium content regulation (Fonseca et al., 2005; H. Liu et al., 1997; Takei et al., 2006). As with several other UPR modulators, WFS1 is localized in mitochondria-associated ER membranes (MAMs), linking it to mitochondrial activity and regulation, including mitochondrial dynamics and degradation rates (Cagalinec et al., 2016). MAMs facilitate ER-mitochondria interactions, which regulate calcium signalling and metabolism, among other processes. Abnormalities in MAM functioning have been implicated in various neurodegenerative and metabolic disorders (J. Liu and Yang, 2022). Furthermore, the absence of WFS1 in fibroblasts leads to disturbances in calcium exchange and reduced formation of ER-mitochondria contacts *in vitro* (Angebault et al., 2018;

La Morgia et al., 2020). The clinical phenotypes of WS overlap with those of mitochondrial diseases, and mitochondrial disturbances have been associated with WS, although the reports are controversial (Barrett et al., 2000; Barrientos et al., 1996; Bu and Rotter, 1993).

1.3 Renin-angiotensin-aldosterone system

Most WS patients suffer from vasopressin-sensitive *diabetes insipidus* due to neuronal loss leading to vasopressin deficiency and defective processing of vasopressin precursors (Gabreëls et al., 1998). Vasopressin is part of a larger osmoregulatory network that functions by interacting with the renin-angiotensin-aldosterone system (RAAS) (Reid et al., 1983). Vasopressin release is stimulated by angiotensin II, and vasopressin, in turn, regulates the secretion of renin. Both angiotensin II and vasopressin increase the release and action of aldosterone (Szczepanska-Sadowska et al., 2018).

The RAAS is a central regulator of critical functions such as fluid-salt balance and blood pressure, and is also involved in the regulation of glucose homeostasis, immunity, inflammation and apoptosis. Dysregulation of the RAAS is implicated not only in cardiovascular complications but also in the pathophysiology of other conditions, including pulmonary fibrosis, diabetes and neurodegeneration (Ghadhanfar et al., 2017; Labandeira-Garcia et al., 2017; Ribeiro-Oliveira et al., 2008).

The RAAS acts through a complex cascade of peptides (Figure 1). A decrease in blood pressure releases the aspartyl protease renin, which converts the precursor angiotensinogen into angiotensin I (ANG I) (Schweda et al., 2007). Angiotensin-converting enzyme (ACE) further processes ANG I into the central peptide hormone of the RAAS, angiotensin II (ANG II), which activates ANG II receptor type 1 (AGTR1) and ANG II receptor type 2 (AGTR2). AGTR1 activation by ANG II leads to aldosterone release, water retention, increased blood pressure, proliferation, and proinflammatory and profibrotic responses. AGTR2 activation has the opposite effect and results in vasodilatation (Ames et al., 2019). ANG I can also be converted to the heptapeptide ANG-(1–9) by angiotensin-converting enzyme 2 (ACE2), and ANG-(1–9), in turn, is processed to ANG-(1–7) by ACE. ANG-(1–7) is a ligand for the G protein-coupled receptor MAS1 proto-oncogene. Like AGTR2, MAS1 acts as a functional antagonist of AGTR1, resulting in vasodilatation (Ghadhanfar et al., 2017; Santos et al., 2003). In addition to vasodilatation, activation of AGTR2 and MAS1 causes antifibrotic, anti-inflammatory and antioxidative responses (Cooper et al., 2021). Furthermore, AGTR2 expression tonically inhibits ACE activity (Hunley et al., 2000) and promotes axonal regeneration in the optic nerves of adult rats (Lucius et al., 1998).

The RAAS is tightly associated with the kallikrein-kinin system (KKS) through kallikrein and ACE. Like the RAAS, the KKS regulates blood pressure and inflammation (Schmaier, 2002). Bradykinin (BK) is a central peptide of KKS that acts via bradykinin receptor 1 and 2 (BDKRB1 and BDKRB2, respectively).

BDKRB2 activation by BK leads to vasodilatation, hypotension and natriuresis. BDKRB1 activation is stimulated by injury, pain and strong inflammatory responses (Cooper et al., 2021; Girolami et al., 2021), and in the central nervous system (CNS), BDKRB1 can mediate Ca^{2+}-dependent microglial migration (Ifuku et al., 2007). Both ANG-(1–9) and ANG-(1–7) can enhance BK receptor signalling through the modification of ACE activity and BDKRB2 sensitivity (Z. Chen et al., 2005). ACE degrades BK, and reduced ACE activity leads to a more active BK-directed hypotensive axis (Hornig et al., 1997; Pellacani et al., 1994).

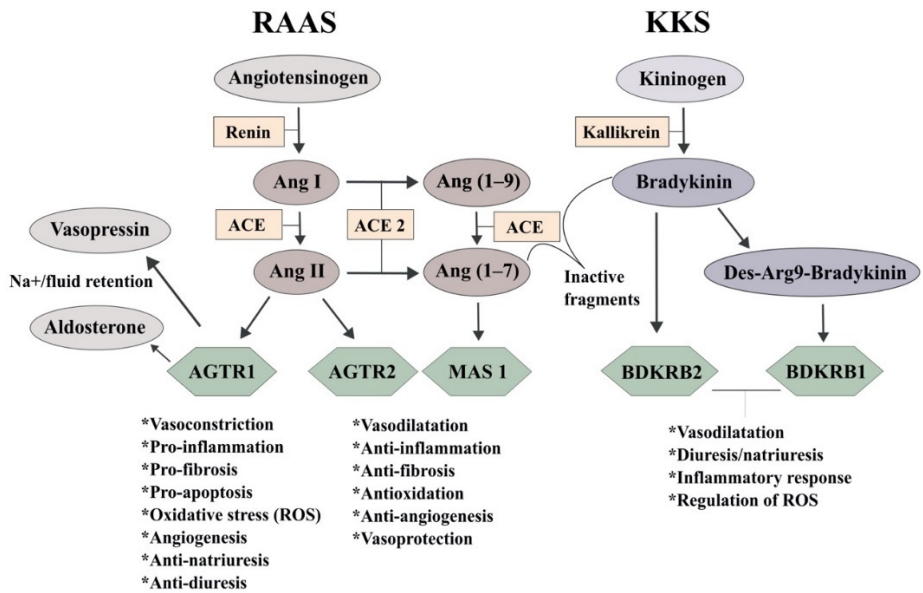

Figure 1. Main components of the classical renin-angiotensin-aldosterone and kallikrein-kinin systems (RAAS and KKS, respectively). The RAAS and KKS maintain homeostatic balance and are activated in response to imbalances in sodium and water content. The KKS is also stimulated by pain, injury and inflammatory responses. Dysregulation of these systems may lead to cardiovascular complications and overactivation of inflammatory processes. Abbreviations: ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Ang I, angiotensin I; Ang II, angiotensin II; Ang (1–9), angiotensin-(1–9); Ang (1–7), angiotensin-(1–7); AGTR1, angiotensin II receptor type 1; AGTR2, angiotensin II receptor type 2; MAS1, MAS1 proto-oncogene, G protein-coupled receptor; BDKRB1, bradykinin receptor B1; BDKRB2, bradykinin receptor B2; ROS, reactive oxygen species. (Prepared according to Cooper et al., 2021; Sugawara et al., 2021)

The RAAS is very complex; in addition to the "classical" systemic RAAS, a tissue-specific "micro-RAAS" has been described for several organs, including the pancreas and brain (Leung and Chappell, 2003; Wright and Harding, 2013). Moreover, key RAAS components are localized in the mitochondria of various tissues, including the kidney, adrenal gland, heart, liver, and brain (specifically

in dopaminergic neurons) (Escobales et al., 2019; Valenzuela et al., 2016). "Micro-RAASs" are part of cellular control systems regulating proliferation and regeneration, vasodilatation and vasoconstriction, and inflammatory responses (Ganten et al., 1971; Guimond and Gallo-Payet, 2012; Leung and Chappell, 2003; Wright and Harding, 2013). Importantly, at the local level, the RAAS is also linked to the regulation of ER stress, mitochondrial function, and MAMs (Cao et al., 2019). RAAS modulation can relieve oxidative and ER stress and improve mitochondrial function (Escobales et al., 2019; Sunanda et al., 2021).

Finally, problems in the heart and lungs, organs that are important parts of the RAAS, have been reported in WS patients. Cases of congenital heart disease, including sinus tachycardia, ventricular septal defects, atrial or ventricular arrhythmias, or pulmonary valvular stenosis, have been described in WS patients (Fabbri et al., 2005; Medlej et al., 2004). WS patients can suffer from early-onset respiratory and breathing problems before developing vision abnormalities (Lynch, 2020).

1.4 Rodent models of Wolfram syndrome

To date, several rodent models have been generated to study WS. In rodents, *Wfs1* has been mapped to chromosome 5 in mice and chromosome 14 in rats (*Ensembl Genome Browser*; Ganie and Bhat, 2009). Human and murine sequences have high homology—the identity at the nucleotide level is 83%, and that at the amino acid level is 87% (Strom et al., 1998). Ishihara and colleagues (Ishihara et al., 2004) created the first whole-body *Wfs1*-deficient mouse model by disrupting exon 2. These mutant mice exhibited pancreatic beta cell loss accompanied by reduced glucose-stimulated insulin secretion, impaired glucose homeostasis, and increased depression-like behaviour with reduced social interaction (Ishihara et al., 2004; Kato et al., 2008). Subsequently, several other groups developed their own *Wfs1*-deficient murine models (Luuk et al., 2008; Plaas et al., 2017; Riggs et al., 2005).

WS mouse and rat models created at the University of Tartu were used in the studies discussed in this thesis. Luuk and colleagues generated a *Wfs1*-deficient mouse model by replacing more than 90% of exon 8 with a beta-galactosidase-encoding (*lacZ*) cassette (Luuk et al., 2008). Compared with their wild-type littermates, these mice, both males and females, have significantly lower body weights and impaired glucose tolerance (Kõks et al., 2009). They display interrupted dopaminergic and serotonergic systems (Matto et al., 2011; Visnapuu, Plaas, et al., 2013; Visnapuu, Raud, et al., 2013) and abnormal behavioural activity in response to stress (Luuk et al., 2009). In addition, they display decreased retinal thickness and degeneration of corneal nerve fibres (Waszczykowska et al., 2020, 2022).

The first rat model of WS was generated by Plaas and colleagues (Plaas et al., 2017) by deleting coding exon 5 of the *Wfs1* sequence. These mutant rats develop primary symptoms of WS: progressive impairment of glucose tolerance leading

to *diabetes mellitus*, fasting hyperglycaemia, decreased pancreatic beta cell mass and reduced glucose-stimulated insulin secretion. In addition, these animals present with decreased medullary volume and degeneration of the optic nerve and develop decreased visual acuity and signs of low-frequency sensorineural hearing loss (Jagomäe et al., 2021; Plaas et al., 2017). To date, the established rat model of WS best mimics the disease symptoms that develop in WS patients.

1.5 Potential treatment options for Wolfram syndrome

Improved treatment strategies are constantly being sought to slow the progression of WS and thereby improve patients' quality of life. Despite years of research, no specific treatment has been identified for WS, and the development of so-called orphan drugs is not attractive. Therefore, there have been increasing attempts to apply drug repurposing strategies in the treatment of WS. These strategies significantly speed up the drug introduction process; nevertheless, every new discovery is still important.

Although, to date, no curative treatments are available for WS, some promising drug candidates have been identified. These candidates include chemical chaperones (e.g., sigma-1 receptor (S1R) agonists), ER stress modulators (e.g., valproate (VPA)) and antidiabetic agents (e.g., glucagon-like peptide 1 receptor (GLP-1R) agonists). S1R is an ER-localized transmembrane chaperone protein that regulates mitochondrial activity via calcium ion transfer and is involved in neuronal plasticity, neurotransmitter release and regulation of the ER stress response (Hayashi and Su, 2007; Ren et al., 2022). S1R agonists have been shown to improve mitochondrial function and ameliorate behavioural deficits in WS animal models (Crouzier et al., 2022). VPA, which was originally used as a mood stabilizer and anticonvulsant, induces WFS1 expression, affects the ER stress response, reduces apoptosis *in vitro* (Batjargal et al., 2020; Kakiuchi et al., 2009), and ameliorates glucose tolerance in patients with epilepsy (Rakitin et al., 2015). Similar to VPA, the ryanodine receptor antagonist dantrolene (a skeletal muscle relaxant) has been shown to suppress ER stress-mediated cell death in WS models (Lu et al., 2014). Dantrolene has already been evaluated in phase Ib/IIa clinical trials (clinical trial identifier: NCT02829268). Although there were no improvements in the parameters assessed, dantrolene was safe and well tolerated by the study participants (Abreu et al., 2021). Several drug candidates approved for the treatment of amyotrophic lateral sclerosis (ALS) have also shown promising results in WS models both *in vivo* and *in vitro*. In *Wfs1*-deficient cerebral organoids, riluzole, a glutamatergic antagonist, was able to restore synapse formation and functionality by regulating aberrant glutamate transporter expression. In addition, it also improved depressive behaviour and spatial memory in *Wfs1* conditional knockout mice (Yuan et al., 2023). Another ALS treatment recently approved in the United States (Mullard, 2022), a combination of 4-phenylbutyrate and tauroursodeoxycholic acid, has attracted increased interest because it elevated WFS1 levels, relieved ER stress and inhibited apoptosis in patient-

derived induced pluripotent stem cells. Moreover, the combination also induced insulin secretion in stem cell-derived beta cells and slowed the progression of diabetes in *Wfs1*-deficient mice (Kitamura et al., 2022). The safety and efficacy of this drug combination (under the name AMX0035) in adult patients with WS are being investigated in a phase II clinical trial (clinical trial identifier: NCT05676034).

Antidiabetic agents, GLP-1R agonists in particular, have demonstrated high potential by delaying WS disease progression in both rodent models (Jagomäe et al., 2021; Kondo et al., 2018; Sedman et al., 2016; Seppa et al., 2019, 2021; Toots et al., 2018) and human patients (Frontino et al., 2021; Scully and Wolfsdorf, 2020). The GLP-1R agonist liraglutide (LIR) slows the progression of diabetes, neurodegeneration and loss of vision and improves cognitive abilities in a rat model of WS (Jagomäe et al., 2021; Seppa et al., 2019, 2021; Toots et al., 2018).

The studies discussed in this work focused on two of the above-described drugs—VPA and LIR. In addition, LIR was used in combination with the brain-derived neurotrophic factor (BDNF) mimetic 7,8-dihydroxyflavone (7,8-DHF), which promotes neuronal regeneration and has neuroprotective properties and has been proposed as a potential treatment strategy for several neuropsychiatric disorders (S. Yang and Zhu, 2022).

1.5.1 Valproate

VPA is a widely used anticonvulsant and a first-line mood stabilizer for patients with bipolar disorder (Chang et al., 2009; Jope and Johnson, 2004). VPA is a 2-chain branched fatty acid consisting of 8 carbons and is a synthetic derivative of valeric acid (Verrotti et al., 2011). Most of its metabolites are biologically active (Chateauvieux et al., 2010).

Since VPA has a wide spectrum of activity due to its multiple targets, the exact mechanism of its action is not well established. Among its many effects, VPA indirectly increases central gamma-aminobutyric acid (GABA) by potentiating glutamate decarboxylase activity and inhibiting GABA transaminase, promotes general gene expression by regulating histone deacetylases (Chateauvieux et al., 2010; Marchion et al., 2005; Marinova et al., 2009) and blocks voltage-gated ion channels, prolonging their recovery time (Marchion et al., 2005; Rosenberg, 2007). Furthermore, VPA has an inhibitory effect on glycogen synthase kinase-3 (GSK3), which is a central kinase involved in a wide range of cellular processes, including insulin signalling (Jope and Johnson, 2004).

VPA is able to modulate the ER stress response, thereby preventing cell death and, notably, inducing WFS1 expression (Chuang, 2005; Huang et al., 2014; Kakiuchi et al., 2009; Kim et al., 2005). Moreover, VPA protects cells from apoptosis in cell lines harbouring autosomal dominant *WFS1* mutations, and acute VPA treatment improves glucose tolerance in epileptic patients (Batjargal et al., 2020; Rakitin et al., 2015).

The clinical trials database *ClinicalTrials.Gov* lists two ongoing phase II trials to investigate the efficacy and safety of VPA in WS paediatric and adult patients (clinical trial identifiers: NCT03717909; and NCT04940572).

1.5.2 Liraglutide

LIR is a member of the group of GLP-1R agonists, together with semaglutide, exenatide and dulaglutide, among others. These agonists are synthetic analogues of endogenous GLP-1, an incretin hormone that is released upon nutrient intake and stimulates glucose-dependent insulin excretion, delays gastric emptying and promotes satiety. GLP-1 has also been shown to reduce inflammation and apoptosis while promoting autophagy and the consequent mitochondrial turnover, improving cognitive abilities and exerting a neuroprotective effect (J. Chen et al., 2018; Müller et al., 2019). Receptors for GLP-1 are mostly localized in the pancreas, brain, kidney and heart (Pyke et al., 2014; Wei and Mojsov, 1995).

LIR (brand name Victoza®, Novo Nordisk) was approved for the treatment of type 2 *diabetes mellitus* by the European Medicines Agency in 2009 (*Victoza | European Medicines Agency*) and by the U.S. Food and Drug Administration in 2010 (*Drug Approval Package: Victoza (Liraglutide [rDNA]) Injection*), because it improved beta cell function and glycaemic control via enhanced glucose-dependent insulin secretion and promoted early satiety (Gough, 2012; Wajcberg and Amarah, 2010). In recent years, LIR has also been employed as a weight loss drug.

Due to their antidiabetic and neuroprotective properties, GLP-1R agonists have also been investigated as potential treatment options for the alleviation of WS. LIR and exenatide have been shown to delay the diabetic phenotype both in WS rats and mice (Jagomäe et al., 2021; Kondo et al., 2018; Sedman et al., 2016; Toots et al., 2018), and dulaglutide improved glycaemic control in WS patients (Frontino et al., 2021; Scully and Wolfsdorf, 2020). Furthermore, long-term treatment with LIR, neurotrophic factor 7,8-DHF, or their combination has neuroprotective effects, alleviates cognitive decline, improves learning, and slows the loss of visual acuity in aged WS rats (Jagomäe et al., 2021; Seppa et al., 2019, 2021). In addition, treatment with the dual GLP-1/glucose-dependent insulinotropic polypeptide (GIP) receptor agonist DA-CH5 in WS rats significantly reverses glucose intolerance, protects beta cells and retinal ganglion cells from death and delays loss of visual acuity (Jagomäe et al., 2023).

The efficacy of a dual-targeted GLP-1/GIP receptor agonist, an antidiabetic agent called tirzepatide, in stimulating insulin production and glycaemic control in WS patients is being clinically evaluated (clinical trial identifier: NCT05659368).

1.6 Concluding remarks

WS is a rare but devastating disease that leads to premature death in approximately the third decade of life. WS patients suffer from diabetes and loss of visual acuity, often accompanied by *diabetes insipidus*, sensorineural hearing loss and several psychiatric diseases. Symptoms develop at an early age and are progressive in nature.

This disease is caused by dysfunctional wolframin, an ER-resident transmembrane glycoprotein involved in ER stress regulation and calcium homeostasis as well as in the normal functioning of mitochondria. Wolframin deficiency affects all organ systems, and the specific cellular processes and mechanisms involved require further investigation.

Currently, no curative treatment is available, although some repurposed drugs with disease-delaying potential are being evaluated, including the antiepileptic/anticonvulsant VPA and the antidiabetic agent LIR, a GLP-1R agonist. Both drugs, especially LIR, have shown promising results, and research into their effects is ongoing.

2. AIMS OF THE STUDY

The aim of this study was to describe the transcriptomic changes driven by WFS1 deficiency, with a focus on the RAAS, using rodent models of Wolfram syndrome created at the University of Tartu and to evaluate the effect of VPA and LIR on those changes.

Specifically, the aims were as follows:

1. To evaluate the effect of acute (single administration) and prolonged VPA treatment on glycaemic control in male *Wfs1*-deficient mice.
2. To investigate prolonged VPA treatment-induced changes in the liver gene expression profile of male *Wfs1*-deficient mice to identify potential targets through which VPA exerts its metabolic effects.
3. To investigate the importance of WFS1 in the integrity of the RAAS and KKS in the heart and lungs of young male *Wfs1*-deficient rats and to characterize the modulatory effect of acute treatment with VPA, LIR, or their combination on these systems.
4. To investigate the importance of WFS1 in the integrity of the hippocampal RAAS in aged male *Wfs1*-deficient rats and to characterize the modulatory effect of chronic treatment with LIR, 7,8-DHF, or their combination.

3. MATERIALS AND METHODS

3.1 Experimental animals

3.1.1 *Wfs1*-deficient mice (Papers I, II)

Wfs1-deficient mice, specifically *Wfs1* exon 8 knockout mice, were used in the first and second studies (Papers I–II), the generation of which has been thoroughly described by Luuk et al. (Luuk et al., 2008, 2009). Briefly, most of the 8th exon of *Wfs1* was substituted by an in-frame NLSLacZNeo cassette, creating a functional knockout of wolframin 1.

All experiments described in Paper I were performed with 5–6-month-old male F2 hybrids [(129S6/SvEvTac × C57BL/6J) × (129S6/SvEvTac × C57BL/6J)] (mice were originally obtained from Scanbur BK, Sollentuna, Sweden). Hybrids, i.e., those with mixed backgrounds, were chosen for better translational value (to better mimic human genetic variation). In Paper II, 1–1.5-month-old (at the beginning of the experiment) male F2 hybrids [(129S6/SvEvTac × C57BL/6J) × (129S6/SvEvTac × C57BL/6J)] (mice were originally obtained from Scanbur BK, Sollentuna, Sweden) were used. Both studies compared male homozygous *Wfs1*$^{-/-}$ (*Wfs1* KO) and heterozygous *Wfs*$^{+/-}$ (*Wfs1* HZ) mutant mice with their wild-type *Wfs1*$^{+/+}$ (WT) littermates as controls (except in Paper I, where HZ were included only for VPA treatment). Mice were housed according to standard laboratory conditions in groups of eight to nine at 20 ± 2 °C under a 12/12-h light/dark cycle (lights on at 7 a.m.) with unlimited access to food (R70 Lantmännen, Sweden) and water (reverse osmosis-purified).

All experiments were performed between 9 a.m. and 5 p.m. Breeding and genotyping of mice using multiplex PCR were conducted at the Department of Physiology, Institute of Biomedicine and Translational Medicine, University of Tartu as described previously (Luuk et al., 2008).

All animal experiments described in Papers I–II were approved by the Estonian Project Authorization Committee for Animal Experiments (No. 86, August 28, 2007 for Paper I; No. 39, October 7, 2005 for Paper II) and complied with the European Communities Directive (86/609/EEC).

3.1.2 *Wfs1*-deficient rats (Papers III, IV)

In Paper III and Paper IV male *Wfs1*-deficient rats and wild-type rats were used. The generation and phenotype of the *Wfs1*-deficient (*Wfs1* coding exon 5 knockout) rats were extensively described previously by Plaas et al. (Plaas et al., 2017). Briefly, zinc-finger technology was used to delete coding exon 5 of the *Wfs1* gene, resulting in the removal of 27 amino acids from the WFS1 protein sequence (amino acids 212–238) and a serine-to-alanine substitution at position 239. Outbred CD® (Sprague–Dawley) IGS male homozygous *Wfs1* mutant (*Wfs1*-ex5-KO232) rats (WS rat) and their wild-type control littermates (WT) were used. The outbred rat line was selected for better translational value. In

Paper III, the animals used were 3.5–4-month-old. This is the age at which WS rats have not fully developed the WS phenotype (Plaas et al., 2017) and early changes (herein in RAAS and KKS) can be detected. In Paper IV, the animals were 9 months old at the start of the experiment. Older rats were chosen as the focus was on changes in the local RAAS in the hippocampus and in brain stem under conditions of neurodegeneration. The animals were randomly allocated into groups of up to eight. In addition, 12.5–13-month-old treatment-naïve animals were used in Paper IV.

The animals were housed in groups of four under a 12/12-h light/dark cycle (lights on at 7 a.m.). Rats had unlimited access to food (Sniff universal mouse and rat maintenance diet, Ssniff #V1534, ssniff Spezialdiäten, Germany) and water (reverse osmosis-purified). All experiments were performed between 9 a.m. and 5 p.m. Breeding and genotyping were performed at the Laboratory Animal Centre at the University of Tartu extensively described previously (Plaas et al., 2017). The experimental protocols for Papers III–IV were approved by the Estonian Project Authorization Committee for Animal Experiments (No. 165, 3 April 2020 for Paper III and No. 155, 6 January 2020 for Paper IV). All experiments were performed in accordance with the European Communities Directive of September 2010 (2010/63/EU) and complied with the ARRIVE guidelines.

An overview of the animals and treatments used (Paper I–IV) can be found in Table 1.

Table 1. Animals and treatments used

Paper	Species	Genotypes	Age (months)†	Gender	Background	Treatments	Other manipulations
I	Mouse	WT *Wfs1* HZ* *Wfs1* KO	5-6	Male	129S6/SvEvTac x C57BL/6J	Single injection of one of the following: Valproate (300 mg/kg, i.p) Lithium chloride (200 mg/kg, i.p) Diazepam (3 mg/kg, i.p) Rosiglitazone (3 mg/kg, i.p) Saline (10 mL/kg, i.p)	IPGTT
II	Mouse	WT *Wfs1* HZ *Wfs1* KO	1-1.5	Male	129S6/SvEvTac x C57BL/6J	For 3 months daily one of the following: Valproate (300 mg/kg, i.p) Saline (10 mL/kg, i.p)	IPGTT
III	Rat	WT *Wfs1* KO	3.5-4	Male	CD® (Sprague Dawley) IGS	For 8 days daily one of the following: Liraglutide (0.4 mg/kg, s.c) Valproate (300 mg/kg, s.c) Liraglutide + valproate (s.c) Saline (1 mL/kg, s.c)	N.A
IV	Rat	WT *Wfs1* KO	9 12.5-13	Male	CD® (Sprague Dawley) IGS	For 3.5 months daily one of the following: Liraglutide (0.4 mg/kg, s.c) 7,8-dihydroxuflavone (5 mg/kg, s.c) Nontreated	Not part of the Paper IV (see 3.2.4)

* In Paper I *Wfs1* HZ were only treated with VPA.
† Age at the beginning of the experiment.

3.2 Animal experiments

3.2.1 Acute valproate and lithium chloride treatment in mice (Paper I)

Mice (Section 3.1.1) were acutely treated with a single injection of valproic acid sodium salt (valproate, VPA) (300 mg/kg, i.p.), lithium chloride (LiCl) (200 mg/kg, i.p.), diazepam (3 mg/kg i.p.), rosiglitazone (3 mg/kg i.p.), or vehicle (0.9% sodium chloride, hereafter referred to as saline, 10 mL/kg, i.p.) during the intraperitoneal glucose tolerance test (Section 3.2.6). All drugs were dissolved in saline.

3.2.2 Chronic valproate treatment in mice (Paper II)

For three months, mice (Section 3.1.1) received a daily dose of valproic acid sodium salt (VPA, Sigma Aldrich, 300 mg/kg i.p. daily) dissolved in saline, or vehicle (saline 10 mL/kg i.p. daily). The dose of VPA used for the chronic study was chosen as previously described (B. Chen et al., 2000). A glucose tolerance test (glucose injection of 2 g/kg i.p.) (Section 3.2.6) was performed 24 hours after the last VPA administration. Mice were euthanized 24 hours after the glucose tolerance test at 4–4.5 months of age. Each experimental group consisted of eight animals. The liver was dissected, immediately rinsed with saline, snap frozen in liquid nitrogen, and stored at −80°C until further analysis of relative gene expression. The liver was chosen as it is a metabolically (including glucose metabolism) relevant tissue (Lin and Accili, 2011).

3.2.3 Acute valproate and liraglutide treatment in rats (Paper III)

In Paper III, 3.5–4-month-old WS rats and WT control rats (Section 3.1.2) were randomly allocated into treatment groups of liraglutide (LIR, n = 8), valproic acid sodium salt (VPA, n = 8) or liraglutide + valproate cotreatment (LIR + VPA, n = 8), or the control group of saline (vehicle) (SAL, n = 8). Each animal received a daily subcutaneous injection of the indicated drug at a volume of 1 mL/kg for eight consecutive days. The following effective and safe doses of the drugs were chosen based on previous studies (Toots et al., 2018): LIR, 0.4 mg/kg/day (Novo Nordisk, Denmark); VPA, 0.3 g/kg/day (Sigma–Aldrich P4543); LIR + VPA and SAL, 1 mL/kg (0.9% NaCl, saline). All drugs were dissolved in saline.

Rats were euthanized with an injection of Euthasol vet (300 mg/kg, i.p.) after eight days of treatment and within 4 hours of the last drug administration. The lower lobe of the lung and left ventricle of the heart were dissected, immediately rinsed with saline and snap frozen in liquid nitrogen. Lungs and heart were chosen as organs that are important parts of the systemic RAAS, which also have considerable WFS1 levels, especially in the heart (Cooper et al., 2021; Hofmann et al., 2003; Strom et al., 1998).

The collection of trunk blood and serum separation were conducted as previously described (Toots et al., 2018). The samples were stored at −80 °C for further analysis of relative gene expression and peptide levels.

3.2.4 Chronic liraglutide treatment in rats (Paper IV)

The samples used in Paper IV were collected as part of a previous long-term treatment study (Seppa et al., 2021). Specifically, 9-month-old rats (Section 3.1.2) were randomly assigned to the liraglutide (LIR, n = 5–7), 7,8-dihydroxyflavone (7,8-DHF, n = 5–7), or liraglutide + 7,8-dihydroxyflavone (LIR + 7,8-DHF, n = 6–8) treatment groups or the control group (vehicle) (VEH, n = 5–7). Drugs were prepared as follows: LIR (Novo Nordisk, Denmark) was dissolved in saline; 7,8-DHF (#D1916, Tokyo Chemical Industry Co., Ltd., Japan) was first dissolved in 100% dimethyl sulfoxide (DMSO) to 400 mg/mL, followed by a 1:20 dilution in polyethylene glycol-300 (PEG-300)/PBS mix (1:1), resulting in a final solution of 20 mg/mL 7,8-DHF in 5% DMSO/47.5% PEG- 300/47.5% PBS. Animals received a daily subcutaneous dose of LIR (0.4 mg/kg), 7,8-DHF (5 mg/kg), LIR + 7,8-DHF, or the corresponding vehicle (1 mL/kg of saline or 0.25 mL/kg of 5% DMSO/47.5% PEG-300/47.5% PBS) for 3.5 consecutive months. All drug injections were performed between 8 a.m. and 11 a.m. (Seppa et al., 2021).

Over the study period, these animals also underwent several other experimental manipulations, including routine blood glucose measurements, visual acuity evaluation, cataract scoring, Morris water maze and MRI under isoflurane anaesthesia (Seppa et al., 2021), which are not part of the Paper IV.

To control for the effect of the repeated experimental manipulations noted above, 12.5–13-month-old naïve WS rats and their WT littermates (n = 8, both groups) (Section 3.1.2) were used specifically for Paper IV. These animals were not subjected to any treatment or manipulation (hereafter referred to as "treatment-naïve") and were directly euthanized from their home cages.

Both treated (within 24 hours following the last injection) and treatment-naïve animals (taken directly from their home cages for downstream analyses) were sacrificed by decapitation. The hippocampi and brain stems were dissected, immediately rinsed with saline and snap frozen in liquid nitrogen. The tissue samples were stored at −80 °C for further analysis of relative gene expression (Paper IV; Seppa et al., 2021).

3.2.5 Generation of type 1 diabetic mice (Paper I)

Male *Wfs1*-deficient (both homozygous (KO) and heterozygous (HZ)) mice and their WT littermates (Section 3.1.1) received an intraperitoneal injection of the selective beta cell toxin streptozotocin (STZ; Sigma Aldrich, 170 mg/kg). The development of hyperglycaemia (blood glucose level > 250 mg/dL ~14 mmol/L; determined via blood glucose measurement by a handheld glucose metre) seven days later was considered a confirmation of type 1 diabetes.

3.2.6 Intraperitoneal glucose tolerance test (IPGTT) (Papers I, II)

Briefly, food was removed 60 minutes prior to the experiment, and access to food, except water, was prevented during the entire test. In Paper I, basal levels of blood glucose were determined after fasting, after which VPA (300 mg/kg, i.p.), LiCl (200 mg/kg, i.p.), diazepam (3 mg/kg i.p.), rosiglitazone (3 mg/kg i.p.), or vehicle (0.9% NaCl, 10 mL/kg, i.p.) was injected. Blood glucose levels were measured 15 minutes posttreatment, and a glucose solution (2 g/kg, i.p.) was administered. Blood glucose levels were measured 30, 60, 120, and 180 minutes following the glucose challenge. All chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA), except diazepam (Grindex, Latvia), and were injected at a dose of 10 mL/kg. Each group consisted of 6–16 animals. In Paper II, basal blood glucose levels were measured, followed by glucose injection (2 g/kg, i.p.), after which the levels were determined again at 30, 60, and 90 minutes after the injection. Each group consisted of eight animals. Blood glucose concentrations were measured from the tail vein using a commercial handheld glucose metre (Accu-Check Go, Roche, Mannheim, Germany).

3.2.7 Insulin tolerance test (Paper I)

The insulin resistance (tolerance) test was methodologically similar to the glucose tolerance test, except that bovine insulin (Sigma Aldrich, 2 U/kg, i.p., dissolved in 0.9% saline) was administered instead of glucose.

3.3 Immunohistochemistry, biochemical and molecular analyses

3.3.1 Immunohistochemistry (Paper I)

Mice were anaesthetized and perfused transcardially with 20 mL of prewarmed phosphate-buffered saline (PBS) followed by 20 mL of prewarmed 2% paraformaldehyde in PBS. The pancreas was removed and postfixed in 2% paraformaldehyde in PBS overnight at +4 °C, followed by incubation in 20% sucrose for 2 days at +4 °C. The tissue was frozen, and 40-µm-thick sections were cut using a cryostat (Leica) and thaw-mounted onto gelatine-coated microscope slides. Slides of tissue sections were stained with an anti-insulin antibody (insulin H-86, dilution 1:200, Santa Cruz Biotechnology) using the Vectastain ABC system and DAB peroxidase substrate according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA).

3.3.2 Urine chemistry (Paper I)

Urine (300 µL per mouse) was collected during the light phase from nonfasting animals. Urine creatinine and glucose levels were measured using standardized procedures at the United Laboratories of Tartu University Hospital.

3.3.3 Blood insulin levels (Paper I)

Insulin levels were measured in blood samples collected from VPA-treated and control mice at the 30-minute time point of the glucose tolerance test using a mouse insulin ELISA kit (Chrystal Chem) according to the manufacturer's protocol. The LiCl, diazepam, or rosiglitazone groups were not tested.

3.3.4 Microarray hybridization (Paper II)

For microarray hybridization analysis, total RNA was extracted from liver tissue homogenates using TRIzol reagent (Ambion, Life Technologies) according to the manufacturers' protocol. The integrity of the total RNA was determined using an Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). An RNA integrity number (RIN) of 7 to 9 was considered acceptable, and 300 ng of total RNA per sample was further processed to produce fragmented biotin-labelled cRNA using the Ambion WT expression kit according to the manufacturer's instructions. cRNA samples were then hybridized to Affymetrix GeneChip Mouse Gene 1.0 ST arrays and quantified.

3.3.5 Culturing of rat primary cortical neurons (Paper III)

For the *Wfs1* siRNA experiments, primary cultures of rat cortical neurons were prepared from the brains of neonatal Wistar rats less than one day old (Wistar Hannover, Taconic, Rensselaer, NY, USA; n = 3), as previously described (Cagalinec et al., 2013). Briefly, cortices were dissected in ice-cold Krebs–Ringer solution containing 0.3% bovine serum albumin, trypsinized in 0.8% trypsin for 10 minutes at 37 °C, and triturated in a 0.008% DNase solution containing 0.05% soybean trypsin inhibitor. Next, the cells were resuspended in Basal Medium Eagle with Earle's salts containing 10% heat-inactivated foetal bovine serum, 25 mM KCl, 2 mM glutamine, and 100 μg/mL gentamicin. Resuspended cells in a volume of 2 mL were plated in 35-mm glass-bottom dishes (MatTek, Ashland, MA, USA) precoated with poly-L-lysine at a density of ~10^6 cells/mL. After incubation for 3 hours, the medium was exchanged for NeurobasalTM-A medium supplemented with B-27, 2 mM GlutaMAXTM-I and 100 μg/mL gentamicin (transfection with *Wfs1* siRNA is described in section 3.3.6).

Second, primary cultures of cortical neurons were also prepared as described above from the brains of *Wfs1* KO rats (Sprague–Dawley IGS rats, Charles River, Wilmington, MA, USA; n = 6), and control CD® (Sprague–Dawley) IGS rats (Charles River) (not wild-type littermates; n = 7). After plating (~ 3.5×10^6 cells in 60-mm dishes), the cells were incubated for 24 hours (1 day) or 48 hours (2 days), washed with PBS and resuspended in TRIzol, after which the cells were immediately frozen in liquid nitrogen. The samples were kept at −80 °C until RNA isolation.

3.3.6 Transfection with *Wfs1* siRNA (Paper III)

After 72 hours (3 days) of plating, the rat primary cortical neurons (plated at a lower density of 2.5×10^5 cells/mL) were transfected with 20 nM validated siRNA against *Wfs1* (Sigma–Aldrich: SASI_Rn02_00265296 Rat NM_031823) using the N-TERTM Nanoparticle siRNA Transfection System (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

In brief, a mixture of target or scrambled (control) siRNA (20 nM) diluted in siRNA buffer and N-TER transfection reagent diluted in ddH_2O was preincubated at room temperature for 20 minutes. For transfection, the growth medium was exchanged for Opti-MEM I medium containing the target or scrambled siRNA mixture. After 3 hours of incubation at 37 °C, the Opti-MEM I was replaced with NeurobasalTM-A medium supplemented with B-27, 2 mM GlutaMAXTM-I, and 100 μg/mL gentamicin. The cells were then incubated for 48 hours at 37 °C in a humidified 5% CO2/95% air incubator until cell lysis for RNA isolation.

3.3.7 RNA isolation, cDNA synthesis and gene expression analyses (Papers II–IV)

In Paper II, total RNA was extracted from liver tissue homogenates using TRIzol reagent (Ambion, Life Technologies) according to the manufacturer's protocol.

The lower lung lobes and left ventricles of the heart in Paper III and the hippocampi and brain stems in Paper IV were homogenized using a Precellys system (Precellys lysing Kit CK14 + Precellys homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France)). Total RNA from the tissue lysates was isolated using Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA, USA) according to the manufacturers' protocol. For total RNA extraction from the rat primary cortical neurons (in Paper III), the Qiagen RNeasy Mini Kit was used.

cDNA was synthesized from total RNA (500 ng) using random hexamers and SuperScriptTM III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). cDNA was further used for RT–qPCR analyses via TaqMan Gene Expression Assays (Papers II–IV).

RT–qPCR was performed on an Applied Biosystems instrument (Applied Biosystems, Waltham, MA, USA) and an ABI PRISM 7900HT Fast Real-Time PCR System with the ABI PRISM 7900 SDS 2.2.2 Software in Paper II and a QuantStudio 12K Flex Real-Time PCR System in Papers III and IV. All reactions were conducted using TaqMan Gene Expression Mastermix and TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' instructions. All the samples to be compared were subjected to the same procedure. In Paper II, the following genes were selected for microarray hybridization data validation: *Ppard*, *Fmo2*, *Sult3a1*, *Lepr* and *Wfs1*; cytoplasmic *Actb* was used as the endogenous reference gene. In Papers III and IV, the relative gene expression levels of *Ace*, *Ace2*, *Agtr1a*, *Agtr1b*, *Agtr2*, *Bdkrb1*, *Bdkrb2*, *Mas1* and *Wfs1* were analysed, and the expression levels of the target genes were normalized to those of *Hprt1* or *Tbp* depending on the cycle threshold

(Ct) value of the target gene analysed. Target genes with higher Ct value were normalized to *Tbp* (Cabiati et al., 2012). Normalization for all experiments was performed using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001) (Papers II–IV).

The complete list of assays used can be found in Table 2.

Table 2. List of the TaqMan assays used for RT–qPCR analysis

Gene	Gene Name	Species	Assay ID	Paper
Actb	*β*- actin	mouse	Mm00607939_s1	II
Fmo2	Flavin containing monooxygenase 2	mouse	Mm0049019_m1	II
Lepr	Leptin receptor	mouse	Mm0040181_m1	II
Ppard	Peroxisome proliferator activator receptor delta	mouse	Mm00803184_m1	II
Sult3a1	Sulfotransferase family 3A, member 1	mouse	Mm00491057_m1	II
*Wfs1**	Wolfram syndrome 1 (wolframin)	mouse	Mm01220326_m1	II
Ace	Angiotensin I converting enzyme	rat	Rn00561094_m1	III, IV
Ace2	Angiotensin I converting enzyme 2	rat	Rn01416293_m1	III, IV
Agtr1a	Angiotensin II receptor, type 1a	rat	Rn02758772_s1	III, IV
Agtr1b	Angiotensin II receptor, type 1b	rat	Rn02132799_s1	III, IV
Agtr2	Angiotensin II receptor, type 2	rat	Rn00560677_s1	III, IV
Bdkrb1	Bradykinin receptor B1	rat	Rn02064589_s1	III, IV
Bdkrb2	Bradykinin receptor B2	rat	Rn01430057_m1	III, IV
Hprt	Hypoxanthine-guanine phosphoribosyltransferase	rat	Rn01527840_m1	III, IV
Mas1	MAS1 proto-oncogene, G protein-coupled receptor	rat	Rn00562673_s1	III, IV
Tbp	TATA box binding protein	rat	Rn01455648_m1	III, IV
*Wfs1**	Wolfram syndrome 1 (wolframin)	rat	Rn00582735_m1	III, IV

* Mouse *Wfs1* assay binds exon 7-8 boundary; rat *Wfs1* assay binds exon 1-2 boundary.

3.3.8 Determination of peptide levels in serum (Paper III)

Serum peptide levels were determined using specific ELISA kits for angiotensin-(1–7) (#CSB-E14241r, Cusabio, Houston, TX, USA), angiotensin-(1–9) (#BM-EKU08764, Hölzel Biotech, Köln, Germany), angiotensin II (#ADI-900-204, Enzo Life Sciences), aldosterone (#ADI-900-173, Enzo Life Sciences, Farmingdale, NY, USA), bradykinin (#ADI-900-206, Enzo Life Sciences) and renin 1 (#RAB1162, Sigma–Aldrich), according to the manufacturers' protocols.

3.3.9 Determination of Na^+ and K^+ levels in serum (Paper III)

The levels of sodium (Na^+) and potassium (K^+) in the serum were quantified using standardized procedures at the United Laboratories of Tartu University Hospital. Briefly, an ion-selective electrode (ISE) indirect Na-K-Cl for Gen.2 on a Roche/ Hitachi Cobas C501 analyser (Roche Diagnostics, Basel, Switzerland) was used following the manufacturers' instructions.

3.4 Data analysis

3.4.1 Microarray data analysis (Paper II)

Images from microarray hybridization were processed, and cell intensity files (CEL files) were generated in GeneChip Command Console Software (Affymetrix). CEL files were further processed using Expression Console v.1.1.2800. 28061 to yield RMA-summarized log2-transformed expression values for the probesets (CHP files). The normalized expression data were analysed using two-way ANOVA (genotype x treatment) with the R software package Bioconductor. A gene list for genotype effects was created that contained genes with significant ($P < 0.001$) changes in expression greater than 2-fold. For the effect of VPA treatment, the P value cut-off was 0.001, and for the genotype-treatment interaction, the P value cut-off was 0.003, given the small number of genes for which the latter was established. Differentially expressed genes were annotated to determine their molecular function using the web-based Mouse Genome Informatics Gene Ontology (MGI GO) database and the UniProt Knowledgebase (UniProtKB/Swiss-Prot).

The raw data in Paper II have been deposited in NCBI's Gene Expression Omnibus (*GEO – NCBI*) and are accessible through GEO Series accession number GSE55143 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE55143).

3.4.2 Statistics (Papers I–IV)

Statistical analyses were performed, and the data were visualised using STATISTICA version 8 or higher (StatSoft Ltd., Bedford, UK/StatSoft Inc., Tulsa, OK, USA) and GraphPad Prism software version 5 or higher (GraphPad Software Inc., San Diego, CA, USA).

In Paper I, the data were compared by one-way or multiple-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test when the ANOVA was statistically significant.

In Paper II, the data were compared by two-way ANOVA (with treatment and genotype as independent factors) followed by Tukey's HSD test.

In Paper III, the data were compared using either factorial ANOVA followed by Fisher's least significant difference (LSD) test or an unpaired t test.

In Paper IV, the data were compared using either one-way ANOVA followed by Dunnett's multiple comparisons test or an unpaired t test.

All the data in Papers I–IV are presented as the mean and standard error of the mean (±SEM); a p value < 0.05 was considered to indicate statistical significance.

4. RESULTS AND DISCUSSION

4.1 Effect of acute VPA treatment in male *Wfs1*-deficient mice (Paper I)

4.1.1 Male *Wfs1*-deficient mice were smaller and had slightly elevated nonfasting blood glucose levels

For basic characterization, the animals were weighed, and nonfasting blood glucose, urinary creatinine and glucose content, and plasma insulin levels were measured. The body weight of 6-month-old male *Wfs1* homozygous (*Wfs1* KO) mice was significantly lower than that of *Wfs1* heterozygous (*Wfs1* HZ) or wild-type (WT) mice (Figure 2a). This observation is consistent with previous reports showing impaired growth in *Wfs1* KO mice (Kõks et al., 2009). Growth hormone deficiency and short stature have also been described in some WS patients (Simsek et al., 2003; Soliman et al., 1995), although these features are not commonly reported.

The nonfasting blood glucose levels were only slightly elevated in *Wfs1* KO mice (Figure 2b), while the plasma insulin levels were notably lower than those in *Wfs1* HZ and WT mice (Figure 2d). Similar to WS patients, mice with dysfunctional WFS1 have exhibited beta cell loss and disrupted insulin secretion (Ishihara et al., 2004). Although statistically insignificant, plasma insulin levels in *Wfs1* HZ mice were slightly lower than those in WT mice, consistent with a gene dosage effect (Figure 2d). This finding supports the theory that *WFS1* mutation carriers have an increased risk of developing insulin-dependent diabetes (Ohata et al., 1998). Nevertheless, near-normal nonfasting blood glucose levels in *Wfs1* KO mice indicate that these mice do not develop overt diabetes with nonfasting hyperglycaemia as WS patients do (Barrett et al., 1995; Barrett and Bundey, 1997). Thus, there might be an alternative insulin-independent mechanism responsible for balancing blood glucose levels in *Wfs1*-deficient mice.

Urinary glucose levels were several times greater in *Wfs1* KO animals (Figure 2c), and there was no significant difference in urinary creatinine levels between the groups (Figure 2e). Glycosuria, an abnormal increase in urinary glucose levels, is a common symptom of diabetes (Ferrannini, 2011). It is plausible that the impaired glucose reabsorption observed in *Wfs1* KO mice may aid in maintaining normal blood glucose levels while plasma insulin is decreased. The majority of renal glucose reuptake is mediated by the glucose transporters GLUT2 and SGLT2 (Idris and Donnelly, 2009), the inhibition of which is used as a treatment strategy for diabetes-related hyperglycaemia (Kshirsagar et al., 2020). As WFS1 is moderately expressed in the kidney (Hofmann et al., 2003), it is possible that WFS1 dysfunction impairs renal glucose reabsorption through these transporters.

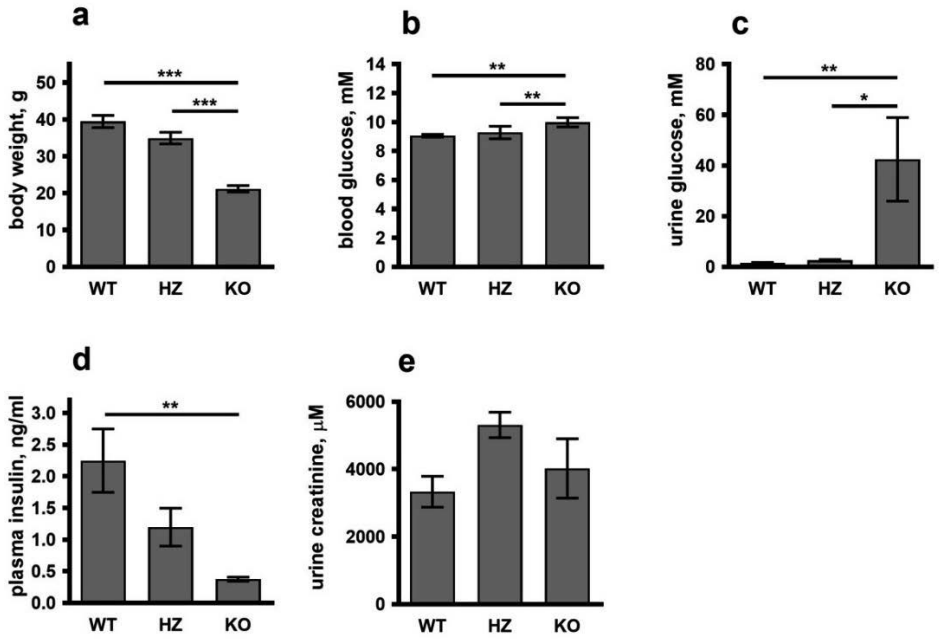

Figure 2. Male *Wfs1* KO mice exhibited retarded growth, decreased insulin and glycosuria. **a** Body weight of 6-month-old wild-type (WT), *Wfs1* heterozygous (HZ) and *Wfs1* knock-out (KO) mice (n=15–16). **b** Blood glucose levels in the nonfasting state (n=115 for WT, n=16 for HZ, and n=99 for KO mice). **c** Urine glucose concentration in the nonfasting state (n=7–8). **d** Plasma insulin levels in the nonfasting state (n=15–16). **e** Urine creatinine concentration in the nonfasting state (n=7–8). The data were compared using one-way ANOVA followed by Tukey's HSD test; *p < 0.05, **p < 0.01, ***p < 0.001 compared to the respective WT group. The data are presented as the mean ± SEM. (Modified from Paper I)

4.1.2 Acute VPA treatment improved glycaemic control in male *Wfs1*-deficient mice

Glycaemic control in WS patients is compromised, and *diabetes mellitus* is the earliest manifestation of WS (Barrett et al., 1995; Barrett and Bundey, 1997). Diabetes in WS patients is controlled with insulin injections, but there is an ongoing search for treatment options that could both improve glucose tolerance and alleviate other dysfunctions. Therefore, in Paper I, the acute effect of the selected drugs on glucose tolerance was analysed.

Glucose challenge (2 g/kg, i.p.) in these animals led to an increase in blood glucose levels, which reached a maximum at 30 minutes following glucose administration in all genotypes, with the highest concentration occurring in *Wfs1* KO mice (Figure 3). These results are consistent with other reported data in these mice (Kõks et al., 2009; Toots et al., 2019). Thus, *Wfs1* KO mice display

disturbed glycaemic control, as observed in WS patients (Barrett et al., 1995; Barrett and Bundey, 1997).

VPA, a widely used antiepileptic, anticonvulsant, and mood stabilizer, has numerous targets: it modulates GABA receptors (Landmark, 2007), stimulates the peroxisome proliferator-activated receptor gamma (PPARG) receptors (Manji and Zarate, 2002), and inhibits glycogen synthase kinase 3β (GSK3) (G. Chen et al., 1999). GSK3 is located downstream of the insulin receptor pathway (IR/PI3K/Akt/GSK3), the activation of which leads to GSK3 inhibition (D. A. Cross et al., 1995). This signalling cascade was hypothesized to be compromised in *Wfs1*-deficient mice. Since VPA has been shown to inhibit GSK3 *in vivo* (Sintoni et al., 2013), modulate blood glucose levels in rodents (Thurston et al., 1985; Turnbull et al., 1985) and induce WFS1 expression thereby facilitating ER stress response (Kakiuchi et al., 2009), its effect on the impaired glucose tolerance in *Wfs1* KO mice was evaluated.

To differentiate between the aforementioned actions of VPA, another inhibitor of GSK3, LiCl (Beaulieu et al., 2004), PPARG agonist rosiglitazone and the GABA-A agonist diazepam, were also used.

To explore the acute effect of these drugs on glucose tolerance, mice were subjected to a single injection of VPA (300 mg/kg, i.p.), diazepam (3 mg/kg, i.p.), LiCl (200 mg/kg, i.p.), or rosiglitazone (3 mg/kg, i.p.) 15 minutes prior to glucose administration. Compared with saline treatment, pretreatment with VPA prior to glucose challenge significantly reduced the peak blood glucose concentration at 30 minutes in the *Wfs1* KO and HZ groups but had no effect in the WT group. Acute VPA treatment normalized the blood glucose concentration to a level similar to that in WT mice. In contrast, pretreatment with diazepam deteriorated glycaemic control in both *Wfs1* KO and WT mice (HZ mice were not tested), leading to increased blood glucose levels (Figure 3). Diazepam, like other benzodiazepines, mostly acts as a positive allosteric modulator of GABA, which can affect insulin secretion, and overactivation of GABA may disrupt insulin release, resulting in elevated blood glucose levels (Haefely, 1984). A single dose of diazepam has been shown to increase blood glucose levels in healthy volunteers (Syvälahti and Kanto, 1975), and daily chlordiazepoxide treatment can aggravate hyperglycaemia in diabetic patients (Zumoff and Hellman, 1977). Moreover, stimulation of peripheral-type benzodiazepine receptors has been shown to suppress glucose-stimulated insulin secretion and reduce insulin sensitivity (Chevassus et al., 2004). Thus, the acute effect of VPA seems to be independent of GABA modulation but cannot be completely excluded considering the controversial effects of benzodiazepines on blood glucose levels (Goudarzi et al., 2021). Pretreatment with LiCl or rosiglitazone in *Wfs1* KO or WT mice had no effect on blood glucose levels compared to those in the respective saline groups (data not shown). Thus, the effect of VPA on blood glucose concentration is most likely not mediated through GSK3 inhibition and/or PPARG activation.

Similarly, a single exposure to VPA can cause a significant blood glucose lowering effect in patients with epilepsy during oral GTT (Rakitin et al., 2015). Furthermore, patients with epilepsy on VPA treatment have fasting hyperinsuli-

naemia accompanied by lower fasting plasma glucose levels (Pylvänen et al., 2006). VPA treatment has also been associated with hypoglycaemia in adult patients even at therapeutic doses (Rota et al., 2021).

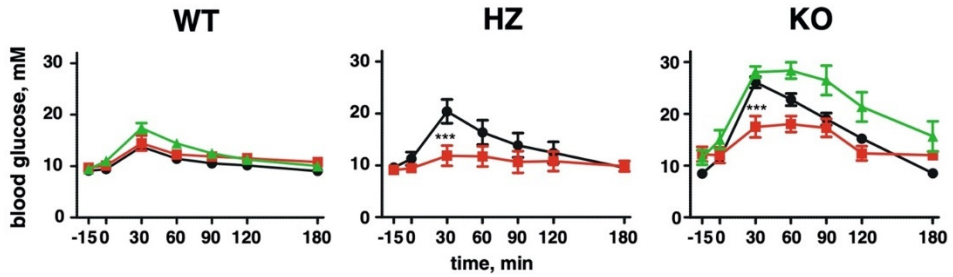

Figure 3. Acute valproate treatment normalized glucose tolerance in male *Wfs1*-deficient mice. Wild type (WT), *Wfs1* heterozygous (HZ) and *Wfs1* knock-out (KO) mice were pretreated with saline (vehicle, black circles), valproate (300 mg/kg, i.p., red squares) or diazepam (KO and WT only; 3 mg/kg, i.p., green triangles) 15 min before glucose administration (2 g/kg, i.p.). The data were compared using one-way ANOVA followed by Tukey's HSD test; ***$p < 0.001$ compared to the saline group of the respective genotype. The data are presented as the mean ± SEM, n = 6–16 mice per group. (Paper I)

4.1.3 Acute VPA treatment reduced plasma insulin levels

In WS patients, insulin sensitivity varies depending on the type of mutation, but overall, the response to insulin treatment is better than that in type 1 diabetic patients (Omkarappa et al., 2021; Paris et al., 2015). To evaluate insulin sensitivity in *Wfs1* KO mice, the effect of insulin (2 U/kg, i.p.) on blood glucose levels was measured. Insulin had similar glucose-lowering effects in both male *Wfs1* KO and WT mice (*Wfs1* HZ mice were not tested), indicating a normal response to insulin in *Wfs1* KO mice (Figure 4a). This finding is consistent with other reports showing that *Wfs1*-deficient mice have lower insulin content due to reduced beta cell mass, but their insulin sensitivity is comparable to that of normal mice (Gorgogietas et al., 2023; Tanji et al., 2015).

Long-term VPA exposure has been shown to increase insulin levels (Demir and Aysun, 2000; Luef et al., 2003; Manaka et al., 2013; Pylvänen et al., 2006). To determine insulin levels in response to a single administration of VPA, blood samples were collected at 30 minutes after the IPGTT. Compared with *Wfs1* HZ or WT mice, *Wfs1* KO mice did not exhibit any difference in baseline insulin levels. Unexpectedly, VPA administration before glucose challenge reduced plasma insulin levels at the 30-minute time point regardless of genotype (Figure 4b). Thus, the modulatory effect of acute VPA treatment on the insulin pathway seems to be independent of WFS1 functionality and differs from its known chronic effect. Therefore, it is plausible that the increase in glycaemic control in *Wfs1* KO mice following a single administration of VPA likely results from the

potentiation of insulin sensitivity rather than modulating its release. To test this hypothesis, mice were treated with the beta cell toxin streptozotocin to generate hypoinsulinaemic diabetic mice (Kolb, 1987). STZ is an antibiotic that results in pancreatic beta cell death (Furman, 2021).

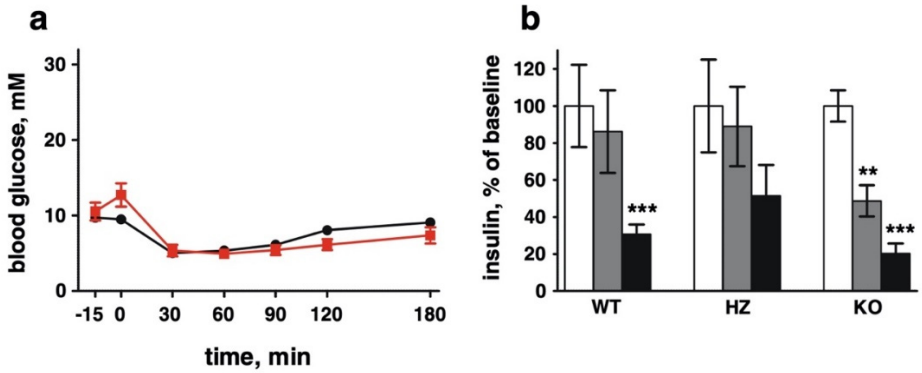

Figure 4. Acute valproate treatment decreased plasma insulin levels regardless of genotype. **a** The effect of insulin (2 U/kg, i.p.) on blood glucose concentration was similar in wild type (WT; black circles) and *Wfs1* homozygous (KO; red squares) mice. **b** The levels of plasma insulin before the experiment (light bars), 30 minutes after the administration of glucose (2 g/kg, i.p., grey bars), and after pretreatment with valproate (300 mg/kg, i.p., dark bars) prior to glucose challenge. The data were compared using one- or two-way ANOVA followed by Tukey's HSD test; **p < 0.01, ***p < 0.001, compared to the baseline value of the respective genotype. The data are expressed as the mean ± SEM, n = 7–15 mice per group. (Paper I)

4.1.4 Acute VPA treatment lowers blood glucose levels in streptozotocin-induced diabetic mice

To determine the importance of insulin in the glucose-lowering effect of acute VPA treatment, mice were treated with streptozotocin (STZ) to generate a mouse model of insulin-dependent type 1 *diabetes mellitus* (Kolb, 1987). Mice were administered a single dose of STZ (170 mg/kg, i.p.) and assessed 7 days later to verify the onset of diabetes.

As expected, after 7 days, STZ had induced hyperglycaemia in both WT and *Wfs1* HZ mice, accompanied by a significant increase in the urinary glucose level and a decrease in the urinary creatinine level, indicating the development of diabetes. However, STZ treatment had no apparent effect on blood glucose or urinary glucose or creatinine levels in *Wfs1* KO mice (Figure 5). In addition, the size of the islets of Langerhans in the pancreas was observed by immunostaining. Nontreated *Wfs1* KO mice had smaller islets of Langerhans than nontreated WT mice; however, the size of the islets of Langerhans in WT mice was reduced following STZ (in Paper I: Figure 6a, b). This STZ-induced reduction in the size of the islets was not detected in *Wfs1* KO mice (in Paper I: Figure 6c, d).The lack of STZ toxicity in *Wfs1* KO mice might be explained by the possible impairment

of GLUT2 in these mice, as GLUT2 in pancreatic beta cells is required for STZ efficiency (Schnedl et al., 1994). Interestingly, STZ resistance is also observed in glucagon receptor knockout mice (Conarello et al., 2007). Diabetes is accompanied by excess glucagon produced by alpha cells, and elevated glucagon levels have also been observed in a rat model of WS (Jia et al., 2022; Toots et al., 2018). Excess glucagon negatively regulates the expression of glucagon receptors; thus, glucagon receptors might also be downregulated in patients with WFS1 deficiency (Charron and Vuguin, 2015), which may decrease sensitivity to STZ.

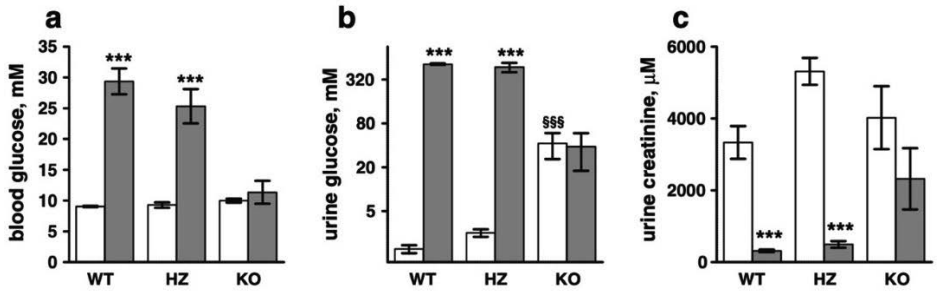

Figure 5. A single dose of streptozotocin induced diabetes in WT and *Wfs1* HZ mice but not in *Wfs1* KO mice. **a** Blood glucose levels in the nonfasting state. **b** Urine glucose levels. **c** Urine creatinine levels. Light bars correspond to baseline values, and dark bars correspond to day 7 after streptozotocin (170 mg/kg, i.p.) administration. The data were compared using Student's t test; ***$p < 0.001$, compared to the baseline value of the respective genotype; §§§$p < 0.001$, compared to the baseline value of the WT group. The data are expressed as the mean ± SEM; n = 6–8 mice per group. (Paper I)

Both exogenous insulin and pretreatment with VPA led to a notable decrease in blood glucose levels compared to the baseline values in STZ-induced diabetic WT and *Wfs1* HZ mice and in *Wfs1* KO mice. Pretreatment with VPA prior to insulin administration resulted in an abrupt decrease in blood glucose concentration, inducing hypoglycaemic coma in some STZ-treated WT and *Wfs1* HZ animals and in all *Wfs1* KO animals; thus, these animals were removed from further analysis (Figure 6). The plasma glucose-lowering effect of VPA treatment has been observed in STZ-treated apoE−/− mice (Bowes et al., 2009) and in spontaneously diabetic BB/E rats (Turnbull et al., 1985). Hence, acute VPA modulates blood glucose levels in insulin deficiency. Although *Wfs1* KO mice had reduced insulin levels in response to acute VPA treatment, long-term exposure to VPA has been reported to cause increased insulin levels (Demir and Aysun, 2000; Luef et al., 2003; Manaka et al., 2013; Pylvänen et al., 2006). One possible explanation for the glucose-lowering effect of VPA is the enhancement of insulin activity, which explains the outcome of VPA and insulin cotreatment, but does not explain why VPA alone is that effective in STZ-induced diabetic mice. It is likely an insulin-independent mechanism involved in glucose excretion and/or assimilation. VPA can decrease glucose reabsorption in the kidneys (Beger et al., 2009)

and inhibit gluconeogenesis (Khan et al., 2016), and inhibition of HDAC (a known action of VPA) has been shown to facilitate glucose utilization, probably via GLUT4 (Raichur et al., 2012). However, this finding does not explain why acute VPA treatment in WT mice had no effect, while it reduced blood glucose levels in both *Wfs1* KO and HZ and in STZ-treated mice. Thus, the mechanisms by which VPA affects blood sugar levels are not yet clear.

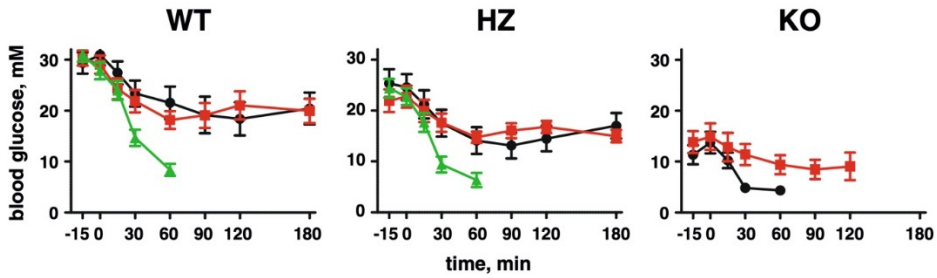

Figure 6. Both exogenous insulin and acute valproate lowered blood glucose levels in streptozotocin-treated mice. The effect of insulin on blood glucose levels was potentiated by pretreatment with valproate (VPA). Wild type (WT), *Wfs1* heterozygous (HZ) and *Wfs1* homozygous (KO) mice were administered insulin (2 U/kg, i.p., black circles) at 0 min, VPA (300 mg/kg, i.p., red squares) at -15 min (without exogenous insulin), or a combination of VPA (15 min prior to insulin challenge) and insulin (green triangles). Cotreatment induced rapid hypoglycaemic coma in *Wfs1* KO mice, and this experiment was terminated. The data are expressed as the mean ± SEM; n = 6–8 mice per group. (Paper I)

4.1.5 Paper I summary discussion

In Paper I, the acute effect of VPA was evaluated in mice harbouring *Wfs1* gene mutations. *Wfs1* KO mice had significantly lower body weight compared to *Wfs1* HZ and WT mice, consistent with previous data. While nonfasting blood glucose levels were only slightly elevated in *Wfs1* KO mice, plasma insulin levels were notably lower, mirroring the beta cell loss and impaired insulin secretion observed in mice with dysfunctional WFS1, consistent with the characteristics of WS patients. However, *Wfs1* KO mice had a normal response to insulin. Urinary glucose levels were markedly greater in *Wfs1* KO mice, resembling glycosuria in diabetic patients.

Pretreatment with VPA significantly reduced the peak blood glucose concentration during glucose challenge in *Wfs1* KO and HZ mice despite reduced plasma insulin levels. The potential of acute VPA treatment to improve glucose tolerance is consistent with observations in patients with epilepsy. The effect of VPA seems to be independent of GSK3 inhibition or PPARG activation, as LiCl and rosiglitazone had no effect on blood glucose levels. Although diazepam worsened glycaemic control, unlike VPA, the role of GABA in the effect of VPA cannot be completely ruled out. Diazepam acts by modulating both the GABA

receptor and mitochondrial translocator protein TSPO (Yue et al., 2022), whose modulatory effects on glucose metabolism have yielded conflicting results.

Like exogenous insulin, VPA also effectively decreased blood glucose levels in STZ-induced diabetic WT and *Wfs1* HZ mice. Moreover, VPA coadministration with insulin had a cumulative effect, causing hypoglycaemic coma in some STZ-induced diabetic mice and in all *Wfs1* KO mice.

Overall, the ability of acute VPA to modulate blood glucose levels independently of insulin suggests that it may affect the levels either by directly regulating the activity of kinases, or alter glucose excretion and/or assimilation. Nevertheless, the exact molecular mechanism involved remains to be elucidated.

4.2 Effect of chronic VPA treatment on the hepatic gene expression profile in male *Wfs1*-deficient mice (Paper II)

4.2.1 VPA had no effect on mean body weight

VPA has been linked to disturbances in metabolic control, and one of the common secondary metabolic side effects of VPA treatment is weight gain, which is usually noticeable within the first 3 months of treatment (Biton et al., 2001; Demir and Aysun, 2000; Verrotti et al., 2009). Therefore, to determine whether long-term VPA treatment affects body weight and whether genotype has any effect on body weight, the weights of healthy (wild-type, WT), *Wfs1* heterozygous (*Wfs1* HZ), and *Wfs1* homozygous (*Wfs1* KO) male mice were recorded weekly over a period of 14 weeks. From 8 to 9 weeks of age, the growth rates began to differ markedly between the genotypes. *Wfs1* KO mice exhibited stunted growth, reaching a plateau at approximately 20 grams of body weight, while WT and *Wfs1* HZ mice continued to grow normally. By the age of 16 weeks, treatment-naïve *Wfs1* KO mice had a significantly lower mean body weight than treatment-naïve WT or *Wfs1* HZ mice (Figure 7). This finding is consistent with Paper I in which 5- to 6-month-old *Wfs1* KO mice were markedly smaller than their healthy littermates, weighing approximately 20 grams. The reason for the dependency of the growth rate on age in *Wfs1* KO mice might be associated with insufficient hormone production and delayed sexual maturation seen in WS patients (Frontino et al., 2023; Rigoli et al., 2022). Alternatively, mild glucosuria in *Wfs1* mutant mice may contribute to the slower growth of these animals. Administration of VPA for 3 consecutive months had no effect on weight gain regardless of genotype (Figure 7); hence, the effect differed from that reported in human patients (Biton et al., 2001; Demir and Aysun, 2000; Verrotti et al., 2009).

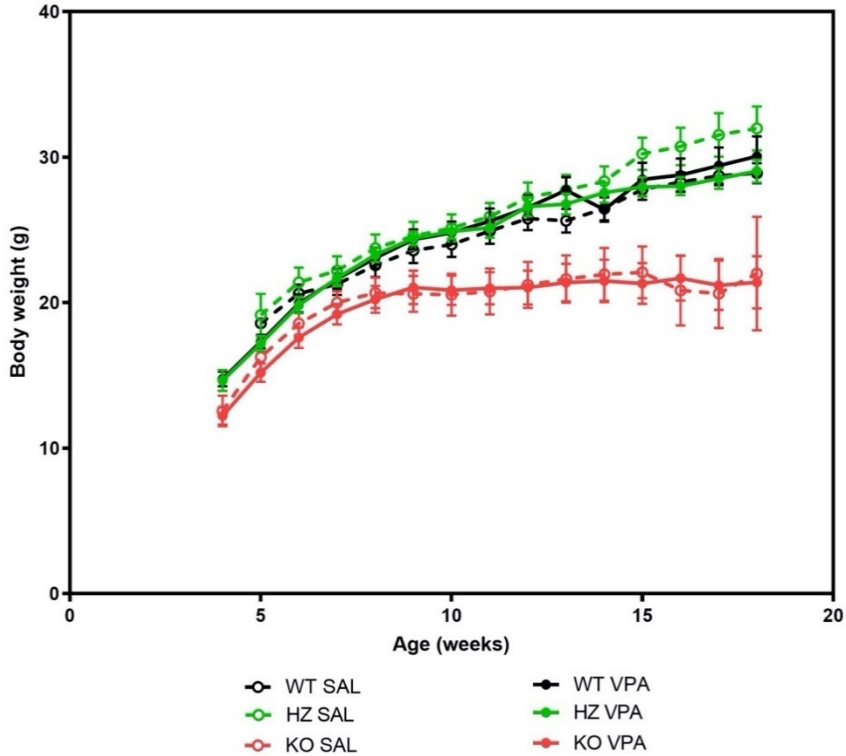

Figure 7. **The growth rate of male *Wfs1* KO mice slowed down at 8–9 weeks of age and reached a plateau**. By the age of 16 weeks, *Wfs1* KO mice (KO, red circles) had significantly lower body weights than wild-type (WT, black circles) or *Wfs1* heterozygous (HZ, green circles) mice (p = 0.0014). Chronic administration of valproate (VPA) for 3 months (300 mg/kg/day, i.p. solid symbols) had no effect on body weight regardless of genotype (p = 0.2393). The data are presented as the mean ± SEM, n=8. (Modified from Paper II)

4.2.2 Chronic VPA treatment did not improve glucose tolerance in male *Wfs1*-deficient mice

Previously (in Paper I), a single administration of VPA was shown to improve glycaemic control in both *Wfs1* KO and HZ mice. In Paper II, the effect of long-term VPA treatment on glucose tolerance was also evaluated.

Saline-treated animals had a baseline fasting blood glucose concentration of approximately 10 mM regardless of genotype. Administration of glucose (2 g/kg i.p.) to these mice resulted in increased blood glucose levels, with a peak at 30 minutes in all mouse groups; the most prominent increase was approximately 30 mM observed in *Wfs1* KO mice (Figure 8). This finding is consistent with previously reported data indicating disturbed glycaemic control in these mice (Kõks et al., 2009; Paper I). Unexpectedly, in contrast to the effect of acute VPA treatment, chronic VPA treatment failed to improve glycaemic control regardless

of genotype. In fact, chronic VPA treatment led to worsened glucose tolerance in WT mice, but the effect was not as remarkable in *Wfs1* HZ or *Wfs1* KO mice (Figure 8). Although the majority of the literature indicates that VPA has a glucose-lowering effect (Demir and Aysun, 2000; Pylvänen et al., 2006), hyperglycaemia has been reported in overweight patients treated with VPA (Elmslie et al., 2009). *Wfs1* KO mice have increased anxiety in novel stressful conditions (Luuk et al., 2009). High anxiety can lead to elevated cortisol, decreased insulin and increased glucose levels (Sharma et al., 2022). VPA has shown anxiolytic properties in both rodents and humans (Aliyev and Aliyev, 2008; Cintado et al., 2023). It is plausible that the higher peak glucose levels observed in *Wfs1*-deficient mice were partly due to greater anxiety induced by the novel GTT procedure, and the hypoglycaemic effect of acute VPA treatment was instead caused by reduced anxiety. However, that does not explain glucose-lowering effect in STZ-induced type 1 diabetic mice. Nevertheless, the impact of VPA on glycaemic and metabolic control remains somewhat controversial, and the outcome seems to be strongly influenced by study design, genetics and other factors.

Previously (Paper I), single injection of VPA reduced insulin levels and also showed a glucose-lowering effect in STZ-induced type 1 diabetic mice; thus, acute VPA did not act by increasing insulin. The effect of chronic VPA on insulin levels was not measured herein, but it is known that long-term VPA therapy induces increased insulin levels and fasting hyperinsulinaemia (Demir and Aysun, 2000; Luef et al., 2003; Manaka et al., 2013; Pylvänen et al., 2006), which supports VPA as a blood glucose-lowering agent.

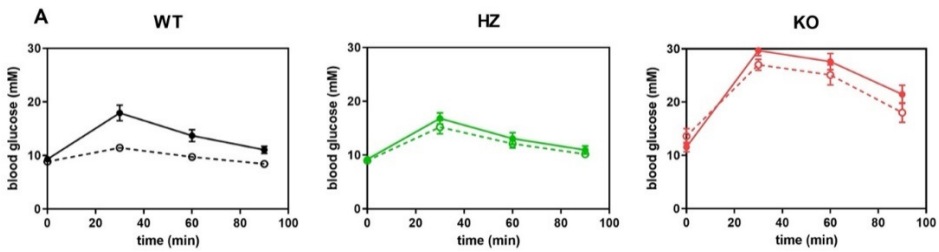

Figure 8. **Valproate treatment for 3 months did not improve glycaemic control in male *Wfs1* mutant mice**. A glucose tolerance test was conducted in male wild-type (WT), *Wfs1* heterozygous (HZ) and *Wfs1* homozygous (KO) mice after 3 months of VPA treatment (300 mg/kg/day). Blood glucose levels in the vehicle group (0.9% saline, 10 mL/kg, i.p., empty circles) and VPA treated group (solid circles) immediately before and at 30, 60, and 90 minutes following glucose challenge (2 g/kg, i.p.). The data are presented as the mean ± SEM, n = 8. (Modified from Paper II)

4.2.3 Hepatic gene expression after chronic VPA treatment

In addition to the long-term effect of VPA on glycaemic control, the impact of VPA on gene expression in the liver of male *Wfs1* KO mice was investigated. The liver is a metabolically relevant tissue, and insulin affects glucose metabolism in hepatocytes (Lin and Accili, 2011). WFS1 is also expressed in hepatocytes (De Falco et al., 2012). A postprandial increase in insulin levels shifts the liver towards glucose consumption and lipid synthesis (Lin and Accili, 2011). The liver is also the main contributor to the clearance of peripheral insulin, the impairment of which has been associated with type 2 diabetes (Najjar and Perdomo, 2019). In Paper II, the goal was to identify differentially expressed genes that might be involved in the effects on glucose metabolism and metabolic alterations induced by VPA treatment. The liver transcriptome was analysed using the Affymetrix GeneChip Mouse Gene 1.0 ST Array.

First, the expression of *Wfs1* was analysed. As expected, downregulation of *Wfs1* gene expression was not observed. The Affymetrix GeneChip Mouse Gene 1.0 ST Array contains a probe for each exon of the gene, and most of the exons in *Wfs1* KO mice are intact (Kõks et al., 2009). However, exon-specific analysis revealed notably lower expression of *Wfs1* exons 7 and 8 in *Wfs1* KO mice (data not shown), as confirmed by RT–qPCR using a TaqMan probe bounding exons 7 and 8 (Figure 9). The expression in *Wfs1* HZ mice was reduced by approximately 50% in comparison to that in WT mice. Thus, a considerable percentage of the expressed *Wfs1* gene is truncated, and both *Wfs1* KO and HZ mice exhibit a severe deficiency of functional WFS1. Treatment with VPA led to elevated *Wfs1* mRNA expression regardless of genotype (Figure 9); the ability of VPA to induce *Wfs1* mRNA and WFS1 protein expression *in vitro* has been previously demonstrated (Kakiuchi et al., 2009).

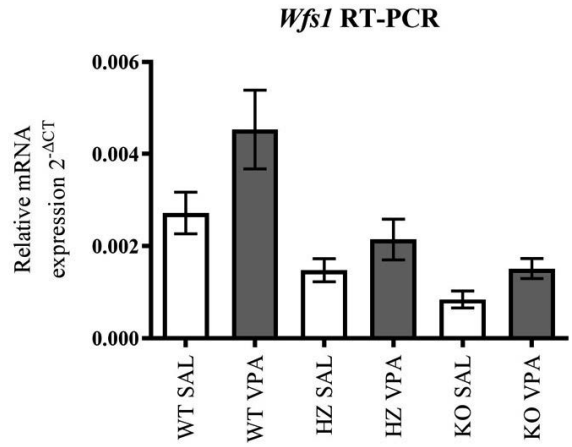

Figure 9. ***Wfs1*** **mRNA expression in the liver of male wild-type and** ***Wfs1*** **mutant mice**. The relative mRNA expression level of *Wfs1* (based on exons 7 and 8 of the mRNA sequence) was analysed in the livers of 4- to 4.5-month-old animals after 3 months of

treatment with valproate (VPA) (presented as $2^{-\Delta CT}$ relative to the housekeeping gene *Actb*). The base expression of *Wfs1* mRNA was substantially lower in *Wfs1* homozygous (KO) mice than in *Wfs1* heterozygous (HZ) or wild-type (WT) mice (saline (SAL) group animals). Chronic VPA treatment induced increased *Wfs1* expression regardless of genotype. *Wfs1* expression levels correlate well with the gene dosage effect. Statistical significance was determined by two-way ANOVA. The data are presented as the mean ± SEM, $n = 8$. (Modified from Paper II)

Microarray analysis of the liver revealed numerous genes whose expression was affected by genotype. In *Wfs1* KO mice, 23 genes were upregulated and 19 genes were downregulated compared to those in WT mice ($p < 0.05$ and fold change > 2.0) (Table 3). Ten genes were regulated by VPA treatment ($p < 0.05$ and fold change > 1.2) (in Paper II: Table 2), and an additional 9 genes exhibited an interaction between genotype and VPA treatment ($p < 0.05$ and fold change > 1.0) (Table 4).

The majority of differentially expressed genes in the liver of *Wfs1* KO mice are cytochromes, which mediate electron transport and oxidative processes and are mainly involved in steroid metabolism and fatty acid/lipid metabolism (upregulated: *Cyp2b13*, *Cyp2a22*, *Cyp17a1*, *Cyp2c38*, *Cyp4a14*; downregulated: *Cyp7b1*, *Cyp8b1*, *Cyp2u1*, *Cyp4a12b*, *Cyp4a12a*) (Table 3). Most of these cytochromes also participate in drug metabolism, as do two of the most prominently upregulated genes (*Sult3a1*, *Fmo3*). The liver is a major organ involved in oxidative processes, as the metabolism of drugs and other substances occurs there. Thus, it was expected that these genes would be most prominent. In addition to cytochromes several other genes upregulated (*Slco1a4*, *Hao2*, *Pnpla3*, *Abhd1*, *Acot3*) and downregulated (*Hsd3b5*, *Fitm1, Elovl3*) in *Wfs1* KO mice are involved in steroid and fatty acid/lipid metabolism pathways. Previous findings in these mice have indeed indicated a shift in lipid metabolism with a trend towards lipolysis (Porosk et al., 2017); however, the lipid profile of WS patients has been reported to be normal (Haghighi et al., 2013). Among the upregulated genes were also *Lepr* and *Ppargc1a*, which are important in energy metabolism, including glucose metabolism (Amitani et al., 2013; Puigserver et al., 2003).

Table 3. List of genes whose expression in the mouse liver is regulated by the *Wfs1* KO genotype, as measured by the Affymetrix GeneChip Mouse Gene 1.0 ST Array. (Modified from Paper II)

P value	Fold change	Gene	Gene description	Biological process
			Upregulated in *Wfs1* KO mice	
				Drug metabolism
6.32*E*-05	131.3	*Sult3a1*	Sulfotransferase family 3A, member 1	
4.94*E*-06	112.2	*Fmo3*	Flavin containing monooxygenase 3	
9.44*E*-07	4.4	*Fmo2*	Flavin containing monooxygenase 2	
1.29*E*-04	2.1	*Abcb1a*	ATP-binding cassette, sub-family B, member 1A	
				Steroid metabolism
3.81*E*-04	37.6	*Cyp2b13*	Cytochrome P450, family 2, subfamily b, polypeptide 13	+drug metabolism
7.93*E*-05	10.3	*Cyp2a22*	Cytochrome P450, family 2, subfamily a, polypeptide 22	+drug metabolism
4.10*E*-04	4.4	*Cyp17a1*	Cytochrome P450, family 17, subfamily a, polypeptide 1	+glucocorticoid biosynthesis
6.13*E*-05	2.1	*Cyp2c38*	Cytochrome P450, family 2, subfamily c, polypeptide 38	+drug metabolism
4.56*E*-04	2.0	*Slco1a4*	Solute carrier organic anion transporter family, member 1a4	
				Fatty acid/lipid metabolism
3.50*E*-06	9.8	*Slc22a27 [AB056442]*	Solute carrier family 22, member 27	+drug metabolism
1.07*E*-04	6.3	*Hao2*	Hydroxyacid oxidase 2	
7.17*E*-04	5.0	*Pnpla3*	Patatin-like phospholipase domain containing 3	
5.54*E*-15	4.8	*Abhd1*	Abhydrolase domain containing 1	
1.19*E*-04	3.5	*Acot3*	Acyl-CoA thioesterase 3	
6.50*E*-04	2.0	*Cyp4a14*	Cytochrome P450, family 4, subfamily a, polypeptide 14	
				Other
8.82*E*-06	14.0	*Slc22a26 [BC014805]*	Solute carrier family 22 (organic cation transporter), member 26	Putative integral membrane transport
1.46*E*-05	5.2	*Lepr*	Leptin receptor	Energy metabolism
7.39*E*-04	3.5	*Mt1*	Metallothionein 1	Heavy metal metabolism, negative regulation of growth
7.42*E*-04	2.9	*Cux2*	Cut-like homeobox 2	Cognition, neuronal proliferation and differentiation
3.59*E*-04	2.8	*Rgs16*	Regulator of G-protein signaling 16	G protein-coupled receptor signaling pathway
2.81*E*-04	2.4	*Gm6192*	Predicted gene 6192	
2.97*E*-04	2.2	*Rsph4a*	Radial spoke head 4 homolog A (Chlamydomonas)	Cilium movement
3.97*E*-06	2.1	*Ppargc1a*	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	Energy metabolism, circadian rhythm regulation

P value	Fold change	Gene	Gene description	Biological process
			Downregulated in *Wfs1* KO mice	
				Steroid metabolism
1.25*E*-04	2.8	*Hsd3b5*	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 5	
3.81*E*-07	3.9	*Cyp7b1*	Cytochrome P450, family 7, subfamily b, polypeptide 1	+circadian rhythm regulation, memory
1.21*E*-05	2.0	*Cyp8b1*	Cytochrome P450, family 8, subfamily b, polypeptide 1	
				Fatty acid/lipid metabolism
5.25*E*-06	2.2	*Cyp2u1*	Cytochrome P450, family 2, subfamily u, polypeptide 1	+drug metabolism
2.82*E*-05	2.6	*Fitm1*	Fat storage-inducing transmembrane protein 1	
2.10*E*-04	3.0	*Cyp4a12b*	Cytochrome P450, family 4, subfamily a, polypeptide 12B	
1.99*E*-04	3.1	*Cyp4a12a*	Cytochrome P450, family 4, subfamily a, polypeptide 12a	
1.06*E*-07	6.4	*Elovl3*	Elongation of very long chain fatty acids (FEN1/E102, SUR4/E103, yeast)-like 3	
				Other
1.68*E*-04	2.0	*Mtnr1a*	Melatonin receptor 1A	Circadian rhythm regulation, regulation of insulin secretion
1.42*E*-05	2.0	*Ttc39c*	Tetratricopeptide repeat domain 39C	Cilium assembly
9.12*E*-06	2.4	*Rarres1*	Retinoic acid receptor responder (tazarotene induced) 1	
2.47*E*-05	2.5	*Neb*	Nebulin	
4.58*E*-05	2.6	*Cib3*	Calcium and integrin binding family member 3	Calcium ion homeostasis, mechanoelectrical transduction by cochlear hair cells
2.33*E*-06	2.6	*Alas2*	Aminolevulinic acid synthase 2, erythroid	Heme biosynthesis, response to hypoxia
1.53*E*-04	2.7	*Slc15a5*	Solute carrier family 15, member 5	
4.13*E*-06	3.1	*Susd4*	Sushi domain containing 4	Complement activation regulation
9.25*E*-05	3.1	*Mup21*	Major urinary protein 21	
4.37*E*-07	3.5	*Nat8*	N-acetyltransferase 8 (GCN5-related, putative)	Glutathione metabolism
5.38*E*-05	4.6	*Nat8f5* *[Cml5]*	N-acetyltransferase 8 (GCN5-related) family member 5 [Camello-like 5]	Regulation of gastrulation, heart development

The main interest was to identify genes whose expression is affected by chronic VPA treatment in a genotype-dependent manner (Table 4). The genes with the greatest difference in expression between WT and *Wfs1* KO mice treated with VPA were peroxisome proliferator activator receptor beta/delta (*Ppard*), interleukin-3 regulated nuclear factor (*Nfil3*), and nuclear receptor subfamily 1, group D, member 2 (*Nr1d2*); all these genes are also associated with the regulatory network of circadian rhythms (Yan et al., 2008). The identification of genes involved in circadian rhythm is not surprising since VPA can alter the expression of transcription factors regulating rhythmicity (Griggs et al., 2018). Moreover, Drosophila neuronal *Wfs1* knockdown results in reduced sleep and disrupted circadian rhythms (Hao et al., 2023), indicating an already disturbed circadian rhythm regulatory network in WFS1 deficiency.

Table 4. List of genes whose expression in the mouse liver after chronic VPA treatment is dependent on the *Wfs1* KO genotype, as measured by the Affymetrix GeneChip Mouse Gene 1.0 ST Array. (Modified from Paper II)

P value	Fold change	Gene symbol	Gene description	Biological processs
				Circadian rhythm regulation
1.1*E*-04	2.26	*Ppard*	Peroxisome proliferator activator receptor delta	+fatty acid/lipid metabolism, inflammatory response, insulin secretion, myelination
2.1*E*-03	2.17	*Nfil3*	Nuclear factor, interleukin 3, regulated	+immune response
5.6*E*-04	2.03	*Nr1d2*	Nuclear receptor subfamily 1, group D, member 2	+fatty acid/lipid metabolism, inflammatory response
				Other
1.9*E*-03	1.58	*Cyp2j9*	Cytochrome P450, family 2, subfamily j, polypeptide 9	Drug metabolism
9.2*E*-04	1.32	*Lnx2*	Ligand of numb-protein X 2	Neuron differentiation
1.9*E*-03	1.26	*Zfp334*	Zinc finger protein 334	Regulation of transcription
1.3*E*-03	1.25	*Piezo2 [Fam38b]*	Piezo-type mechanosensitive ion channel component 2 [Family with sequence similarity 38, member B]	Cation transport, sensory perception
1.7*E*-03	1.20	*Aldh9a1*	Aldehyde dehydrogenase 9, subfamily A1	Carnitine metabolism
2.1*E*-03	1.18	*Fnbp4*	Formin binding protein 4	

VPA treatment induced an increase in *Ppard* expression regardless of genotype. However, the upregulation of hepatic *Ppard* in VPA-treated WT mice was notably greater than that in VPA-treated *Wfs1* KO mice compared to that in the corresponding vehicle group. These results (as well as the expression of *Sult3a1*, *Fmo2* and *Lepr*) were verified by RT–qPCR analysis (Figure 10). This observation is consistent with previous findings in CHO and F9 cell lines, where VPA increased *Ppard* gene expression (Göttlicher et al., 1998; Lampen et al., 1999). Moreover, PPAR reporter assays have classified VPA as a “triple Ppar -alpha, -beta/delta, -gamma agonist” (Szalowska et al., 2014).

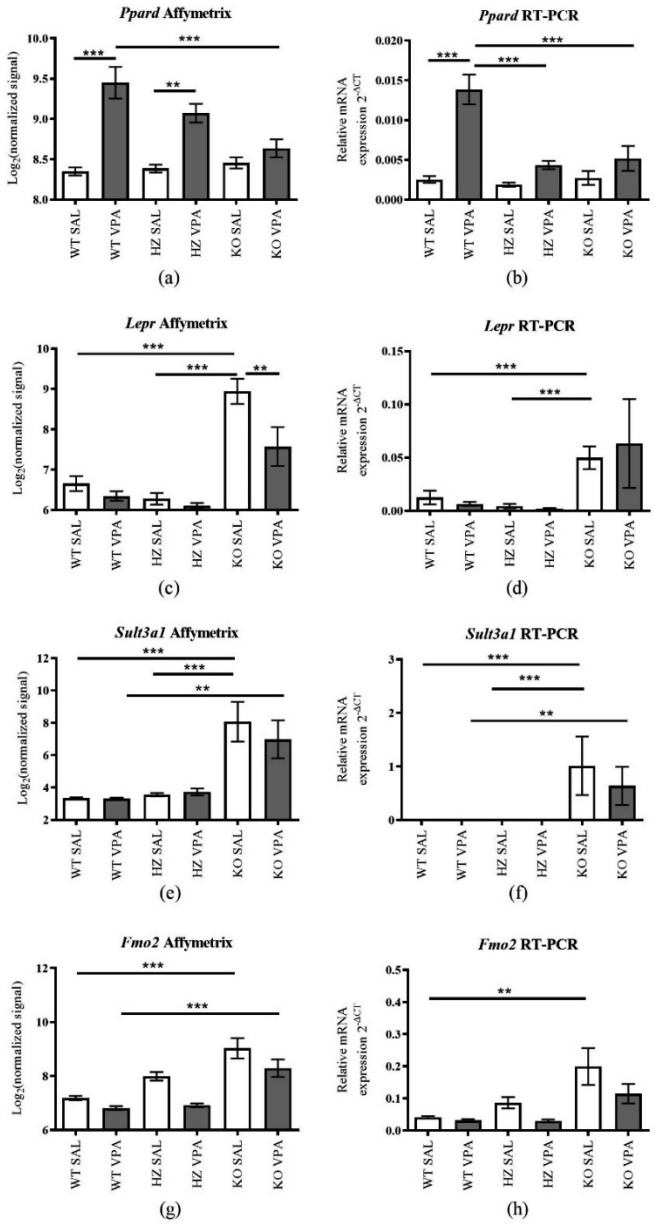

Figure 10. **Comparison of the results from the Affymetrix GeneChip Mouse Gene 1.0 ST Array and RT–qPCR analysis in the livers of 4- to 4.5-month-old male wild-type (WT), *Wfs1* heterozygous (HZ) and *Wfs1* homozygous (KO) mice after 3 months of VPA treatment (300 mg/kg/day) and vehicle (0.9% saline, 10 mL/kg, i.p; SAL).** Relative mRNA expression is represented as the mean of quadruplicate samples normalized to the reference gene *Actb*. (a) and (b) *Ppard*; (c) and (d) *Lepr*; (e) and (f) *Sult3a1*; (g) and (h) *Fmo2*. Statistical significance was determined by two-way ANOVA followed by Tukey's post hoc test; *** $p < 0.001$, ** $p < 0.01$. The data are presented as the mean ± SEM, $n = 8$ per group. (Modified from Paper II)

PPARD, together with PPARA and PPARG, are nuclear transcription factors activated by lipids that exert several biological activities, including the regulation of fatty acid metabolism (Grimaldi, 2005). PPARD is widely expressed, and its physiological activities include skin healing, the regulation of fatty acid oxidation and hepatic lipogenesis and the enhancement of fatty acid uptake in muscle (Grimaldi, 2005; J. Lee and Chung, 2011; S. Liu et al., 2013). PPARD agonists may serve as effective tools to fight obesity and metabolic syndrome (J. Lee and Chung, 2011). The PPARD agonist GW501516 prevents high-fat diet-induced hyperglyceridaemia and boosts the lipin-1-PGC-1a-dependent pathway, thereby intensifying hepatic fatty acid oxidation (Barroso et al., 2011). Notably, a gene encoding PGC-1a (*Ppargc1a*) was also upregulated in *Wfs1* mutant mice (Table 3).

Furthermore, PPARD activation increases glycolysis and pentose phosphate shunt function, ameliorates insulin sensitivity and has been proposed as a potential treatment option for type 2 diabetes (C.-H. Lee et al., 2006; Serrano-Marco et al., 2011). It is plausible that the beneficial effects of PPARD on insulin sensitivity and peripheral glucose uptake might partly contribute to the effect of VPA on glucose levels.

4.2.4 Paper II Summary discussion

VPA treatment is associated with weight gain and metabolic disturbances; however, the mechanisms underlying its metabolic effects are not well understood. Since VPA has been proposed as a treatment option for WS, the impact of chronic VPA treatment on growth/body weight; metabolic control, specifically glucose tolerance; and hepatic gene expression was evaluated in *Wfs1*-deficient mice.

Untreated *Wfs1* KO mice exhibited stunted growth, and by the age of 16 weeks, they had significantly lower body weight than untreated WT or *Wfs1* HZ mice, consistent with previous findings. Chronic VPA treatment for 3 months had no significant effect on body weight in any of the genotypes.

Wfs1 KO mice exhibited disturbed glycaemic control, with significantly elevated blood glucose levels after glucose challenge. Acute VPA treatment was previously shown to improve glycaemic control in these mice, but chronic VPA treatment did not have the same positive effect. Conversely, chronic VPA treatment tended to impair glucose tolerance in WT mice.

The hepatic gene expression profile revealed that *Wfs1* deficiency affects the expression of genes related to oxidative processes, lipid metabolism, and xenobiotic (drug) metabolism. Notably, *Ppard*, a gene involved in fatty acid metabolism, insulin sensitivity and glycaemic control, was upregulated by VPA treatment in a genotype-dependent manner. The upregulation of *Ppard* could partly contribute to the metabolic effects of VPA.

Chronic VPA treatment did not increase body weight but also did not improve glycaemic control in mice regardless of genotype. Given the conflicting results on the effect of VPA on blood glucose levels, an ameliorating effect of long-term VPA treatment on glucose tolerance or other positive effects, e.g., neuropro-

tection, cannot be completely ruled out. Nevertheless, VPA has been in clinical use for decades, and investigations of its efficacy and safety in WS patients are ongoing.

4.3 The transcriptional profile of key RAAS components in a rat model of Wolfram syndrome (Paper III)

4.3.1 *Agtr2*, *Agtr1b* and *Bdkrb1* were downregulated in the peripheral tissues of male WS rats

Although WFS1 and its physiological role have been extensively examined, its involvement in the renin-angiotensin-aldosterone system (RAAS) and bradykinin pathways, also known as the kallikrein-kinin system (KKS), remains unexplored. To detect early alterations in the RAAS and KKS before the WS phenotype was fully developed, in Paper III, the expression levels of its key components in the lower lung lobe and left ventricle of the heart of young 3.5–4-month-old WS rats and their WT littermates were determined.

Compared with those in their WT littermates, the lungs and heart of WS rats had notably lower expression levels of *Agtr2*, *Agtr1b* and *Bdkrb1* mRNA (Figures 11d, f, g and 12d, f, g). Similarly, the levels of *Mas1* were substantially downregulated in the lungs of WS animals; conversely, in the heart, *Mas1* levels were slightly upregulated (Figure 12c). *Ace* expression was increased in the heart (Figure 12a) but did not differ significantly in the lungs of WS animals (Figure 11a). *Ace2* expression, by contrast, was decreased in the heart (Figure 12b), but tended to be elevated in the lungs (Figure 11b) compared to the levels in WT rats. The expression of *Agtr1a* and *Bdkrb2* did not differ significantly between the two genotypes in either organ (Figures 11e, h and 12e, h). Notably, in the lungs, gene expression differences between genotypes were more pronounced.

In addition to its role in the RAAS, AGTR2 promotes the regeneration of axons in the optic nerve in adult rats (Lucius et al., 1998). Furthermore, the ability of AGTR2 to form active heterodimers with tropomyosin receptor kinase B (TRKB) and BDKRB2, thereby enhancing receptor-mediated signal transmission, has been described (Abadir et al., 2006; Diniz et al., 2018). It is plausible that although *Bdkrb2* expression is normal in WS rats, BDKRB2 sensitivity may be compromised, given the marked reduction in *Agtr2* expression in these rats. Thus, downregulation of *Bdkrb1* and a potential decrease in BDKRB2 receptor sensitivity may lead to dysregulated Ca^{2+} homeostasis. This disruption could interfere with oxidative stress, ER stress, and the immune response in individuals with WS.

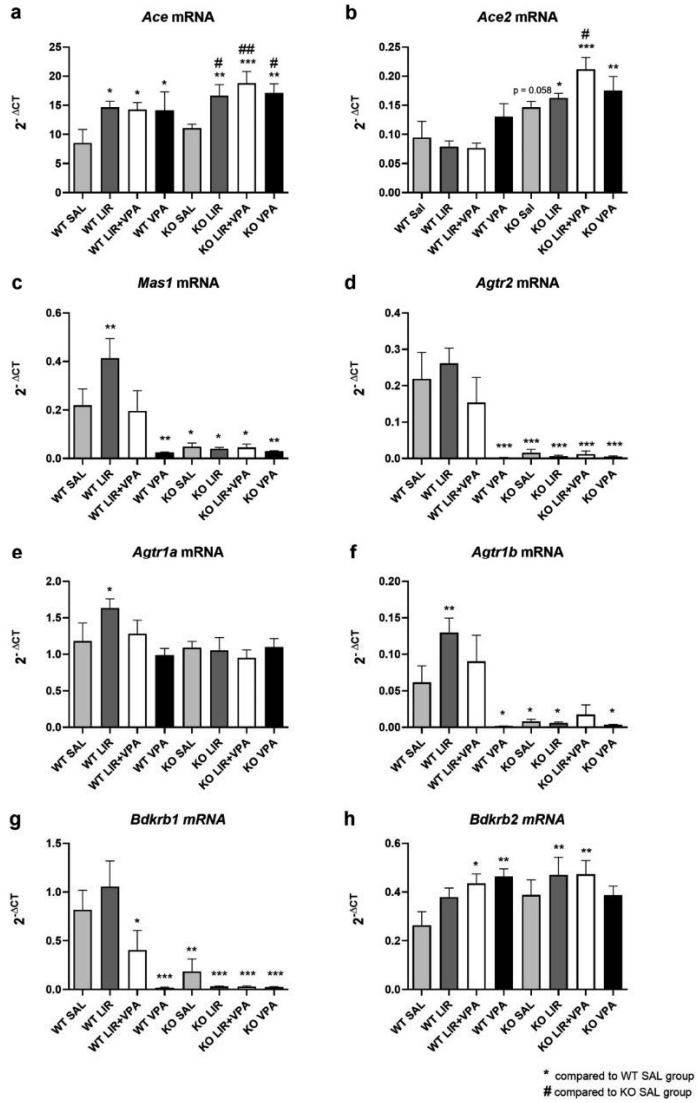

Figure 11. **In the lungs of male WS rats, *Mas1*, *Agtr2*, *Agtr1b* and *Bdkrb1* were significantly downregulated compared to the wild type rats (WT).** Gene expression analyses were performed on the lungs of 3.5- to 4-month-old animals after 8 days of treatment with liraglutide (LIR), valproate (VPA), liraglutide + valproate (LIR + VPA), or saline (SAL). The relative expression levels of (a) *Ace* mRNA, (b) *Ace2* mRNA, (c) *Mas1* mRNA, (d) *Agtr2* mRNA, (e) *Agtr1a* mRNA, (f) *Agtr1b* mRNA, (g) *Bdrkb1* mRNA and (h) *Bdrkb2* mRNA were detected. The gene expression level is presented as $2^{-\Delta CT}$ relative to the housekeeping gene *Tbp*. The data were compared using factorial ANOVA, followed by Fisher's LSD post hoc tests; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to the WT vehicle (SAL) group; and # $p < 0.05$; ## $p < 0.01$ compared to the KO vehicle (SAL) group. The data are presented as the mean ± SEM, n = 7–8 per group. (Modified from Paper III)

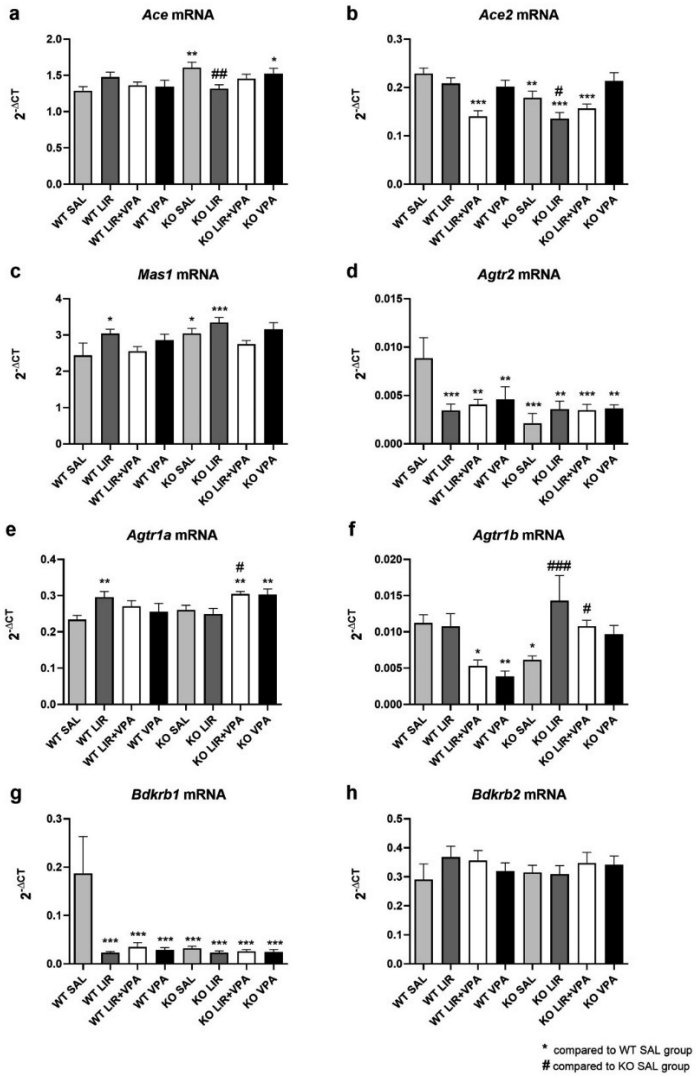

Figure 12. **In the heart of male WS rats (KO), *Agtr2* and *Bdkrb1* were significantly downregulated compared to the wild type rats (WT).** Gene expression analyses were performed on the hearts of 3.5- to 4-month-old animals after 8 days of treatment with liraglutide (LIR), valproate (VPA), liraglutide + valproate (LIR + VPA), or saline (SAL). The relative expression levels of (a) *Ace* mRNA, (b) *Ace2* mRNA, (c) *Mas1* mRNA, (d) *Agtr2* mRNA, (e) *Agtr1a* mRNA, (f) *Agtr1b* mRNA, (g) *Bdrkb1* mRNA and (h) *Bdrkb2* mRNA were detected. The gene expression level is presented as $2^{-\Delta CT}$ relative to the housekeeping gene *Tbp*. The data were compared using factorial ANOVA followed by Fisher's LSD post hoc tests; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to the WT vehicle (SAL) group; and # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ compared to the KO vehicle (SAL) group. The data are presented as the mean ± SEM, n = 8 per group. (Modified from Paper III)

In summary, the expression of the RAAS components studied in Paper III exhibited significant genotype-dependent differences, with considerable discrepancies between the lungs and heart.

4.3.2 *Agtr2* and *Bdkrb1* were downregulated in the primary cortical neurons of male WS rats

To assess the relevance of the observed differences in *Agtr2* and *Bdkrb1* expression in the lungs and heart, expression analysis of *Agtr2*, *Bdkrb1* and *Wfs1* was conducted in primary cortical neurons isolated from WS rats.

In 1-day-old primary cortical neurons from WS rats, the expression levels of both *Agtr2* and *Bdkrb1* were significantly lower and the level of *Wfs1* was slightly decreased, compared to those in neurons from healthy rats (Figure 13). Interestingly, in 2-day-old primary cortical neurons from the control rats, the expression of these receptors decreased to levels comparable to those observed in WS rats. It can be speculated that the increased expression of *Agtr2* and *Bdkrb1* in 1-day-old WT cells was due to stress-related feedback associated with tissue dissection and subsequent culture, but this stress response was absent in *Wfs1*-deficient cells. Under normal circumstances, *Wfs1* expression remains stable, and the protein is degraded by the ubiquitin ligase SMURF1 if it is not needed. In instances of stress, the degradation of SMURF1 allows WFS1 to perform its function (Guo et al., 2011). WFS1 acts as a negative regulator of ER stress, leading to the speculation that functional WFS1 triggers the expression of *Agtr2* and *Bdkrb1*, potentially aiding in adaptation to stressful conditions. Thus, cells lacking WFS1 would be incapable of increasing *Agtr2* and *Bdkrb1* levels in response to stress, and there would be no discernible differences between the groups under nonstress conditions.

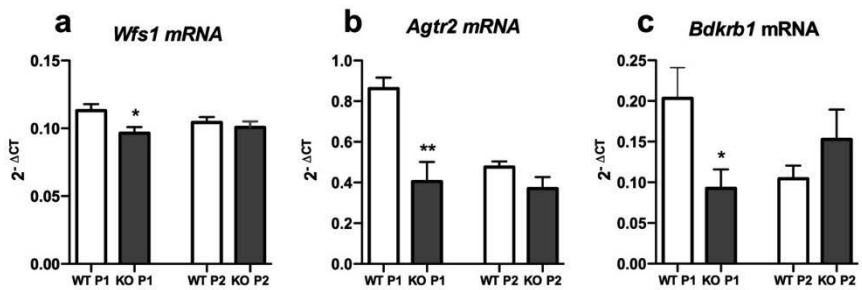

Figure 13. ***Agtr2* and *Bdkrb1* mRNA levels were downregulated in primary cortical neurons from male WS rats *in vitro*.** Rat primary cortical neurons were isolated from healthy control (WT) or WS rats (KO) and cultured for 24 hours (P1) or 48 hours (P2). Relative gene expression levels of (a) *Wfs1* mRNA, (b) *Agtr2* mRNA, and (c) *Bdkrb1* mRNA were measured. Gene expression levels are presented as $2^{-\Delta CT}$ relative to the housekeeping gene *Hprt*. Statistical significance was determined by an unpaired *t* test; * $p < 0.05$, ** $p < 0.01$ compared to the respective WT group. The data are presented as the mean ± SEM, n = 6–7 per group. (Paper III)

4.3.3 *Agtr2* and *Bdkrb1* were downregulated in *Wfs1* knockdown

To further confirm the changes in the RAAS observed in *Wfs1*-deficient animals, *Wfs1* was knocked down. To knock down *Wfs1*, primary rat cortical neurons were transfected with *Wfs1* siRNA. Notably, when cortical neurons were cultured for previous qPCR experiments, a significant reduction in the number of *Wfs1* KO cells was observed in P1 culture compared to that of WT cells. This finding indicates that a portion of the KO cell population did not survive. Thus, using WT cells for *Wfs1* knockdown via siRNA also minimized the differences in cell populations.

Subsequently, the relative expression levels of *Wfs1*, *Agtr2*, *Bdkrb1*, *Ace* and *Ace2* were measured. Following transfection with *Wfs1* siRNA, the levels of *Wfs1* in the neurons decreased by more than half (Figure 14a). Furthermore, in *Wfs1* knockdown neurons, the expression of both *Agtr2* and *Bdkrb1* was reduced by approximately 50% in addition to a decrease in *Wfs1* (Figures 14b, c). These findings suggest a potential dependence of the expression of these receptors on the levels of *Wfs1*. The expression of *Ace* and *Ace2* remained unchanged after *Wfs1* knockdown (Figures 14d, e). Although the primary culture of cortical neurons may have contained some glial cells, both cell types express components of the RAAS and KKS (Luuk et al., 2008).

Taken together, the results obtained in Paper III indicate that the expression of *Agtr2* and *Bdkrb1* is downregulated not only *in vivo* in the lungs and heart of WS rats but also *in vitro* in primary cortical neurons from the WS rat brain and in *Wfs1* knockdown primary cortical neurons.

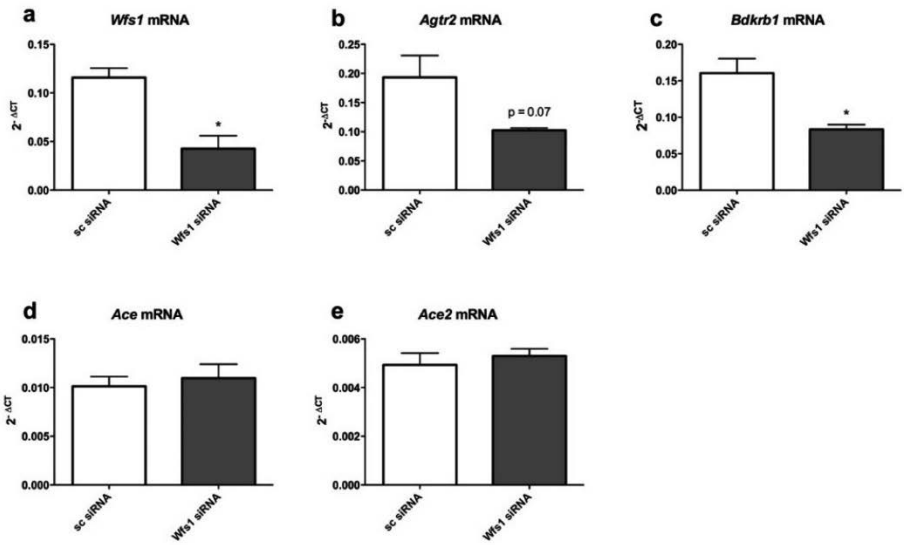

Figure 14. **The levels of *Agtr2* and *Bdkrb1* mRNA were downregulated in *Wfs1* knockdown *in vitro*.** Rat primary cortical neurons were transfected with control (sc siRNA) or *Wfs1* siRNA using the N-TER nanoparticle siRNA transfection system.

Relative gene expression levels of (a) *Wfs1* mRNA, (b) *Agtr2* mRNA, (c) *Bdkrb1* mRNA, (d) *Ace* mRNA, and (e) *Ace2* mRNA were measured. Gene expression levels are presented as $2^{-\Delta CT}$ relative to the housekeeping gene *Hprt*. Statistical significance was determined by unpaired *t* test; * $p < 0.05$ compared to the scramble siRNA group. The data are presented as the mean ± SEM, n = 3 per group. (Paper III)

4.3.4 LIR and VPA had a modulatory effect on the expression of RAAS components

The antidiabetic drug LIR, a GLP-1R agonist, and the antiepileptic drug VPA have demonstrated great potential in preclinical studies. However, the specific targets through which LIR and/or VPA exert their inhibitory effects on WS progression remain unknown. Previous studies have shown that both of these drugs can influence the expression of RAAS components (Cui et al., 2020; Romaní-Pérez et al., 2015; Seppa et al., 2020). To determine how acute exposure to LIR, VPA, or their combination affects components of the RAAS and KKS in the lungs and heart, gene expression analyses were conducted.

In the lungs, acute VPA treatment reduced the expression of *Mas1*, *Agtr2*, *Agtr1b*, and *Bdkrb1* (Figures 11c, d, f, g) in all the genotypes. However, in WT animals, but not in WS animals, VPA-induced downregulation of these genes was reversed when coadministered with LIR (Figures 11c, d, f, g). Compared with saline treatment, LIR treatment alone tended to elevate the mRNA levels of *Ace*, *Mas1* and *Agtr1a* (Figures 11a, c, e) in the lungs of the WT animals. In WS rats, LIR-induced increase in gene expression was mostly absent, except for that of *Ace* (Figures 11a).

In the heart of WT animals, all the treatments decreased the expression of *Agtr2* and *Bdkrb1*, while *Agtr1b* was downregulated by VPA and LIR + VPA cotreatment but not by LIR treatment. In the heart of WT animals, unlike in the lungs, the VPA-induced downregulation in the expression of *Agtr2*, *Agtr1b* and *Bdkrb1* was not reversed by LIR + VPA cotreatment (Figures 12d, f, g). These results are consistent with those of a previous study indicating that VPA can reduce the expression of *Agtr2* (Cui et al., 2020). In addition, *Agtr1b* expression in the heart of WS but not WT rats increased in response to all treatments (Figure 12f). In the heart of WT animals, similar to the lungs, LIR modestly upregulated the expression of *Ace*, *Mas1* and *Agtr1a* (Figures 12a, c, e). In WS rats subjected to acute LIR or LIR + VPA cotreatment, the expression levels of *Ace* and *Ace2* in the lungs were slightly elevated, whereas those in the heart were slightly decreased (Figures 11a, b and 12a, b).

In summary, these observations in Paper III suggest that acute pharmacological challenge with LIR and VPA can affect the expression of key components of the RAAS and KKS, which is influenced by both genotype and specific tissue.

4.3.5 Bradykinin levels were increased and aldosterone levels were decreased in male WS rats

To investigate whether genotype and acute treatment with LIR or VPA impact the levels of aldosterone and bradykinin, key hormonal components of the RAAS and KKS pathways, serum peptide levels were analysed.

The levels of aldosterone, the downstream component of the RAAS, were approximately four times lower in male WS rats compared to the levels in WT rats (Figure 15e). Aldosterone has profibrotic and proinflammatory effects that are, in part, mediated by inflammation and oxidative stress pathways (Fourkiotis et al., 2012). Patients with type 1 diabetes have also been reported to have reduced plasma aldosterone levels (Luik et al., 2003), whereas elevated aldosterone levels have been linked to type 2 diabetes via impact on insulin sensitivity (Colussi et al., 2007; Lastra-Lastra et al., 2009). In diabetic patients, however, reduced aldosterone production is due to lower renin levels, which were also decreased in WS rats, albeit not significantly (Figure 15d). Interestingly, in WS patients, impaired vasopressin biosynthesis and release, potentially caused by neuronal loss, have been observed (Gabreëls et al., 1998; Medlej et al., 2004). The expression of the vasopressin receptor 1A (*V1aR*), through which vasopressin stimulates the release of aldosterone from the adrenal glands, was also found to be affected in *Wfs1* KO mice (Ivask et al., 2018). In *V1aR* KO mice, the levels of aldosterone were decreased several-fold (Aoyagi et al., 2009), and it was recently shown that overexpression of *Wfs1* increases the level of aldosterone (Ma et al., 2023). Orthostatic hypotension has been described in some WS patients, including the asympathicotonic subtype (Mathis et al., 2011), in which the levels of plasma catecholamines are reduced (Thulesius, 1976). Since aldosterone stimulates the biosynthesis of catecholamines (Goto et al., 2009), the lower levels of aldosterone observed in *Wfs1* deficiency may play a role in this process.

Cotreatment with both LIR and VPA elevated aldosterone levels, by approximately threefold, nearly normalizing to the levels observed in WT rats (Figure 15e). Interestingly, in WT animals, all drug treatments reduced the levels of aldosterone compared to those in the respective control group (Figure 15e). Similar findings were reported in healthy individuals treated with LIR and exenatide, where acute LIR administration tended to inhibit aldosterone, whereas chronic LIR dosing led to increased aldosterone levels. In contrast, a single therapeutic dose of exenatide distinctly suppressed aldosterone (Heinla et al., 2021; Sedman et al., 2017).

Conversely, bradykinin levels were increased in WS rats; however, both LIR and VPA, as well as their combination, were able to reduce bradykinin levels to those observed in WT (saline) animals (Figure 15f). This finding aligns with the treatment-dependent decrease in *Bdkrb1* expression levels (Figures 11g and 12g) (Sun et al., 2020). The basal levels of angiotensin II (ANG II), angiotensin-(1–7) (ANG-(1–7)), angiotensin-(1–9) (ANG-(1–9)) and renin were similar between genotypes (Figure 15a–d). However, ANG II levels were increased by all treatments in both WS and WT animals (Figure 15a), as were the levels of renin

(Figure 15d, statistically nonsignificant), which helps to generate angiotensin I (ANG I), the precursor of ANG II. Furthermore, cotreatment with LIR + VPA increased ANG-(1–9) levels regardless of genotype (Figure 15c). ANG-(1–7) levels exhibited a slight, albeit statistically insignificant, increase in response to cotreatment in WT but not in WS rats (Figure 15b).

ANG I is processed into ANG II through ACE activity (Ames et al., 2019). ANG II prompts adrenal glands to release aldosterone by activating the receptors AGTR1 and AGTR2 (Wilkinson-Berka et al., 2019; Yatabe et al., 2011). Acute administration of LIR, VPA and especially their combination elevated ANG II levels while normalizing serum aldosterone and bradykinin concentrations in WS rats. Considering that bradykinin is mostly degraded by ACE (Hornig et al., 1997; Pellacani et al., 1994), it is plausible that all these treatments enhance ACE activity in WS animals. Decreased levels of bradykinin may contribute to reduced neuroinflammation and ER stress, as observed in WS rats following LIR treatment (Seppa et al., 2021).

Furthermore, both *Bdkrb1* and *Agtr2*, which were significantly downregulated in *Wfs1*-deficient animals across the tissue types investigated, are known to influence bradykinin sensitivity. Bradykinin is involved in inflammation (Qadri and Bader, 2018) and has been demonstrated to trigger BDKRB1-mediated Ca^{2+}-dependent microglial migration in the CNS via the reverse mode of the sodium-calcium exchanger (NCX1) (Ifuku et al., 2007). Notably, NCX1 is considerably downregulated in the cardiomyocytes of WS rats (Kureková et al., 2020). In addition, following bradykinin stimulation, fibroblasts from WS patients release reduced amounts of Ca^{2+} from the ER compared to those from control individuals (La Morgia et al., 2020). This suggests a diminished responsiveness of bradykinin receptors and a decrease in NCX1 activity.

Previous studies have demonstrated that LIR and other GLP-1R agonists can boost ACE2 activity (Romaní-Pérez et al., 2015). ACE2 is responsible for converting ANG I into ANG-(1–9); thus, ANG-(1–9) levels indicate ACE2 activity. The findings in Paper III suggest that LIR and VPA alone do not increase serum ANG-(1–9) levels; however, their combination leads to elevated ANG-(1–9) levels regardless of genotype, indicating increased ACE2 activity.

Overall, the rat model of WS exhibited decreased serum aldosterone levels and increased bradykinin levels compared to those in the WT control group. Acute treatments with LIR or LIR + VPA induced increased serum levels of aldosterone and angiotensin II, while decreased bradykinin concentrations, indicating enhanced ACE activity. Serum levels of ANG-(1–9) were elevated in both genotypes treated with the combination of LIR and VPA, suggesting increased ACE2 activity.

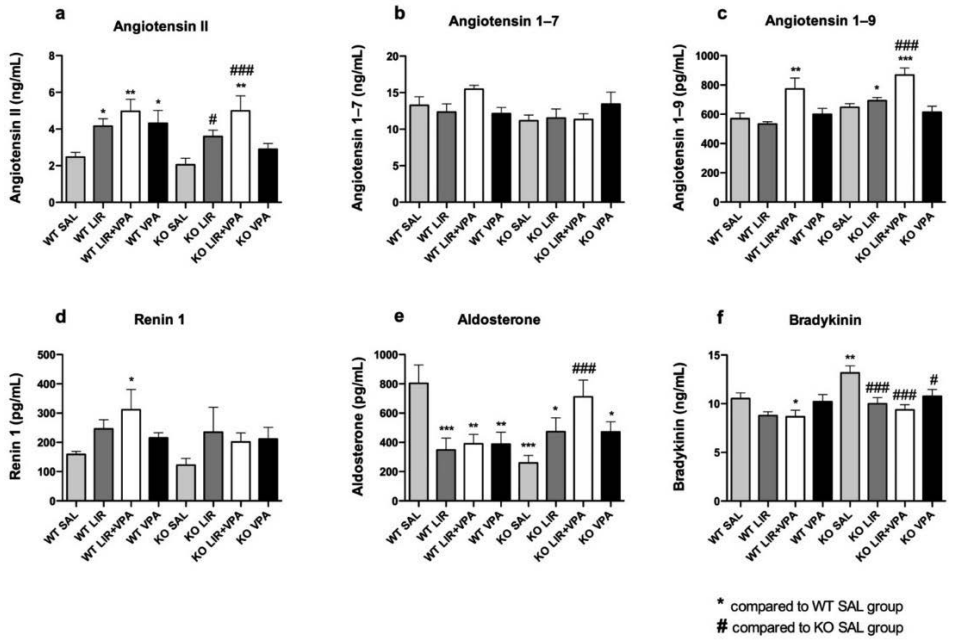

Figure 15. ***Wfs1*** **deficiency led to a significant decrease in aldosterone and increase in bradykinin serum levels.** Neurohormone levels were measured in the serum of 3.5- to 4-month-old animals after 8 days of treatment with liraglutide (LIR), valproate (VPA), liraglutide + valproate (LIR + VPA), or saline (SAL). ELISA was used to measure the serum levels of (a) angiotensin II, (b) angiotensin 1–7, (c) angiotensin 1–9, (d) renin 1, (e) aldosterone and (f) bradykinin. The data were compared using factorial ANOVA, followed by Fisher's LSD tests; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to the WT vehicle (SAL) group; and # $p < 0.05$; ### $p < 0.001$ compared to the KO vehicle (SAL) group. The data are presented as the mean ± SEM, n = 7–8 per group. (Paper III)

4.3.6 Sodium (Na^+) and potassium (K^+) levels remained unaffected

The RAAS responds to fluctuations in blood pressure and the concentrations of sodium (Na^+) and potassium (K^+). Activation of the RAAS typically occurs under low-Na^+ and/or high-K^+ conditions (Lehoux et al., 1994; Tremblay et al., 1992). Alterations in the RAAS may mirror changes in electrolyte levels, and vice versa. Therefore, the levels of Na^+ and K^+ were measured.

No differences in the levels of Na^+ or K^+ were detected between the WS and WT rats. One possible explanation is the use of young animals whose phenotype has not yet fully developed; thus, the changes in electrolytes may be balanced by alternative routes. Furthermore, neither LIR nor VPA treatment nor their combination had a substantial impact on the concentrations of these electrolytes (Figure 16).

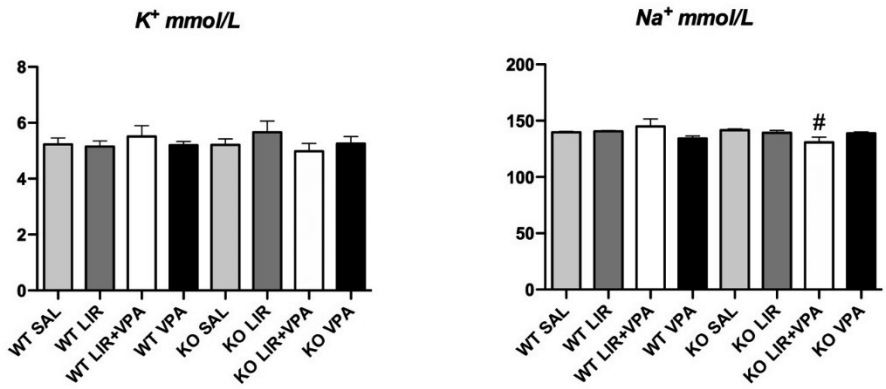

Figure 16. **Na^+ and K^+ levels were measured in the serum of 3.5- to 4-month-old animals after 8 days of treatment with liraglutide (LIR), valproate (VPA), liraglutide + valproate (LIR + VPA), or saline (SAL).** Electrolyte levels were determined using an ion-selective electrode (ISE) indirect Na-K-Cl for Gen.2 on a Roche/Hitachi Cobas c system (Roche Diagnostics) and are presented as mmol/L. The data were compared using factorial ANOVA followed by Fisher's LSD post hoc tests; # $p < 0.05$ compared to the KO vehicle (SAL) group. The data are presented as the mean ± SEM, n = 6–8 per group. (Paper III)

4.3.7 Paper III Summary discussion

Paper III examined early alterations in the RAAS and bradykinin pathway (KKS) in the lungs and heart of young WS rats.

WS rats had significantly lower expression levels of *Agtr2*, *Agtr1b*, and *Bdkrb1* mRNA in their lungs and heart, along with notable differences in other components of the RAAS and KKS. Downregulation of *Agtr2* and *Bdkrb1* expression was confirmed in primary cortical neurons from WS rats and further validated by *Wfs1* knockdown. Acute exposure to LIR, VPA, or their combination affected the RAAS and KKS components with genotype- and tissue-specific variations. In addition, WS rats displayed several-fold lower serum aldosterone levels and higher bradykinin levels, while drug treatments nearly normalized these concentrations.

Surprisingly, sodium (Na^+) and potassium (K^+) levels were unchanged, suggesting that these critical electrolytes remained relatively stable despite genotype or drug treatment.

While heart malformations have been identified in some WS patients (Medlej et al., 2004), cardiovascular conditions are relatively infrequent and not distinctly evident. Nevertheless, these findings suggest the potential importance of performing more comprehensive cardiac assessments during routine patient check-ups. Second, the ER response is often evaluated using bradykinin, which mobilizes the release of Ca^{2+} from the ER by stimulating inositol trisphosphate receptor (IP3R) (G. Li et al., 2018). In light of the present data demonstrating significantly decreased *Bdkrb1* expression in *Wfs1* deficiency, a degree of caution is

warranted when interpreting data derived from employing bradykinin in *Wfs1*-deficient cells.

In conclusion, this study demonstrates for the first time the effect of WFS1 on components of the RAAS and KKS and highlights the potential impact of drug treatments such as LIR and VPA on these pathways.

4.4 The transcriptional profile of key RAAS components in a rat model of Wolfram syndrome appears to be affected by stress (Paper IV)

4.4.1 *Agtr1a*, *Agtr1b*, *Agtr2* and *Bdkrb1* were downregulated in the hippocampus but not in the brain stem of male WS rats receiving chronic treatment

Cardiovascular homeostasis is maintained not only by the peripheral RAAS but also by central angiotensinergic pathways, i.e., the so-called "local" RAAS of the brain. RAAS modulators have demonstrated effects other than hypotensive effects, such as cognitive improvement and neuroprotection (Jackson et al., 2018), and their dysregulation has been implicated in neurodegenerative disorders (Labandeira-Garcia et al., 2017).

Previously (Paper III), the peripheral RAAS was found to be significantly impacted in *Wfs1*-deficient rats. The expression of the key RAAS receptors *Agtr2* and *Bdkrb1* was markedly downregulated. Additionally, WS rats exhibited reduced aldosterone and increased bradykinin serum levels, and LIR demonstrated the ability to modulate these hormone levels, consistent with prior findings that LIR can affect RAAS components (Romaní-Pérez et al., 2015; Sedman et al., 2017).

Thus, this study (Paper IV) aimed to determine whether the RAAS is altered in the CNS of aged WS rats, when the brain already has more extensive neurodegenerative changes, particularly in the brain stem and hippocampus, regions significantly affected by WS (Hershey et al., 2012; Shannon et al., 1999), and because the hippocampus is closely linked to cognitive abilities (Sweatt, 2004). Brain stem and hippocampal tissues collected from aged WS rats as part of a previous long-term treatment study that demonstrated the beneficial effects of the GLP-1R agonist LIR and BDNF mimetic 7,8-DHF were used for this study (Seppa et al., 2021).

GLP-1R activation exerts neuroprotective effects in part by alleviating ER stress and enhancing cell survival and mitochondrial function (J. Chen et al., 2018; Nuamnaichati et al., 2020), potentially including through an ACE2-mediated RAAS compensatory axis. The ACE2/ANG-(1–7)/MAS1/AGTR2 axis, stimulates a strong ER stress response and initiates anti-inflammatory and regenerative pathways, thereby supporting cell function (Rodrigues Prestes et al., 2017; G. Yang et al., 2018). 7,8-DHF has also been shown to exert neuroprotective effects by activating TRKB (Jang et al., 2010). Thus, it was also evaluated

whether LIR, 7,8-DHF, or their combination could modulate the expression of key RAAS components in these brain tissues. Given that LIR treatment has previously demonstrated neuroprotective effects and enhanced cognitive function (Seppa et al., 2021), it was speculated that these beneficial effects may be partly linked to neural RAAS modulation.

First, in vehicle-treated WS rats, the hippocampal levels of *Agtr1a*, *Agtr1b*, *Agtr2* and *Bdkrb1* mRNA were significantly lower than those in vehicle-treated WT littermates (Figures 17a–d). These changes persisted in WS rats across all treatment groups, suggesting that none of the drugs (LIR, 7,8-DHF, or their combination) were able to modulate expression of those genes in these animals. This finding is consistent with earlier observations in the lungs and heart, which exhibited lower levels of *Agtr2*, *Agtr1b* and *Bdkrb1* that were unaffected by LIR treatment, except *Agtr1b* in the heart (Figures 11d, f, g and 12d, f, g).

In contrast, a treatment-related effect was evident in WT rats; the expression of hippocampal *Agtr1a*, *Agtr1b*, *Agtr2* and *Bdkrb1* mRNA was significantly lower across all treatment groups compared to that in the vehicle group (Figures 17a–d). In part, this phenomenon could be due to the requirement of functional WFS1 for these drugs to regulate the RAAS during an extended period of stress induced by long-term experimental manipulations. In addition, it is plausible that in WT animals, the neuroprotective effects of these drugs reduce the need for RAAS engagement, even under chronic stress conditions. In other words, the upregulation of RAAS components is not needed with these drugs (as observed in vehicle-treated WT animals). Finally, no notable differences related to treatment or genotype were detected for the expression levels of *Bdkrb2*, *Ace*, *Ace2* or *Mas1* (Figures 17e–h).

In the brain stem, however, no significant differences were revealed in any of the target genes in either the between-genotype or between-treatment group comparisons (Figure 18).

To summarize, consistent with previous findings in the periphery, key RAAS components notably differed in the hippocampus but not in the brain stem of WS rats compared to their WT counterparts following prolonged treatment. In particular, the differences in *Agtr2* and *Bdkrb1* were again notable. The protective potential of AGTR2 is well established; its activation leads to both anti-inflammatory and antifibrotic effects and can promote the regeneration of axons (Lucius et al., 1998). In the CNS, AGTR2 activation can facilitate BDNF/TRKB-mediated signalling, which in turn promotes various cellular processes, including cell proliferation, survival, and adaptability. Dysregulation of the BDNF/TRKB axis has been linked to several neuropsychiatric conditions (Diniz et al., 2018). Both inflammation and trauma can lead to BDKRB1 activation (Hofman et al., 2016). Once activated, BDKRB1 subsequently exerts neuroprotective effects by facilitating Ca^{2+}-dependent bradykinin-induced migration of microglia (Ifuku et al., 2007). Collectively, the absence of functional WFS1 may disrupt AGTR2- and BDKRB1-mediated signalling, hindering their ability to provide neuroprotection, which includes processes such as cell regeneration, coping with ER stress, and inflammatory responses. This disruption could exacerbate WS progression.

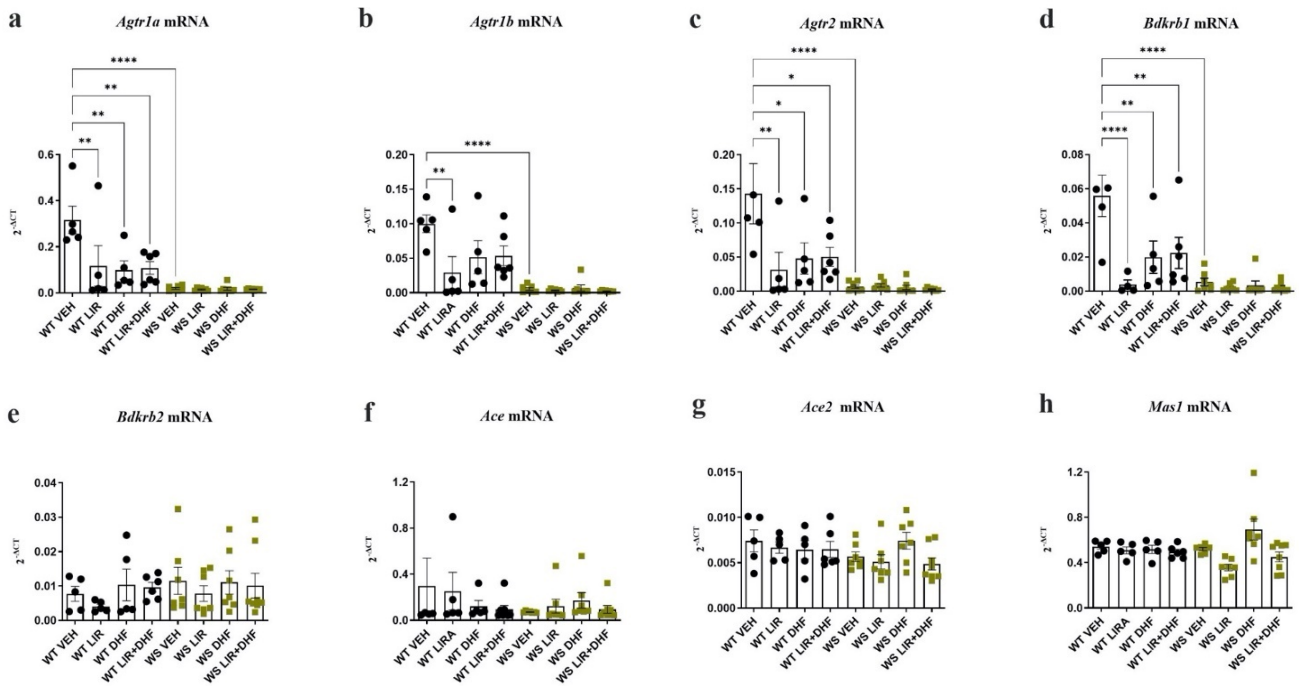

Figure 17. **The expression of *Agtr1a*, *Agtr1b*, *Agtr2* and *Bdkrb1* was significantly downregulated in the hippocampus of chronically treated aged male *Wfs1*-deficient rats compared to the wild type controls.** Gene expression was analysed in the hippocampi of 12.5-month-old male wild type (WT), *Wfs1* knock-out (KO) animals after 3.5 months of treatment with liraglutide (LIR), 7,8-dihydroxyflavone (DHF), liraglutide + 7,8-dihydroxyflavone (LIR + DHF) or vehicle (VEH). Relative gene expression levels of (a) *Agtr1a*, (b) *Agtr1b*, (c) *Agtr2*, (d) *Bdkrb1*, (e) *Bdkrb2*, (f) *Ace*, (g) *Ace2* and (h) *Mas1* (presented as $2^{-\Delta CT}$ relative to the housekeeping gene *Hprt*). Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparisons test; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. The data are presented as the mean ± SEM, n = 5–8 per group. (Modified from Paper IV)

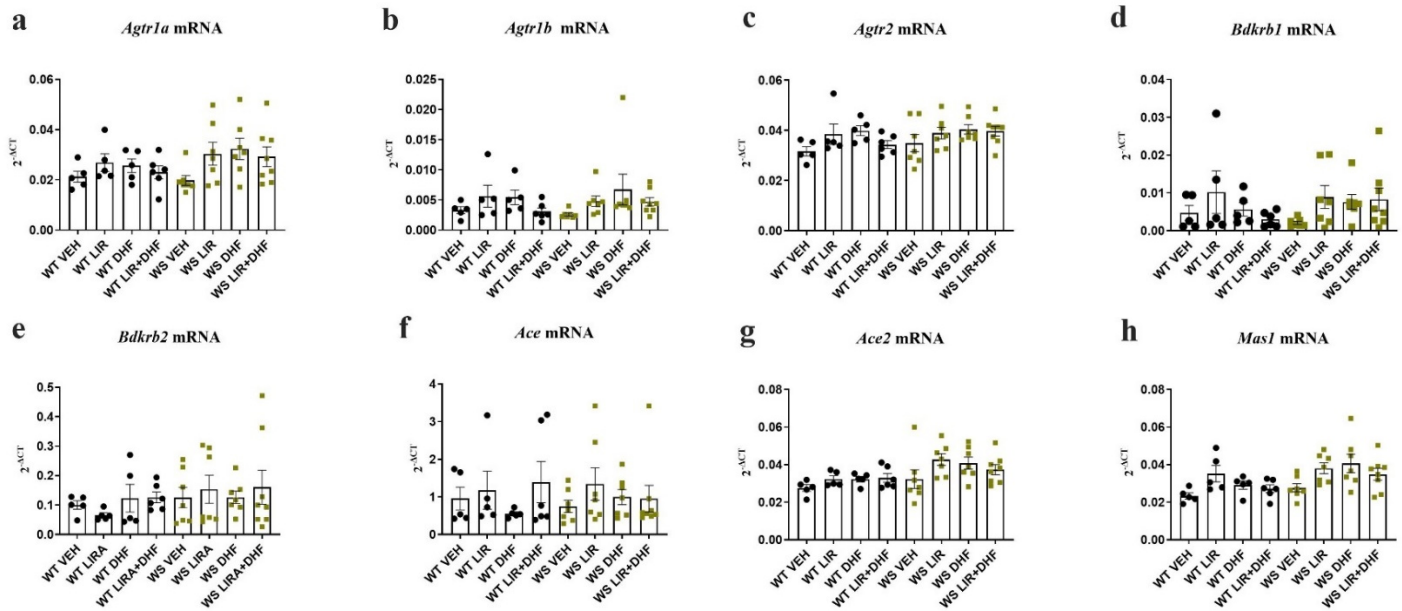

Figure 18. **No significant between-genotype or between-treatment group differences in the expression of key RAAS components were observed in the brain stem of chronically treated aged male *Wfs1*–deficient rats compared to the wild type controls.** Gene expression was analysed in the brain stems of 12.5-month-old male wild type (WT), *Wfs1* knock-out (KO) animals after 3.5 months of treatment with liraglutide (LIR), 7,8-dihydroxyflavone (DHF), liraglutide + 7,8-dihydroxyflavone (LIR + DHF) or vehicle (VEH). Relative gene expression levels of (a) *Agtr1a*, (b) *Agtr1b*, (c) *Agtr2*, (d) *Bdkrb1*, (e) *Bdkrb2*, (f) *Ace*, (g) *Ace2* and (h) *Mas1* (presented as $2^{-\Delta CT}$ relative to the housekeeping gene *Hprt*). Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparisons test. The data are presented as the mean ± SEM, n = 5–8 per group. (Modified from Paper IV)

Surprisingly, chronic drug treatment induced alterations in the hippocampal expression of WT animals but did not affect the levels in WS animals or in the brain stem of either genotype (Figures 17 and Figure 18). This suggests that changes in key RAAS components may be specific to certain brain regions.

4.4.2 Gene expression profile in the hippocampus and brain stem of treatment-naïve male WS rats differed from that in rats receiving chronic treatment

Several psychiatric complications, such as heightened anxiety and depression, have been documented in both WS patients and animal models (Munshani et al., 2021). Furthermore, both preclinical and clinical studies have linked alterations in the RAAS with mood disorders (Mohite et al., 2017). This led to the questions of whether the stress induced by prolonged treatment and handling could have influenced the differences in the expression of key RAAS components observed in the between-genotype group comparisons and whether fully functional WFS1 is required for effective functioning of the RAAS during chronic stress. To explore this, the expression of RAAS components was measured in age-matched treatment-naïve male WS and WT rats taken directly from their home cages.

The hippocampal RAAS expression profile differed significantly between treated (manipulated) and treatment-naïve (nonmanipulated) rats. Specifically, the expression of hippocampal *Agtr1a*, *Agtr1b*, *Agtr2* and *Bdkrb1* did not differ between treatment-naïve WT and WS rats, in contrast to the significant down-regulation observed in vehicle-treated WS rats *versus* vehicle-treated WT rats (Figure 19a–d vs. Figure 17a–d). Instead, compared with their WT counterparts, treatment-naïve WS rats exhibited lower levels of *Ace*, *Ace2* and *Mas1* levels (Figures 19f–h).

Reduced levels of *Ace* and *Ace2* in the hippocampus of treatment-naïve WS rats may suggest disruptions in the processing of angiotensin, potentially compromising AGTR1-, AGTR2- and MAS1-mediated signalling (ACE2/ANG-(1–7)/MAS1/AGTR2 axis). Alterations in the activity of neural ACE and ACE2 increase neuronal vulnerability to inflammation and ER stress. These alterations can also facilitate the accumulation of substances such as bradykinin, tau and amyloid-beta, all of which are linked to neurodegenerative pathologies (Hemming and Selkoe, 2005; Kehoe et al., 2016; Petek et al., 2018; Singh et al., 2020). The inhibition of ACE has been shown to delay neurodegeneration by slowing tau hyperphosphorylation (AbdAlla et al., 2015), while activation of ACE2 and AGTR2 can protect against cognitive decline (Fouda et al., 2019). ACE inhibitors may enhance cognitive functions, including learning and memory, by stimulating the ANG-(1–7)/MAS1 axis (Hellner et al., 2005). Intriguingly, a recent study showed that WFS1-positive neurons in the entorhinal cortex express tau and mediate its transfer to the hippocampal *cornu ammonis* 1 (CA1) pyramidal cells (Delpech et al., 2021). Increased vulnerability to tau pathology in WFS1 deficiency suggests that, similar to ACE, WFS1 interacts with tau and influences its

effects (S. Chen et al., 2022; S. Chen et al., 2020). Thus, the modulation of RAAS components may affect cognitive processes.

To determine whether the expression of RAAS components in treatment-naïve rats displayed the same regional specificity as that in treated rats, the analysis was extended to the brain stem. The expression of *Ace* was increased, and the expression of *Agtr2* was decreased in the brain stem of treatment-naïve WS rats compared with that in the brain stem of WT rats (Figures 20f, c). In addition, there were slightly lower levels of *Agtr1a*, *Agtr1b* and *Bdkrb1* in WS rats, albeit statistically insignificant (Figures 20a, b, d). Similar to that in the hippocampus, *Mas1* and *Ace2* expression was slightly decreased—although not significantly—in treatment-naïve WS rats (Figures 20g, h).

Overall, region-specific differences in treatment-naïve rats were not as prominent as those in treated animals. Nevertheless, the hippocampal RAAS expression profile differed notably between treated and treatment-naïve WS and WT animals, suggesting that RAAS functioning might be influenced by a potential interplay between WFS1 availability and chronic stress induced by prolonged experimentation.

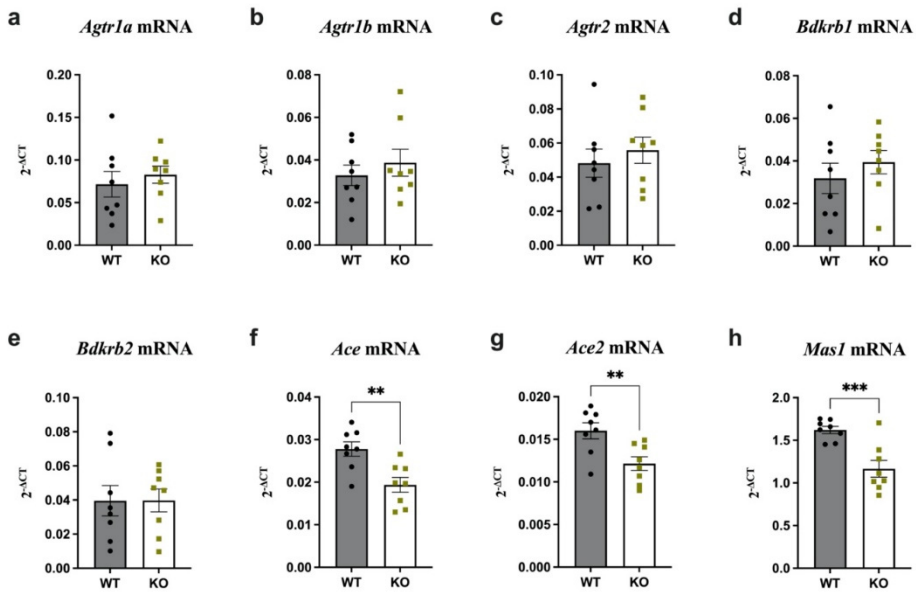

Figure 19. **The expression of *Ace*, *Ace2* and *Mas1* was substantially downregulated in the hippocampus of treatment-naïve aged male *Wfs1*-deficient rats compared to the wild type controls.** Gene expression was analysed in the hippocampi of 12.5- to 13-month-old male wild type (WT), *Wfs1* knock-out (KO) animals taken directly from their home cages. Relative gene expression levels of (a) *Agtr1a*, (b) *Agtr1b*, (c) *Agtr2*, (d) *Bdkrb1*, (e) *Bdkrb2*, (f) *Ace*, (g) *Ace2* and (h) *Mas1* (presented as $2^{-\Delta CT}$ relative to the housekeeping gene *Hprt*). Statistical significance was determined using an unpaired t test; ** $p < 0.01$; *** $p < 0.001$. The data are presented as the mean ± SEM, n = 8 per group. (Paper IV)

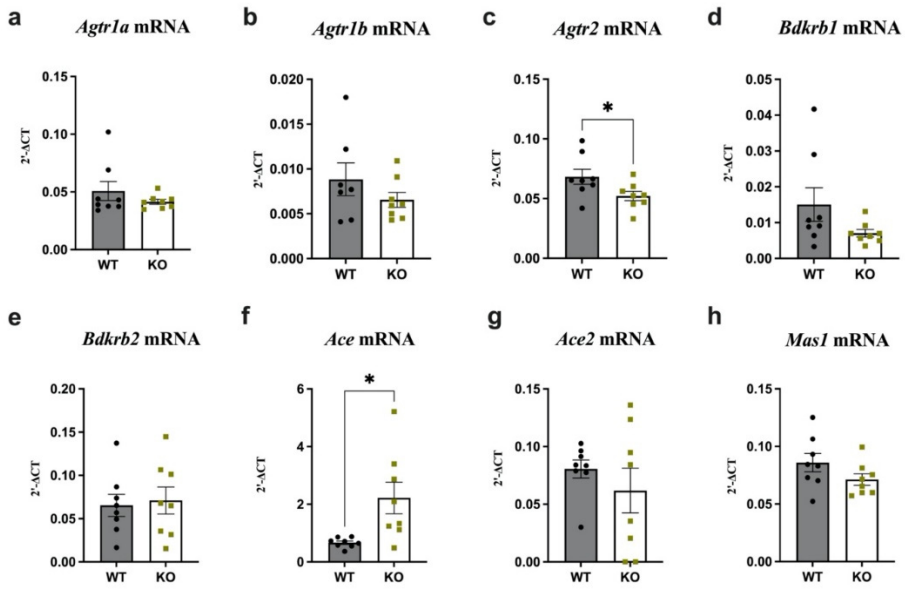

Figure 20. ***Ace* and *Agtr2* were significantly upregulated and downregulated, respectively, in the brain stem of treatment-naïve aged male *Wfs1*-deficient rats compared to the wild type controls.** Gene expression was analysed in the brain stems of 12.5- to 13-month-old male wild type (WT), *Wfs1* knock-out (KO) animals taken directly from their home cages. Relative gene expression levels of (a) *Agtr1a*, (b) *Agtr1b*, (c) *Agtr2*, (d) *Bdkrb1*, (e) *Bdkrb2*, (f) *Ace*, (g) *Ace2* and (h) *Mas1* (presented as $2^{-\Delta CT}$ relative to the housekeeping gene *Hprt*). Statistical significance was determined using an unpaired t test; * $p < 0.05$. The data are presented as the mean ± SEM, n = 8 per group. (Paper IV)

4.4.3 Paper IV Summary discussion

Paper IV described the RAAS at the transcriptional level within the CNS, focusing on the brain stem and hippocampus, which are critical regions affected in WS, the latter of which is also known for its profound impact on cognitive abilities. RAAS modulation has been recognized for its potential benefits beyond improving blood pressure, including cognitive improvement and neuroprotection. Dysregulation of RAAS components has been linked to neurodegenerative disorders.

Consistent with previous findings in peripheral tissues and in primary cortical neuron culture (Paper III), hippocampal levels of *Agtr2* and *Bdkrb1* were significantly lower in vehicle-treated WS rats (exposed to extended experimental stress) than in their WT counterparts. In addition, levels of *Agtr1a* and *Agtr1b* were substantially decreased. Remarkably these changes persisted across all treatment groups (LIR, 7,8-DHF, or their combination). This finding indicates that lack of fully functional WFS1 might aggravate AGTR2- and BDKRB1-mediated signalling, diminishing their neuroprotective effects.

Interestingly, in contrast to those in WS rats, treatment-induced changes in RAAS gene expression in the hippocampus, but not in the brain stem, were observed in WT rats across all treatment groups compared to their vehicle-treated counterparts. This indicates that the impact of these drugs may be influenced by the availability of functional WFS1, especially under conditions of prolonged stress.

The lack of significant alterations in RAAS gene expression in the brain stem of treated rats for both between-genotype and between-treatment group comparisons suggested that the interplay between WFS1 and the RAAS may be influenced by both region and environmental conditions.

Unlike in vehicle-treated WS rats, in treatment-naïve WS rats, which had not experienced prolonged experimental stress, the hippocampal levels of *Agtr2*, *Agtr1a*, *Agtr1b*, and *Bdkrb1* did not differ significantly from that of their WT counterparts. Instead, compared with their WT littermates, treatment-naïve WS rats exhibited reduced *Ace*, *Ace2* and *Mas1* levels. Changes in the RAAS in treatment-naïve animals displayed regional specificity (hippocampus *versus* brain stem) similar to that in treated animals. WFS1 is widely expressed in the CA1 region of the hippocampus and is expressed at lower levels in the medulla of the brain stem, which might explain the less pronounced alterations in the latter.

Reduced hippocampal *Ace* and *Ace2* levels in treatment-naïve (nonmanipulated) WS rats could imply disruptions in angiotensin processing, potentially compromising the effects mediated by AGTR1, AGTR2, and MAS1. This in turn may increase neuronal vulnerability to inflammation and ER stress and facilitate the accumulation of neurodegeneration-associated proteins. Interestingly, disruption of brain RAAS functioning is linked to ageing-related alterations and neurodegeneration by exacerbating oxidative stress and neuroinflammation (Labandeira-Garcia et al., 2017). For example, excess angiotensin II has been demonstrated to enhance oxidative stress and decrease the survival of dopaminergic neurons (Rodriguez-Pallares et al., 2008). Crucially, pharmacological manipulation of RAAS components can alleviate ER and oxidative stress as well as improve the function of mitochondria (Escobales et al., 2019; Sunanda et al., 2021).

Cells in the hippocampus are replaced to some extent throughout life. In peripheral tissues, where WFS1 is broadly expressed, the effect of WFS1 deficiency in the WS model can be detected already at a young age, when symptoms have not yet fully developed. In the hippocampus, for example, the effect becomes apparent only in aged animals (but not in young, data not shown). This can probably be affected by other external factors, such as chronic stress. WFS1 appears to not be constantly required everywhere but rather under certain conditions and at certain stages of development.

The present and previous findings (Papers IV and III) indicate that a lack of functional WFS1 might disrupt RAAS function, as evidenced by alterations in its key components, both in the periphery and in the CNS. These disturbances may consequently increase oxidative stress, impair inflammatory responses and Ca^{2+}

homeostasis, affect cognition and contribute to the development of neuropsychiatric complications. WFS1 may affect RAAS regulation under stressful conditions and facilitate the functioning of the system's stress-response compensatory axis. Disturbances in this axis could therefore exacerbate the course of WS disease (Figure 21).

The inability of LIR and 7,8-DHF to modulate the hippocampal RAAS suggests that the positive effects of LIR on neuroprotection and cognitive function observed in earlier studies (Seppa et al., 2019) do not depend on the RAAS. GLP-1Rs are abundant in pyramidal neurons, and their expression can be induced by injury to GABAergic interneurons and astrocytes (Cork et al., 2015; Hamilton and Hölscher, 2009; C. H. Lee et al., 2011). GLP-1R agonists have been shown to abate microglial activation in WS rats (Seppa et al., 2019) and enhance GABAergic neurotransmission in various pathological conditions, including ischaemia (Korol et al., 2014; C. H. Lee et al., 2011). Intriguingly, activation of the GABA receptor delays neuronal death in injuries induced by ischaemia (Zhou et al., 2008). Consequently, while the precise mechanisms underlying the neuroprotective effects of LIR in WS are not fully understood, it is plausible that these mechanisms involve mitigating reactive gliosis by modulating GABAergic signalling and/or enhancing ACE2 activity (Romaní-Pérez et al., 2015).

Overall, this study indicates that the RAAS might be compromised in WFS1 deficiency (a summary of the results of Paper III and Paper IV can be found in Table 5) and highlights the need for further research into the complex interplay among WFS1, the RAAS, and chronic stress in WS.

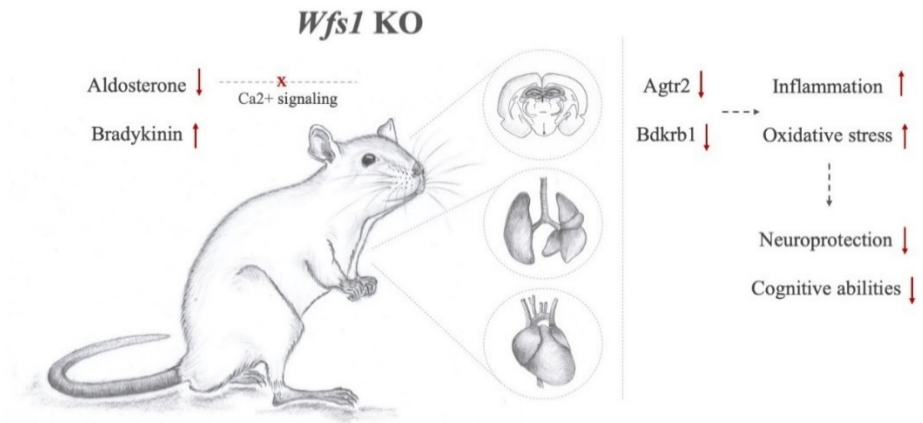

Figure 21. The expression of key components of the RAAS is altered in the rat model of WS (*Wfs1* KO). RAAS imbalance may lead to aggravation of inflammatory and oxidative processes, which decreases neuroprotection and exacerbates the course of WS disease. Recently, low aldosterone was directly linked to WFS1 deficiency, hypothetically due to a disturbed Ca^{2+} axis and impaired mitochondrial function (Ma et al., 2023). (Original by Marite Punapart)

4.5 Limitations of the studies

First, a major limitation of all the studies discussed here is the use of only male animals, and there might be a sex effect on the effects caused by functional WFS1 deficiency regarding changes in RAAS. Female *Wfs1* KO mice develop symptoms such as diabetes later than male *Wfs1* KO mice and their metabolic disturbances are milder (Luuk et al., 2009; Noormets et al., 2011). Thus, use of male *Wfs1* KO mice allows investigation in younger animals. In addition, most of the studies on WS have been conducted in male animals, which facilitates comparison of results.

Second, gene expression levels do not always correlate with protein levels or functional activity, as there are several stages of regulation that depend on current physiological needs. However, the aim was to describe alterations at the transcription level, as the studies were exploratory, and changes in protein levels were beyond the scope of these studies.

Furthermore, in Paper III and Paper IV, analyses were restricted to specific tissues based on the presence of WFS1 and the relevance of RAAS in these tissues. Future studies should also consider investigating transcriptomic alterations in the RAAS within other tissues, as the RAAS displays complex cross-regulated communication. The temporal development of RAAS imbalance across the course of WS also requires investigation.

Finally, it is apparent that the results reported in Paper IV may have been significantly influenced by chronic stress inadvertently induced by prolonged experimental handling. Thus, additional investigations using classical stress paradigms should be conducted to verify the results described here.

Table 5. Summary of the results from Paper III and Paper IV.

	Treated 3.5-4-month-old rats								Treated 12.5-month-old rats				Treatment-naive 12.5-13-month-old rats
	Heart				Lungs				Hippocampus				Hippocampus
Genes	VEH	LIR	VPA	LIR+VPA	VEH	LIR	VPA	LIR+VPA	VEH	LIR	DHF	LIR+DHF	No treatment
Ace	↑	– ↓	—	—	—	↑ ↑	↑ ↑	↑ ↑	—	—	—	—	↓
Ace2	↓	– ↓	—	↓ –	—	—	—	– ↑	—	—	—	—	↓
Agtr1a	—	↑ –	—	– ↑	—	↑ –	—	—	↓	↓ –	↓ –	↓ –	—
Agtr1b	↓	– ↑	↓ –	↓ ↑	↓	↑ –	↓ –	—	↓	↓ –	—	—	—
Agtr2	↓	↓ –	↓ –	↓ –	↓	—	↓ –	—	↓	↓ –	↓ –	↓ –	—
Bdkrb1	↓	↓ –	↓ –	↓ –	↓	—	↓ –	↓ –	↓	↓ –	↓ –	↓ –	—
Bdkrb2	—	—	—	—	—	—	↑ –	↑ –	—	—	—	—	—
Mas1	↑	↑ –	—	—	↓	↑ –	↓ –	—	—	—	—	—	↓

	Treated 3.5-4-month-old rats			
Serum peptides	VEH	LIR	VPA	LIR+VPA
Angiotensin II	—	↑ ↑	↑ –	↑ ↑
Angiotensin 1-7	—	—	—	—
Angiotensin 1-9	—	—	—	↑ ↑
Renin 1	—	—	—	↑ –
Aldosterone	↓	↓ –	↓ –	↓ ↑
Bradykinin	↑	– ↓	– ↓	↓ ↓

* BLACK ARROWS indicate significant changes in WT animals compared to their corresponding vehicle group.
* RED ARROWS indicate significant changes in WS animals compared to their corresponding vehicle group
* VEH section indicate significant changes in vehicle WS animals compared to vehicle WT animals

5. MAIN CONCLUSIONS

The studies discussed herein characterized the impact of both acute and chronic VPA treatment on glycaemic control in male *Wfs1*-deficient mice, as well as the impact of chronic VPA treatment on the hepatic gene expression profile in these mice. In addition, the effects of WFS1 deficiency and treatment with VPA, the GLP-1R agonist LIR and the BDNF mimetic 7,8-DHF were investigated on key components of the RAAS in a rat model of WS.

The main conclusions are as follows:

1. Male *Wfs1* KO mice had lower body weight and slightly elevated nonfasting blood glucose levels accompanied by notably reduced levels of insulin, while response to insulin was normal (Paper I). Pretreatment with acute VPA decreased the peak blood glucose concentration during glucose challenge in both *Wfs1* KO and HZ mice. The effect of acute VPA treatment on glucose tolerance appears to be independent of GSK3 inhibition, PPARG, or GABA receptor modulation, as LiCl and rosiglitazone did not exert similar effects, and diazepam worsened glycaemic control. The ability of VPA to modulate blood glucose levels in insulin deficiency suggests an insulin-independent mechanism; VPA may affect glucose excretion and/or assimilation rather than affect insulin release. In addition, the effect of VPA on components of the GABAergic system cannot be excluded because experiments involving different GABA receptor modulators on glycaemic control have yielded controversial results. However, the precise molecular mechanism underlying the effect of acute VPA treatment on glucose metabolism remains to be elucidated.
2. Consistent with previous data, male *Wfs1* KO mice exhibited stunted growth. In contrast to acute VPA treatment, prolonged treatment with VPA for 3 months in *Wfs1*-deficient mice had no significant effect on glycaemic control or body weight (Paper II). The hepatic gene expression profile indicated that invalidation of the *Wfs1* gene affects the expression of genes related to oxidative processes and lipid and fatty acid metabolism. Notably, the expression of *Ppard*, a gene involved in fatty acid metabolism, insulin sensitivity, and glycaemic control, was upregulated by VPA in a genotype-dependent manner. Modulation of *Ppard* may contribute to the metabolic effects of VPA. This study underscores the complex interplay between genetic factors and drug response in metabolic regulation.
3. In a rat model of WS, changes in the RAAS and KKS were already detectable at a young age, when the primary symptoms have not yet fully developed (Paper III). Specifically, the expression of *Agtr2* and *Bdkrb1* was downregulated both *in vitro* (in primary cortical neurons) and *in vivo* (in the heart and lungs), and the serum concentrations of aldosterone and bradykinin were significantly altered without any detectable changes in sodium or potassium levels. Acute (1 week) exposure to the potential WS drug candidates LIR, VPA, or their combination did not normalize the gene expression levels

observed in WS rats. Nevertheless, these drugs have notable modulatory effects on the RAAS. These data indicate for the first time the involvement of WFS1 in the regulation of RAAS and KKS components, which might interfere with the inflammatory response and further disturb Ca^{2+} homeostasis, thereby accelerating the progression of WS. In addition, these findings suggest the practicality of more comprehensive cardiac assessments in WS patients, despite relatively infrequent cardiovascular conditions.

4. Consistent with previous observations in the periphery, significant decreases in hippocampal *Agtr2* and *Bdkrb1* expression were observed in male WS rats in a long-term treatment trial (Paper IV). Chronic treatment with LIR, 7,8-DHF, or their combination had no significant effect on reversing the changes in the hippocampal expression of RAAS components in WS rats, suggesting that modulation of RAAS does not contribute to the positive effects of these drugs on neuroprotection and cognitive function. Crucially, chronic stress (induced by extended experimental manipulations) may have contributed to the observed changes in the RAAS, as the expression profile of RAAS components differed in treatment-naïve animals. Stress might further exacerbate the effects of WFS1 deficiency on the RAAS, potentially worsening disease progression due to a compromised compensatory axis. Furthermore, these data re-emphasize the influence of the experimental design and environment, e.g., procedural stress.

SUMMARY IN ESTONIAN

Valproaadi ja liraglutiidi mõju Wolframi sündroomi loommudelites: fookuses transkriptsioonilised muutused reniin-angiotensiin-aldosterooni süsteemis

Wolframi sündroom on autosoom-retsessiivselt päranduv neurodegeneratiivne harvikhaigus (sagedus sõltuvalt populatsioonist ligikaudu 1:770 000 kuni 1:55 000), mis on tingitud homosügootsetest või liitheterosügootsetest mutatsioonidest *WFS1* geenis, mis kodeerib valku wolframiin. Haigust kirjeldasid esmakordselt 1938. aastal D. J. Wolfram ja H. P. Wagener. Seda iseloomustab kombinatsioon insuliinsõltuvast suhkrudiabeedist (*diabetes mellitus*), progresseeruvast nägemisnärvi atroofiast, magediabeedist (*diabetes insipidus*) ja sensorineuraalsest kuulmislangusest, mis enamasti arenevad välja esimese paarikümne eluaasta jooksul. Lisaks võivad kaasuda psühhiaatrilised häired ning mitmed teised neuroloogilised tüsistused. Wolframi sündroomiga patsientide keskmine eluiga on 30–40 eluaastat ning peamiseks surmapõhjuseks on ajutüve degenereerumisest tulenev hingamiskeskuse kahjustus. Kuigi Wolframi sündroom on harvikhaigus, on *WFS1* mutatsiooni heterosügootsetel kandjatel oluliselt suurem risk psühhiaatriliste haiguste, sensorineuraalse kuulmislanguse või diabeedi esinemiseks. Uued andmed viitavad ka sellele, et lapseea monogeense diabeedi korral on retsessiivne etioloogia, nagu nt mutatsioonid *WFS1* geenis ilma teiste Wolframi sündroomile iseloomulike kaasuvate sümptomiteta sagedasemad kui seni arvatud.

Funktsionaalse wolframiini puudumisel tekib rakkudes endoplasmaatilise retiikulumi (ER) stress, kaltsiumi tasakaalu häirumine ja seeläbi suureneb rakkude suremus. Viimastel aastatel on wolframiini tugevalt seostatud ka ER ja mitokondrite vahelise suhtluse hõlbustamisega, mis toimub spetsiaalsete struktuuride kaudu, mida nimetatakse mitokondritega seotud ER-membraanideks. Leitud on, et wolframiin mõjutab mitokondriaalset dünaamikat ja funktsioneerimist.

Wolframi sündroomi jaoks täna efektiivne ravi puudub. Patsientidel esinev insuliinsõltuv suhkrudiabeet ja magediabeet alluvad küll hästi vastavalt insuliini asendus- ja desmopressiini ravile, kuid haiguse tõhusaks kontrolli all hoidmiseks on oluline pidurdada neurodegeneratiivseid protsesse. Wolframi sündroomi progresseerumise aeglustamiseks on uurimisel mitmed juba laialt kasutusel olevad ravimid, sh epilepsia ja bibolaarse häire ravis kasutatav valproaat ja diabeedivastane ravim, glükagoonitaolise peptiid-1 retseptori (GLP-1R) agonist liraglutiid. Liraglutiid on näidanud suurt potentsiaali haiguse kulu pidurdamisel nii Wolframi sündroomi loommudelites kui ka patsientides. Valproaat suudab tõsta WFS1 hulka ja vähendada ER-stressist tulenevat rakusurma ning mõjutada veresuhkru taset veres. Valproaat on Wolframi sündroomi ravimi kandidaadina ka kliinilistes uuringutes.

Käesolevas väitekirjas kirjeldatud uuringud keskendusid valproaadi ja liraglutiidi toime iseloomustamisele Wolframi sündroomi loommudelites transkriptoomi tasemel. Täpsemalt uuriti pikaajalise valproaadi ravi mõju maksa geeniekspressioonile *Wfs1* – puudulikes hiirtes, mille eesmärk oli tuvastada valproaadist tulenevate metaboolsete muutuste võimalikud mehhanismid. Wolframi sündroomi hiiremudelis leiti, et valproaadil on sõltuvalt ravi kestusest nende hiirte vere glükoositasemele erinev mõju. Valproaadi ühekordne manustamine parandas glükeemilist kontrolli, samas kui pikaajalisel ravil valproaadiga efekt puudus. Pikaajaline ravi valproaadiga mõjutas *Wfs1* – puudulike hiirte maksakoes peamiselt geenide avaldumist, mis on seotud oksüdatiivsete protsesside, lipiidide metabolismi ning ööpäevarütmi reguleerimisega. Enim oli mõjutatud geeni *Ppar d* ekspressioon, millelt kodeeritaval valgul on ulatuslik roll rasvade ainevahetuses ning millel on kirjeldatud ka mõju insuliini tundlikkusele ja seeläbi glükoosi ainevahetusele.

Töö teise poole eesmärk oli välja selgitada WFS1 olulisus reniin-angiotensiin-aldosterooni süsteemis (RAAS) ning valproaadi ja liraglutiidi moduleeriv toime sellele süsteemile Wolframi sündroomi rotimudelis. Leiti, et RAAS-i võtmeretseptorite angiotensiin II tüüp 2 retseptori ja bradükiniini retseptori B1 ekspressioon oli märkimisväärselt vähenenud nii *in vitro* kui ka *in vivo* tingimustel. Lisaks täheldati Wolframi sündroomi rottide seerumis aldosterooni taseme vähenemist ja bradükiniini taseme tõusu. Lisaks meile seostati hiljuti WFS1 puudulikkust aldosterooni madalama tasemega ka kartsinoomi mudelis. On oluline märkida, et muutused RAAS-is olid täheldatavad juba noortes rottides, kui haigusnähud polnud veel täielikult avaldunud. Kuigi katsed valproaadi ja liraglutiidiga näitasid RAAS-i moduleerivat potentsiaali, ei suutnud nad nendes rottides geeniekspressiooni taset normaliseerida. Samas nii valproaat kui ka liraglutiid olid võimelised märkimisväärselt tõstma aldosterooni taset ja vähendama bradükiniini taset.

Tulemustele tuginedes võib väita, et kuigi Wolframi sündroomi rottidel ei esine RAAS-i häiretele viitavaid füsioloogilisi ilminguid, on RAAS-i komponentide tasakaal transkriptsiooni tasemel häirunud. Lisaks viitavad tulemused sellele, et RAAS-i komponentide ekspressiooni mõjutab tugevalt stress. Tasakaalustamata RAAS võib viia põletikuliste protsesside ja oksüdatiivse stressi ägenemiseni ja seeläbi suurendada rakkude apoptootilist aktiivsust. Sellest tulenevalt on võimalik, et Wolframi sündroomi korral on RAAS-i kompensatoorne telg häiritud, eriti stressi tingimustes, mistõttu võib haiguse kulg kiireneda, sh vananemisega seotud protsessid. Kuigi kumbki antud uurimustöös kasutatud ravim (valproaat, liraglutiid) ei suutnud RAAS-i komponentide ekspressiooni normaliseerida, ei ole välistatud nende positiivne mõju alternatiivsetele signalisatsiooniradadele, mis kompenseerivad RAAS-i võimalikku puudulikkust. Nende tulemuste valguses tuleks ehk ümber hinnata kliinilised juhised ja kriteeriumid nii monogeense diabeedi kui ka Wolframi sündroomi kohta, võimaldades seeläbi paremaid ravivõimalusi.

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Haridus

2011–...	Tartu Ülikool, neuroteadused, PhD
2009–2011	Tartu Ülikool, biomeditsiin, MSc, *cum laude*
2006–2009	Tartu Ülikool, geenitehnoloogia, BSc, *cum laude*

Töökogemus

04.01.2021–...	Tartu Ülikool, Loodus- ja täppisteaduste valdkond, tehnoloogiainstituut, bioohutuse tuumiklabori spetsialist (1,00)
13.08.2018–03.01.2021	SA TÜ Kliinikum Ühendlabor Kliinilise geneetika keskus, Laborianalüütik (1,00)
01.02.2016–06.02.2020	Sports Gene OÜ, Laborant (0,50)
01.01.2014–30.06.2017	Synlab Eesti OÜ, Synlab Eesti OÜ; asenduslaborant (0,10)

Kursused

2014	Välislabori külastus Kopenhaageni Ülikoolis (Neuroteaduste ja Farmakoloogia instituut, Neuroplastilisuse labor, Anders Hay-Schmidt); immuunohistokeemia ja in situ hübridisatsiooni meetodite omandamine (Euroopa Liidu Euroopa Sotsiaalfond programmi DoRa raames, mida viib ellu Sihtasutus Archimedes)
2012	"Näriliste käitumise hindamine: meetodid ja rakendamine" (FinPharmaNet ja Soome Biokeskus)
2011	Kursus "Competence on care and use of laboratory animals" (Turu Ülikool) (Euroopa Liidu Euroopa Sotsiaalfond programmi DoRa raames, mida viib ellu Sihtasutus Archimedes)

Uurimistöö põhisuunad

3. Terviseuuringud; 3.1 Biomeditsiin; B470 Füsioloogia

Publikatsioonid

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