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ALINA ALTPERE

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Targeting of mechanisms of elevated anxiety in female Wfs1-deficient mice

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Press

Department of Physiology, Institute of Biomedicine and Translational Medicine, University of Tartu, Tartu, Estonia

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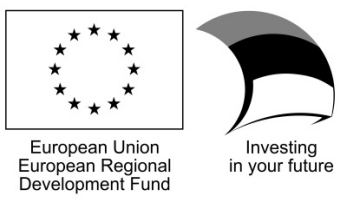

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Paper I

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Paper II

Sütt S*, **Altpere A***, Reimets R, Visnapuu T, Loomets M, Raud S, Salum T, Mahlapuu R, Kairane C, Zilmer M, Vasar E. Wfs1-deficient animals have brain-region-specific changes of Na+, K+-ATPase activity and mRNA expression of α1 and β1 subunits. J Neurosci Res. 2015 Mar;93(3):530–7. doi: 10.1002/jnr.23508.

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Paper III

Raud S, Reimets R, Loomets M, Sütt S, **Altpere A**, Visnapuu T, Innos J, Luuk H, Plaas M, Volke V, Vasar E. Deletion of the Wolfram syndrome-related gene Wfs1 results in increased sensitivity to ethanol in female mice. Neuropharmacology. 2015 Aug;95:59–67. doi: 10.1016/j.neuropharm.2015.02.019.

Contributions of the author:

Paper I. The author participated in the study design, dissected the brain regions, performed all gene expression analyses, carried out the statistical analysis and was responsible for writing and publishing the manuscript.

Paper II. The author participated in the study design, dissected the brain regions, performed all qRT-PCR gene expression analyses and protein expression analyses, carried out the statistical analysis and participated in writing the manuscript.

Paper III. The author performed qRT-PCR gene expression experiments and participated in planning and writing the manuscript.

ABBREVIATIONS

ANOVA	analysis of variance
Atf6α	activating transcription factor 6α in species other than humans
ATF6α	activating transcription factor 6α in humans
cDNA	complementary DNA
Chop	C/EBP homologous protein or gene in species other than humans
CHOP	C/EBP homologous protein or gene in humans
DA	dopamine
Ddit3	DNA damage inducible transcript 3 (CHOP)
E	embryonic day
Eif2ak3	eukaryotic translation initiation factor 2 alpha kinase 3 (PERK)
eIF2α	eukaryotic initiation factor 2 alpha
EPM	elevated plus-maze
ER	endoplasmic reticulum
GABA	gamma-aminobutyric acid
$GABA_A$	GABA receptor A
Gabra1	alpha 1 subunit of $GABA_A$ receptor
Gabra2	alpha 2 subunit of $GABA_A$ receptor
Gabra3	alpha 3 subunit of $GABA_A$ receptor
Grp78	78 kDa, glucose regulated protein
Grp94	94 kDa, glucose regulated protein
Hprt1	hypoxanthine phosphoribosyltransferase 1 gene
Ire1α	inositol-requiring transmembrane kinase and endonuclease 1α in species other than humans
IRE1α	inositol-requiring transmembrane kinase and endonuclease 1α in humans
LORR	loss of righting reflex
Na-pump	sodium-potassium adenosine triphosphatase
Na^+-K^+ ATPase	sodium-potassium adenosine triphosphatase
NMDA	N-methyl-D-aspartate receptor
Perk	protein kinase-like ER kinase protein or gene in species other than humans
PERK	protein kinase-like ER kinase protein or gene in humans
PTSD	post-traumatic stress disorder
qRT-PCR	quantitative real-time PCR
UPR	unfolded protein response
Wfs1	Wolfram syndrome 1 gene or protein in species other than humans
WFS1	Wolfram syndrome 1 gene or protein in humans
WS	Wolfram syndrome
Xbp1	X-box binding protein 1 gene in species other than humans

XBP1	X-box binding protein 1 gene in humans
Xbp1s	spliced Xbp1 RNA in species other than humans
XBP1s	spliced XBP1 RNA in humans
Xbp1t	total Xbp1 RNA

INTRODUCTION

Wolfram syndrome (WS) is a genetic neurodegenerative disorder that is characterized by early-onset diabetes, optic nerve atrophy, deafness and retardation. It has been demonstrated that WS is caused by the mutations in WFS1 gene (Khanim *et al.* 2001). WFS1 is a recessive gene that encodes an endoplasmic reticulum (ER) membrane protein – wolframin (Takei *et al.* 2006). Absence of functional wolframin has been linked to impaired Ca^{2+} homeostasis in ER and faulty regulation of ER stress in pancreatic β-cells, leading to cell death (Takei *et al.* 2006; Yamada *et al.* 2006). It has been shown that heterozygous carriers of defective WFS1 gene are 26 fold more likely to be hospitalized with different psychiatric disorders, including mood disorders, tendency to violence, sleeplessness and anorexia (Strom *et al.* 1998; Swift *et al.* 1998). Wfs1 gene has been suggested as a marker for potential of developing post-traumatic stress disorder (PTSD). Kesner and colleagues have demonstrated elevated Wfs1 expression in the hippocampus and amygdala of rats that developed PTSD-like symptoms, but not in rats that were exposed to the same traumatic event and did not develop PTSD behavior (Kesner *et al.* 2009). Nonetheless, the exact role of WFS1 gene in the development of neuropsychiatric disorders remains to be established.

In order to investigate the function of Wfs1 in the body, transgenic Wfs1-deficient mice have been generated (Luuk *et al.* 2008). It was revealed that mice lacking functional Wfs1 gene were more anxious and displayed impaired ability to cope with novel and stressful situations compared to wild-type mice (Luuk *et al.* 2009). Since deficient function of WFS1 gene plays a role in the development of emotional disorders in humans (Strom *et al.* 1998; Swift *et al.* 1998) the major goal of the present study was to extend our knowledge about the mechanisms underlying the elevated anxiety in Wfs1-deficient mice. For potential target validation we explored the interactions of Wfs1 protein with ER stress response, sodium–potassium adenosine triphosphatase (Na^+-K^+ ATPase) and GABAergic system.

It has been shown, that aberrant ER-stress response is an upstream event in the pathophysiology of mood disorders (Xiang *et al.* 2017). As Wfs1-deficiency caused elevated expression of ER stress markers in cell culture studies (Fonseca *et al.* 2010), we decided to explore whether the elevated levels of ER stress markers were also related to the increased anxiety in Wfs1-deficient mice. It has been shown that Wfs1-deficient mice display increased anxiety in the elevated plus-maze test (EPM) (Luuk *et al.* 2009) and thus we chose EPM to induce anxiety-like behavior in mice for this study. Elevated plus-maze is considered to be a useful tool to investigate both anxiolytic and anxiogenic agents. It is widely used to measure exploratory activity and anxiety-like behavior in rodents (Lister 1987), but the effect of EPM itself on the expression of ER stress markers has not been studied yet.

In cell culture studies WFS1 has been identified as a molecular partner of Na^+-K^+ ATPase β1 subunit (Zatyka *et al.* 2008). The impairment of Na^+-

K^+ ATPase activity leads to neuronal dysfunction and is associated with an increased risk of developing depression, anxiety, and bipolar disorder in humans (Goldstein *et al.* 2006; Crema *et al.* 2010; Kirshenbaum *et al.* 2011). These findings led us to the hypothesis that the disturbed function of Na^+-K^+ ATPase can play a role in the elevated anxiety in Wfs1-deficient mice. To study this hypothesis, we measured Na^+-K^+ ATPase activity and expression levels of its subunits α1 and β1 in different brain areas after exposing the animals to the EPM.

Re-uptake of γ-amino-butyric acid (GABA), the principal inhibitory neurotransmitter in the amygdala that modulates anxiety-related behavior, is impaired in Na^+-K^+ ATPase α2 subunit-deficient mice indicating a connection between Na^+-K^+ ATPase and the GABAergic system (Ikeda *et al.* 2003). Indeed, we have established in our previous studies compromised function of GABAergic system in Wfs1-deficient mice (Luuk *et al.* 2009; Raud *et al.* 2009). As GABA A ($GABA_A$) receptors have been shown to mediate the effects of ethanol in the central nervous system and low concentrations of ethanol possess anxiolytic-like effect (Davies 2003), we decided to study anxiolytic-like as well as sedative/hypnotic effects of ethanol on the behavior of Wfs1-deficient mice. For further clarification of the involvement of $GABA_A$ receptors, the gene expression levels of receptor subunits were investigated in the brain structures after ethanol treatment.

We found a change in the expression levels of some ER stress markers in the brain regions responsible for the dominant behavioral traits of mice due to the EPM exposure. On the other hand, no changes in the expression of ER stress markers were seen in experimentally naïve Wfs1-deficient mice, showing that Wfs1-deficiency itself does not cause ER stress in young adult animals, but rather modifies the biochemical response of an animal to an external stressor. We were also able to identify changes in Na^+-K^+ ATPase activity in some brain regions, providing further evidence of the involvement of cation imbalance in the development of anxiety. Moreover, we established increased sensitivity of Wfs1-deficient mice to anxiolytic-like action of ethanol. This behavioral finding was associated with an altered expression of $GABA_A$ receptor subunits in genetically modified mice.

In conclusion, this study suggests that the increased anxiety-like behavior displayed by Wfs1-deficient mice in this model is most probably due to the compromised function of GABA system. Besides that, it underlies the suitability of Wfs1-deficient mouse as an animal model in studying the molecular mechanisms behind anxiety.

1. REVIEW OF LITERATURE

1.1. Wolfram syndrome and Wfs1 gene

Wolfram syndrome (WS) is a genetic disorder that was first reported in 1938 by Wolfram and Wagener, who described four siblings with juvenile diabetes mellitus and optic nerve atrophy (Wolfram & Wagener 1938). WS is caused by mutations in the WFS1 gene and characterized by diabetes mellitus, diabetes insipidus, optic nerve atrophy, deafness and retardation (Khanim *et al.* 2001). The prognosis of this syndrome is poor and most patients die prematurely with the median age at death being 30 years, usually from respiratory failure as a result of brainstem atrophy (Barrett *et al.* 1995). Severe neurological symptoms usually become evident in the late twenties to early thirties, but recent research suggests that neurodegenerative changes are already apparent in the early childhood (Hershey *et al.* 2012) and will become fully manifested by the age of 15 (Chaussenot *et al.* 2011). It has been shown that certain neuronal cells are selectively destroyed as the consequence of the mutations in the WFS1 gene (Urano 2014).

Wolframin, a protein encoded by the WFS1 gene is localized to the endoplasmic reticulum (ER), suggesting that ER dysfunction may be a major pathogenic component of WS. In cell culture studies, WFS1 mutations lead to the elevated ER stress levels, pancreatic β-cell dysfunction, and initiation of ER stress-associated cell death (Fonseca *et al.* 2005; Fonseca *et al.* 2010).

The expression level of Wfs1 is the highest in the brain, liver, spleen, heart, kidney, lung, skeletal muscles and neurosecretory tissues, including pancreatic insulin-secreting β-cells (Inoue *et al.* 1998; Strom *et al.* 1998; Hofmann *et al.* 2003). Luuk *et al.* (2008) detected a strong expression of the Wfs1 gene in the ventral striatum and central extended amygdala. Noticeable Wfs1 expression was detected in the hippocampal CA1 region, hypothalamic magnocellular neurosecretory system, parasubiculum, superficial part of the second and third layers of the prefrontal cortex and proisocortical areas, and central auditory pathway. Wfs1 was found in the nerve fibers of the medial forebrain bundle, globus pallidus, reticular part of the substantia nigra, lateral lemniscus, alveus, posterior caudate putamen, fimbria, dorsal hippocampal commissure, subiculum, and in smaller quantities the central sublenticular extended amygdala and ventral tegmental area (Luuk *et al.* 2008). It has also been shown that neurons expressing Wfs1 gene product project from the nucleus accumbens to midbrain dopaminergic neurons and the expression level of Wfs1 is definitely the highest in brain structures that belong to or are associated with the limbic system (Takeda *et al.* 2001; Luuk *et al.* 2008). It seems that in mice, the gene expression level of Wfs1 is the highest in the brain structures related to learning, memory and emotions.

These neuroanatomical findings raise a theory that the lack of WFS1 protein is probably related to the development of psychiatric symptoms. Indeed, WFS1 heterozygotes have 26 times higher risk for psychiatric hospitalization primarily

due to severe depression (Swift *et al.* 1998). Carriers of the mutant WFS1 gene have been shown to be more likely to commit suicide and suffer from generalized anxiety (Swift *et al.* 1998). Some WFS1 polymorphisms have been shown to be associated with increased risk for mood disorders (Koido *et al.* 2005). Kesner and colleagues have demonstrated elevated Wfs1 expression in the hippocampus and amygdala of rats that were more prone to develop PTSD-like symptoms, but not in rats that were exposed to the same traumatic event without developing PTSD-like behavior (Kesner *et al.* 2009).

In mice, the effect of Wfs1-deficiency on emotional behavior has been investigated in knock-outs with targeted deletion of the 2nd or 8th exon of the Wfs1 gene. Knock-out mice with deleted 2nd exon displayed only increased freezing behavior during the training period in the fear conditioning test (Kato *et al.* 2008), whereas mice with disrupted 8th exon of the Wfs1 gene demonstrated remarkably elevated anxiety in various tests of anxiety (Luuk *et al.* 2009). One has to take into account that the genetic background of mice in these two studies was different: the first study employed Bl6 mice (Kato *et al.* 2008), whereas in the second study F2 hybrids of mixed Bl6 and 129Sv background were used (Luuk *et al.* 2008).

1.2. Emerging targets of elevated anxiety in Wfs1-deficient mice

1.2.1. Wfs1 and endoplasmic reticulum stress

Endoplasmic reticulum plays a significant role in a variety of cellular functions. It coordinates the synthesis, folding, and posttranslational modification of proteins, cytoplasmic and mitochondrial metabolism, calcium (Ca^{2+}) storage and cell death. Disruption of ER protein folding or overloading the ER capacity to fold proteins effectively causes ER stress (Kaufman *et al.* 2002; Xu *et al.* 2005), which is sensed by three major ER stress sensors: activating transcription factor 6α (ATF6α), inositol-requiring transmembrane kinase and endonuclease 1α (IRE1α) and protein kinase-like ER kinase (PERK). When activated, each of these sensors can initiate a cascade of events called unfolded protein response (UPR), either leading to the recovery of ER homeostasis or triggering apoptosis when the ER stress is prolonged or the degree of ER stress is too severe to be corrected (Xu *et al.* 2005; Lai *et al.* 2007; Schonthal 2012; Wang & Kaufman 2012; Mekahli *et al.* 2016). When cells encounter ER stress, ATF6 leads to the production of chaperones GRP78 and GRP94, and the activation of X-box binding protein 1 (XBP1), while PERK phosphorylates translation initiation factor elF2α, leading to the inhibition of translation as well as to the activation of C/EBP homologous protein (CHOP) (Gold *et al.* 2013). Under ER stress, IRE1α is activated to splice the mRNA encoding XBP1. Spliced XBP1 (XBP1s) induces UPR target genes encoding factors involved in ER protein folding and

degradation (Jiang *et al.* 2015) (Figure 1). Induction of GRP78 is also regulated by IRE1α pathway (Yamagishi *et al.* 2007; Xiang *et al.* 2017).

Aberrant UPR which could lead to cell death is an upstream event in the pathophysiology of a variety of diseases like neurodegeneration, cardiac diseases, cancer, diabetes and mood disorders (Chakrabarti *et al.* 2011; Xiang *et al.* 2017). WS is also linked to impaired ER stress response (Urano 2014). In general, the elevated expression of both pro-apoptotic (CHOP) and cell survival related ER stress markers (GRP78, GRP94, XBP1) is accompanied with diabetes, neurodegeneration and depression (Fonseca *et al.* 2010; Nevell *et al.* 2014; Shang *et al.* 2014; Scheper & Hoozemans 2015), the disease symptoms present in patients with WS.

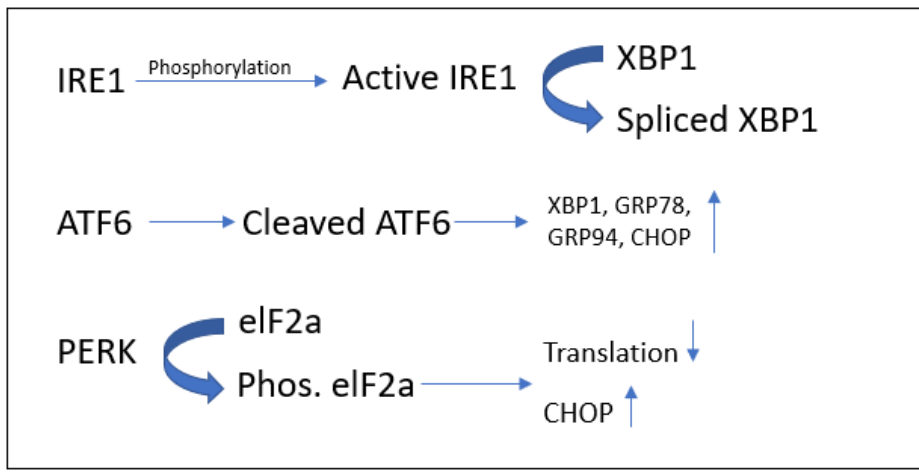

Figure 1. Simplified schematic representation of UPR response pathways based on the above-mentioned materials and summarized by the author of this thesis. Phos. – phosphorylated.

Besides the pathological aspect of ER stress, it is known that psychological stress can also induce ER stress activation (Pavlovsky *et al.* 2013). In most of the previous animal studies the expression of ER stress markers has been investigated in depression models which are caused by moderate or severe stressors (Ishisaka *et al.* 2011; Pavlovsky *et al.* 2013; Tan *et al.* 2015; Timberlake & Dwivedi 2015). Most of the ER stress markers investigated have been elevated in these studies. The effect of mild stress on ER stress activation is not yet known.

EPM is considered to be a useful tool to investigate both anxiolytic and anxiogenic agents. It is widely used to measure exploration and anxiety-like behavior in rodents (Lister 1987), but the effect of EPM itself on the expression of ER stress markers has not been studied yet.

1.2.2. Wfs1 and Na^+-K^+ ATPase

WFS1 is a molecular partner of the sodium-potassium adenosine triphosphatase (Na^+-K^+ ATPase) β1 subunit. Their interaction occurs in the ER, and probably plays a role in the maturation of the β1 subunit (Zatyka *et al.* 2008). Na^+-K^+ ATPase is a membrane protein responsible for translocating sodium and potassium ions across the cell membrane. Using ATP for energy, three Na^+ ions are transported out of the cell for every two K^+ ions that are pumped in (Lingrel & Kuntzweiler 1994). Therefore, Na^+-K^+ ATPase is responsible for the restoration and maintenance of intracellular gradient of Na^+ and K^+ and is involved in the regulation of the ability of neurons to maintain resting potential and neuronal firing (Therien & Blostein 2000). The sodium gradient is also used to drive many transport processes, including the translocation of amino acids, glucose and other nutrients into cells (Lingrel & Kuntzweiler 1994).

The Na^+-K^+ ATPase consists of a catalytic α subunit and a regulatory β subunit (Kaplan 2002). The α subunit contains the ATP binding site, the phosphorylation site, and amino acids essential for the binding of cations, while the β subunit appears to be involved in the maturation of the enzyme and localization of the ATPase to the plasma membrane (Lingrel & Kuntzweiler 1994). There are four different isoforms of the Na^+-K^+ ATPase α subunit (α1, α2, α3, α4) and three isoforms of the β subunit (β1, β2, β3). The α1 subunit is expressed both in the glia and the neurons in the central nervous system (Dobretsov & Stimers 2005; Romanovsky *et al.* 2005), α2 is found predominantly in the glial cells, and α3 is expressed only in the neurons (Moseley *et al.* 2003). The impairment of Na^+-K^+ ATPase activity and/or mutations in its subunits in different brain structures lead to neuronal dysfunction and are associated with an increased risk of developing depression, anxiety, and bipolar disorder (Goldstein *et al.* 2006; Crema *et al.* 2010; Kirshenbaum *et al.* 2011). Therefore, different psychiatric abnormalities, such as depression and psychosis, in WS patients may be related to the disturbed Na^+-K^+ ATPase regulation and maturation of its subunits in the central nervous system. There are also some studies suggesting that Na^+-K^+ ATPase can sub-serve information processing role in some neurons (Forrest 2014).

1.2.3. Wfs1 and GABA system

GABA is a major inhibitory neurotransmitter in the human brain and GABAergic inhibitory postsynaptic potentials play an important role in controlling the excitability and responsiveness of cortical neurons (McCormick 1989).

There are at least two distinct classes of GABA receptors: $GABA_A$ and $GABA_B$. The $GABA_A$ receptors belongs to the superfamily of ligand-gated ion channels (Macdonald & Olsen 1994). $GABA_A$ receptor is also one of the targets for the action of ethanol in the central nervous system (Kumar *et al.* 2009). It

has been shown that ethanol affects the expression of genes that encode components of the $GABA_A$ receptor (Biggio *et al.* 2007).

Ethanol affects several neurological pathways and causes significant changes in the brain. Some of the neuronal pathways known to be affected by alcohol consumption are dopaminergic, serotoninergic, GABAergic and glutamatergic pathways (Banerjee 2014). Ethanol at low blood concentrations releases behaviors that are otherwise inhibited and usually produces feelings of relaxation and good mood which may facilitate socializing. However, even low quantities of ethanol affect the ability of hippocampus to process information, which in turn impairs memory formation. Higher doses of ethanol affect the brain further by inducing intoxication wherein the person may experience temporary loss of coordination and judgment (Gilpin & Koob 2008). On the molecular level, low concentration of ethanol affects GABAergic neurotransmission by increased release of GABA (Roberto *et al.* 2004) and other substances that are active at the $GABA_A$ receptors (Barbaccia *et al.* 1999). High concentration of ethanol has a direct effect on $GABA_A$ receptor (Mihic & Harris 1997; Weiner & Valenzuela 2006) and, may affect the expression of the subunits of $GABA_A$ receptor responsible for certain behavioral effects of ethanol.

Re-uptake of GABA is impaired in Na^+-K^+ ATPase α2 subunit-deficient mice indicating a connection between Na^+-K^+ ATPase and GABAergic system (Ikeda *et al.* 2003; Moseley *et al.* 2007). A tight interaction between the GABAergic system and Na^+-K^+ ATPase was also reported by Kaur and colleagues who found that long term blocking of $GABA_A$ receptor resulted in higher Na^+-K^+ ATPase activity in the rats' brain (Kaur *et al.* 2004).

Experimentally naïve Wfs1-deficient mice have been shown to display altered expression of $GABA_A$ receptor subunits alpha 1 and 2 that are related to the sedative and anti-anxiety effects of drugs, respectively (Luuk *et al.* 2009; Raud *et al.* 2009). The alpha 3 subunit of $GABA_A$ receptor may also take part in the regulation of anxiety, as selective and nonselective modulators of alpha 3 $GABA_A$ receptor have been shown to have an anxiolytic effect in mice (Morris *et al.* 2006; Navarro *et al.* 2006). Pharmacological studies have revealed that Wfs1-deficient mice are more sensitive to the anxiolytic-like effect of diazepam, a gamma-aminobutyric acid (GABA) positive allosteric modulator at $GABA_A$ receptor (Luuk *et al.* 2009; Raud *et al.* 2009). GABA and $GABA_A$ receptors have also been found in the pancreas. There is an indication about interaction between Wfs1 protein and GABAergic system in the pancreas. Pancreatic β-cells release insulin alongside with GABA, which inhibits the release of glucagon from pancreatic α-cells by binding to $GABA_A$ receptors. It seems, that GABA and $GABA_A$ receptors also play an important role in the regulation of blood glucose level and metabolism (Xu *et al.* 2006).

2. OBJECTIVES

Mutations in the WFS1 gene have been associated with WS. WFS1 has been identified as a molecular partner of β1 subunit of Na^+-K^+ ATPase, has been associated with elevated ER stress in cell cultures, and interacts with the GABAergic system both in the brain and pancreas. However, the exact function of Wfs1 remains to be established. The goal of this study was to shed more light on the effect of Wfs1-deficiency in the brain of young mice and get closer to understanding the mechanisms behind negative emotions (including anxiety) present in patients with WS. Based on the above presented analysis of literature the current dissertation aims to answer the following questions:

1. Does Wfs1-deficiency affect the expression of ER stress markers in the brain structures of naïve young mice? How does mild stress, induced by an exposure to the elevated plus-maze, affect the indices of ER stress in the brain structures of wild-type and Wfs1-deficient mice?
2. How do Wfs1-deficiency and stressful manipulations affect the activity of Na^+-K^+ ATPase in the brain structures of mice? How are the genes of Na^+-K^+ ATPase subunits involved in the aberrant stress response of young Wfs1-deficient mice?
3. Are the behavioral effects of ethanol, a drug that modulates the GABAergic system, altered in Wfs1-deficient mice? How are the genes of $GABA_A$ receptor subunits implicated in the altered effects of ethanol in Wfs1-deficient mice?
4. How are the interactions between the GABAergic system, Na^+-K^+ ATPase and ER stress markers in the brain structures related to elevated anxiety observed in Wfs1-deficient mice?

3. MATERIALS AND METHODS

3.1. Animals (Papers I, II, III)

Wfs1-deficient mice were generated by invalidating the 8th exon of the Wfs1 gene (Luuk *et al.* 2008). Breeding and genotyping were carried out at the Institute of Biomedicine and Translational Medicine, University of Tartu. The experiments were performed in young adult (2–3 months) female wild-type and Wfs1-deficient F2 hybrid mice ([129S6/SvEvTac × C57BL/6] × [129S6/SvEvTac × C57BL/6)]. Female mice were chosen, because they display higher activity in the exploratory models of anxiety compared to male mice (Johnston & File 1991; Zimmerberg & Farley 1993; Ferguson & Gray 2005). Also, Wfs1-deficiency affects female animals' health condition to a lesser extent. For example, disturbances in glucose metabolism are less obvious in female Wfs1-deficient mice compared to male Wfs1-deficient mice (Luuk *et al.* 2009). Nevertheless, the mean body weight of Wfs1-deficient mice was significantly lower compared to wild-type littermates (18.6±0.5 grams and 21.7±0.6 grams, respectively). The mice were housed in respective home cages in groups of seven to eight under a 12 h light/dark cycle (lights on at 07:00). Tap water and food pellets were available *ad libitum,* except during testing. Behavioral experiments were carried out between 10:00 and 17:00. Permission for the present study was given by the Estonian National Board of Animal Experiments (No. 88, 25th of August 2011) in accordance with the European Communities Directive of September 2010 (2010/63/EU).

3.2. Behavioral and pharmacological studies

3.2.1. Locomotor activity test (Paper III)

For the study of locomotor activity, the animals were placed singly into transparent photoelectric motility boxes (448 mm × 448 mm × 450 mm) connected to a computer (TSE; Technical & Scientific Equipment GmbH, Germany) for 30 min. The illumination level of the boxes was around 400 lx. The floor of the box was cleaned thoroughly with 5% alcohol and dried after each animal. The distance travelled (m), time in locomotion (s) and the frequency of rearings were registered.

3.2.2. Elevated plus-maze test (EPM) (Papers I, II, III)

The test employs elements of neophobia, exploratory drive and approach/ avoidance conflict (Handley & Mithani 1984; Pellow *et al.* 1985; Lister 1987). According to the Directive 2010/63/EU it is categorized as a mild procedure (http://ec.europa.eu/environment/chemicals/lab_animals/pdf/guidance/directive/en.pdf). The plus-maze was elevated 30 cm from the floor and consisted of two

opposite open arms (17.5 cm × 5 cm, surrounded by a 0.25 cm high border) and two enclosed arms of the same size with 14 cm high sidewalls and an end wall. The arms extended from a common central square (5 cm × 5 cm) and were perpendicular to each other, making the shape of a plus sign. The room was dimly lit to promote the exploratory activity (illumination level 35–40 lux). Pre-experimental social separation of the animals for 15 min was used to increase their exploratory activity (Raud *et al.* 2009). The test lasted 5 minutes. The maze was thoroughly cleaned with damp and dry towels between the subjects. Test sessions were video-recorded, and the video files were analyzed by a trained observer unaware of the experimental conditions. The following measures were observed: % of open arm entries [number of open arm entries / total arm entries x 100]; % on open arm [time on open arm / time on total arm x 100]; number of unprotected head-dippings; risk assessment behavior (sum of the number of stretch-attend postures and the number of attempts to enter the central platform, located between open and closed arms); the number of line crossings; the number of closed arm entries.

3.2.3. Ethanol treatment (Paper III)

In the elevated plus-maze and locomotor activity test, the effects of three different doses of ethanol (0.5, 1 and 2 g/kg) were studied. Only one dose of ethanol (2 g/kg) was used for the rotarod test. Mice received and injection of ethanol [5% (v/v) for 0.5 and 1 g/kg or 20% (v/v) for 2 g/kg] 20 min prior to testing. To study ethanol metabolism, mice were treated with ethanol [2 or 4 g/kg 20% (v/v)] 30 min before measuring of the ethanol concentration in the blood. In the loss of righting reflex test, mice were treated with ethanol (4 g/kg) or pentobarbital sodium salt (Sigma/Aldrich, 45 mg/kg). For gene expression studies, ethanol (2 g/kg) was injected 30 or 60 min before decapitation. Ethanol and pentobarbital sodium salt used in this study were diluted with 0.9% NaCl solution (B. Braun Melsungen AG, Germany) and injected intraperitoneally.

3.2.4. Motor coordination in rotarod test (Paper III)

Rotarod is one of the standard tests to measure coordination, balance and procedural learning in rodents. This test is also used to evaluate sedation (Soderpalm *et al.* 1989; Tang *et al.* 1995; Steiner *et al.* 2011). The equipment consisted of a motor-driven drum (3 cm in diameter) rotating at a fixed speed (9 rpm). Five minutes before the first trial on the rotarod, mice were habituated to stay on the drum for one minute. In later trials, habituation was not used. Each mouse was given three trials (second after 2 h and third 24 h after). The effect of ethanol on motor coordination was measured on the fourth trial (after 48 h). The time of maximal performance for each trial was set at 120 s. The latency (s) to the first fall and the total number of falls were registered (Koks *et al.* 2001).

3.2.5. Loss of righting reflex (LORR) (Paper III)

After an intraperitoneal injection of 4 g/kg of ethanol (20%, v/v) or pentobarbital (45 mg/kg), the mice were placed in a supine position in a V-shaped cardboard trough and tested for the onset and duration of LORR. LORR was defined as the inability of a mouse to right itself three times within 30 s. The righting reflex was considered regained when the mouse could fully right itself three times within 30 s (Lasek *et al.* 2011). The onset and the duration of LORR (min) were measured.

3.3. Total RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR) (Papers I, II, III)

The animals were decapitated in a separate room 30 and 60 min after ethanol or 30 min after vehicle injection (ethanol 30, ethanol 60 and vehicle group, respectively) for the ethanol effect study, right after behavioral experiments for ER stress markers and Na^+-K^+ ATPase activity study or right after taking them out of the home cage as experimentally naïve controls. The brain was rapidly removed from the skull and different brain structures (the frontal cortex [including the prefrontal cortex], ventral striatum, temporal lobe [including the amygdaloid complex], midbrain and hippocampus) were dissected and snap-frozen in liquid nitrogen. Brain was dissected according to the coordinates provided in the mouse brain atlas by Franklin and Paxinos (1997).

Total RNA extraction, cDNA synthesis and qRT-PCR were performed as described before (Raud *et al.* 2009). Comparison of different housekeeping genes showed that Hprt1 is the most stably expressed gene in wild-type and Wfs1-deficient mice (Raud *et al.* 2009) and, therefore, it was applied in this study. qRT-PCR was performed in four parallel reactions for each sample. The results were normalized to Hprt1 expression and presented according to Livak and Schmittgen (Livak & Schmittgen 2001). The acute effect of ethanol (2 g/kg) was investigated on Gabra1, Gabra2 and Gabra3 mRNA expression in wild-type and Wfs1-deficient mice. The effects of Wfs1-deficiency and EPM were investigated on ER stress markers and the effect of genotype on Na^+-K^+ ATPase subunits was also investigated.

The assays and the sequences of primers and probes are given in Table 1.

Table 1. The Taqman assays and probes used in the study

Gene Symbol	Assay ID or sequence (5'-3')	Gene ID
Atp1a1	Mm00523263_m1	NM_011716
Atp1b1	Mm00437612_m1	NM_031823
Gabra1	Mm00433435 m1	NM_010250
Gabra2	Mm00439046_m1	NM_008066
Gabra3	Mm01294271_m1	NM_008067
Grp94	Mm00441927_m1	NM_011631
Grp78_for Grp78_rev Grp78_probe	CATAAACCCCGATGAGGCTGTA CACCTGTATCCTGATCACCAGAGA FAM-TGGTGCCGCTGTCCAGGCTG	NM_022310
Xbp1_total_for Xbp1_total_rev Xbp1_total_probe	AAGAACACGCTTGGGAATGG ACTCCCCTTGGCCTCCAC FAM-CACGCTGGATCCTG	NM_013842
Xbp1_spliced_for Xbp1_spliced_rev Xbp1_spliced_probe	TGCTGAGTCCGCAGCAGG TCAGAGTCCATGGGAAGATGT FAM-CAGGCCCAGCAGTTGCT	NM_013842
Hprt1_for Hprt1_rev Hprt1_probe	GCAGTACAGCCCCAAAATGG AACAAAGTCTGGCCTGTATCCAA VIC-AAGCTTGCTGGTGAAAAGGACCTCTCG	NM_013556
Ddit3_for Ddit3_rev Ddit3_probe	CCAGGAAACGAAGAGGAAG CCTCTGTCAGCCAAGCTAG FAM-TTCACTACTCTTGACCCTGCG	NM_007837
Eif2ak3_for Eif2ak3_rev Eif2ak3_probe	TGGTTGGAGGGAAATCTCTG CATCCCAAGGCAGAACAGAT FAM-GCTTACAGTGGAAAGCTGAGG	NM_010121
Atf6α	Mm0295317_m1	NM_001081304

Gabra1 – alpha 1 subunit; Gabra2 – alpha 2 subunit; Gabra3 – alpha 3 subunit of $GABA_A$ receptor; Grp94 – 94 kDa, glucose regulated protein, Grp78 – 78 kDa, glucose regulated protein, Xbp1 – X-box binding protein, Hprt1 – hypoxanthine phosphoribosyltransferase 1 gene, Ddit3 – DNA damage inducible transcript 3 (Chop), Eif2ak3 – eukaryotic translation initiation factor 2 alpha kinase 3 (Perk).

3.4. Biochemical studies

3.4.1. Measurement of plasma corticosterone levels (Paper II)

The animals were decapitated immediately after completing the experiment in the EPM test or after taking them from their home cage. Mice were transported to a separate room for decapitation. Blood from the trunk of the body (mixed arterial and venous blood) was collected into heparinized tubes. For separation of serum, probes were centrifuged for 10 min at 1,500g, and serum was stored at –20°C. Corticosterone measurement was performed with an Octeia corticosterone HS ELISA kit from Immunodiagnostic Systems (Boldon Tyne & Wear, United Kingdom) according to the manufacturer's instructions. A brief description of the measurement was reported previously (Luuk *et al.* 2009).

3.4.2. Measurement of Na^+-K^+ ATPase activity (Paper II)

Na^+-K^+ ATPase membrane preparations were isolated from the mouse brain by a previously described method (Karel'son *et al.* 1985). Briefly, the tissues were homogenized at 4°C in a medium containing 0.32 M sucrose, 1 mM EDTA, 0.1% deoxycholate (DOC), and 37.5 mM imidazole-HCl (pH 7.4 at 8°C). The homogenate was centrifuged for 10 min at 10,000g, and the supernatant was removed and centrifuged for another 30 min at 24,000g. The enzyme preparation was obtained by resuspension of the final sediment in the buffer described above (without DOC). Total Na^+-K^+ ATPase activity was measured by incubation of membrane proteins (~20 µg) in 375 µl medium containing 100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 4 mM ATP, and 25 mM imidazole-HCl (pH 7.4 at 37°C). The reaction was carried out for 10 min and terminated with 3.5% of sodium dodecyl sulfate (SDS). The released inorganic phosphate (Pi) was determined as described elsewhere (Karel'son *et al.* 1985). Na^+-K^+ ATPase activity was established as a difference between the release of Pi from ATP with and without NaCl and KCl in the incubation medium. We used the ATP molecule as an acid purified by column chromatography, which removes Na^+ ions from Na_2ATP (Sigma, St. Louis, MO). The specific activity of the enzyme was expressed as micromoles Pi released per minute per milligram protein. The protein content was determined by the Lowry method, with bovine serum albumin as the standard (Lowry *et al.* 1951). Under all experimental conditions, the activity of Na^+-K^+ ATPase was linear as a function of incubation time and enzyme amount.

3.4.3. Blood ethanol concentrations (Paper III)

Five microliters of blood were taken by puncturing the tail vein of the mice immobilized in a 50 ml tube restrainer. Blood was collected 30, 60, 120 and 240 min after intraperitoneal injection of 2 or 4 g/kg of ethanol. Immediately after collection, the samples were analyzed by enzymatic color test using LKM 139 and mini-photometer LP 20 (Dr. Bruno Lange GmbH, Germany) according to manufacturer's instructions.

3.5. Western blot analysis (Paper II)

Brains were dissected into different brain structures (dorsal striatum, temporal lobe [including the amygdaloid complex], and midbrain), and tissues were dissolved in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium DOC, and 1% Triton X-100) including 1 mM protease inhibitors (Sigma). The proteins were sonicated and centrifuged for 10 min at 12,000g at 4°C. The supernatant was kept at -70°C, and the concentration of protein was measured by using a BCA kit (Pierce, Rockford, IL). Equal amounts of protein (20 mg) were separated by 10% SDS-polyacrylamide gel electrophoresis,

transferred to PVDF membranes, and incubated with primary antibodies ATP1A1 (1:1,000; Sodium Potassium ATPase Alpha 1 Antibody; Novus Biologicals, Littleton, CO), ATP1B1 (1:500; Na1/K-ATPase b1 H-115; sc-25709; Santa Cruz Biotechnology, Santa Cruz, CA), or β-actin (1:10,000, Cell Signaling Technology, Danvers, MA) overnight at 4°C. The membranes were washed three times with phosphate-buffered saline Tween-20 and incubated with HRP-conjugated goat anti-mouse or anti-rabbit IgG secondary antibody (1:10,000, Jackson Immunoresearch, West Grove, PA) for 1h at room temperature. The signal was detected with an enhanced chemiluminescence system (Pierce, Rockford, IL). Band intensities were analyzed in MultiGauge (Fujifilm), and protein amount was calculated as a ratio with β-actin.

3.6. Statistical Analysis

Results are expressed as mean values ± S.E.M. Statistical analyses were done using the Statistica V8 (Statsoft Inc., Oklahoma, USA) (Papers I and II) and V10 (Paper III) software.

Paper I: The EPM results were analyzed using nonparametric Mann-Whitney's U-test. For gene expression studies, two-way ANOVA (genotype and EPM exposure as independent variables) was applied. *Post hoc* comparisons were performed by means of Tukey's HSD test.

Paper II: The EPM results were analyzed using nonparametric Mann-Whitney's U-test. Corticosterone and Na-pump activity analysis were performed by two-way ANOVA. For gene and protein expression studies, one-way ANOVA was applied. *Post hoc* comparisons were performed by means of unequal N Tukey's HSD test.

Paper III: The EPM and locomotor activity results were analyzed using two-way ANOVA with genotype and ethanol treatment as independent measures. Three-way mixed design ANOVA (genotype × ethanol dose × time) with two between-subjects variables (genotype and ethanol dose) and one within-subjects variable (time) was applied for the statistical analysis of blood ethanol concentrations. Rotarod results were analyzed using repeated measures ANOVA with one within-subjects variable (trial) and two-way ANOVA (genotype x ethanol treatment). Data from the LORR test were analyzed with one-way ANOVA. Two-way ANOVA was applied to analyze gene expression results (genotype × ethanol treatment). *Post hoc* comparisons between individual groups were performed by means of Newman-Keuls test.

4. RESULTS

4.1. ER stress and Wfs1-deficiency (Paper I)

4.1.1. Elevated plus-maze

Wfs1-deficient mice displayed lower exploratory activity in the EPM compared to wild-type mice. Mann-Whitney's U-test established that % of OA entries ($Z=1.97$, $p=0.05$) as well as % on OA ($Z=2.11$, $p=0.03$) and unprotected head-dippings ($Z=1.98$, $p=0.05$) were significantly decreased in Wfs1-deficient mice. Additionally, Wfs1-deficient mice displayed remarkably higher risk assessment behavior ($Z=-2.49$, $p=0.01$) compared to wild-type mice. However, no significant difference was seen if the number of closed arm entries was evaluated in two genotypes ($Z=0.15$, $p=0.88$) (Fig. 2).

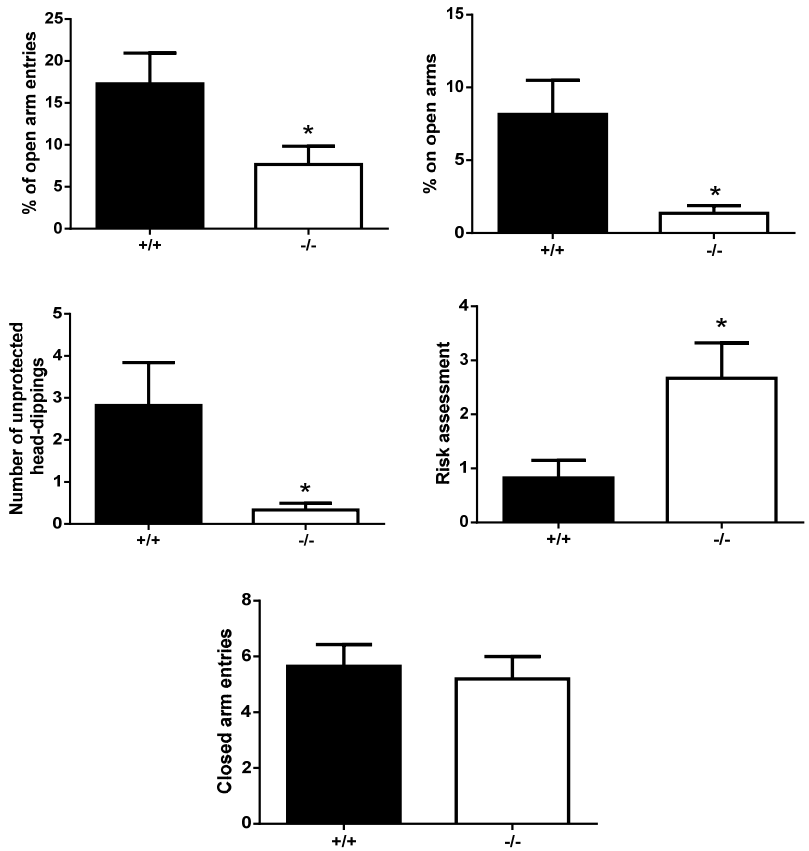

Figure 2. Exploratory behavior of wild-type (+/+) and Wfs1-deficient (-/-) mice in the EPM. * $p<0.05$ compared with wild-type animals, Mann-Whitney U test. n = 9.

4.1.2. Gene expression of ER stress markers

ER stress related gene expression was studied in the ventral striatum, temporal lobe, and hippocampus. Comparison of groups not exposed to EPM did not reveal any significant differences in the ER stress related genes in the brain structures examined. Statistically significant differences were observed after exposure of mice to the EPM in the ventral striatum, hippocampus and temporal lobe.

4.1.2.1. Ventral striatum

Xbp1t and Xbp1s. Significant effect of exposure ($F(1,20)=4.44$; $p<0.05$) was established for Xbp1t. Both genotypes exposed to the EPM demonstrated an increase in Xbp1t expression level compared to naïve mice of the same genotype, but this difference did not reach statistical significance (for wild-type $p=0.26$ and Wfs1-deficient $p=0.38$) (Fig. 3A).

Xbp1s mRNA was significantly affected by exposure ($F(1,20)=8.14$; $p<0.01$) and genotype × exposure ($F(1,20)=10.95$; $p<0.01$). The following *post hoc* comparison indicated that wild-type mice exposed to the EPM had significantly higher expression of Xbp1s mRNA compared to naïve wild-type mice and Wfs1-deficient mice exposed to the EPM (Fig. 3A).

Grp94. Two-way ANOVA indicated significant effects of exposure ($F(1,20)=4.94$; $p<0.05$) and genotype × exposure ($F(1,20)=4.40$; $p<0.05$). Wfs1-deficient mice exposed to the EPM had significantly higher levels of Grp94 expression compared to naïve mice of the same genotype. There was no such change in wild-type mice (Fig. 4).

Grp78 and Atf6α. No genotype- or exposure-related differences were observed in the expression of these genes (Fig 4, Atf6α data not shown).

Chop. Chop mRNA level was significantly influenced by genotype ($F(1,20)=7.6$; $p<0.01$). Comparison of groups exposed to the EPM showed significantly lower level of Chop mRNA in Wfs1-deficient mice compared to the respective group of wild-type mice (Fig. 4).

Perk. A significant effect of exposure ($F(1,20)=5.66$; $p<0.05$) was established. The following *post hoc* comparison showed higher level of Perk mRNA in Wfs1-deficient mice exposed to the EPM compared to respective mice of the same genotype, but this difference did not meet statistical significance ($p=0.07$) (Fig. 4).

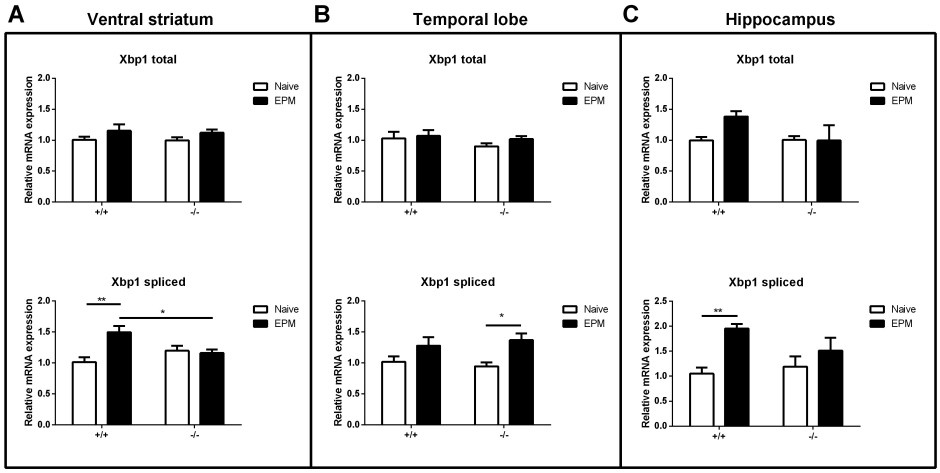

Figure 3. Relative mRNA expression of total and spliced Xbp1 in ventral striatum (A), temporal lobe (B) and hippocampus (C) of WT (+/+) and Wfs1KO (-/-) mice non-exposed (naïve) and exposed to EPM. n=6–9. * - p<0.05 in Tukey HSD test after two-way ANOVA. ** - p<0.01 in Tukey HSD test after two-way ANOVA.

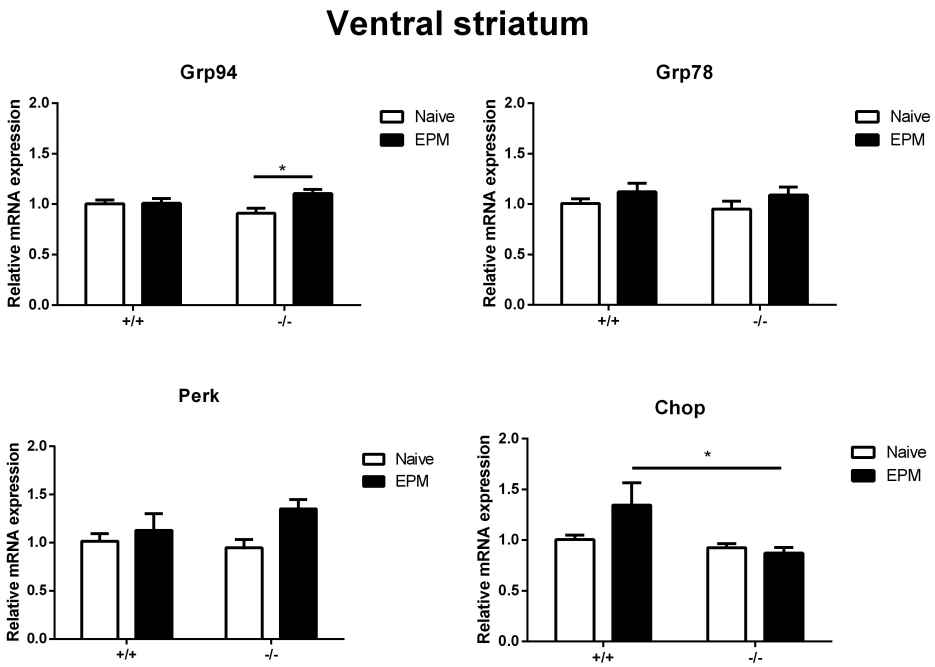

Figure 4. Relative mRNA expression of Grp78, Grp94, Chop and Perk in the ventral striatum of WT (+/+) and Wfs1KO (-/-) mice non-exposed (naïve) and exposed to EPM. n=6–9 * - p<0.05 in Tukey HSD test after two-way ANOVA.

4.1.2.2. Temporal lobe

Xbp1t and Xbp1s. Expression of Xbp1t mRNA was not affected by genotype or exposure (Fig. 3B). Statistically significant effect of exposure was established in Xbp1s mRNA expression level ($F(1,20)=11.06$; $p=0.039$). The following *post hoc* comparison showed that Wfs1-deficient mice exposed to the EPM had significantly increased level of Xbp1s mRNA compared to naïve Wfs1-deficient mice. This effect of EPM exposure was not observed in wild-type mice (Fig. 3B).

Grp78, Grp94, Chop, Atf6α. No genotype- or exposure-related differences were observed in the expression of these genes (Fig. 5; Atf6α.data not shown).

Perk. Significant effect of exposure ($F(1,20)=5.66$; $p<0.05$) was established in the expression of this gene. However, no significant differences between groups were established by *post hoc* comparison of means (Fig. 5).

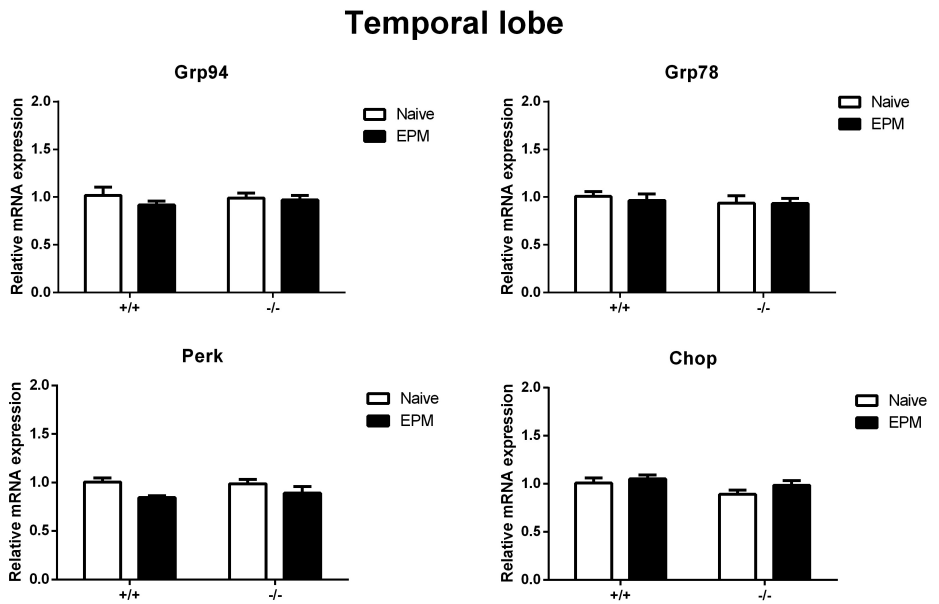

Figure 5. Relative mRNA expression of Grp78, Grp94, Chop and Perk in the temporal lobe of WT (+/+) and Wfs1KO (-/-) mice non-exposed (naïve) and exposed to EPM. n=6–9

4.1.2.3. Hippocampus

Xbp1t and Xbp1s. Expression of Xbp1t mRNA was not affected by genotype or exposure (Fig. 3C). Significant effect of exposure ($F(1,31)=11.02$; $p<0.01$) was seen in the level of Xbp1s mRNA. Compared to naïve wild-type mice, wild-

type mice exposed to the EPM had significantly higher level of Xbp1s mRNA ($p<0.01$) (Fig. 3C).

Grp78. Significant effect of exposure ($F(1,32)=8.40$; $p<0.01$) was established in the expression of this gene. The following *post hoc* comparison showed that wild-type mice exposed to the EPM had significantly increased level of Grp78 mRNA compared to naïve wild-type mice (Fig. 6).

Grp94, Chop and Atf6α. No genotype- or exposure-related differences were observed in the expression of these genes (Fig. 6; Atf6α data not shown).

Perk. Significant effect of genotype ($F(1,20)=8.82$; $p<0.01$) was revealed in the expression of this gene. However, no statistically significant differences between groups were established by *post hoc* comparison of means (Fig. 6).

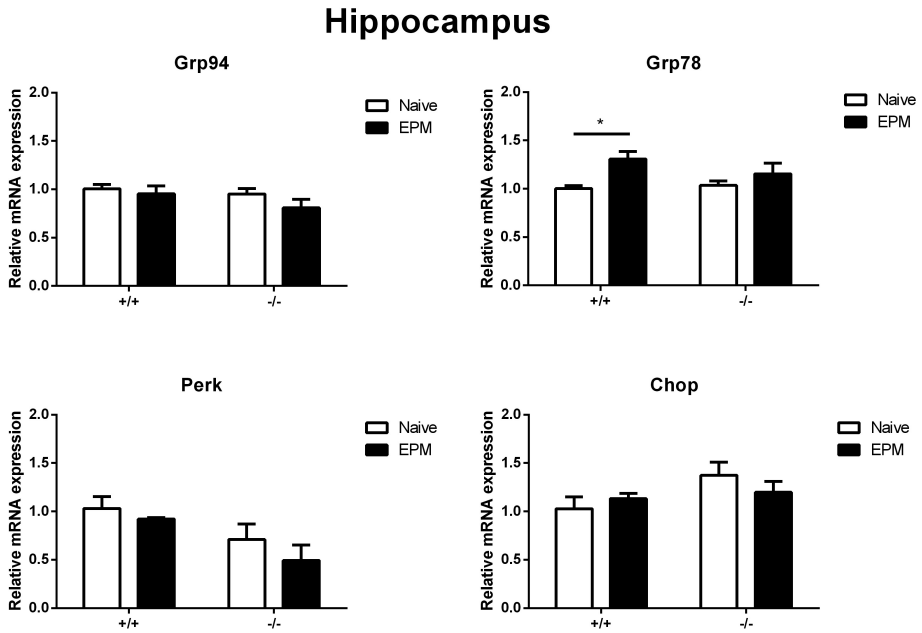

Figure 6. Relative mRNA expression of Grp78, Grp94, Chop and Perk in the hippocampus of WT (+/+) and Wfs1KO (-/-) mice non-exposed (naïve) and exposed to EPM. n=6–9 * - $p<0.05$ in Tukey HSD test after two-way ANOVA.

4.2. Wfs1 and Na^+-K^+ ATPase activity (Paper II)

4.2.1. Elevated plus-maze

As in the previous part of this dissertation, lower exploratory activity of Wfs1-deficient animals in the EPM was detected (Fig. 7). Mice lacking the Wfs1 gene spent significantly less time in the open arms compared to wild-type littermates ($Z=1.98$, $p=0.05$). For the number of closed arm entries, no significant diffe-

rence between the genotypes was observed (Z=1.02, p=0.31). Wfs1-deficient mice performed remarkably fewer unprotected HDs than wild-type mice (Z=2.94, p=0.003). The frequency of protected HDs was also somewhat lower, but did not reach the level of statistical significance (Z=1.59, p=0.11). For the total number of line crossings, no significant difference between the genotypes was seen (Z=1.47, p=0.14).

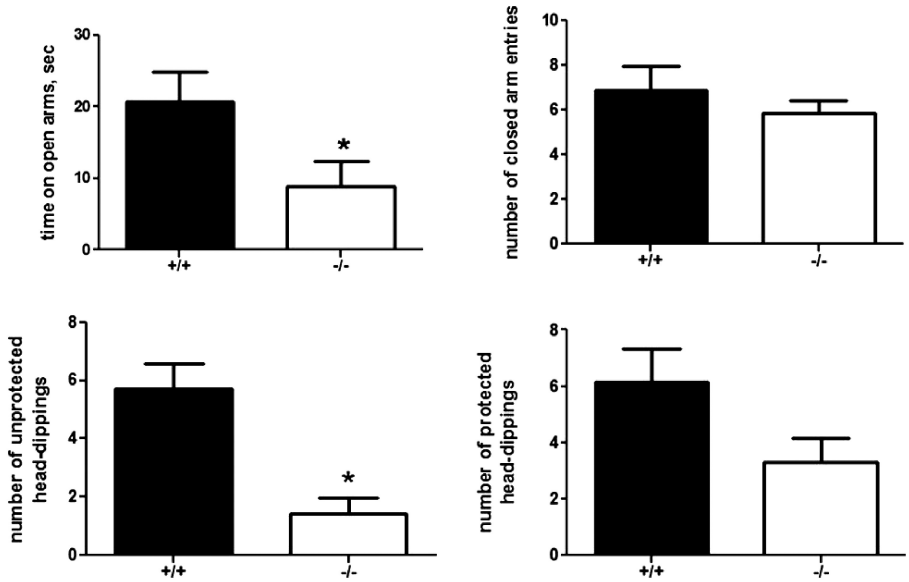

Figure 7. Exploratory behavior of Wfs1-deficient mice in the EPM. Solid bars represent wild-type mice; open bars represent Wfs1-deficient mice. Mean values ± SEM; n = 7 animals in each group. * - $p<0.05$ compared with wild-type animals, Mann-Whitney's U-test.

4.2.2. Measurement of plasma corticosterone level

Plasma corticosterone level was measured in naïve animals and in animals after plus maze exposure. Two-way ANOVA established the following effects on corticosterone levels: exposure ($F(1,16)=95.37$; $p<0.0001$), genotype ($F(1,16)=23.06$; $p<0.001$) and exposure × genotype ($F(1,16)=14.1$; $p<0.01$). Further analysis with *post hoc* unequal-N Tukey's HSD test showed similar baseline corticosterone level in wild-type and Wfs1-deficient mice. The plus maze exposure induced a significant increase in the stress hormone levels in both genotypes. Moreover, comparison of groups exposed to the plus maze test demonstrated that this stressful challenge caused a remarkably greater increase in corticosterone levels of Wfs1-deficient mice as compared to wild-type mice (Fig. 8).

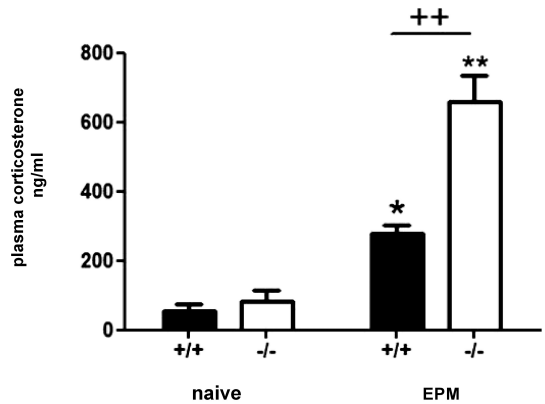

Figure 8. Plasma corticosterone levels in wild-type (solid bars) and homozygous Wfs1-deficient mice (open bars). Mean values ± SEM; n = 4–6. * - $p<0.01$, ** – $p<0.001$ compared with unstressed individuals, unequal-N Tukey's HSD test after significant two-way ANOVA.
++ – $p<0.001$ compared with respective group of wild-type mice.

4.2.3. Na^+-K^+ ATPase activity

We studied Na^+-K^+ ATPase activity in different brain structures (temporal lobe, dorsal and ventral striatum, midbrain, hippocampus, and prefrontal cortex) and found the highest activity of the enzyme in the midbrain and the lowest activity in the temporal lobe of wild-type animals (Fig. 9). The plus maze exposure established the following significant effects of Na^+-K^+ ATPase activity in the temporal lobe: $F(2,42)=14.9$; $p<0.001$ (genotype) and $F(2,42)=6.97$; $p<0.01$ (genotype × exposure). A *post hoc* test showed that naïve Wfs1-deficient mice had significantly higher activity of the Na^+-K^+ ATPase in the temporal lobe compared with wild-type mice (Fig. 10). The anxiogenic-like challenge significantly increased Na^+-K^+ ATPase activity in the temporal lobe of wild-type mice, but not in Wfs1-deficient animals. In the dorsal striatum, Na^+-K^+ ATPase activity was also influenced by genotype and exposure: $F(2,53)=19.5$; $p<0.001$ (genotype); $F(1,53)=50.0$; $p<0.001$ (exposure); and $F(2,53)=94.2$; $p<0.001$ (genotype × exposure) (Fig. 10). Again, Wfs1-deficient mice had remarkably increased activity of Na^+-K^+ ATPase compared with wild-type mice. After plus maze exposure, significantly lower Na^+-K^+ ATPase activity was found in Wfs1-deficient mice compared with wild-type animals. Exposure to the plus maze also remarkably decreased Na^+-K^+ ATPase activity in the ventral striatum ($F(2,35)=5.14$; $p<0.05$ [genotype]; $F(1,35)=26.8$; $p<0.001$ [exposure]; and $F(2,35)=4.31$; $p<0.05$ [genotype × exposure]) and in the midbrain; $F(1,42)=27.43$; $p<0.001$ [exposure]; and $F(2,42)=7.19$; $p<0.01$ [genotype × exposure]) in Wfs1-deficient homozygous animals, but not in wild-type mice (Fig. 10).

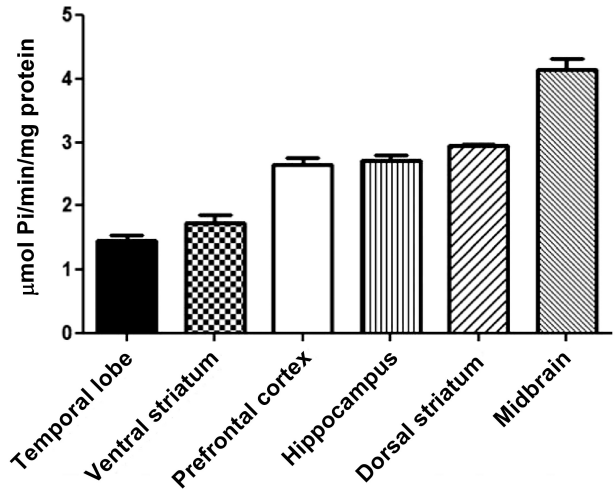

Figure 9. Comparison of Na^+-K^+ ATPase activity in different brain structures in wild-type animals. The highest activity of the Na^+-K^+ ATPase was in the midbrain and the lowest activity was in the temporal lobe.

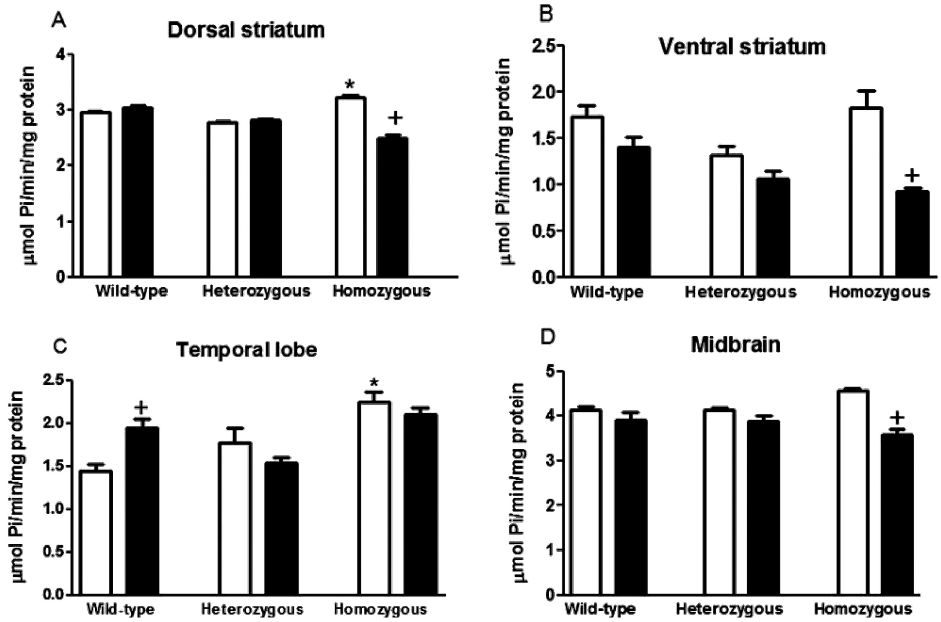

Figure 10. Effect of Wfs1 gene invalidation and exposure to the EPM on Na^+-K^+ ATPase activity in the dorsal striatum (**A**), ventral striatum (**B**), temporal lobe (**C**), and midbrain (**D**). Open bars represent naïve animals; solid bars represent exposure to the EPM. Mean values ± SEM; n = 8–10 in each group. *$p < 0.001$ compared with wild-type mice, unequal-N Tukey's HSD test after significant two-way ANOVA; $^+p<0.001$ compared with the non-exposed group, unequal-N Tukey's HSD test after significant two-way ANOVA.

4.2.4. Expression of Atp1a1 and Atp1b1 in the temporal lobe, dorsal and ventral striatum, and midbrain

We detected a statistically significant decrease in the mRNA expression level of Atp1a1 in the dorsal striatum ($F(2,26)=4.02$; $p<0.05$) and midbrain ($F(2,33)=4.5$; $p<0.05$) of Wfs1-deficient homozygous animals compared with wild-type animals, whereas no significant changes in Atp1a1 mRNA levels between the genotypes were detected in the temporal lobe or ventral striatum (Fig. 11). In the case of Atp1b1 gene expression, we found a statistically significant effect for the temporal lobe ($F(2,19)=4.65$; $p<0.05$; Fig. 11), but not for other brain regions. An elevation of Atp1b1 mRNA expression was established in Wfs1-deficient homozygous mice compared with wild-type animals. However, we did not detect any statistically significant changes in protein amount of α_1 or β_1 subunit between Wfs1-deficient homozygous mice and wild-type animals in the temporal lobe, dorsal and ventral striatum, or midbrain (data not shown).

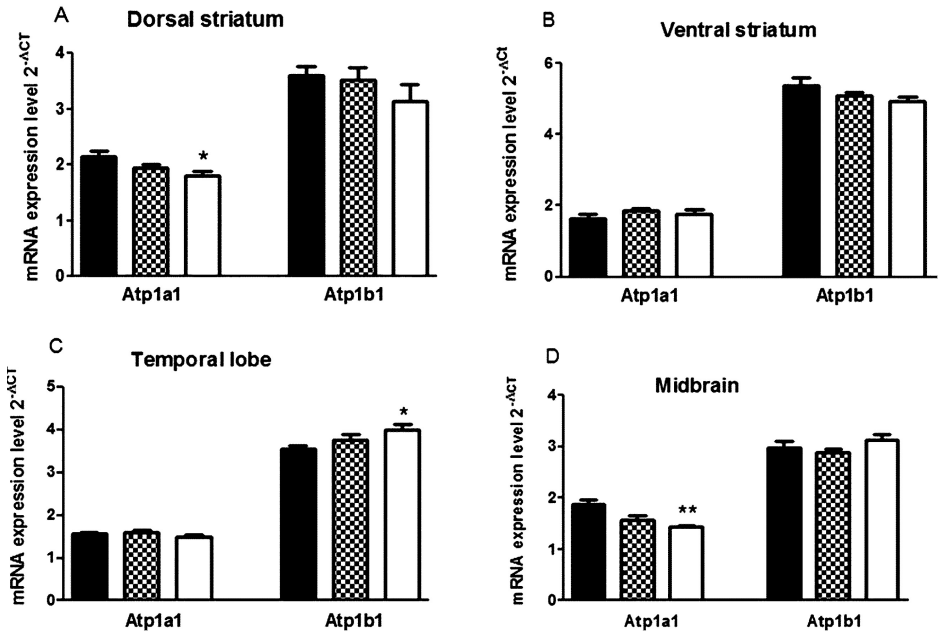

Figure 11. Effect of Wfs1 gene invalidation on the expression of Atp1a1 and Atp1b1 genes in the dorsal striatum (A), ventral striatum (B), temporal lobe (C), and midbrain (D). Solid bars represent wild-type mice; hatched bars represent Wfs1-deficient heterozygous animals; open bars represent Wfs1-deficient homozygous mice. Mean values ± SEM; n = 7–12 in each group. *$p<0.05$ compared with wild-type animals, unequal-N Tukey's HSD test after significant one-way ANOVA, **$p<0.001$ compared with wild-type animals, unequal-N Tukey's HSD test after significant one-way ANOVA.

4.3. Ethanol and Wfs1 (Paper III)

4.3.1. Elevated plus-maze

Ethanol induced a significant increase in open arm exploratory behavior in homozygous Wfs1-deficient mice (Fig. 12). For % of open arm entries, a significant effect of genotype ($F(2,80)=3.0$; $p<0.05$) was seen. Further analysis with unequal N Tukey HSD *post hoc* test showed that ethanol at a dose of 2 g/kg tended to increase the % of open arm entries in Wfs1-deficient mice compared to vehicle-treated Wfs1-deficient mice ($p=0.06$). In wild-type ethanol-treated mice no such difference was observed. Ethanol at a dose of 2 g/kg significantly increased the % of open arm entries in Wfs1-deficient mice compared to the respective group of wild-type mice (Fig. 12A).

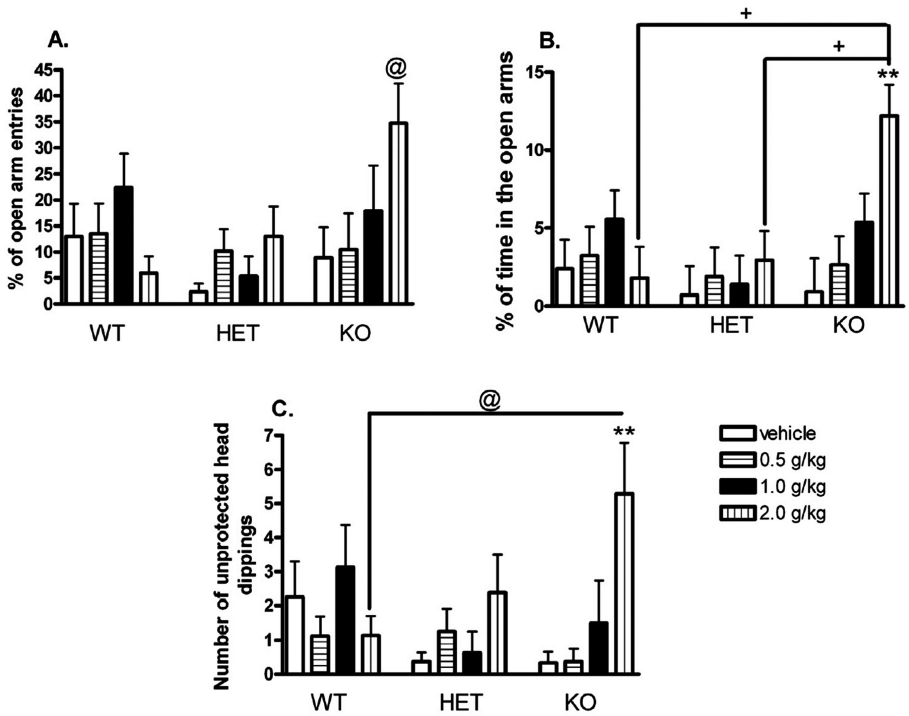

Figure 12. Changes in exploratory behavior of wild-type (WT), heterozygous (HET) and homozygous (KO) Wfs1-deficient mice in the EPM in response to ethanol injection (0.5–2.0 g/kg). n = 7–8 per group. @ – $p<0.05$ compared to wild-type mice treated with ethanol (2 g/kg). ** – $p<0.01$ compared to heterozygous mice treated with saline. + – $p<0.01$ compared to homozygous mice treated with ethanol (2 g/kg).

For % of time spent on the open arms, significant main effects of genotype ($F(2,80)=3.4$; $p<0.05$), treatment ($F(3,80)=2.8$; $p<0.05$) and genotype × treatment ($F(6,80)=2.3$; $p<0.05$) were seen. Wfs1-deficient mice treated with ethanol (2 g/kg) spent significantly higher % of time in the open arms compared to saline-treated Wfs1-deficient mice (Fig. 12B). In wild-type and heterozygous mice, ethanol did not cause a remarkable change in this parameter at any dose used. Comparison of groups treated with ethanol (2 g/kg) showed that the % of time in the open arms was significantly larger in Wfs1-deficient mice than the respective wild-type and heterozygous groups.

For unprotected head-dippings, a significant effect of treatment ($F(3,80)=3.2$; $p<0.05$) and genotype × treatment ($F(6,80)=2.7$; $p<0.05$) was established. Ethanol at a dose of 2 g/kg induced a significant increase in the number of unprotected head-dippings in Wfs1-deficient mice compared to saline-treated Wfs1-deficient littermates (Fig. 12C). There was no such change in this parameter in wild-type and heterozygous mice following ethanol treatment. Comparison of ethanol-treated groups showed that ethanol at the dose of 2 g/kg induced a significant increase in the number of unprotected head-dippings in Wfs1-deficient mice as compared to wild-type mice.

For closed arm entries, significant effect of genotype ($F(2,80)=3.1$; $p<0.05$) was seen. However, no remarkable differences between groups were established by means of post hoc comparison of means (data not shown).

4.3.2. Locomotor activity in motility test

For time in locomotion a significant effect of genotype ($F(2,71)=4.5$; $p<0.01$) was found. Comparison of saline-treated animals revealed lower baseline activity in Wfs1-deficient mice in this parameter compared to wild-type and heterozygous littermates. However, the following analysis with Tukey HSD *post hoc* test did not establish any significant differences between the genotypes. Comparison of ethanol-treated groups showed that the administration of ethanol (0.5–2 g/kg) did not induce any noticeable differences between the genotypes (Fig. 13A).

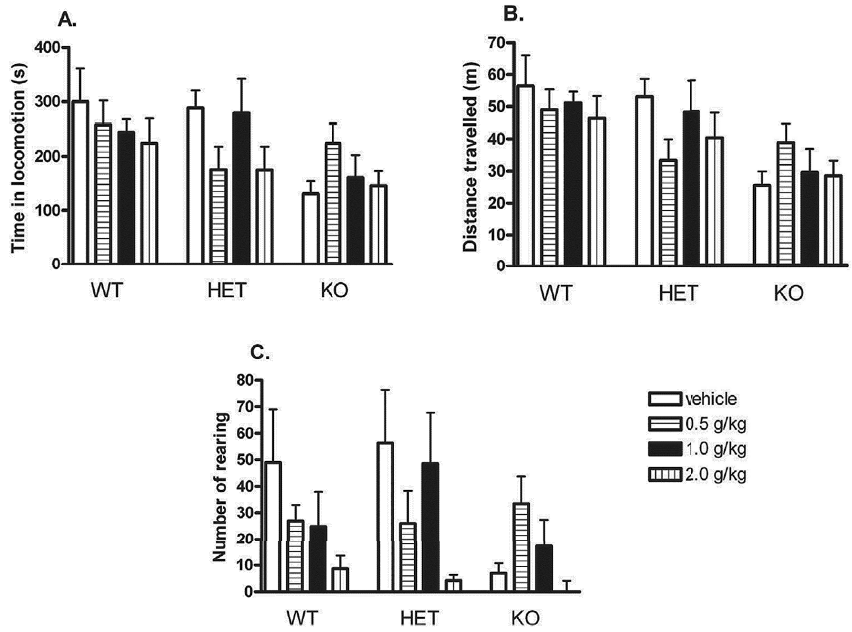

Figure 13. The effect of treatment with ethanol (0.5–2.0 g/kg) on the motor activity of wild-type (WT), heterozygous (HET) and homozygous (KO) Wfs1-deficient mice in the locomotor activity test. n = 7–8 per group.

For distance travelled, there was a significant effect of genotype ($F(2,71)=8.5$; $p<0.01$). Again, the baseline activity of Wfs1-deficient mice tended to be lower compared to other genotypes. However, the following *post hoc* analysis showed that this difference did not reach statistical significance. Treatment with ethanol (0.5–2 g/kg) induced a similar effect on distance travelled in various genotypes (Fig. 13B).

For the number of rearings, a significant effect of treatment ($F(3,71)=3.7$; $p<0.05$) was established, but the following Newman-Keuls *post hoc* test showed no differences between the genotypes (Fig. 13C).

4.3.3. Motor coordination in the rotarod test

There was a significant effect of trial ($F(2,88)=9.2$; $p<0.01$) on the latency to the first fall from the drum. Motor skill performance of Wfs1-deficient mice improved substantially over trials as they displayed longer latency to fall from the rotarod on the third trial compared to the first trial (Table 2). There was no such change in heterozygous and wild-type mice over trials. A significant effect of ethanol treatment ($F(1,44)=35.3$; $p<0.01$) was demonstrated for the latency to fall from the drum. Ethanol (2 g/kg) induced a notable decrease in this parameter in all the genotypes (Fig. 14A).

Table 2. The effect of trial on the number of falls and latency to fall from the drum of wild-type (WT), heterozygous (HET) and homozygous Wfs1-deficient (KO) mice. n = 7–8.

	Trial	WT	HET	KO
Latency to fall from the drum	1	64±14	57±13	47±11
	2	53±12	68±12	67±11
	3	77±12	80±10	101±8*
Number of falls	1	3,1±0,9	2,6±0,6	2,9±0,61
	2	3,7±0,7**	3,1±0,8	1,9±0,4
	3	1,7±0,6	1,6±0,5	1,1±0,6

* – p<0.01 compared to the first trial in Wfs1-deficient mice. ** – p<0.05 compared to the third trial of wild-type mice

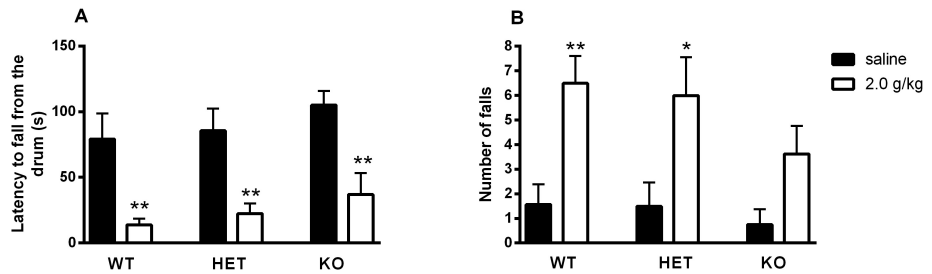

Figure 14. The effect of treatment with ethanol (2.0 g/kg) on the performance of wild-type (WT), heterozygous (HET) and homozygous (KO) Wfs1-deficient mice in the rotarod test. n = 7–8 per group. * – p<0.05 compared to the saline-treated group of the same genotype. ** – p<0.01 compared to the saline-treated group of the same genotype.

For the number of falls, a significant effect of trial ($F(2,88)=9.5$; $p<0.01$) was seen. Wfs1-deficient mice showed a gradual decrease in the number of falls over trials. However, following *post hoc* test did not establish any significant differences between trials. There were no statistically significant improvements in the performance of Wfs1-deficient mice over trials. For wild-type mice, the number of falls decreased significantly on the third trial when compared to the second trial ($p<0.05$) (Table 2). A significant effect ($F(1,44)=21.4$; $p<0.01$) of treatment with ethanol was detected for the number of falls. Ethanol at the dose of 2 g/kg induced less incoordination in mice lacking the Wfs1 gene as alcohol did not cause a notable increase in the number of falls from the drum in Wfs1-deficient mice (Fig. 14B).

4.3.4. Loss of righting reflex (LORR)

4.3.4.1. Ethanol

According to one-way ANOVA, a significant genotype effect for both onset of LORR ($F(2,19)=11.9$; $p<0.01$) and duration of LORR ($F(2,19)=3.4$; $p<0.05$) was established. Treatment with ethanol (4 g/kg) significantly increased both the latency to LORR and the duration of LORR in Wfs1-deficient mice as compared to wild-type littermates (Fig. 15A). Homozygous mice displayed a longer latency to LORR when compared to heterozygous mice that received an injection of ethanol.

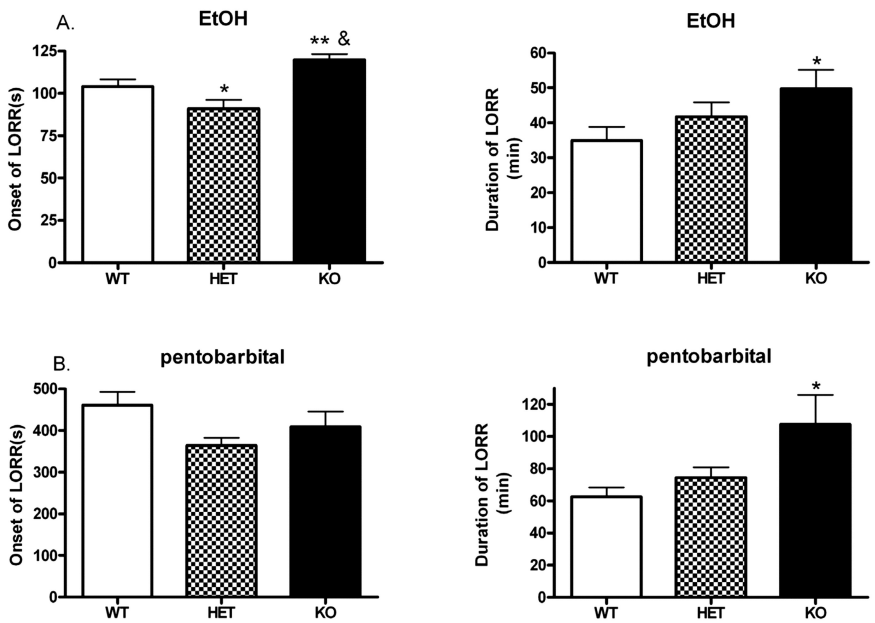

Figure 15. The onset and the duration of LORR in wild-type (WT), heterozygous Wfs1-deficient (HET) and homozygous Wfs1-deficient (KO) mice after injection of ethanol (4.0 g/kg) (A) and pentobarbital (45 mg/kg) (B). n = 7–8 per group. ** – $p<0.01$, * – $p<0.05$ compared to wild-type mice. & – $p<0.01$ compared to heterozygous mice.

4.3.4.2. Pentobarbital

The duration of LORR after injection with pentobarbital was significantly affected by genotype ($F(2,19)=4.1$; $p<0.05$). The following *post hoc* comparison with Newman-Keuls test showed that pentobarbital-treated Wfs1-deficient mice remained asleep for a significantly longer period compared to wild-type littermates (Fig. 15B). No remarkable change in the onset of LORR was seen between the genotypes.

4.3.5. Blood ethanol concentrations

Three-way ANOVA established the following significant effects on blood ethanol concentration: treatment ($F(1,28)=354.1$; $p<0.01$), time ($F(3,84)=161.1$; $p<0.01$), time × treatment ($F(3,84)=16.1$; $p=0.01$). However, there were no significant differences between the genotypes in blood ethanol concentrations after treatment with 2 or 4 g/kg of ethanol (Fig. 16).

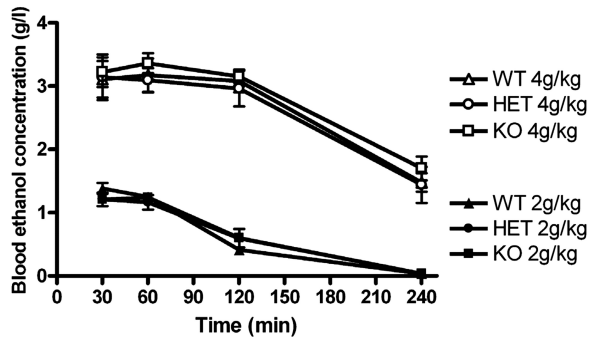

Figure 16. Blood ethanol concentrations (g/l) in wild-type (WT), heterozygous Wfs1-deficient (HET) and homozygous Wfs1-deficient (KO) mice after the administration of 2.0 or 4.0 g/kg of ethanol. n = 7–8 per group.

4.3.6. Gene expression of Gabra1, Gabra2 and Gabra3

In our previous study, comparisons between Wfs1-deficient homozygous and wild-type mice in the expression levels of GABAergic system related genes were performed (Raud *et al.* 2009). The inclusion of heterozygotes would have increased the number of mice used while not necessarily providing more insight into the matter. Therefore, in the gene expression studies only wild-type and Wfs1-deficient homozygous mice were used.

To establish the effect of acute ethanol administration on the expression of Gabra1, Gabra2 and Gabra3 genes, the levels of mRNA expression of these genes were measured in the temporal lobe and frontal cortex at 30 and 60 min after ethanol (2 g/kg) injection.

4.3.6.1. Temporal lobe

Gabra1. Two-way ANOVA did not establish any significant effects for Gabra1 (data not shown).

Gabra2. There was a significant effect of ethanol treatment ($F(2,39)=6.9$; $p<0.01$) for Gabra2. *Post hoc* analysis with unequal N Tukey HSD test established that 60 min after the injection of ethanol homozygous mice displayed a remarkable increase in the levels of Gabra2 as compared to the Wfs1-deficient saline group (Fig. 17A).

Gabra3. Two-way ANOVA did not establish any significant effects for Gabra3 (data not shown).

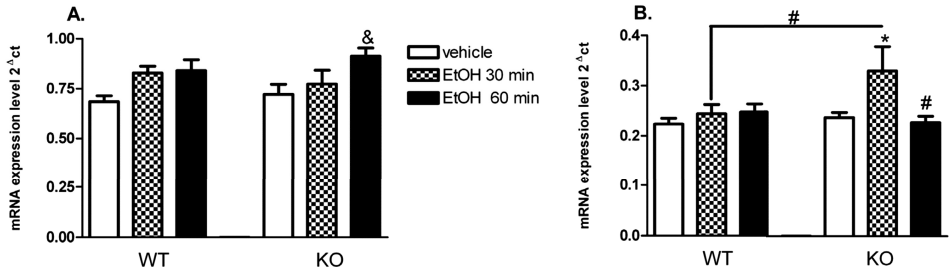

Figure 17. The effect of ethanol on the expression level of Gabra2 subunit of $GABA_A$ receptor in the temporal lobe (A) and frontal cortex (B) in Wfs1-deficient mice 30 and 60 minutes after injection. n = 6–8 per group. * – $p<0.05$ compared to saline-treated homozygous mice. # – $p<0.01$ compared to ethanol 30 min group of homozygous mice. & – $p<0.05$ compared to saline-treated homozygous mice. WT – wild-type mice, KO – Wfs1-deficient homozygous mice.

4.3.6.2. Frontal cortex

Gabra1. Two-way ANOVA did not reveal any significant effects for Gabra1 (data not shown).

Gabra2. There were significant effects of ethanol treatment ($F(2,39)=4.9$; $p<0.01$) and genotype × ethanol treatment ($F(2,39)=3.8$; $p<0.05$) seen for Gabra2. According to *post hoc* analysis, Wfs1-deficient homozygous mice had notably higher levels of Gabra2 mRNA 30 min after ethanol (2 g/kg) administration compared to the respective group of wild-type mice ($p<0.01$) (Fig. 17B). There was also a significant increase in the level of Gabra2 mRNA in homozygous mice 30 min after treatment with ethanol compared to respective vehicle-treated mice. However, at 60 min post injection of ethanol, the levels of Gabra2 in homozygous mice returned to the level of vehicle-treated mice.

Gabra3. Two-way ANOVA did not reveal any significant effects for Gabra3 (data not shown).

5. DISCUSSION

Epidemiological and gene polymorphism studies in humans indicate a relationship between WFS1 and psychiatric disorders (Koido *et al.* 2005; Swift & Swift 2005). Currently the mechanisms underlying development of neuropsychiatric symptoms in patients with WS are poorly understood. Presuming that Wfs1 animal model adequately reflects the symptoms of the human disease, this work was devoted to studying the molecular mechanisms of increased anxiety in Wfs1-deficient mice. In the three articles, comprising this dissertation, we studied interactions related to anxiety between Wfs1 protein and various molecular targets – ER stress markers (Paper I), Na+-K+ ATPase (Paper II), and the GABA system (Paper III) – in the brain structures implicated in emotional behavior in Wfs1-deficient mice.

In the EPM, Wfs1-deficient mice displayed augmented anxiety-like behavior compared to wild-type mice. This was reflected by a significant decrease in open arm entries, reduced time spent on the open arms and diminished frequency of unprotected head-dippings without a significant decrease in locomotor activity (number of closed arm entries). Additionally, Wfs1-deficient mice displayed increased risk assessment behaviors compared to wild-type littermates. These behavioral findings are in line with the results from previous studies (Luuk *et al.* 2009; Raud *et al.* 2009). Exploratory activity on open arms reflects an animal's innate motivation to explore novel environment (Walf & Frye 2007) and risk assessment behaviors can be interpreted as cautious exploration (neophobia) induced by a potentially dangerous novel environment (Blanchard *et al.* 1993). Besides behavioral alterations, hormonal changes reflecting the stress level of animals were detected in EPM-exposed Wfs1-deficient mice. EPM exposure increased corticosterone level significantly in both genotypes, but judging by the differences in stress hormone levels between genotypes, Wfs1-deficient mice were significantly more stressed by the exposure to the EPM than wild-type littermates. These behavioral and hormonal characteristics make Wfs1-deficient mice a suitable model for studying the molecular mechanisms of increased anxiety.

5.1. Stressful situation induces changes in the expression of ER stress markers in brain regions associated with dominant behavioral traits of the genotypes (Paper I)

Physical/psychological stress has been shown to induce ER stress and there are studies demonstrating higher levels of ER stress markers in cells lacking functional Wfs1 (Fonseca *et al.* 2010; Mondal *et al.* 2015). However, the effect of Wfs1-deficiency on ER stress markers in the mouse brain nor the effect of a mild stressor, such as EPM, on the expression of ER stress markers, has not been investigated before. In this study we showed that after exposure to EPM

there was a statistically significant increase in the expression of Grp94 in the ventral striatum of Wfs1-deficient mice, but not in wild-type littermates. Grp94 is a chaperon that in principle could be the downstream target for both Ire1α and Atf6α pathways (Lee *et al.* 2003). Expression of Atf6α remained unchanged in the ventral striatum after exposure to EPM in both genotypes, but the amount of spliced Xbp1 that indicates the splicing activity on Ire1α, was significantly increased in the ventral striatum of wild-type mice after exposure to EPM. No such change was seen in Wfs1-deficient mice. A similar increase in Ire1α activity was observed in the hippocampus of wild-type mice, but not in Wfs1-deficient mice. Interestingly, Ire1α splicing activity was higher in the temporal lobe of Wfs1-deficient mice. In terms of behavior-brain relationships, neophobic and/or low exploratory behavior, seen in Wfs1-deficient mice, is regulated by the amygdala (situated in the temporal lobe) (Adamec *et al.* 2001; Martin *et al.* 2009; Davis *et al.* 2010). Increased exploratory drive, observed in wild-type mice, is regulated by the ventral striatum (Rebec *et al.* 1997; Cardinal *et al.* 2002; Liljeholm & O'Doherty 2012; Schmidt *et al.* 2012). Both reward- and goal-directed as well as emotion-associated functions are related to the hippocampus (Kennedy & Shapiro 2009; Royer *et al.* 2010; Sotres-Bayon *et al.* 2012). These findings can indicate aberrant coping ability with a stressful situation in the case of deficient function of the Wfs1 gene.

Wfs1-deficient mice exposed to the EPM had a significantly lower expression of Chop in the ventral striatum compared to the respective group of wild-type mice. Additionally, compared to naïve mice, the EPM tended to increase the expression of Chop in wild-type mice, but not in Wfs1-deficient mice. Interestingly, similar Chop dynamics, as in Wfs1-deficient mice, was found in patients with bipolar disorder. Namely, no differences were seen in basal transcript levels of CHOP between patients and healthy controls. However, a significant elevation of CHOP levels was seen after treatment with ER stressors only in controls, indicating impaired CHOP induction in patients with bipolar disorder (So *et al.* 2007). Our previous studies have shown that Wfs1-deficient mice display passive coping style and decreased exploratory drive, analogous to depressive and anxiety symptoms in humans (Luuk *et al.* 2009; Reimets *et al.* 2016), suggesting that Wfs1-deficient mouse could be a suitable animal model for anxiety/depression studies. Impaired Chop induction, among other findings in the current study, seems to strengthen this suggestion.

The EPM test induces mild and short-term stress and this could explain why changes were detected only in some ER stress markers. This is different from other studies, where more severe stressors induced an increase in the expression of most of the ER stress markers in different brain regions (Pavlovsky *et al.* 2013; Tan *et al.* 2015; Timberlake & Dwivedi 2015). A mild stressor, on the contrary, allows to study the induction of earlier and more sensitive ER stress markers. Among them, Xbp1s mRNA was significantly increased in both genotypes after exposure to the EPM, whereas higher expression of Xbp1s was found in brain regions responsible for dominant behavioral trend of the genotype, i.e. in anxious Wfs1-deficient mice in the temporal lobe and in

curious wild-type mice in the ventral striatum. A correlation between ER stress activation in the amygdala and behavioral trends was also demonstrated by Huang and colleagues (Huang *et al.* 2013), showing the effect of social defeat stress (moderate stress) on the protein level of Grp78 and Chop. They found significantly higher levels of Grp78 and Chop in the amygdala of mice susceptible to stress, but not in unsusceptible mice.

It is proposed that ER stress activation by psychological stressors is triggered by oxidative stress and oxidative stress could be activated by neurotransmitters (Hayashi *et al.* 2009). For example, oxidative stress can be triggered by auto-oxidation of dopamine (DA) which generates H_2O_2 (Cadet & Brannock 1998), or by tryptophan, precursor of serotonin (5-HT), which induces lipid peroxidation and decreases antioxidative capacities (Feksa *et al.* 2006). Our previous studies have shown significantly altered function of dopaminergic and serotonergic systems in Wfs1-deficient mice since naïve Wfs1-deficient mice displayed increased density of dopamine D1 receptors (Tekko *et al.* 2017) and decreased levels of dopamine and 5-HT transporters mRNA (Visnapuu *et al.* 2013; Reimets *et al.* 2016). Importantly, the two latter studies also showed that mild stress caused increased levels of DA and 5-HT in the dorsal and ventral striatum of wild-type mice, but not in Wfs1-deficient mice. This finding may explain the elevation of Xbp1s in the ventral striatum of wild-type mice. Extending these causal connections to the temporal lobe, differences in the dynamics of neurotransmitters between genotypes would be expected, i.e. the EPM exposure could induce higher levels of neurotransmitters in Wfs1-deficient mice leading to higher expression of Xbp1s mRNA, whereas in wild-type mice the levels of neurotransmitters and ER stress markers could remain unaffected. This suggestion is supported by the finding that exposure to the EPM increases the level of 5-HT in the amygdala of anxious, but not in curious rats (Andersen & Teicher 1999).

Concerning the hippocampus, a structure that is associated with motivations and neophobia, the expression of ER stress markers in wild-type mice tended to follow the expression pattern in the ventral striatum and in Wfs1-deficient mice the expression pattern of the temporal lobe. Thus, the dynamics of hippocampal ER stress markers seems to correlate with the brain region that determines the behavioral trends of mice in the EPM.

Induction of Xbp1s, the downstream target of ER stress sensor Ire1α, implicates increased activity of Ire1α mediated pathway (Yoshida *et al.* 2001; Wang & Kaufman 2012; Xie *et al.* 2015). Thus, in wild-type mice, the EPM exposure activated only Ire1α ER stress pathway. Concerning other signaling pathways, Perk and Atf6α, mild stress even tended to decrease their expression in wild-type mice. For example, contrary to Xbp1s, the EPM induced decreasing trends in the level of Atf6α mRNA in all studied brain regions and a similar decreasing trend was evident for Perk in the hippocampus of wild-type mice. Therefore, we connect the EPM-induced elevation in Grp78 mRNA expression in the hippocampus of wild-type also with the Ire1α pathway. In Wfs1-deficient mice exposed to the EPM, Ire1α signaling cascade was also

activated, but in the temporal lobe, and additionally Atf6α pathway's activation in the ventral striatum could be suggested. Indeed, Wfs1-deficient mice had a significant EPM-induced increase in the ventral striatum Grp94 mRNA, a chaperon which in principle could be the downstream target for both Ire1α and Atf6α pathways (Lee *et al.* 2003), but since there was an increasing trend in Atf6α and a decreasing trend in Xbp1s expression in the ventral striatum of Wfs1-deficient mice, the higher expression of Grp94 mRNA could be primarily connected with the Atf6α pathway. According to Fonseca and colleagues (Fonseca *et al.* 2010), Atf6α ER stress signaling pathway is suppressed via formation of Atf6α-Wfs1 complex. This conclusion is also supported by a study by Morikawa and colleagues, showing that the mutation of WFS1 increased the active form of ATF6α (Morikawa *et al.* 2017). Therefore, Atf6α pathway activation in the ventral striatum of Wfs1-deficient mice is likely connected with the impaired function of the Wfs1 gene.

Studying naïve Wfs1-deficient mice at the age of three months, we did not detect a significant increase in the indices of ER stress compared to naïve wild-type littermates. In fact, the expression pattern of ER stress markers of adult Wfs1-deficient mice was similar to the one seen in their early postnatal period (Tekko *et al.* 2014). As the pathogenesis of WS is related to chronic ER stress (Lu *et al.* 2014; Urano 2014), it raises the question why the level of ER stress markers in the brain does not differ between young Wfs1-deficient mice and young wild-type mice and why significant ER stress activation can be induced only by a psychological stressor. One of the possibilities is that the signs of ER stress occur in naïve mice older than three months. A recent study with Wfs1-deficient fruit flies showed that Wfs1-deficiency itself does not induce an activation of ER stress in old Drosophila brain (Sakakibara *et al.* 2018). However, Wfs1-deficient flies were notably vulnerable to various stressors e.g. oxidative stress related to neurodegeneration, an alteration also seen in patients with WS. Therefore, the most probable explanation for unchanged ER stress indices in young Wfs1-deficient mice is that deficient function of the gene alone does not disrupt cellular functions. Rather, Wfs1 has a crucial role in behavioral adaptation mechanisms to novel and stressful situations and Wfs1-deficiency in mice essentially disturbs adequate stress response.

5.2. Wfs1-deficiency leads to elevated activity of Na^+-K^+ ATPase in brain regions associated with anxiety (Paper II)

It has been established that changes in Na^+-K^+ ATPase activity and/or mutations in its subunits in different brain structures lead to neuronal dysfunction and are associated with an increased risk of developing depression, anxiety, and bipolar disease (Goldstein *et al.* 2006; Crema *et al.* 2010; Kirshenbaum *et al.* 2011). In this study, elevated Na^+-K^+ ATPase activity was measured in the temporal lobe (including amygdala) and dorsal striatum (including caudate putamen) of Wfs1-deficient mice when compared to wild-type mice. These brain structures have

previously been shown to have a very high Wfs1 mRNA expression level, first detected as early as embryonic day (E) 15.5. Synaptophysin 1, a neuronal differentiation marker, is also highly expressed in the amygdala and caudate putamen at E 15.5, indicating that Wfs1 may be involved in the differentiation of neurons in the developing brain (Tekko *et al.* 2014). It can be speculated that Wfs1-deficient animals have defects in the formation of synapses, especially in the extended amygdala. In wild-type mice, the elevation of Na^+-K^+ ATPase activity in the temporal lobe was seen after exposure to EPM. Given that the amygdala and striatum are both involved in the regulation of anxiety, our results of elevated Na^+-K^+ ATPase activity might reflect increased anxiety in Wfs1-deficient mice (Luuk *et al.* 2008).

On the other hand, a challenging environment, such as EPM, reduced the activity of Na^+-K^+ ATPase in the midbrain and ventral and dorsal striatum in Wfs1-deficient animals compared to the non-exposed group. It has been shown that dopamine regulates Na^+-K^+ ATPase activity in the striatum (Wu *et al.* 2007), and it has previously been demonstrated by our group that a novel and aversive environment induces a reduction in dopamine metabolism in homozygous Wfs1-deficient mice compared to heterozygous and wild-type mice (Visnapuu *et al.* 2013). Therefore, impaired dopamine release in Wfs1-deficient mice (Matto *et al.* 2011) may be the key factor of Na^+-K^+ ATPase activity in the striatum and midbrain in response to the EPM exposure.

To investigate the mechanisms of elevated activity of Na^+-K^+ ATPase we decided to measure mRNA expression levels of its relevant subunits.

We found an increase in Na^+-K^+ ATPase β1 subunit mRNA expression in temporal lobe of naïve Wfs1-deficient mice compared to naïve wild-type mice. This finding is correlated with an increase of Na^+-K^+ ATPase activity. β1 sub-unit determines the level of the α1 subunit in the plasma membrane and is responsible for the stability of the α1 subunit during its synthesis in the ER (Rajasekaran *et al.* 2004). The correlation between the elevation of Na^+-K^+ ATPase activity and the increased level of β1 subunit mRNA has previously been shown in the renal cell line LLC-PK_1 (Lescale-Matys *et al.* 1990) and in the liver of diabetic rats (Sennoune *et al.* 2000). One can speculate, that a higher β1 subunit mRNA level in the temporal lobe of Wfs1-deficient mice might help to compensate for the increased ATP1B1 protein degradation rate shown in WFS1-depleted cells (Gharanei *et al.* 2013).

Since WFS1 protein is expressed in the secretory vesicles of neuroblastoma cells and is a molecular partner of the V1A subunit of H^+ V-ATPase (Gharanei *et al.* 2013) it might also regulate the activity of the vacuolar H^+ pump, which is responsible for the acidification of synaptic vesicles and the release of neurotransmitters in the nervous system (Forgac 2007). Therefore, a decrease of mRNA level of the Atp1a1 in the midbrain and dorsal striatum in Wfs1-deficient animals might be related to altered activity of vacuolar H^+ pump and impaired release of dopamine. Also, the phosphorylation/dephosphorylation of catalytic subunits regulates short-term changes of Na^+-K^+ ATPase activity (Poulsen *et al.* 2010). It has been shown that changes of Na^+-K^+ ATPase activity

are regulated through the phosphorylation primarily of their α1 subunit, whereas the total amount of α1 subunit remains unchanged (El-Beialy *et al.* 2010; Marquezan *et al.* 2013). It is also possible that the decrease of Na^+-K^+ ATPase activity might be related to increased phosphorylation level of the α1 subunit in the striatum and midbrain after EPM exposure.

In conclusion, Wfs1-deficient animals display brain-region-specific changes in Na-pump activity and mRNA expression level of α1 and β1 subunits. The present study supports the hypothesis about the role of the Na^+-K^+ ATPase in emotional disorders, because in WS patients several psychiatric disorders are evident, including symptoms of severe depression. However, further studies are required to clarify the molecular mechanisms responsible for the brain-region-specific changes of Na^+-K^+ ATPase activity in Wfs1-deficient animals.

5.3. Changes in the functionality of GABAergic system in the brain of Wfs1-deficient mice (Paper III)

It has been shown that the re-uptake of GABA, the principal neurotransmitter in the amygdala, is impaired in Na^+-K^+ ATPase α2 subunit-deficient mice and long term blockade of $GABA_A$ receptor resulted in higher Na^+-K^+ ATPase activity in rats' brain (Ikeda *et al.* 2003; Kaur *et al.* 2004) indicating a connection between Na^+-K^+ ATPase and the GABAergic system. To further investigate this connection and the involvement of GABA in the behavioral traits associated with Wfs1-deficiency, we used ethanol as a known modulator of GABAergic system. Anxiolytic dose of ethanol significantly increased exploratory activity of Wfs1-deficient mice in the EPM, but not of wild-type mice. This indicates that ethanol has stronger anxiolytic-like effect on Wfs1-deficient mice compared to their wild-type littermates. Since the overall activity of mice, reflected by closed arm entries, did not significantly change after treatment with ethanol, it is evident that anxiolytic-like effect of alcohol is not due to increased locomotor activity in Wfs1-deficient mice. This finding is also supported by the results of the motility box test that revealed no differences in motor activity between wild-type and Wfs1-deficient mice after ethanol administration. To exclude the potential effect of altered metabolism of ethanol on behavior, blood ethanol levels were measured in both genotypes and no significant differences were identified.

In addition to higher sensitivity to anxiolytic-like effect of ethanol, Wfs1-deficient mice have decreased sensitivity to the sedative/ataxic effect of ethanol. Performance in the rotarod test was less affected in Wfs1-deficient mice and the LORR test revealed differences in the sedative response of genotypes to ethanol as the onset of LORR was delayed in Wfs1-deficient mice. On the other hand, Wfs1-deficient mice were more susceptible to the hypnotic effect of ethanol as the duration of LORR was significantly increased in Wfs1-deficient mice. In comparison, pentobarbital (45 mg/kg), a $GABA_A$ receptor modulator, induced a similar onset of LORR, whereas Wfs1-deficient mice remained asleep for a

longer duration than wild-type mice. This shows that changes in the function of $GABA_A$ receptor are probably responsible for the higher sensitivity to the hypnotic effect of ethanol in Wfs1-deficient mice.

Raud and colleagues have found that Gabra2 is down-regulated in the frontal cortex and temporal lobe of naïve Wfs1-deficient mice and an exposure to EPM induced a downregulation of Gabra2 in wild-type mice, making the expression levels of Gabra2 similar between genotypes (Raud *et al.* 2009). To test $GABA_A$ receptor subunit involvement in the modulation of the effect of ethanol, the expression levels of Gabra1, Gabra2 and Gabra3 genes were measured. We found that an anxiolytic-like dose of ethanol had no effect on the mRNA expression levels of Gabra1 and Gabra3, but 30 minutes after the injection, an increase of Gabra2 mRNA was seen in the frontal cortex of Wfs1-deficient mice. In the temporal lobe of Wfs1-deficient mice, changed mRNA expression became evident 60 minutes after ethanol injection. Both brain areas take part in the regulation of emotions, including anxiety (Davis & Shi 2000; Martijena *et al.* 2002; Finn *et al.* 2003; Leistedt & Linkowski 2013). This, and a previous finding, where an exposure of mice to EPM induced a similar downregulation of Gabra2 mRNA in wild-type mice to the level of anxious Wfs1-deficient mice, supports the involvement of Gabra2 mRNA expression level in the modulation of anxiety-like behavior in mice.

In several studies, a tight interaction between GABAergic and dopaminergic systems has been shown (Brambilla *et al.* 2003; Matsumoto *et al.* 2003). Dopamine is known to modulate GABA release in the brain (Delgado *et al.* 2000; Seamans *et al.* 2001), but vice versa interaction has also been shown. A recent study by Nikolaus and colleagues showed that $GABA_A$ receptor agonist muscimol, but not $GABA_A$ receptor antagonist bicuculline, reduces $D_{2/3}$ receptor radioligand binding to the striatum and ventral tegmental area relative to baseline. Interestingly, this study also demonstrated that motor/exploratory behaviors were diminished after muscimol, but elevated after bicuculline administration (Nikolaus *et al.* 2018). Altered function of $GABA_A$ receptors and D_2 receptor-like binding sites have been shown to be implicated in anxiety disorders (Nikolaus *et al.* 2010). Taking into account that Wfs1-deficient mice have lower levels of D_2 receptor mRNA in the dorsal striatum (Visnapuu *et al.* 2013) and altered $GABA_A$ receptor function in the temporal lobe, one may conclude that these molecular changes underlay the elevated anxiety and altered sensitivity to ethanol observed in Wfs1-deficient mice. Precise interactions between dopamine and GABA systems in Wfs1-deficient mice remain to be clarified in further studies.

6. SUMMARY

In the present study, potential mechanisms of elevated anxiety in young female Wfs1-deficient mice were investigated. ER stress, Na^+-K^+ ATPase and GABAergic system were chosen as the molecular targets for this study due to their connection to the Wfs1 gene and previous association of disturbances in these systems with mood disorders (Morris *et al.* 2006; Navarro *et al.* 2006; Crema *et al.* 2010; Xiang *et al.* 2017). Correlations between exploratory behavior in the EPM and the expression of ER stress markers and Na^+-K^+ ATPase activity in wild-type and Wfs1-deficient mice, as well as the effect of Wfs1-deficiency on the functioning of the GABAergic system, were studied. In the EPM, increased anxiety of Wfs1-deficient mice was accompanied with higher corticosterone level, indicating that novel environment is more stressful for Wfs1-deficient animals than for wild-type animals. Mice lacking Wfs1 gene were also more sensitive to the anxiolytic and hypnotic effects of ethanol, which is probably due to the altered function of the GABAergic system. Besides that, Wfs1-deficiency seriously disturbs the function of the dopaminergic system in the forebrain structures (Visnapuu *et al.* 2013). The altered functions of GABA- and dopaminergic systems seem to be related to the compromised release of insulin (IGF-1) in Wfs1-deficient mice (Figure 18).

EPM, a mild stressor, induced only the expression of earlier and/or the most sensitive ER stress markers. Among them, the most abundantly expressed was Xbp1s, a downstream target of the Ire1α pathway. In wild-type mice, elevated expression of Xbp1s mRNA was found in the ventral striatum, a brain region connected with motivations (including curiosity), while in Wfs1-deficient mice, increased Xbp1s expression was detected in the temporal lobe, which is related to anxious behavior. Such a correlation between the activated brain region and Xbp1s-Ire1α cascade could be related to the affected function of GABA- and monoaminergic activity in Wfs1-deficient mice (Figure 18).

For Na^+-K^+ ATPase, a correlation between the activated brain region and enzyme activity also seems to be related to differences in GABA- and dopaminergic activity between the genotypes (Figure 18). Again, Wfs1-deficient mice had significantly higher Na^+-K^+ ATPase activity in the temporal lobe. On the other hand, lower level of Na^+-K^+ ATPase was detected in the dorsal and ventral striatum of Wfs1-deficient mice after exposure to EPM.

Altogether, it is likely that the reduced activity of the GABAergic system plays a central role in increased anxiety, whereas the reduced activity of the dopaminergic system is responsible for reduced motivations established in Wfs1-deficient mice. The alterations in Na^+-K^+ ATPase activity and levels of ER stress markers in the temporal lobe are probably associated with the impaired function of GABAergic neurotransmission in this brain structure, whereas the same is true for the dopaminergic system in the dorsal and ventral striatum. Wfs1-deficient mice display altered glucose metabolism because of deficient release of insulin from β-cells in the pancreas. A recent study

demonstrated that GABAergic synaptic transmission is a target in the amygdala for insulin receptor signaling that may underline insulin modulation of emotion- and feeding-related behaviors (Korol *et al.* 2018). One may speculate that insufficient function of insulin in Wfs1-deficient mice may be responsible for the reduced activity of GABAergic transmission in the temporal lobe and thus for the increased anxiety established in these mice. A similar inhibition of the function of the dopaminergic system occurs in the dorsal and ventral striatum due to reduced production of insulin (or IGF-1) in Wfs1-deficient mice (Visnapuu *et al.* 2013). Altogether, the compromised exploratory drive in Wfs1-deficient mice is due to the severe alterations in both motivational (curiosity) and emotional (neophobia) mechanisms based on GABA- and dopaminergic neurotransmission in the forebrain (Figure 18). Further studies are needed to establish the intimate mechanisms of these relevant interactions.

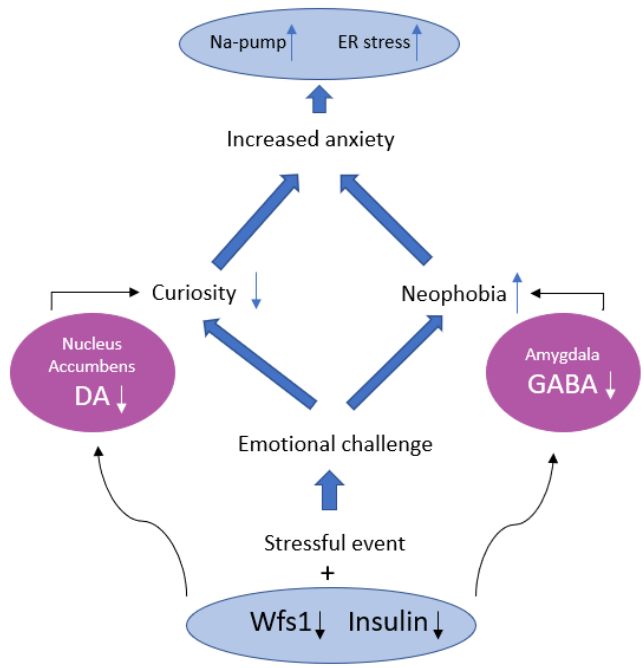

Figure 18. Schematic representation of the mechanisms behind elevated anxiety in Wfs1-deficient mice, based on this study and our previous findings (Raud *et al.* 2009; Visnapuu *et al.* 2013) summarized by the author of this study. DA – dopamine; Na-pump – Na^+-K^+ ATPase activity;

CONCLUSIONS

1. We found no changes in the expression levels of ER-stress markers in naïve Wfs1-deficient mice compared to respective wild-type mice. Differences between genotypes became evident after exposure to the elevated plus-maze acting as a mild stressor. An elevation in the expression of the earliest and most sensitive ER-stress markers occurred in brain structures responsible for the dominant behavioral trait of the genotype. In anxious Wfs1-deficient mice Xbp1s mRNA expression level was elevated in the temporal lobe, whereas in curious wild-type mice it occurred in the ventral striatum.
2. Anxious Wfs1-deficient mice had increased activity of Na^+-K^+ ATPase in brain structures involved in the regulation of emotional behavior – temporal lobe. Elevated Na^+-K^+ ATPase activity was associated with higher mRNA expression level of Na^+-K^+ ATPase β1 subunit found in the temporal lobe of Wfs1-deficient mice. An increase in gene expression level is likely to be a compensatory mechanism to deal with higher protein degradation level of Atp1b1, a molecular partner of Wfs1. Exposure to the EPM reduced the activity of Na+-K+ ATPase in brain areas implicated in dopaminergic neurotransmission. This supports the evidence for disturbed function of the dopaminergic system in Wfs1-deficient mice and, therefore, reflects altered motivations in these animals.
3. Wfs1-deficient mice displayed higher sensitivity to anxiolytic-like and hypnotic effects of ethanol than wild-type mice. After the administration of an anxiolytic dose of ethanol, Wfs1-deficient mice showed less anxiety-like behaviors in the EPM, spending longer time in exploring the aversive open parts of the apparatus. Moreover, genetically modified mice lost the righting reflex for a longer time after the administration of a hypnotic dose of ethanol. On the other hand, Wfs1-deficient mice showed decreased sensitivity to the sedative/ataxic effect of ethanol. The mRNA expression level of Gabra2, a subunit of $GABA_A$ receptor, was increased after ethanol injection in the frontal cortex and temporal lobe of Wfs1-deficient mice, but not in wild-type mice. These differences in Gabra2 levels are most probably related to the different reaction of the genotypes to ethanol administration.
4. It became evident that the ER stress response, Na^+-K^+ ATPase activity, and function of the GABAergic system were all affected in young Wfs1-deficient mice. Remarkably, most prominent changes occurred in the temporal lobe, a brain structure containing the amygdala, and in the ventral/dorsal striatum. Both brain regions are associated with the regulation of anxiety and motivations. These structures are under the modulatory role of the GABA- and dopaminergic systems, which in turn have an impact on the behavioral and molecular changes in Wfs1-deficient animals. Altered function of GABAergic neurotransmission in the temporal lobe is likely a reason for the increased anxiety in Wfs1-deficient mice, whereas the reduced function of the dopaminergic system in the ventral/dorsal striatum is linked

to reduced motivations. Wfs1-deficient mice in 129Sv/Bl6 background display features which make them a useful model of increased anxiety in mice.

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SUMMARY IN ESTONIAN

Ärevuse molekulaarsete mehhanismide uurimine emastel Wfs1-puudulikkusega hiirtel

Antud töö keskendub emastel Wfs1-puudulikkusega hiirtel esineva ärevuse tekkimise mehhanismide uurimisele. Uuringu sihtmärkideks said valitud ER stress, Na^+-K^+ pump ja GABAergiline süsteem, kuna muutusi nendes süsteemides on varasemalt seostatud meeleoluhäirete tekkimisega (Morris *et al.* 2006; Navarro *et al.* 2006; Crema *et al.* 2010; Xiang *et al.* 2017).

Uurisime tõstetud pluss-puuri abil uudistamiskäitumise seoseid endoplasmaatilise retiikulumi (ER) stressi markerite ekspressiooni ja Na+-K+ pumba aktiivsusega, aga ka Wfs1-puudulikkuse mõju gamma-aminovõihappe (GABA) süsteemile. Tõstetud pluss-puuris oli ärevamatel Wfs1-puudulikkusega hiirtel vere kortikosterooni tase kõrgem, mis viitab sellele, et uus keskkond tekitab Wfs1-puudulikkusega hiirtel suuremat stressi kui metsiktüüpi hiirtel. Hiired, kellel puudub funktsionaalne Wfs1 geen, olid tundlikumad ka etanooli rahustava ja hüpnootilise efekti suhtes. Selline suurenenud tundlikkus on ilmselt seotud muutustega GABAergilises süsteemis. Lisaks sellele mõjutab Wfs1-puudulikkus drastiliselt dopamiinergilist süsteemi (Visnapuu et al. 2013). Muutused dopamiinergilises ja GABAergilises süsteemis võivad olla seotud insuliini häirunud sekretsiooniga Wfs1-puudulikkusega hiirtel (Figure 18).

Selles töös kasutasime tõstetud pluss-puuri nõrga stressorina, mis indutseeris ainult kõige varasemate ja/või tundlikumate ER stressi markerite ekspressiooni. Nende hulgas võis kõige suuremat tõusu täheldada splaissitud Xbp1 (Xbp1s) ekspressioonis. Metsiktüüpi hiirtel suurenes Xbp1s ekspressioon ventraalses striaatumis – ajuosas, mis on seotud motivatsiooniga (sh uudishimu). Wfs1-puudulikkusega hiirte puhul oli Xbp1s ekspressioon suurenenud temporaalsagaras, mis sisaldab amügdalat ja on seotud ärevusega. Selline korrelatsioon Xbp1s ekspressiooni ja genotüübi domineeriva käitumismalli eest vastutava ajuosa vahel võib olla seotud erinevustega GABA- ja monoamiinergilises aktiivsuses.

Korrelatsioon aktiveeritud ajuosa ja Na+-K+ pumba aktiivsuse vahel võib samuti olla seletatav erinevustega GABAergilise ja dopamiinergilise süsteemi aktiivsuses. Wfs1-puudulikkusega hiirtel oli Na+-K+ pumba aktiivsus samuti suurenenud temporaalsagaras. Madalam aktiivsus oli aga detekteeritav dorsaalses ja ventraalses striaatumis pluss-puuri järgselt.

Kokkuvõtvalt võib järeldada, et Wfs1-puudulikkusega hiirtel omab GABAergilise süsteemi aktiivsuse langus keskset rolli suurenenud ärevuse kujunemises ning dopamiinergilise süsteemi aktiivsuse langus põhjustab ilmselt vähenenud motivatsiooni. Muutused Na+-K+ pumba aktiivsuses ja ER stressi markerite ekspressioonis temporaalsagaras on ilmselt seotud häirunud GABAergilise närviülekandega antud aju piirkonnas. Varasemalt on näidatud, et Wfs1-puudulikkusega hiirtel on muutunud glükoosi metabolism, mis tuleneb vähenenud insuliini sekretsioonist. Teine hiljutine uurimustöö on näidanud, et insuliin

mõjutab GABAergilist süsteemi amügdalas ja see võib mõjutada emotsionaalset ja toitumisega seotud käitumist (Korol et al. 2018). Võib oletada, et ebapiisav insuliini tase Wfs1-puudulikkusega hiirtel vähendab GABAergilise süsteemi aktiivsust temporaalsagaras ja seeläbi suurendab nende loomade ärevust. Sarnane inhibitsioon toimub Wfs1-puudulikkusega hiirtel ka dopamiinergilises süsteemis dorsaalses ja ventraalses striaatumis.

Nii võib järeldada, et Wfs1-puudulikkusega hiirtel esinev vähenenud uudistamiskäitumine tuleneb motivatsioonilise (uudishimu) ja emotsionaalse (neofoobia) süsteemi muutustest, mis on tingitud kõrvalekalletest GABA- ja dopamiinergilises süsteemis (Figure 18).

Edasised uuringud on vajalikud, et selgitada nende interaktsioonide täpseid mehhanisme.

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