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# Animal model of Wolfram Syndrome in mice: behavioural, biochemical and psychopharmacological characterization

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# Animal model of Wolfram Syndrome in mice: behavioural, biochemical and psychopharmacological characterization

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Institute of Molecular and Cell Biology, University of Tartu

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**Contribution of the author:**
The author made all the embryonic stem cell culture work and microinjections for making Wfs1-deficient $Wfs1^{bgal/bgal}$ mice model. He participated in establishing of breeding strategies. Author performed the experiments and analysis of glucose tolerance test, motility test and psychopharmacological studies, and participated in writing the manuscript.

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# I. ABBREVIATIONS

| | |
|---|---|
| AC1 | adenylyl cyclase 1 |
| AC8 | adenyl yl cyclase 8 |
| ADP | Adenosine diphosphate |
| AKT | A serine/threonine protein kinase |
| ATP | Adenosine triphosphate |
| cAMP | cyclic adenosine monophosphate |
| CNS | central nervous system |
| DA | dopamine |
| DAT | dopamine transporter |
| DNA | 2-desoxyribonucleic acid |
| DOPAC | 3,4-Dihydroxyphenylacetic acid |
| Drd1 | Family member of dopamine receptor $D_1$ |
| Drd2 | family member of dopamine receptor $D_2$ |
| D1 | Dopamine receptors witch activate adenyl cyclase and production of cyclic adenosine monophosphate, also known as DRD1 |
| D2 | Dopamine receptor $D_2$, wich inhibits the production of cyclic adenosine monophosphate also known as DRD2 |
| ER | endoplasmic reticulum |
| EPAC2 | exchange protein, directly activated by cAMP 2 also known as RAPGEF4 |
| GABA | gamma-aminobutyric acid |
| $GABA_A$ | gamma aminobutyric acid (GABA) receptor A |
| GABRA1 | gene that codes $GABA_A$ receptor subunit alpha-1 |
| GABRA2 | gene that codes $GABA_A$ receptor subunit alpha-2 |
| GAD1 | Glutamate decarboxylase 1 |
| GAD2 | Glutamate decarboxylase 2 |
| GIP | Gastric inhibitory polypeptide, member of incretins |
| GLP1 | glucagon-like peptide 1, member of incretins |
| GLUT2 | Glucose transporter 2 |
| GPCR | G protein-coupled receptors |
| Gs | heterotrimeric G protein subunit that activates the cAMP-dependent pathway by activating adenylate cyclase |
| GTP | guanosine monophosphate |
| HVA | Homovanillic acid |
| L-DOPA | L-3,4-dihydroxyphenylalanine, precursor to the neurotransmitters dopamine, norepinephrine (noradrenaline), and epinephrine (adrenaline) |
| MAO | monoamine oxidase, breaks down free dopamine |

| | |
|---|---|
| mRNA | messenger ribonucleic acid (RNA) |
| NLS | nuclear localization signal *or* sequence |
| NMDA | *N*-methyl-D-aspartate, an selective agonist that binds to NMDA receptors but not to other glutamate receptors |
| PI3K | Phosphatidylinositide 3-kinases |
| PKA | cAMP-dependent protein kinase A |
| PKC | Protein kinase C (PKC) is a family of serine- and threonine-specific protein kinases that can be activated by calcium |
| PKG | cGMP-dependent protein kinase or Protein Kinase G |
| UPR | unfolded protein response |
| VMAT2 | The vesicular monoamine transporter 2 |
| Wfs1 | Wolfram syndrome 1 gene or protein in any species other than human |
| WFS1 | Wolfram syndrome 1 gene or protein in human |
| $Wfs1^{bgal/bgal}$ | Wfs1 deficient mouse strain expressing truncated Wfs1 protein fused to beeta galactosidase reporter enzyme |
| WS | Wolfram syndrome |
| 3MT | 3-Methoxytyramine |

# 2. INTRODUCTION

Wolfram syndrome (WS, Online Mendelian Inheritance in Man [OMIM] 222300) is a rare autosomal recessive neurodegenerative disorder characterized by early-onset diabetes mellitus, progressive optic atrophy, diabetes insipidus, and deafness (Domenech *et al*., 2006). WS was first characterized by Wolfram and Wagener in 1938. WFS1/wolframin has been identified as the causative gene (Strom *et al*., 1998; Inoue *et al*., 1998). There are a number of considerations from the viewpoint of nervous system function making Wfs1 gene and protein attractive objects for studies (Luuk, 2009). First, WS is accompanied by a variety of neurological and psychiatric symptoms, including severe depression, psychosis, impulsivity, and aggressiveness (Barrett *et al.*, 1995; Swift *et al.*, 1991; Swift *et al.*, 1990). Secondly, carriers of WfS1 gene mutations, who are not affected with Wolfram Syndrom, have a 26-fold higher likelihood of psychiatric hospitalization mainly due to depression (Swift *et al.,* 1998). Third, Wfs1 has been implicated in fear and anxiety-related behaviors in rodents (Kesner *et al.*, 2009; Koks *et al.*, 2002) and its polymorphisms are possibly associated with increased risk for mood disorders (Koido *et al.*, 2005). The WfS1 gene locates at 4p16.1 (Strom *et al.,* 1998; Inoue *et al.,* 1998), a replicated linkage locus of bipolar disorder (Ewald *et al.,* 1998, 2002; Detera-Wadleigh *et al.*, 1999). Some studies showed that bipolar disorder with psychosis (Als *et al.,* 2004; Cheng *et al*., 2006) or suicidal behavior (Cheng *et al.,* 2006) are linked with this locus. These lines of evidence suggested the possible role of WfS1 gene mutations in the pathophysiology of bipolar disorder and related phenotypes (Kato *et al*., 2008).

Moreover, there are studies demonstrating the mutations of WFS1 gene in patients suffering from neuropsychiatric disorders (Ohtsuki *et al.,* 2000; Martorell *et al.,* 2003; Torres *et al.,* 2001; Crawford *et al.,* 2002; Evans *et al*., 2000). It is important to stress that none of these patients had mutations causing WS. Despite the fact that WFS1 gene mutations are probably not frequent causes of mental disorders, understanding the mechanism of how WFS1 gene mutations lead to mental symptoms in patients with WS will shed light on the pathophysiology of neuropsychiatric disorders (Kato *et al*., 2008). Indeed, the neurobiological mechanisms of neuropsychiatric alterations due to WFS1 gene deficiency are not clear. However, by taking into account the psychiatric symptoms accompanying WS (Barrett *et al.*, 1995; Swift *et al.*, 1991; Swift *et al.*, 1990) as well as the localization of Wfs1 gene and protein in the brain (Luuk *et al*., 2008) may suggest that the impaired function of γ-aminobutyric acid (GABA)- and dopaminergic systems could be among the reasons for the development of mental disorders in these patients. Extensive comorbidity among major depressive disorder and anxiety disorders suggests related disease etiologies (Murphy *et al*., 2004; Gamez *et al*., 2007). Reduced brain concentrations of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) and altered function or reduced expression of its principal $GABA_A$ receptors are increasingly implicated in depressive and anxiety disorders (Luscher *et al.,*

2011). On the other hand, it has been shown that the hypoactivity of dopaminergic system, particularly in the prefrontal cortex, has been associated with the negative symptoms of depression and schizophrenia (apathy, loss of interest, anhedonia etc.) (Davis *et al.,* 1991). By contrast, the hyperactivity of the dopaminergic system, especially in the ventral and dorsal striatum, has been linked to the positive symptoms of schizophrenia such as hallucinations, delusions, psychomotor restlessness and uncontrollable behaviour (Davis *et al.,* 1991). These neuropsychiatric symptoms related to the altered function of GABA- and dopaminergic system very much resemble that occurring in relation to WFS1 deficieny in humans (Swift *et al.,* 1998).

The major goal of this doctoral thesis was to generate a model of WS in laboratory animals. For this purpose a mutant mouse line was created by deleting exon 8 of the Wfs1 gene and by inserting a beta galactosidase expression cassette (NLS-LacZ) in its place. The 8th exon was chosen for deletion, because our aim was to create a C-terminal „loss of function" mouse model of the Wfs1 protein as the majority of the mutations observed in WS patients are located in that exon. This enabled to create a WS-like condition in mice to study the pathologies caused by WS. The first part of study was dedicated to the behavioural phenotyping of Wfs1 deficient mice. According to the performed studies the impaired function of GABA- and dopaminergic system was revealed in these mutant mice. The following steps were taken to characterize more thoroughly these neurochemical alterations occurring in Wfs1-deficient mice. First, the relation between the impaired adaptation in the stressful environment and functional activity of $GABA_A$ receptors, the major targets of anxiolytic drugs, was analyzed in Wfs1 deficient mice. Second, in order to reveal the possible deviations in the functioning of dopaminergic system in Wfs1-deficient mice the experiments were performed using dopamine agonists with the different mechanism of action: amphetamine (undirect agonist) and apomorphine (direct agonist) as well as the measurments of dopamine and its metabolite levels in the dorsal and ventral striatum. Finally, based on the performed experiments the validity of mutant mice as the model WS was evaluated.

# 3. REVIEW OF LITERATURE

## 3.1. Wolfram syndrome

Wolfram syndrome (WS) is a very rare autosomal recessive disease, described first in 1938 by Wolfram and Wagener, who studied eight siblings with a juvenile diabetes mellitus accompanied by vision impairment (Wolfram and Wagener 1938). This syndrome is also known as DIDMOAD (*diabetes insipidus, diabetes mellitus, optic atrophy, deafness*). In addition to endocrinological disturbances, WS is characterised by optic atrophy, deafness, dementia, urinary tract atony, ataxia, mental retardation, peripheral neuropathy, glaucoma, epilepsy, bipolar disorder, depression and schizophrenia (Swift *et al.*, 1990; Swift *et al.*, 1991).

The first symptoms of WS are diabetes and optic atrophy that appear during the first two decades. Renal tract abnormalities appear in the third decade and, as a rule, in the fourth decade several severe neurological complications will be evident (Barrett *et al.*, 1995; Hansen *et al.*, 2005; Cano *et al.*, 2007). However, the recent evidence suggests that the neurodegeneration is already evident in early childhood (Hershey *et al.,* 2012) and it is fully manifested for a age 15 (Chaussenot *et al.*, 2011). Moreover, recent study by Hershey *et al.* (2012) demonstrates abnormalities in the brainstem and cerebellum, as well as increase in intracranial volume already in young patients. This finding suggests that WFS has a pronounced impact on early brain development in addition to later neurodegenerative effects, representing a significant new insight into the WFS disease process. Generally the average life expectancy of WS patients is 30 years, the death being caused by neurodegeneration and urinary tract atony.

WS is caused by mutations in the Wfs1 gene, which in humans is located on chromosome 4p16.1 (Inoue *et al.*, 1998). The prevalence of WS in the UK has been estimated to be 1 in 770,000 and in North America 1 in 100,000 (Fraser *et al.*, 1977; Barrett *et al.*, 1995). The frequency of heterozygous Wfs1 mutation carriers is 1 in 345 in the UK and about 1 in 100 in the USA (Swift *et al.*, 1991; Barrett *et al.*, 1995).

Mutations in 4p16 region where the Wfs1 gene is located have also been observed in patients with Huntington's disease, Wolf-Hirschhorn syndrome, night blindness and psychiatric diseases like bipolar disorder, depression and schizophrenia (Blackwood *et al.*, 1996; Asherson *et al.*, 1998; Ewald *et al.*, 2002; Koido *et al.*, 2005; Christoforou *et al.*, 2007). Depressive and psychotic symptoms have been found in about 60% of WS patients. It has been estimated that even heterozygous mutations in the Wfs1 gene elevate the risk of psychiatric hospitalization 26 times (Swift *et al.*, 1998).

In WS patients over 100 mutations have been identified (71 point mutations, 37 deletions and 14 insertions )(Figure 1). The majority of them are located in the 8th exon that modifies the hydrophilic C-terminal part of the protein. Generally the mutations in the Wfs1 gene are patient-specific (there are also compound heterozygotes), which makes the timely diagnosis of the disease much more difficult as the whole sequence of the gene must be studied (Cryns *et al.*, 2003; Cano *et al.*, 2007).

## 3.2. WFS1 gene and protein

In humans the Wfs1 gene is located on the short arm of the 4th chromosome (4p16.1) and in mice on the long arm of the 5th chromosome (5qB3). There is a 83% overlap in the nucleotide sequence of the Wfs1 gene and a 87% overlap in the amino acid sequence of its protein in humans and mice (Strom *et al.*, 1998). The length of the gene is 33.4 kb. The gene consists of eight exons the first of which is noncoding, thus the transcription starts from the second exon (Inoue *et al.*, 1998). The 8th exon is the largest (2.8 kb), containing about 60% of the whole protein-coding sequence of the Wfs1 gene. The majority of the mutations (deletions, insertions, nonsense and missense mutations) described in WS patients are located in this exon. On rare occasions, mutations have also been found in exons 3, 4, 5 and 6. The Wfs1 mRNA consists of 3,640 nucleotides and the coding sequence is 2,673 nucleotides long (Cryns *et al.*, 2003). The product of the Wfs1 gene, wolframin, consists of 890 amino acids and has a molecular weight of approximately 100 kDa. Wolframin is a transmembrane protein that is located in the membrane of the endoplasmatic reticulum (ER). Its amino-terminal domain is in the cytoplasm and carboxy-terminal domain in the ER lumen. Wolframin has nine transmembrane segments (Fig. 1) (Hofmann *et al.*, 2003; Inoue *et al.*, 1998; Strom *et al.*, 1998).

The expression level of Wfs1 is the highest in the brain, heart, lung, liver, spleen, skeletal muscles, kidney and neurosecretory tissues, including pancreatic insulin-secreting β-cells (Inoue *et al.*, 1998; Strom *et al.*, 1998; Hofmann *et al.*, 2003). It has been found that in the mouse brain, the Wfs1 gene expression level is higher in brain structures related to emotions, learning and memory. Luuk *et al.* (2008) have showed that a very strong expression of the Wfs1 gene is detectable in the central extended amygdala and ventral striatum. Prominent Wfs1 expression was detected in the hippocampal CA1 region, parasubiculum, superficial part of the second and third layers of the prefrontal cortex and proisocortical areas, hypothalamic magnocellular neurosecretory system, and central auditory pathway. Wfs1 expression was also detected in numerous brainstem nuclei and in laminae VIII and IX of the spinal cord. Wfs1-positive nerve fibers were found in the medial forebrain bundle, reticular part of the substantia nigra, globus pallidus, posterior caudate putamen, lateral lemniscus, alveus, fimbria, dorsal hippocampal commissure, subiculum, and to a lesser extent in the central sublenticular extended amygdala, compact part of substantia nigra, and ventral tegmental area (Luuk *et al.*, 2008). Besides, it has been shown that neurons expressing the Wfs1 gene project from the nucleus accumbens to the midbrain dopaminergic neurons. Therefore, the Wfs1 protein could be one of the most important regulators of the function of mesolimbic dopaminergic neurons (Luuk *et al.*, 2008). In conclusion, the expression level of Wfs1 is the highest in brain structures that belong to or are closely associated with the limbic system (Takeda *et al.*, 2001; Luuk *et al.*, 2008).

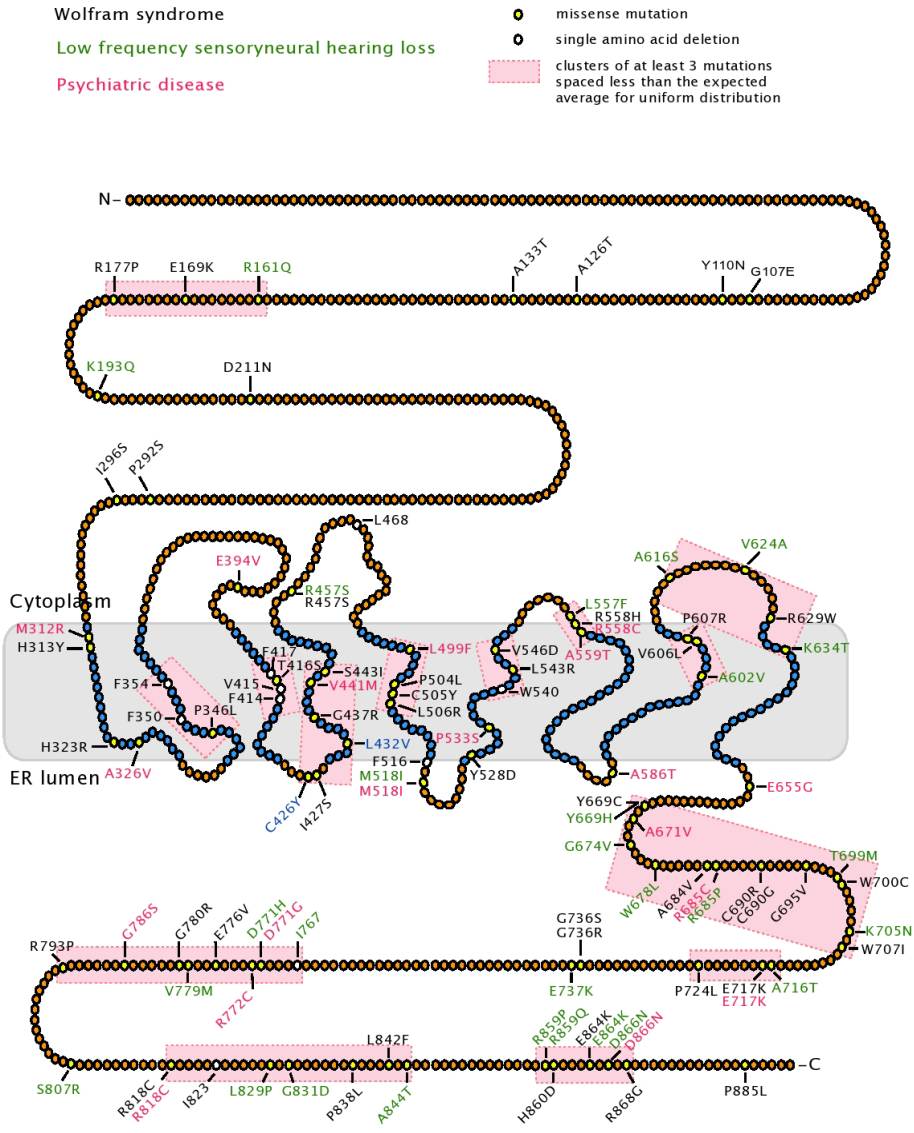


**Figure 1.** Schematic overview of the Wfs1 gene in humans. The hypothetical structure of the WFS1 protein that consists of nine transmembrane segments. Mutations cousing the single amino acid changes in WFS1 protein. Mutation definition – the first letter denotes the wild type amino acid, the number after first letter denotes its position in the WFS1 protein sequence. The last letter denotes mutanted form. If there is deletion, the last letter is absent (Figure adopted from the Ph.D. dissertation of Hendrik Luuk, 2009).

## 3.3. The functions of the Wfs1 gene

The Wfs1 protein is localized in the ER, where proteins are folded and modified, and thus obtain a proper three-dimensional structure necessary for normal functioning. ER is also involved in transporting proteins, lipids, $Ca^{2+}$ ions and many other materials needed by the different parts of the cell (Osman *et al.*,2003; McBain and Morgan, 2003; Ishihara *et al.*, 2006).

On the other hand, WFS1 takes part in the regulation of intracellular $Ca^{2+}$ homeostasis and influences the viability of insulin-secreting β-cells (including insulin secretion) both *in vitro* and *in vivo* experiments (Osman *et al.*,2003; McBain and Morgan, 2003; Ishihara *et al.*, 2006). In pancreas, Wfs1 plays a role in the processing and secretion of insulin in response to glycose stimuli that are presented after a meal (Osman *et al.*, 2003; Ishihara *et al.*,2004). Recent studies have revealed that the Wfs1 protein is present in the secretory granules of pancreatic β-cells, where it could be directly implicated in the release of insulin (Hatanaka *et al.,* 2011). The transmembrane part of the Wfs1 protein and its C-terminal domain interact with the $Na^+/K^+$-ATPase β1 subunit, calmodulin and adenylate cyclase 8 (Osman *et al.*, 2003; Zatyka *et al.*, 2007, Fonseca *et al.,* 2012).

Recently Fonseca *et al.* (2012) showed that WFS1 plays a really important role in regulation of insulin secretion and UPR response (Fonseca *et al.,* 2012). Insulin secretion (in pancreatic β-cells) is typically initiated by a rise in extracellular glucose in the pancreatic β-cells (GLUT2 mediated glucose intake), which is detected by a metabolic signalling pathway. Aerobic glycolysis and mitochondrial oxidation produce metabolic signals, such as a rise in the ATP to ADP concentration ratio, witch closes $K^+_{ATP}$-channels, depolarizes the plasma membrane, and causes calcium influx that stimulates exocytosis (Fonseca *et al.,* 2012). At the same, the GLP1 and GIP (incretin hormones) bind to their receptors on on pancreatic β-cells witch causes the activation of $G_s$ proteins. The glucose and GLP1 signalling pathways cross on AC8. AC8 is fully activated when both $G\alpha_s$-GTP and calcium-calmodulin are bound (Fonseca *et al.,* 2012). AC8 generates cAMP from ATP which is essential for insulin secretion. The subsequent rise in cellular cAMP triggers exocytosis by cAMP-dependent protein kinase A (PKA) and EPAC2, resulting in an increase in the number of vesicles that are highly sensitive to $Ca^{2+}$ (Fonseca *et al.,* 2012; Lemaire *et al.,* 2012 ). WFS1 interacts with calmodulin and AC8 in glucose-dependent manner to stimulate cAMP synthesis and calcium activation of enzymatic flux. It is possible that the physical interaction between AC8 and calmodulin is regulated by WFS1. Furthermore, Fonseca *et al.* (2012) showed that WS associated mutations inhibited the formation of AC8-calmodulin complex and insulin secretion. They demonstrated that glucose stimulation causes the translocation of WFS1 from the ER to the plasma membrane, where it interacts with AC8. ER stress blocks these translocations and interactions (Fonseca *et al.*, 2012; Lemaire *et al.,* 2012). Fonseca *et al.* (2012) proposed that WSF1 is not only a molecular „switch" for insulin release, but it also regulates insulin synthesis. As long as ER can manage with protein folding (after glycose stimulated insulin secretion),

WFS1 mediated stimulation of AC8 continues and cAMP increases insulin mRNA synthesis (Lemaire *et al.,* 2012)

There is less information about the role of WFS1 in neurotransmission. It is not clear yet whether WFS1 generates the cAMP signal for exocytosis or regulates the genes involved in synaptic plasticity. AC8 and AC1 (calmodulin activated adenylate cyclase 1) are expressed in neurons and are implicated in synaptic plasticity and long-term memory (Wang and Storm, 2002). Also it has been shown that WFS1 binds to calcium-calmodulin in the rat neurons through its N-terminal cytoplasmatic domain and this interaction was lost by WS associated mutations (Yurimoto *et al.,* 2009). However, the role of WFS1 in neurons and neurotransmission is far from being clear and it can be more complicated than established for the pancreatic β-cells.

## 3.4. Wfs1-deficient mouse as a model for Wolfram syndrome and other diseases

In order to study Wfs1 deficiency *in vivo*, two Wfs1-deficient mouse models have been created so far by different research groups. The first one was created by Riggs and colleagues (2005). Their model is a conditional Wfs1 exon 8 knock-out mouse (in a 129SVJ genetic background) with a pancreatic cells specific deletion of exon 8 of the Wfs1 gene. These mice are viable and fertile and display no deviances from Mendelian genotypic distribution. Male mice were used in the experiments. Riggs and colleagues (2005) found that this mouse had a progressive glucose intolerance and insulin-deficiency and by 4 months of age these mice have developed a type 2 diabetes-like condition. At 6 months of age, the body weight of homozygous mice is significantly lower than in wild-type controls. Also, smaller mass of insulin-producing pancreatic β-cells and increased apoptosis and ER stress level of these cells were observed in mutant mice, which reflects a type 2 diabetes-like condition (Riggs *et al.*, 2005). The second model was published by Ishihara and colleagues in 2004. Their mouse model is a so-called „full knock-out" as exon 2 of the Wfs1 gene has been deleted in these mice. Preliminary studies revealed that mutant mice responded with decreased insulin secretion in the glucose tolerance test and as the result of which by the increased blood sugar levels. Progressive loss of pancreatic β-cells, caused by apoptosis and ER stress, manifests in these mutants as type 2 diabetes-like condition only in 129/SVEV x C57BL6 F2 genetic background. In C57 background, these mice do not develop overt diabetes; thus it can be concluded that this background protects mice to certain extent against diabetes caused by Wfs1-deficiency. In conclusion, these authors argued that WFS1 protein played an important role in insulin exocytosis and the maintenance of β-cell mass. In behavioural experiments, these knock-out mice had a significantly shorter escape latency in the habituation phase of the passive and active avoidance tests. They displayed increased freezing in the training phase of the fear conditioning test and longer latency to find the platform in the learning phase of the Morris water maze test (Ishihara *et al.*, 2004; Kato *et al.*, 2008).

## 3.5. Potential neurochemical targets of Wfs1-deficiency

Our preliminary sudies established that Wfs1 deficient mice displayed the augmented anxiety-like responses and significant inhibition in the locomotor activity tests. Therefore, the next part of literature overview is dedicated to the GABA- and dopaminergic systems as the key neurochemical mechanisms in the regulation of emotional behaviour and locomotor activity.

### 3.5.1. GABA-ergic mechanisms

GABA is widely distributed neurotransmitter in the brain. Its effects are mediated both via $GABA_A$ and $GABA_B$ receptor. $GABA_A$ receptor is an ionotropic receptor and a ligand-gated ion channel (Ortells and Lunt 1995). This transmembrane ion channel opens or closes only when a „messenger" chemical (e.g. neurotransmitter) binds to it. $GABA_B$ receptor is a metabotropic transmembrane receptor linked via G-proteins to potassium channels (Chen *et al.,* 2005). The changing potassium concentrations hyperpolarize the cell at the end of an action potential. GABA is synthesized from glutamate by means of glutamate decarboxylase, which is coded in humans by Gad1 and Gad2 genes. Both are expressed mainly in the central nervous system, but Gad2 expression has also been found in the pancreas. The deficiency of these two genes has been linked with Parkinson's disease, schizophrenia, bipolar disorders and type 1 diabetes (Ludvigsson *et al.*, 2008; Woo *et al.*, 2004; Lewitt *et al.*, 2011).

GABA itself is not an inhibitory neurotransmitter as it stimulates the $GABA_A$ receptor (inhibition is caused by receptor activation). $GABA_A$ receptor activation results in the efflux of chloride ions and thereby the membrane of the neuron becomes hyperpolarized. This inhibits neurotransmission as the generation of action potential is suppressed (Richter *et al.*, 2012).

$GABA_A$ receptor has several ligand-binding sites. GABA itself binds between the alpha and beta subunits (which is also the binding site for muscimol, gaboxadol and bicuculline). However, the binding sites for benzodiazepines are located between the alpha and gamma subunits (for those types of $GABA_A$ receptors that have these subunits). The majority of $GABA_A$ receptors are sensitive to benzodiazepines and those that are not, are generally sensitive to other neurostimulators such as ethanol, barbiturates, anesthetics and neurosteroids (Richter *et al.*, 2012).

Moderate $GABA_A$ receptor activation causes an anxiolytic effect in patients and powerful inhibition of these receptors induces general anesthesia. Binding of benzodiazepines changes the conformation of $GABA_A$ receptors, which in its own increases the affinity of GABA for $GABA_A$ receptors. Due to this change the transport of chloride ions intensifies and the hyperpolarization of the membrane of neurons increases. This amplifies the anxiolytic or sedative effect of GABA itself. Different benzodiazepines have a different affinity to $GABA_A$ receptor and they bind to different alpha subunits. For example, benzodiazepines that have high affinity to alpha1 or alpha5 subunits have a sedative

effect, whereas benzodiazepines that have high affinity to alpha2 or alpha 3 subunits exert an anxiolytic effect (Richter *et al.*, 2012).

$GABA_A$ receptor subunits have many different isoforms. Nineteen different $GABA_A$ receptor subunits have been identified in the mammal central nervous system (α1–6, β1–3, γ1–3, δ, ε, π, θ, ρ1–3) that are coded by different genes (Richter *et al.*, 2012).

Classical benzodiazepine diazepam binds to $GABA_A$ receptors that consist of the following subunits (the binding site is on the outer layer of the subunits, between alpha and gamma subunits): α1βγ2, α2βγ2, α3βγ2 and α5βγ2. It has also been shown that when diazepam binds to alpha1 subunit, it has a sedative effect and when it binds to alpha2 subunit, it has a stimulating effect. Therefore, in studying the effect of diazepam, the genes coding these subunits (Gabra1 and Gabra2) are of utmost importance (Richter *et al.*, 2012; Olsen *et al.*, 2009; Wafford *et al.*, 2004).

Besides the central nervous system, GABA and $GABA_A$ receptors are also present 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. Therefore, besides neurotransmission in the central nervous system, GABA and $GABA_A$ receptors play (similarly to Wfs1 protein) a very important role in the regulation of blood sugar level and metabolism (Xu *et al.*, 2006).

## 3.6. Dopaminergic mechanisms

### 3.6.1. Dopamine and its receptors in the central nervous system and pancreas

Dopamine (DA) is a catecholamine, an important neurotransmitter and a neurohormone that is produced already in the early stages of embryonic development and plays a major role in the development of the central nervous system. Generally, dopamine is produced in neurons (Ohtani *et al.*, 2003). Dopamine is a water-soluble signaling molecule that exerts its effects by binding to the receptors on the cell surface, thereby influencing the functioning of cells. Dopamine activates five different types of dopaminergic receptors (D1–D5). All dopamine receptors belong to the family of G protein-coupled receptors (GPCR) and are pharmacologically classified into two major groups, based on how they influence the activity of adenyl cyclase (dopamine D1 and D2 receptors). Doopamine (Drd1) D1 receptors activate adenyl cyclase, whereas dopamine D2 (Drd2) receptors inhibit the production of cAMP (Missale *et al.,* 1998).

In the brain, Drd1 receptors are the most widely expressed. Drd1 mRNA has been identified in the dorsal striatum (dorsolateral caudate-putamen), ventral striatum (incl. nucleus accumbens and olfactory tubercle), hypothalamus and thalamus. Drd2 receptors (D2S, D2L, D3 ja D4) can be found primarily in the striatum, nucleus accumbens, hypothalamus, substantia nigra and cortex (Missale *et al.*, 1998). In addition, it has been shown that Drd2 receptors are also expressed in pancreatic β-cells, where they play an important role in insulin secretion (Rubi *et al.*, 2005).

In the brain dopamine is mainly produced in the dopaminergic neurons located in the midbrain's ventral tegmental area and substantia nigra. The midbrain's dopaminergic neurons are the primary source of dopaminergic terminals (Chinta *et al.*, 2005). Dopamine synthesis starts from amino acid tyrosine. Tyrosine is assimilated from food or converted from phenylalanine by phenylalanine hydroxylase in the liver and by tyrosine hydroxylase in the dopaminergic neurons. The influx of tyrosine into neurons is mediated by amino acid transporters (Bressan and Crippa, 2005). Tyrosine is synthesized by tyrosine hydroxylase into dihydroxyphenylalanine (L-DOPA, which is a precursor of dopamine and which passes differently from dopamine the blood-brain barrier). L-DOPA is converted by L-amino acid decarboxylase into dopamine. Dopamine is a precursor of noradrenaline and therefore also of adrenaline. Dopamine is degraded by monoamine oxidases (MAO) and catechol-O-methyltransferase into 3-methoxy-tyramine (3MT), 3,4-dihydroxy-phenylacetic acid (DOPAC) and homovanillic acid (HVA) (Cooper *et al*., 1996). Dopamine reuptake into presynaptic neurons is mediated by dopamine transporter (DAT) (Afonso *et al.*, 2010).

Dopaminergic neurotransmission regulates motor activity (including the fluency of movements and speech), pleasure responses (sexual and feeding behaviour, different addictions). In the peripheral nervous system dopamine controls the functioning of the cardiovascular system, influences the release of hormones etc (Feldman *et al.*, 1997). Excessive dopamine levels in the brain have been associated with the positive symptoms of schizophrenia and development of alcoholism. On the other hand, hypoactivity of the dopamine system may contribute to the negative symptoms characteristic to psychiatric disorders (lack of motivation, anhedonia, difficulties in adaptation, withdrawal from society etc) (Velligan and Alphs, 2008).

Dopamine is preserved and held in the synaptic vesicles and also in the endoplasmatic reticulum of neuronal dendrites (Bressan and Crippa, 2005). Vesicular monoamine transporter VMAT2 is a transporter protein, which is located primarily in the membrane of the intracellular vesicle of presynaptic neurons and participates in transporting monoamines into synaptic vesicles. In neurons, VMAT2 accumulates dopamine, serotonin, norepinephrine and histamine. The activity of VMAT2 is necessary for preserving neurotransmitters for later release into the synapse, otherwise these molecules would be degraded. Thus, in dopaminergic neurons, VMAT2 collects and preserves both freshly synthesized neurotransmitter molecules and molecules that have been eliminated from the synapse by reuptake (Takahashi *et al.*, 1997; Eiden *et al.*, 2011).

The arrival of action potential changes the conformation of the cell membrane and that results in the influx of $Ca^{2+}$ ions. This leads to the fusion of the dopamine-containing vesicle with the membrane of a neuron and dopamine is released into presynapse. Dopamine release is strongly modulated by GABA and glutamate (Bressan and Crippa, 2005).

It is interesting to note that in addition to neurons, VMAT2 is also expressed in the pancreatic insulin-secreting β-cells (being a diagnostic marker for diabetes) (Raffo *et al.*, 2008; Siuta *et al.*, 2010) and is present there along with

insulin (Saisho *et a.l*, 2008). Supposedly VMAT2 regulates glucose homeostasis, insulin production, and the transport and preservation of monoamines (dopamine and serotonin) in the β-cells (Raffo *et al.*, 2008).

It has been shown that besides neural transmission, dopamine and Drd2 receptors play also a role in the regulation of blood sugar level. For example, both dopamine agonists and Drd2 agonists (e.g. L-DOPA and quinpirole) inhibit glucose-stimulated insulin secretion *in vitro* (Rubi *et al.*, 2005; Rosati *et al.*, 1976; Ericson *et al.*, 1977; Zern *et al.*, 1980). Production of Dopamine occurs locally in the β-cells, and it is transported and preserved in insulin containing vesicles. Therefore, the high extracellular dopamine concentration inhibits the release of insulin. During glucose-stimulated insulin secretion also dopamine, serotonin, Drd2 receptors, GABA and $Ca^{2+}$ ions are released that all regulate the further secretion of insulin and blood sugar homeostasis (Raffo *et al.*, 2008; Paulmann *et al.*, 2009).

### 3.6.2. Dopaminergic pathways in the central nervous system (Fig. 2)

The are several dopaminergic pathways in the brain, which transmit signals from one part of the brain to another by means of neurotransmitter dopamine. The nuclei consisting of dopaminergic neurons are labelled A8–A16. Nuclei A8–A10 are located in the midbrain, A11–A14 in the diencephalon, A15 in the forebrain and A16 in the olfactory bulb (Bjorklund *et al.*, 1984; Swanson *et al.*, 1982). Approximately 90% of the dopaminergic neurons are located on the ventral side of the midbrain (Shankar *et al.*, 2005). Several dopaminergic pathways start from these regions. There are eight different dopaminergic systems; the four most important ones are following:

*Nigrostriatal pathway* – The nigrostriatal pathway transmits dopamine from the substantia nigra to the striatum. This pathway is associated with motor control and habit formations (Pierce *et al.*, 2006).

*Mesolimbic pathway* – The mesolimbic dopaminergic pathway connects the midbrain's ventral tegmental area (VTA) with the structures belonging to the forebrain's limbic system (nucleus accumbens, hippocampus, amygdala and prefrontal cortex). Most of the neurons projecting from the VTA are dopamine-producing and their activation results in a massive release of dopamine into nucleus accumbens and prefrontal cortex. This system is related to the motivational aspects of behaviour and is also one of the main targets for addictive drugs. Furthermore, deviations in this system are associated with the symptoms of schizophrenia and depression (Pierce *et al.*, 2006).

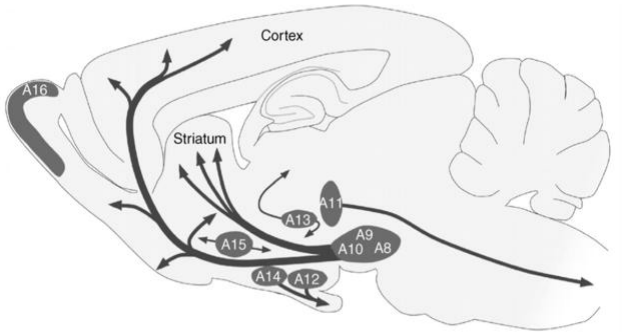


**Figure 2.** Distribution of DA neuron cell groups in the rodent brain in a schematical sagittal view. The principal projections of the DA cell groups are illustrated by arrows. Drawing is modified from Bjorklund and Dunett (2007).

*Mesocortical pathway* – The mesocortical pathway transmits dopamine from the VTA to the frontal cortex. This pathway is associated with the negative symptoms of schizophrenia (Pierce *et al.*, 2006).

*Tuberoinfundibular pathway* – The tuberoinfundibular pathway transmits dopamine from the hypothalamus to the pituitary gland. This pathway influences the secretion of certain hormones, including prolactin. This pathway is associated with hyperprolactinaemia (Pierce *et al.*, 2006; Bjorklund *et al.*, 1984; Swanson *et al.*, 1982).

### 3.6.3. Relevance of dopamine transporter for dopaminergic neurotransmission

Dopamine transporter (DAT) is a transmembrane glucoprotein that pumps dopamine from the synaptic cleft back into the presynaptic cytoplasm (or the other way round), where it is packed again into monoamine vesicles (VMAT2 vesicles) for preservation and later release (Afonso *et al.*, 2010). DAT-mediated dopamine reuptake is powered by the ion concentration gradient created by the Na-pump in the plasma membrane. The function of DAT in the cell membrane is regulated by different secondary signalling molecules, e.g. protein kinase A, protein kinase C, protein kinase G, tyrosine kinases, phosphatases, calcium and calmodulin-dependent kinases, and arachidonic acid (Gonzalo *et al.*, 2006). DAT is expressed primarily in dopaminergic neurons, but its expression has been found also in the peripheral tissues, including lymphocytes (Ramamoorthy *et al.*, 2011). DAT is the most abundant in those brain regions that are related to dopaminergic neurotransmission: nucleus accumbens, nucleus caudatus, substantia nigra, amygdala and ventral tegmental area (Staley *et al.*, 1994; Torres *et al.*, 2003). Impairments in the functioning of DAT have been described in several neuropsychiatric diseases like Parkinson's disease, bipolar disorder, schizophrenia, depression and different addictions (Kahling *et al.*, 2006).

Dopamine release from the axon terminals of neurons is dependent on neural activity, $Ca^{2+}$ channels and also insulin (Adachi *et al.*, 2005). Insulin has a direct effect on the brain's mesolimbic dopaminergic system and this effect is mediated primarily by DAT. Namely, insulin plays a role in the transport of DAT from the plasma membrane into cytosol and vice versa (Owens *et al.*, 2005). For example, in hypoinsulinemic rats the DAT-mediated dopamine reuptake from the synapse has decreased (Patterson *et al.*, 1998). In mice with streptozotocin-induced diabetes, dopamine reuptake is about 45% lower than in the control group (Owens *et al.*, 2005). These results show that lower level of insulin is directly associated with impaired dopamine reuptake.

Psychostimulant amphetamine releases dopamine from the presynapse of neurons via DAT and blocks dopamine reuptake (essentially it puts DAT to work only one-way). Released dopamine activates Drd2 receptors to the greatest extent in the ventral and dorsal striatum. It was shown already in 1970s that in rats with a streptozotocin-induced type 1 diabetes amphetamine failed to increase locomotor activity, because amphetamine was not able to release dopamine. This effect was reversible by injecting insulin into the brain (Marshall, 1978).

## 3.7. State of art and definition of tasks for present study

According to the existing clinical studies majority of WS patients suffer from neuropsychiatric symptoms. On the other hand, heteozygous mutations of WFS1 gene significantly increase the risk to be hospitalized due to the neuropsychiatric disease (Swift *et al.,* 1998). Moreover, the mutations of WFS1 gene have been described in patients suffering from bipolar disorder, major depressive disorder and schizophrenia (Kato *et al.,* 2008). Besides that WFS1 gene belongs to the genes which mutations increase risk for type 2 diabetes. Interestingly, this is in line with the findings that the frquency of type 2 diabetes is significantly elevated among the patients suffering from bipolar depression and schizophrenia (Coclami and Cross 2011).

Taken together, it is apparent that the genetically modified knock out mouse model is a valid model to reflect the disturbances in glucose metabolism present in WS patients (Ishihara *et al.,* 2004; Riggs *et al.,* 2005). Since most of the mutations in WFS1 gene are located in exon 8 in WS patients suffering from the psychiatric symptoms a new Wfs1 exon 8 knock-out mice model would have a potential for establishing of connections between metabolic and neuropsychiatric disorders. Therefore, the major goal of the present work was to generate Wfs1 exon 8 knock-out mice. The initial step was the phenotyping of these mice keeping in mind both the neuropsychiatric and metabolic symptoms occurring in humans suffering from WS and neuropsychiatric disoders. Since the disturbances of emotional behaviour and locomotor activity were dominating in these animals the psychopharmacological approach was taken to reveal the alterations of GABA- and dopaminergic systems in animals lacking Wfs1 gene.

# 4. AIMS OF THE STUDY

Based on the literature review an attempt was done to generate the animal model of WS and to study the possible alterations in the behaviour and neurochemistry caused by Wfs1 deficiency. The majority of mutations causing WS are located in the 8th exon of WFS1 gene. Therefore, the approach following the human pathology was used for the invalidation of Wfs1 gene. Namely the 8th exon of Wfs1 gene was corrupted using the transgenic technology. After building up the animal colony the behavioural and psychopharmacological characterization of these mouse was started. For that purpose following specific tasks were set:

1. The initial phenotyping of Wfs1-deficient mouse to describe the mutants in adaptation-, learning- and anxiety-related behavioural experiments and to measure the levels of stress hormones compared to wild-type controls. Simultaneously with the behavioural measures the alteration of glucose metabolism was studied in mice lacking Wfs1 gene.
2. Since the increased anxiety was dominating feature of behavioural phenotype in Wfs1 deficient mice an attempt was taken to characterize the parameters of GABAergic system. The expression levels of alpha1 and alpha2 subunits of $GABA_A$ receptors and the Gad1 and Gad2 genes were measured in the brain structures associated with the regulation of behavioural adaptation, learning and anxiety (temporal lobe, frontal cortex, mesolimbic system and hippocampus). This study was performed both in the experimentally naive mice and in animals challenged to the elevated plus-maze model of anxiety.
3. The suppressed locomotor activity was the other behavioural feature of Wfs1 deficient mice. Therefore, the changes in the activity of dopaminergic system, playing a central role in the regulation locomotor activity, were characterized in Wfs1 deficient mice. For that purpose the behavioural and biochemical effects of dopamine agonists (amphetamine, apomorphine) were analyzed. In order to confirm the established alterations in the behavioural and biochemical levels, the gene and protein expression experiments, involving DAT and Drd2 receptor, were performed in the midbrain, ventral and dorsal striatum of wild-type and Wfs1 deficient mice.
4. Finally, based on the performed behavioural, biochemical and psychopharmacological studies the validity of Wfs deficient mice for modelling of metabolic and neuropsychiatric symptoms in WS patients was evaluated.

# 5. MATERIALS AND METHODS

## 5.1. Animals and generation of Wfs1 gene deficient mice (Study 1, 2, 3)

The third model of Wfs1 deficient mice was generated at the University of Tartu (see the description of other two in the review of literature). In these conventional Wfs1 knock-out mice, exon 8 in the Wfs1 has been deleted. This is the exon where most of the mutations have been found in WS patients. In mutants, exon 8 has been replaced by NLS-LacZ-Neo expression cassette, which enables to observe the expression patterns of the Wfs1 gene (Fig. 3). By contrast, the N-terminal domain of the protein remained functional.

Wfs1 targeting construct was created by subcloning a 8.8 kb BamHI fragment from 129SvEv/TacfBr mouse genomic PAC clone 391-J24 (RPCI21 library, MRC U.K. HGMP Resource Centre, U.K.) including introns 6–7 and exons 7–8 of Wfs1 gene into pGem11 cloning plasmid (Promega). A 3.7 kb NcoI fragment was replaced by an in-frame NLSLacZNeo cassette, deleting more than 90% of the 8th exon and 60% of the total coding sequence including 8 of the 9 predicted transmembrane domains. A pgk-TK negative selection cassette was cloned upstream of 5' genomic arm. NotI-linearized targeting construct was electroporated into W4/129S6 embryonic stem (ES) cells (Taconic) which were selected for resistance to Neomycin and Gancyclovir. ES cell colonies were tested for homologous recombination by PCR using recombination-specific primer pair NeoR1 5' GACCGCTATCAGGACATAGCG and Wfs1_WTR1 5' AGGACTCAGGTTCTGCCTCA. PCR-product was sequenced to verify the integration site. ES cell clone 8A2 was injected into C57BL/6 blastocysts and heterozygous F1 mice were established by mating male chimeras with C57BL/6 female mice. F2 generation homozygous Wfs1-deficient animals were obtained by crossing heterozygous F1 mice. Mice were genotyped by multiplex PCR for both alleles using primers WfsKO_wtF2 5' TTGGCTTGTATTTGTCGGCC, NeoR1 5' GACCGCTATCAGGACATAGCG and WfsKO_uniR2 5' CCCATCCTGCTCTCTGAACC (Luuk *et al.,* 2009).

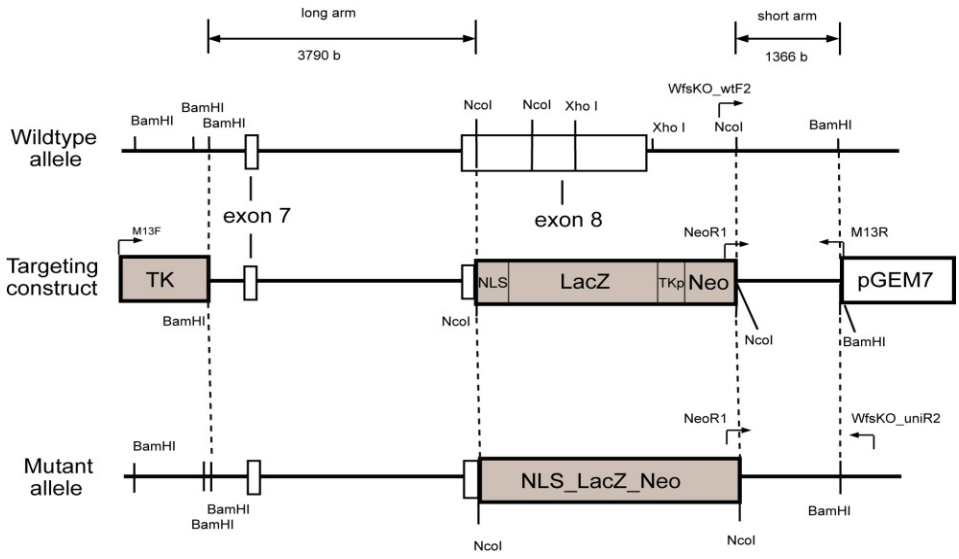


**Figure 3.** The strategy of making Wfs1 *knock-out* mouse. Exon 8 in the Wfs1 gene was replaced with NLS-LacZ-Neo expression cassette (Luuk *et al.*, 2008).

Breeding and genotype analysis of mice were performed in the Department of Physiology, University of Tartu. Mice were F2 hybrids with 129S6/SvEvTac and C57BL/6J mixed genetic background. The mixed background was chosen to eliminate gene-gene interaction effects specific to a single inbred background. Instead, we sought to unravel general behavioural effects of Wfs1-deficiency apparent in a genetically heterogeneous population. In the behavioural, biochemical and gene/protein expression studies only littermates were used and compared. The mice were housed in groups of eight under a 12 hour light/dark cycle with lights on at 7:00 a.m. All three genotypes (wild-type, heterozygous and homozygous Wfs1-deficient) were housed in their respective home cages. The studies were performed between 9:00 a.m. and 7:00 p.m. The animals had free access to food and water except during testing. Mutant mice were always used in parallel with wild-type mice and the animals were taken into groups randomly. The permissions for the present study was given by the Estonian National Board of Animal experiments (no. 39, 7 October 2005; No. 13 from 16.09.2009) in accordance with the European Communities Directive of November 1986 (86/609/EEC) (Luuk *et al.,* 2009).

## 5.2. Drugs (study 1, 2, 3)

All injections were performed in a volume of 10 ml/kg. Amphetamine (2.5, 5, 7.5 mg/kg, amphetamine sulfate, Sigma-Aldrich, St Louis, MO, USA.), an indirect agonist of dopamine, a compound increasing the release of neurotransmitter from the presynaptic terminals. Amphetamine was diluted in 0.9%

NaCl (B. Braun Melsungen AG, Germany) and given intraperitoneally (i.p.) 30 min prior to the experiment. Apomorphine (3 mg/kg, apomorphine hydrochloride, Sigma-Aldrich, St Louis, MO, USA), an unselective Drd1 and Drd2 receptor agonist, was also dissolved in physiological saline. Ascorbic acid was added to the final concentration of 0.1% to prevent the oxidation of apomorphine. Apomorphine was given subcutaneously (s.c.) 15 min before the experiment. Diazepam (1 mg/kg, Grindex, Latvia), an anxiolytic $GABA_A$ receptor agonist, was diluted in saline. Diazepam was given i.p. 30 min prior to testing.

## 5.3. Behavioral Studies (Study 1, 2, 3)

**Capturing and analysis of vocalizations**
AT803b omnidirectional condenser microphone (Audio-Technica, Japan) with a working frequency response range of 100 Hz to 10 kHz was attached above the Plexiglas chamber used for monitoring locomotor activity of single mice. Audio recordings (44.1 kHz sampling frequency, 16 bit depth) were captured on computer hard disc using US122 audio interface (Tascam, U.S.A.) and adjusted for volume using CoolEdit Pro 2.0 (Syntrillium, U.S.A.). Sonogram was generated with SasLab Light application (Avisoft, Germany) (Luuk *et al.,* 2009).

**Rota-rod test**
Motor performance and coordination was assessed by rota-rod. A 1-min training session was given to each mouse on the rota-rod (diameter 8 cm, 9 rpm) 5 min before the first measurement. Motor performance (time until the first fall) was registered during a 2-min session (Luuk *et al.,* 2009).

**Stress-induced analgesia**
Stress was induced by electric foot-shocks (0.2, 0.4, 0.6 mA, alternating current) during 3 min in the apparatus used for active and passive avoidance testing (TSE Systems, Germany). Each group of mice received electric foot-shocks of a single intensity only. Withdrawal latencies in the radiant-heat tail flick test (Plantar Test 7371, TSE Systems) were measured before (baseline) and after stress treatment. The post-stress latences were measured immediately after termination of foot-shocks. Removal of the tail from the heat source terminated the application of thermal stimulation. Ceiling tail-flick latencies were 30 s. Restraint tubes (opaque plastic cylinders, inner diameter 28 mm, length 90 mm, the closed end of the tube had a small hole for breathing) were used for the immobilization of mice during the measurement. Mice were habituated with the tubes for 5 days prior to the measurements (Luuk *et al.,* 2009).

**Locomotor activity**
Locomotor activity of single mice was measured for 30 min in sound-proof photoelectric motility boxes (448 mm × 448 mm × 450 mm) connected to a computer (TSE, Technical & Scientific Equipment GmbH, Germany). In the first study we investigated the effect of illumination on locomotor activity of

Wfs1-deficient mice. The experiment was performed both in dimly (20 lx) and brightly (450 lx) lit conditions. Half of the animals were first exposed to brightly lit motility boxes and the second half to dimly lit motility boxes. On the second day the treatments were reversed so that each animal experienced both conditions. The results from the two experiments were collapsed. In the second study we investigated the effects of amphetamine (2.5–7.5 mg/kg) and apomorphine (3 mg/kg) on locomotor activity. Amphetamine and apomorphine were injected i.p. at 30 and 15 min, respectively, prior to the measurement of locomotor activity in a brightly lit environment (450 lx). The schedule of experiments is presented in Table 1. For each individual, the effect of amphetamine was compared to the mean effect of saline treatments performed in the same individual on days 4 and 20. Similarly, the effect of apomorphine was compared to the mean effect of saline treatments performed on days 20 and 28. The floor of the testing apparatus was cleaned with damp towels and dried thoroughly after each mouse. Computer registered the distance travelled, the number of rearings and corner entries, and time spent in the central part of motility boxes (Luuk *et al.,* 2009).

**Table 1.** Pharmacological treatment schedule in locomotor activity experiment

| Day | Treatment |
|---|---|
| **1–3** | Habituation with the motility boxes |
| **4** | Treatment with saline |
| **8** | Treatment with amphetamine (2.5 mg/kg) |
| **12** | Treatment with amphetamine (5 mg/kg) |
| **16** | Treatment with amphetamine (7.5 mg/kg) |
| **20** | Treatment with saline |
| **24** | Treatment with apomorphine (3 mg/kg) |
| **28** | Treatment with saline |

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**Elevated plus-maze test**

The test employs a naturalistic conflict in mice between the tendency to explore a novel environment and aversive properties of a brightly lit, open area (Handley *et al.,* 1984; Lister 1987; Pellow *et al.,* 1985). The plus-maze consisted of two opposite open arms (17.5 cm × 5 cm) without sidewalls 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 entire plus-maze apparatus was elevated to a height of 30 cm and placed in a dim room

(illumination level ~20 lx). In order to encourage open arm exploration, a slightly raised edge (0.25 cm) was put around the perimeter of the open arm, providing a grip for animals. The open arms were divided into three equal parts by lines. The anxiolytic effect of diazepam, a $GABA_A$ receptor agonist, on exploratory activity was studied in Wfs1-deficient mice and their wild-type littermates isolated for 30 min before the experiment. Diazepam was administered 30 min before the study. The control group received saline. Testing began by placing an animal on the central platform of the maze facing an open arm. An arm entry was counted only when all four limbs were within a given arm. Standard 5-min test duration was employed (Lister 1987; Pellow *et al.,* 1985) and the maze was wiped clean with damp and dry towels between the subjects. Test sessions were video-recorded and the videotapes were subsequently blind-scored by a trained observer. The following measures were registered by the observer: (1) time spent on open arms; (2) number of closed and open arm entries (entries into the most distant part of open arms were counted separately); (3) number of line crossings; (4) ratio between open and total arm entries; (5) number of head dips; (6) number of attempts to enter the central platform located between open and closed arms (Luuk *et al.,* 2009, Raud et al., 2009).

**Light-dark box exploration test**

Light-dark box exploration test is another unconditioned test of anxiety-like behaviour designed for mice (Crawley *et al.,* 1980). Since social isolation is known to affect exploratory activity (Abramov *et al.,* 2004), the effect of short-term isolation on the behaviour of Wfs1-deficient mice was tested explicitly. One group of animals was exposed to the exploration test without previous isolation (non-isolated animals), the second group was isolated for 15–20 min before the experiment. The experiments were carried out in a dim room (illumination ~20 lx). Plexiglas box (45 cm × 20 cm × 20 cm) was divided into two parts: 2/3 was brightly illuminated (~270 lx) and 1/3 was painted black, covered by a lid and separated from the white compartment with a partition containing an opening (13 cm × 5 cm). A mouse was placed in the centre of the light compartment facing away from the opening between the two compartments, and, during 5 min, latency to move into the dark compartment, time spent in the light compartment and number of transitions between the two compartments were recorded (Luuk *et al.,* 2009).

**Fear conditioning test**

This is a form of classical conditioning which investigates the establishment of a simple association between a conditioned stimulus (10 kHz tone, CS) with an unconditioned aversive stimulus (0.5 mA electric foot-shock with a duration of 2 s, US). The study was performed according to the method described by Paylor *et al.* (Paylor *et al.,* 1998) with some modifications. Experiments were carried out with a computer-controlled fear conditioning system (TSE). Context and tone-dependent experiments took place in a lit room. During the training period

and on the day of the experiment, mice were kept in their home cages. Training was conducted in a transparent acrylic chamber (110 mm × 160 mm × 160 mm/110 mm × 135 mm × 155 mm) containing 3 mm stainless steel rod floor, spaced 0.5 cm, through which electric foot-shocks could be administered. The test chamber was placed inside a sound-attenuated chamber and was constantly illuminated (~130 lx). Mice were observed through a window in the front wall of the sound-attenuated chamber. Animals were placed in the conditioning context for 120 s and were then exposed to a CS for 30 s. The CS was terminated by a US. 120 s later another CS–US pairing was presented. The mouse was removed from the chamber 15–30 s later and returned to its home cage (Luuk *et al.,* 2009).

The mice were tested for contextual memory 24 h later by placing them back into the test chamber for 5 min with no CS applied. Total time of freezing (defined as the absence of any movements for more than 3 s) was measured using the standard 10 s interval sampling procedure. Four hours later the mouse was tested for freezing behaviour to the auditory CS. Testing was performed in a different acrylic chamber (220 mm × 160 mm × 160 mm/220 mm × 135 mm × 155 mm) the floor of which was covered with white cardboard. The background colour was black. Duration of the test was 6 min: 3 min without the tone (pre-CS phase) and 3 min with the tone (CS phase). Freezing was counted during the CS phase. Additionally, the number of rearings in pre-CS and CS periods was recorded (Luuk *et al.,* 2009).

**Hyponeophagia test**

The experiment was carried out in a brightly lit (~400 lx) room. The mice, food-deprived for 24 h, were taken from their home cage and placed singly in a translucent plastic box (18 cm × 22 cm × 14 cm) filled with a single layer of food pellets (Lactamin AB, Sweden; weighing 1.5–3.5 g) to a depth of approximately 1 cm. To avoid social transmission of behaviour, mice that had already been tested were placed in a separate box. The latency to start eating was measured from the time a mouse was placed in the box. Eating was defined as eating for at least 3 s consecutively. A cut-off score of 180 s was used (Luuk *et al.,* 2009).

**Forced swimming test**

Mice were placed for 6 min into a glass cylinder (diameter 12 cm, height 24 cm, water depth 15 cm) filled with 25 ± 0.5 °C fresh tap water. A 5 s standard interval sampling technique was used for rating behaviour (Cryan *et al.,* 2002) during the last 4 min of the test. Specific behavioural components were distinguished: (1) climbing behaviour, defined as upward-directed movements of the forepaws along the side of the swim chamber; (2) swimming behaviour, the horizontal movement throughout the swim chamber; (3) immobility, defined as no activity other than that required to keep the animal's head above water (Luuk *et al.,* 2009).

**Morris water maze test**

Spatial memory was studied in Morris water maze (TSE Technical & Scientific Equipment GMBH, Germany). The pool (150 cm in diameter, 50 cm in depth) was filled with 22–24 °C water to a depth of 38 cm. Water surface was made opaque with the addition of non-toxic fine-grained white putty. The invisible white escape platform (16 cm in diameter, submerged 1 cm under water) was positioned in the centre of the imaginary Southwest quadrant, 20 cm from the wall. It remained in a fixed position during the training. Subjects' movements were recorder by a computer connected to the video camera placed above the pool. The experimental room was lighted with four symmetrically placed lamps with 25 W light-bulbs and also a table lamp, which filled the room with dim light (ca. 20 lx). Coloured flyers and figures on the walls served as spatial cues. During each trial, a mouse was released into the water facing the wall of the pool from pseudo-randomly chosen cardinal compass points (North, East, South and West). Randomization ensured that all positions were sampled before a given position was repeated. The acquisition phase of the experiment consisted of a series of 20 initial training trials, lasting up to 60 s each with inter-trial interval of approximately 1 h (five trials per day on 4 consecutive days) and 12 reverse training trials (six trials per day on 2 days). A 60 s probe trial without the platform was performed at the end of each training cycle, 1 h after the last training trial. In probe trials, mice were released at a location on the opposite side of where platform had been located (on day 4 from Northeast, on day 6 from Southwest). For statistical purposes, the reversal training sessions were divided into four blocks of three trials. In all trials, mice were allowed to swim until they landed on the platform or until 60 s had elapsed. Mice failing to find the platform within 60 s were gently placed on the platform with a metal escape sieve and left there about 15 s to orient. Mice that found the platform were also left there for about 15 s. After each trial mice were put to their home cage with the escape sieve. Mice quickly learned to associate the sieve with escaping from the pool and consistently oriented to or followed the sieve on its appearance. The ability of mice to orient to or follow the escape sieve represented independent measures of vision and attention. The latency to find the submerged platform, the distance travelled, swim velocity and time spent in the periphery were registered with water maze software (TSE Technical & Scientific Equipment GMBH, Germany) (Luuk *et al.,* 2009).

**Active avoidance test**

Active avoidance is a fear-motivated associative avoidance task providing a simple way to assess associative learning and memory. In this task the mouse has to learn to predict the occurrence of an aversive event (electric foot-shock) based on the presentation of a specific stimulus (tone or light), in order to avoid the aversive event by moving to a different compartment (Luuk *et al.,* 2009).

Active avoidance learning was carried out in a rectangular two-way automated shuttle-box (TSE), consisting of two similar chambers (14 cm × 11 cm × 16 cm) connected by an arched opening (4 cm × 4 cm). The box was

surrounded by a soundproof chamber. The apparatus was located in a quiet, very dimly (5 lx) illuminated room. The shuttle-boxes had a cover with a light-bulb (10 W) attached above each compartment. Foot-shocks could be administered through a stainless steel rod floor (diameter 3 mm, spaced 5 mm). Mice were placed in the dark compartment facing the wall of the chamber and submitted to an active avoidance test for four consecutive days, 30 trials a day. The test started with a habituation time of 10 s. The conditioned stimulus (CS) was a 10 kHz tone with a maximum duration of 20 s accompanied by lighting up of the target compartment (light and sound signal). The unconditioned stimulus (US; 0.3 mA electrical foot-shock for 5 s) was switched on 5 s after CS and was followed by a stronger US (by a 0.6 mA foot-shock for a maximum of 10 s) in case the mouse failed to move to the target compartment. Intertrial interval was 10 s. After 30 trials, mice were taken back to their home cage (Luuk *et al.,* 2009).

**Testing order of animals**

Only experimentally naïve animals were subjected to the experiments marked as first in the testing order (Table 2). We tested all mice several times to reduce the number of animals used. Behavioural tests were divided into two categories, either sensitive or insensitive to previous experimental experience. Plus-maze, forced swimming test, locomotor activity, fear conditioning and hyponeophagia were considered as sensitive, whereas stress-induced analgesia, rota-rod test, Morris water maze test, active avoidance test and dopamine agonist induced hyperlocomotion as insensitive or less sensitive tests (McIlwain *et al.,* 2001; Voikar *et al.,* 2004, Luuk *et al.,* 2009).

**Table 2.** Order of behavioural tests

| Behavioural test | Number in order |
|---|---|
| **Elevated plus-maze** | 1 |
| **Locomotor activity test** | 1, 2 |
| **Forced swimming test** | 1 |
| **Hyponeophagia** | 1 |
| **Fear conditioning** | 1 |
| **Light-dark box exploration test** | 2 |
| **Amphetamine-induced hyperlocomotion** | 2 |
| **Apomorphine-induced hyperlocomotion** | 3 |
| **Rota-rod test** | 2 |
| **Active avoidance test** | 3 |
| **Stress-induced analgesia** | 4 |
| **Morris water-maze test** | 3 |

Luuk *et al.,* 2009

## 5.4. Evaluation of function of dopaminergic system in Wfs1 Deficient mice (Study 3)

Additionally to impaired glucose metabolism, our preliminary pharmacological studies (Study 1) proved the locomotor stimulatory effect of amphetamine, an indirect agonist of dopamine (DA), to be significantly weaker in Wfs1-deficient mice and these mice exhibited an enhanced locomotor response to apomorphine, a direct DA receptor agonist (Luuk *et al.,* 2009). The DA-ergic system in Wfs1-deficient mice was studied in terms of 1) the effect of amphetamine (5 mg/kg) and apomorphine (3 mg/kg) on locomotor activity and DA metabolism, 2) the motor sensitization to the repeated treatment with subthreshold dose of amphetamine (2.5 mg/kg), 3) the expression of Dat and Drd2 receptor mRNAs, 4) the expression of Dat protein. Therefore, the main goal of this study was to establish the possible alterations in the DA-ergic system due to the invalidation of the Wfs1 gene in mice.

The locomotor activity was measures as described above. The distance travelled, time in locomotion and number of corner entries were registered. Illumination level in the motility boxes during the experiments was approximately 400 lux. The higher illumination level was used to suppress spontaneous locomotor activity in mice. This approach enables one to see the motor stimulation at lower doses of dopamine agonists, otherwise being masked with a high exploratory activity of animals. In the beginning, animals were habituated (30 min) to the motility boxes on two consecutive days. This was done in order to reduce any behavioral activation due to novelty seeking. Thereafter the animals were tested in the same boxes with saline and drugs (amphetamine 5 mg/kg, apomorphine 3 mg/kg). Since the behavioral experiments lasted 9 h, precautions were taken to control the possible daily fluctuations in the locomotor activity of animals. Therefore, the experiments were always performed in randomized order, that is, wild-type mice were always used in parallel with genetically modified animals. Sensitization of animals to amphetamine was also measured using the same motility boxes with similar environmental conditions. For this, the animals were habituated for two days and on the third day the experiment started. Animals were injected with 2.5 mg/kg amphetamine, and placed into the motility boxes for 30 min once a day for 6 consecutive days (Visnapuu *et al.,* 2013).

## 5.5. Biochemical measurements

**Blood glucose measurement and glucose tolerance test**

Non-fasted experimentally naïve animals were used for this experiment. In order to reduce the behavioural stress due to the repeated handling, the animals were kept in their home cages. Blood samples were collected by repeated tail vein punctures from mice immobilized in a 50 ml tube restrainer. Intraperitoneal injection of D(+)-glucose (Sigma, 2 g/kg, dissolved in saline) was performed after the first glucose measurement (at 0 min). Additional blood samples

were taken 30, 60 and 120 min after the injection. Blood glucose concentration was measured using Accu-Check GO portable glucometer (Roche, Mannheim, Germany) (Luuk *et al.,* 2009).

**Corticosterone response to stress**

The study was performed between 12:00 and 14:00. An equivalent number of group-housed experimentally naïve animals received either an i.p. injection of saline (stress treatment group) or were left undisturbed in their home cage until decapitation in a separate room after 30 min. Immediately after decapitation, blood from the trunk of the body (mixed arterial and venous blood) was collected into heparinized tubes and centrifuged for 10 min at 1500 × *g*. Sera were stored at −20 °C until the assay using corticosterone HS ELISA kit Octeia from Immunodiagnostic Systems (U.K.) according to manufacturer's instructions. Briefly, 400 µl of buffer was added to 100 µl of calibrator, control or sample serum, followed by 30 min incubation at 80 °C and cooling to the room temperature. One hundred microliters of heat-treated calibrator, control or sample were transferred to antibody-coated plates and 100 µl of enzyme conjugate was added. Plate was covered and incubated at 22 °C for 4 h, then washed three times with wash buffer. Next, 200 µl of TMB substrate was added and incubated at 22 °C for 30 min. Absorbance was measured at 450 nm after adding 100 µl of stop solution (Luuk *et al.,* 2009).

**Monoamine measurements**

Mice were randomly divided into groups that received an injection of only saline, amphetamine (5 mg/kg) or apomorphine (3 mg/kg). After administration of saline or DA agonists the animals were placed into the above described motility boxes where their locomotor activity was assessed. This procedure lasted 30 min after which the mice were decapitated and their brains rapidly removed. There was also one group of mice taken directly from their home-cages in order to measure the effect of exposure of mice to the motility boxes. Two brain regions were dissected – the dorsal (encompassing the caudate putamen) and ventral striatum (encompassing the nucleus accumbens and olfactory tubercle) – and frozen in liquid nitrogen. The dissection was performed according to the coordinates presented in the mouse brain atlas by Franklin and Paxinos (Franklin and Paxinos 1997). DA and its metabolites were assayed by high performance liquid chromatography (HPLC) with electrochemical detection. The tissue samples were homogenized with Bandelin Sonopuls ultrasonic homogenizer (Bandelin Electronic, Berlin, Germany) in ice-cold solution of 0.1 M perchloric acid (10–30 µl/mg) containing 5 mM sodium bisulphite and 0.4 mM EDTA to avoid oxidation. The homogenate was then centrifuged at 17,000 × g for 10 min at 4 °C. Aliquots (10 µl) of the obtained supernatant were chromatographed on a Lichrospher 60 RP Select B column (250 × 3 mm; 5 µm). The separation was done in isocratic elution mode at column temperature of 30 °C using the mobile phase containing 0.05 M sodium citrate buffer at pH 3.7; 0.02 mM EDTA; 1 mM KCl; 1 mM sodium octyl-

sulphonate and 5.6% acetonitrile. The chromatography system consisted of a Hewlett Packard HP 1100 Series isocratic pump, a thermostated autosampler, a thermostated column compartment and an HP 1049 electrochemical detector (Agilent, Waldbronn, Germany) with glassy carbon electrode. The measurements were done at an electrode potential of + 0.7 V versus the Ag/AgCl reference electrode. We measured the tissue levels of dopamine (DA) and its metabolites – 3 methoxytyramine (3-MT), 3,4- dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in pmol/mg of tissue weight. Finally, DA turnover was calculated as the ratio between the tissue levels of DA endmetabolite HVA and DA itself (HVA/DA) (Visnapuu *et al.,* 2013)

## 5.6. Gene expression studies

**Gene expression studies of GABA-related genes in WSF1 deficient mice (Study 2)**

In the study 1 we have found that the genetic invalidation of Wfs1 gene exon 8 impairs the adaptation of mice in a novel and stressful environment in terms of increased anxiety in ethological models. Pre-treatment of mutant mice with diazepam (1 mg/kg), an anxiolytic drug acting via $GABA_A$ receptors, antagonized increased anxiety (in elevated plus-maze test) and stress-induced vocalizations in Wfs1-deficient mice (Luuk *et al.,* 2009). As the pharmacological action of diazepam is to enhance the effect of the neurotransmitter GABA by binding to the benzodiazepine site on the GABAa receptor the expression of Gabra1 and Gabra2 (coding alpha 1 and alpha 2 subunits of GABA receptors) genes was studied in the frontal cortex, temporal lobe, hippocampus and mesolimbic area. All these brain areas show high concentrations of Wfs1 protein and mRNA (Luuk *et al*., 2008, Luuk *et al.,* 2009). To determine the overall impact of Wfs1 deficiency for GABA-ergic system, the expression of Gad1 and Gad2 genes, enzymes responsible for the synthesis of GABA, were studied in the temporal lobe and frontal cortex.

The behavioral experiments lasted from 10 a.m. to 3 p.m. and were performed in two separate rooms. In the first room the elevated plus-maze experiment was performed and in the second room animals were decapitated for dissection of brain structures. The elevated plus-maze test was carried out as described above. Before testing in the elevated plus-maze, animals were kept in isolation for 15 min. According to our previous experience, short-term isolation of mice increases their exploratory activity. During a 5-min observation session the following measures were taken: (1) the number of closed and open arm entries, (2) time spent in exploring the open arms and (3) the number of head-dipping and stretch-attend postures. Subsequently, the ratio between open and total arm entries was calculated. Testing began by placing an animal on the centre of the elevated plus-maze facing the closed arm. An arm entry was counted only when all four limbs of a mouse were within a given arm (Raud et al., 2009).

Experimentally naïve animals and animals exposed to the elevated plus-maze, belonging to both genotypes, were used in parallel. Mice were decapitated immediately after the elevated plus-maze exposure or taking them out from the home-cage. Brains were quickly dissected into four parts (the frontal cortex, mesolimbic area [including the nucleus accumbens and olfactory tubercle], hippocampus and temporal lobe [including the amygdala]) and frozen in liquid nitrogen. The brain dissection was performed according to the coordinates presented in the mouse brain atlas (Franklin and Paxinos 1997). Total RNA was extracted individually from each brain structure of each mouse using Trizol® Reagent (Invitrogen, USA) according to the manufacturer's protocol. First strand cDNA was synthesized by using poly (T)18 oligonucleotides and SuperScript™ III Reverse Transcriptase (Invitrogen, USA). The quantitative real-time PCR (qRT-PCR) experiment for Gabra1 and Gabra2 expression studies was performed using SYBR Green. SYBR® Green technology was chosen, because these primers were previously designed and optimized for this method in our other study (Sütt *et al*., 2008). The primers used are presented in Table 3 (Raud et al., 2009).

**Table 3.** The primers used for analysis of GABA-ergic genes

| Gene symbol | Assay ID or sequence | Gene ID |
|---|---|---|
| **Gabra1 forward** | 5'-TGTACACCATGAGGTTGACCGT-3', | NM 919250.3 |
| ***Gabra 1* reverse** | 5'-GAAGTCTTCCAAGTGCATTGGG-3' | NM 919250.3 |
| ***Gabra 2* forward** | 5'-ATGGTCTCTGCTGCTTGTTCTTCT-3' | NM 008066 |
| ***Gabra 2* reverse** | 5'-AGCACCAACCTGACTGGGTC-3' | NM 008066 |
| ***Hprt* forward** | 5'-GCAGTACAGCCCCAAAATGG-3' | |
| **Hprt reverse** | 5'-AACAAAGTCTGGCCTGTATCCAA-3' | |

Raud *et al.,* 2009.

For Gad1 (ID: NM 008077) and Gad2 (ID: 008078) gene expression, Taqman assays Mm00725661_s1 and Mm01329282_m1 (Applied Biosystems) were used, respectively. In order to select the most stably expressed reference gene we used pairwise comparison approach developed by Vandesompele (Vandesompele *et al.,* 2002). This approach top ranks the candidates with the highest degree of similarity of the expression profile across the sample set. In the present study three candidate normalization genes, HPRT1, GAPDH, B2M ($\beta_2$ - microglobulin), were used as they are considered the most common used housekeeping genes expressed in brain tissues. The amplification efficiency of the reference genes was as follows: HPRT1 96%, GAPDH 67% and B2M 92%. The intra-assay coefficient of variability for B2 M, GAPDH and HPRT1 genes was 0.62%, 0.11% and 0.06%, respectively. The inter-assay variability for B2 M, GAPDH and HPRT1 was 3.4%, 2.3% and 2%, respectively. According to the internal gene-stability measure (*M*) of the candidate gene the most stable genes were HPRT1 ($M = 0.013$) and GAPDH ($M = 0.014$). The *M* value for B2 M was 0.022. However, since the amplification efficiency of HPRT1 was

higher compared to GAPDH and also it was equal to the amplification efficiency on target genes Gabra1 and Gabra2 (data not shown), we chose HPRT1 as the housekeeper for the real-time PCR analysis. Another argument for selecting HPRT1 was the fact that [$C_t$] for GAPDH resulted in higher value between the target and reference gene and this makes following comparison between groups not so effective as in case of HPRT1, because a higher value between the target and reference gene gives smaller differences in ratio calculation.

For the Hprt gene, a housekeeping gene, the following primers were used: 5'-GCAGTACAGCCCCAAAATGG-3' (forward) and 5'-AACAAAGTCTGG CCTGTATCCAA-3' (reverse). The probe for Hprt was 5'-VIC-AAGCTTG CTGGTGAAAAGGACCTCTCG TAMRA-3' (Raud *et al.,* 2009)

**Gene expression studies of dopamine-related gened in Wfs1 deficient mice (Study 3)**

This study was done on a separate group of animals taken directly from their home-cages. The gene expression studies in wild-type, heterozygous and homozygous animals were conducted in parallel. The measurements were performed on the midbrain for dopamine transporter and the dorsal and ventral striatum for dopamine D2 receptors(Drd2). For qRT-PCR analysis, the ABI PRISM 7900HT Fast Real-Time PCR System equipment (PE Applied Biosystems, USA) and the ABI PRISM 7900 SDS 2.2.2 Software were used. Every reaction was made in four parallel samples to minimize possible errors. All reactions were performed in a final volume of 10 µl, using 50–100 ng of cDNA. Taqman assays (PE Applied Biosystems) for genes are given in Table 3 (Visnapuu *et al.,* 2013).

**Table 4.** The Taqman assays and probes used in the study

| Gene symbol | Assay ID or sequence | Gene ID |
|---|---|---|
| **Slc6a3 (*dat*)** | Mm00438396_m1 | NM_010020 |
| ***Drd2*** | Mm00438545_m1 | NM_010077.2 |
| ***Hprt1* for** | 5'-GCAGTACAGCCCCAAAATGG-3' | |
| ***Hprt1* rev** | 5'- AACAAAGTCTGGCCTGTATCCAA-3' | NM_013556 |
| ***Hprt1* probe (VIC_TAMRA)** | 5'-VIC-AAGCTTGCTGGTGAAAAGGACCTCTCG TAMRA-3' | |

Visnapuu *et al.,* 2013

## 5.7. Western blot

As in the case of gene expression study, the protein measurements were performed on a separate group of animals taken directly from their home-cages. Brains were removed and the dorsal and ventral striatum dissected. Protein extraction was performed using the PARIS kit (Life Technologies) supplemented with Protease inhibitor cocktail (Thermo Scientific) as described by Raida *et al*. (2012). The tissues were sonicated and centrifuged for 10 min

12,000g at 4°C. The supernatants were kept on –80°C and the protein concentrations were measured by BCA method. Reagents and equipment for electrophoreses and protein transfer was used according to manufacture's guidelines regarding the NuPAGE system (Life Technologies) and described in detail in Raida *et al.* 2012. For western blotting, membranes were blocked for one hour in 3% BSA in PBS. Following blocking the membranes were incubated with rat anti-Dat (Santa Cruz Biotechnologies cat# sc- 32258 in 1:1,000 dilution) and rabbit anti-ß-actin (Cell Signaling cat# 4970 in 1:15,000 dilution) overnight at 4°C. After primary antibody incubation the membranes were washed 6 times in Milli-Q water and incubated with Alexa 680-conjugated donkey anti-rat and Alexa-790 conjugated donkey anti-rabbit (Jackson Immuno Research cat# 712-625-150 and 711-655-152 in 1:20,000 dilution) for one hour at RT. After secondary incubations the membranes were washed 6 times in Milli-Q water followed by a 20 min wash step in PBS-0,1% Tween-20. Prior to signal detection the membranes were washed 6 times in Milli-Q water. Antibody detection was performed using the Li-Cor Odyssey CLx system (Li-Cor biotechnologies). Images were converted to grayscale and quantification was performed using the Gel-analyzer plugin in ImageJ following NIH guidelines (Visnapuu *et al.,* 2013).

## 5.8. Statistics and analysis of results

Mean values and S.E.M. are presented in the figures and tables. All data were analyzed using Statistica version 8.0 (StatSoft, Inc., USA). Behavioural studies and corticosterone measurements were analysed using one-way or two-way analysis of variance (ANOVA). Two-way mixed design ANOVA was applied to data from glucose tolerance test (genotype, between-subjects × time, within subjects) and fear conditioning studies (genotype, between-subjects × tone, within subjects). Data from studies of locomotor activity as well as the learning curves in active avoidance and Morris water maze tests were analysed by repeated measures ANOVA. Genotype was a between-subjects variable in all ANOVA analyses. Data from forced swimming test was analysed with non-parametric Mann–Whitney *U*-test. Data from hyponeophagia, rota-rod test and context-dependent experiments in fear conditioning paradigm were analysed with Student's *t*-test. A three way repeated measures ANOVA (genotype x treatment x day) was used in the experiment measuring sensitization to amphetamine. The plus-maze studies for evaluation of GABA-related gene expression were analyzed by means of Student's *t*-test for independent samples. As our goal was to compare the effect of the plus-maze exposure and genetic manipulation on the expression of GABA-related genes, the only acceptable way to test the validity of this approach was application of two-way ANOVA (genotype and plus-maze exposure as independent variables). Two-way ANOVA (genotype and treatment as independent measures) was performed in the experiments measuring the locomotor activity and brain dopamine levels. One-way analysis of variance (ANOVA) was applied in the dopamine-related

gene expression experiments and Mann-Whitney U test was used for the protein expression studies. *Post hoc* comparisons between individual groups were performed using either Newman–Keuls or Tukey HSD test (Luuk *et al.,* 2009, Raud *et al.,* 2009, Visnapuu *et al.,* 2013)

# 6. RESULTS

## 6.1. Study 1: Description of behavioral phenotype of Wfs1 deficient mice

**Reproduction and overt appearance**

Analysis of reproduction rates of Wfs1-deficient mice during 5 months in late 2007 and early 2008 (a total of 526 births was recorded during the period) indicated an approximately 5% lower birthrate of homozygous Wfs1-deficient mice than expected (20.8% of all females, 21.8% of all males). In general, homozygous Wfs1-deficient mice were normal in overt appearance, but occasionally very small individuals (weighing around 15 g at 3 months of age) were present in the male population. The most peculiar aspect of the overt phenotype of homozygous Wfs1-deficient population was the presence of individuals producing spontaneous audible vocalizations in the 10 kHz range. The proportion of such individuals varied across different batches from around 15 to 50%, and was comparable in males and females. The vocalizations can be described as "chirps" with a fundamental frequency of 3.1 kHz and prominent harmonics at 6.2 and 9.3 kHz (Fig. 4). It was noted that the number of mice producing audible vocalizations was higher under stressful conditions (bright light, handling/injection stress), that the temporal frequency of these calls could get as high as five times per second, and that the calls could vary in loudness. Additionally, in most individuals, calling was blocked by the administration of diazepam (1 mg/ml) (Luuk *et al.,* 2009).

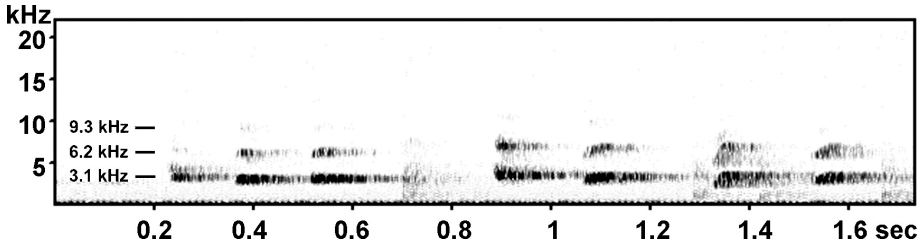


**Figure 4.** Sonogram of audible vocalizations produced by homozygous Wfs1-deficient mice (Luuk *et al.,* 2009).

**Body weight and glucose tolerance test**

In the first batch of 2- and 3-month-old female mice, significantly lower body weight was found in homozygous Wfs1-deficient mice when compared to wild-type littermates (Fig. 5A) ($F(1, 105) = 60.8$, $p < 0.001$ (genotype); $F(1, 105) = 7.9$, $p < 0.01$ (age, between-subjects); $F(1, 105) = 2.46$, $p = 0.12$ (genotype × age)). In contrast to wild-types, there was no significant body weight difference between 2- and 3-month-old Wfs1-deficient mice. In a different batch of mice,

significant body weight difference between female homozygous Wfs1-deficient and wild-type mice was established only at 4 months of age (Fig. 5B). Weight gain between 2 and 4 months of age was more pronounced in wild-type and heterozygous Wfs1-deficient mice when compared to homozygous mice ($F(2, 27) = 6.7$, $p < 0.01$ (genotype); $F(1, 27) = 11.9$, $p < 0.01$ (age, within-subjects); $F(2, 27) = 0.54$, $p = 0.59$ (genotype × age) (Luuk *et al.,* 2009).

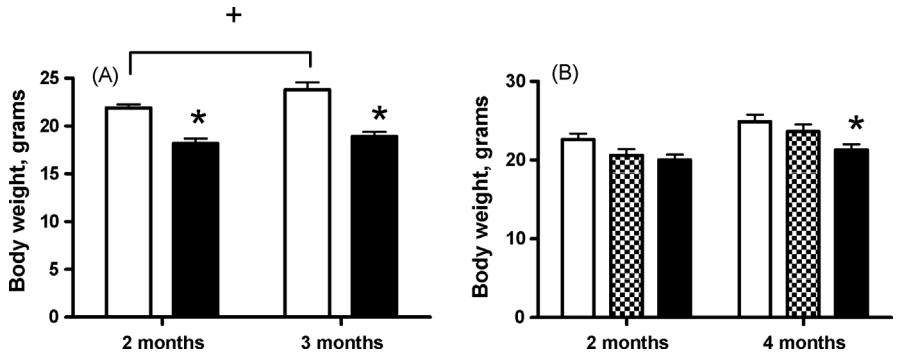


**Figure 5.** Lower body weight in female homozygous Wfs1-deficient mice. (A) Body weight of wild-type (white bars) and homozygous Wfs1-deficient mice (black bars) at 2 and 3 months of age. *$p < 0.05$ (compared to wild-type mice of respective age; Newman–Keuls test after significant two-way ANOVA); $^{+}p < 0.05$ (compared to wild-type mice at 2 months of age). Number of mice in groups ranged from 22 to 30. (B) Weight gain in wild-type (white bars), heterozygous (hatched bars) and homozygous (black bars) Wfs1-deficient mice. *$p < 0.05$ (compared to wild-type mice of respective age; Newman–Keuls test after significant repeated measures ANOVA). Number of mice in each group was 10 (Luuk *et al.,* 2009).

Baseline blood glucose levels did not differ between non-fasted wild-type, heterozygous and homozygous Wfs1-deficient mice (Fig. 6). Intraperitoneal administration of glucose elevated blood glucose levels only in heterozygous and homozygous Wfs1-deficient mice ($F(2, 27) = 154.4$, $p < 0.001$ (genotype); $F(3, 27) = 81.5$, $p < 0.001$ (time, within-subjects); $F(6, 27) = 23.5$, $p < 0.001$ (genotype × time)). Compared to the baseline, in homozygous mice, the concentration of blood glucose was significantly higher at 30 and 60 min ($p < 0.001$), and in heterozygous mice at 30 min ($p < 0.001$) after the administration of glucose (Luuk *et al.,* 2009).

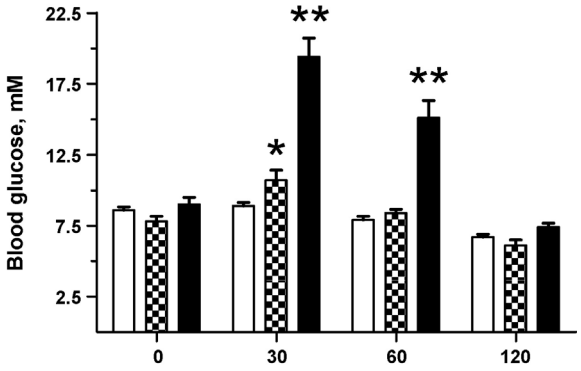


**Figure 6.** Intolerance to intraperitoneal glucose in non-fasted Wfs1-deficient mice. The experiment was performed in female mice. Blood glucose concentration after i.p. administration of glucose (2 g/kg). White bars, wild-type mice; hatched bars, heterozygous Wfs1-deficient mice; black bars, homozygous Wfs1-deficient mice. *$p < 0.05$ (compared to wild-type mice at respective time point; Newman–Keuls test after significant repeated measures ANOVA); **$p < 0.001$. Number of mice in each group was 10 (Luuk *et al.,* 2009).

**Stress-induced changes in corticosterone levels**

No differences in mean blood plasma corticosterone levels were detected in unstressed individuals of different genotypes (Fig. 7). Stress induced by handling and injection of saline induced a significantly larger increase in the plasma corticosterone concentration in female homozygous Wfs1-deficient mice ($p < 0.05$) when compared to their wild-type littermates (Fig. 7) ($F(1, 12) = 30.9$, $p < 0.001$ (genotype); $F(1, 12) = 128.8$, $p < 0.001$ (injection, between-subjects); $F(1, 12) = 26.6$, $p < 0.001$ (genotype × injection)) (Luuk *et al.,* 2009).

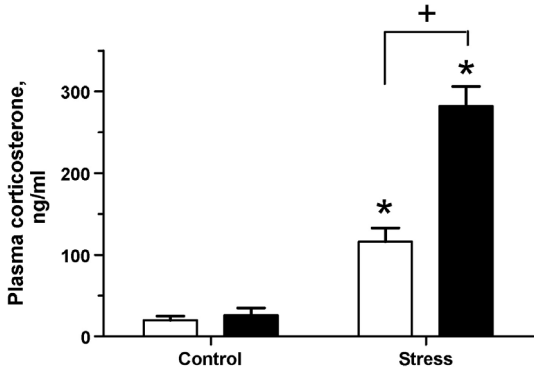


**Figure 7.** Stress induces higher levels of plasma corticosterone in female Wfs1-deficient mice. White bars, wild-type mice; black bars, homozygous Wfs1-deficient mice. *$p < 0.05$ (compared to unstressed individuals of respective genotype; Newman–Keuls test after significant two-way ANOVA); $^{+}p < 0.05$ (compared to respective group of wild-type mice). Number of mice in each group was 4 (Luuk *et al.,* 2009).

**Stress-induced analgesia**

In both, wild-type and homozygous Wfs1-deficient mice, the degree of analgesia was dependent on the intensity of electric foot-shocks (Fig. 8) ($F(3, 77) = 54.4$, $p < 0.001$ (stress, between-subjects)). Foot-shocks of intermediate intensity (0.4 mA) induced a significantly stronger analgetic response in homozygous Wfs1-deficient mice compared to their wild-type littermates ($F(1, 77) = 5.5$, $p < 0.05$ (genotype); $F(3, 77) = 3.3$, $p < 0.05$ (genotype × stress)) (Luuk *et al.,* 2009).

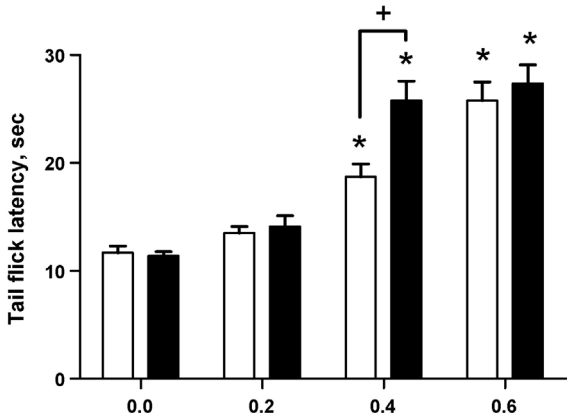


**Figure 8.** Stress-induced analgesia in female Wfs1-deficient mice. White bars, wild-type mice; black bars, homozygous Wfs1-deficient mice. $*p < 0.05$ (compared to unstressed littermates of respective genotype; Newman–Keuls test after significant two-way ANOVA); $^{+}p < 0.05$ (compared to respective group of wild-type animals). Number of mice in groups ranged from 10 to 12 (Luuk *et al.,* 2009).

**Rota-rod test**

Performance of wild-type (119 ± 1 s; $n = 19$) and homozygous Wfs1-deficient mice (110 ± 7 s; $n = 20$) was statistically not different in the rota-rod test (Luuk *et al.,* 2009).

**Locomotor activity in dim and bright environments**

Locomotor activity of wild-type and homozygous Wfs1-deficient mice was compared in a dimly (~20 lx) and brightly (~450 lx) lit room (Fig. 9). Exposure of mice to bright light decreased all locomotor activity parameters in both genotypes in relation to the dim environment (time in locomotion: $F(1, 37) = 116.1$, $p < 0.001$ (environment, within-subjects)); distance travelled: $F(1, 37) = 93.4$, $p < 0.001$; time spent in the centre: $F(1, 37) = 39.5$, $p < 0.001$; number of rearing: $F(1, 37) = 17.1$, $p < 0.001$). In the dark room, homozygous Wfs1-deficient mice made significantly less rearings ($p < 0.05$) while the other parameters were similar in the genotypes. Locomotor activity was significantly lower in Wfs1-deficient mice under bright light (time in locomotion: $F(1, 37) = 10.6$, $p < 0.01$ (genotype); $F(1, 37) = 1.87$, $p = 0.18$ (genotype × environment);

distance travelled: $F(1, 37) = 8.1$, $p < 0.01$ (genotype); $F(1, 37) = 1.70$, $p = 0.20$ (genotype × environment); number of rearing: $F(1, 37) = 7.3$, $p < 0.05$ (genotype), $F(1, 37) = 0.02$, $p = 0.90$ (genotype × environment)) (Luuk *et al.,* 2009).

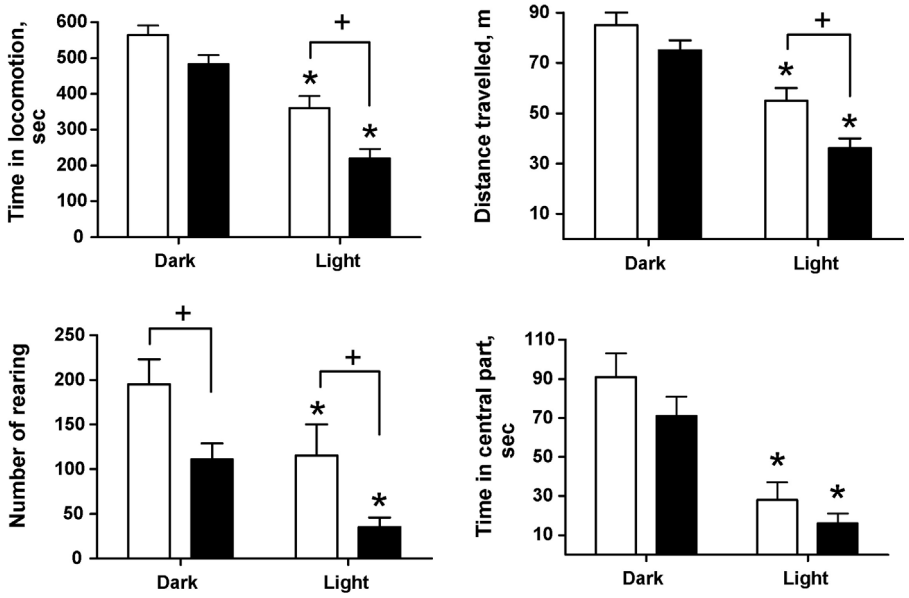


**Figure 9.** Lower exploratory activity in female Wfs1-deficient mice at high illumination levels. White bars, wild-type mice ($n = 20$); black bars, homozygous Wfs1-deficient mice ($n = 19$). *$p < 0.05$ (compared to respective genotype in the dark environment; Newman–Keuls test after significant repeated measures ANOVA); $^{+}p < 0.05$ (compared to respective group of wild-type animals) (Luuk *et al.,* 2009).

**Effect of amphetamine and apomorphine on locomotor activity**

Administration of amphetamine (2.5, 5 and 7.5 mg/kg) induced a dose-dependent activation of locomotor behaviour in female wild-type mice (Fig. 10). The stimulatory effect of amphetamine was weaker in heterozygous and homozygous Wfs1-deficient mice than in wild-type littermates (time in locomotion: $F(2, 27) = 5.78$, $p = 0.008$ (genotype), $F(3, 81) = 109.2$, $p < 0.001$ (treatment, within-subjects), $F(6, 81) = 0.57$, $p = 0.76$ (genotype × treatment); distance travelled: $F(2, 27) = 4.1$, $p < 0.05$ (genotype), $F(3, 81) = 59.3$, $p < 0.001$ (treatment, within-subjects), $F(6, 81) = 1.12$, $p = 0.36$ (genotype × treatment); number of corner entries: $F(2, 27) = 3.1$, $p = 0.06$ (genotype); $F(3, 81) = 34.7$, $p < 0.001$ (treatment, within-subjects); $F(6, 81) = 1.05$, $p = 0.40$ (genotype × treatment)). The stimulatory effect of intermediate dose of amphetamine (5 mg/kg) on locomotor activity was significantly weaker in homozygous Wfs1-deficient mice than in wild-type littermates in terms of distance travelled and number of corner entries ($p < 0.05$). By contrast, treatment of the same mice with apomorphine (3 mg/kg)

induced significantly higher locomotor activation in female homozygous Wfs1-deficient mice when compared to the other genotypes (time in locomotion: $F(2, 27) = 5.62$, $p < 0.01$ (genotype), $F(1, 27) = 29$, $p < 0.001$ (treatment, within-subjects), $F(2, 27) = 0.89$, $p = 0.42$ (genotype × treatment); distance travelled: $F(2, 27) = 5.62$, $p < 0.01$ (genotype), $F(1, 27) = 27.3$, $p < 0.001$ (treatment, within-subjects), $F(2, 27) = 0.54$, $p = 0.59$ (genotype × treatment); number of corner entries: $F(2, 27) = 3.74$, $p < 0.05$ (genotype), $F(1, 27) = 44.6$, $p < 0.001$ (treatment, within-subjects), $F(2, 27) = 0.18$, $p = 0.83$ (genotype × treatment)). A gene dose effect of Wfs1 was apparent for all parameters presented in Fig. 10 (Luuk *et al.,* 2009).

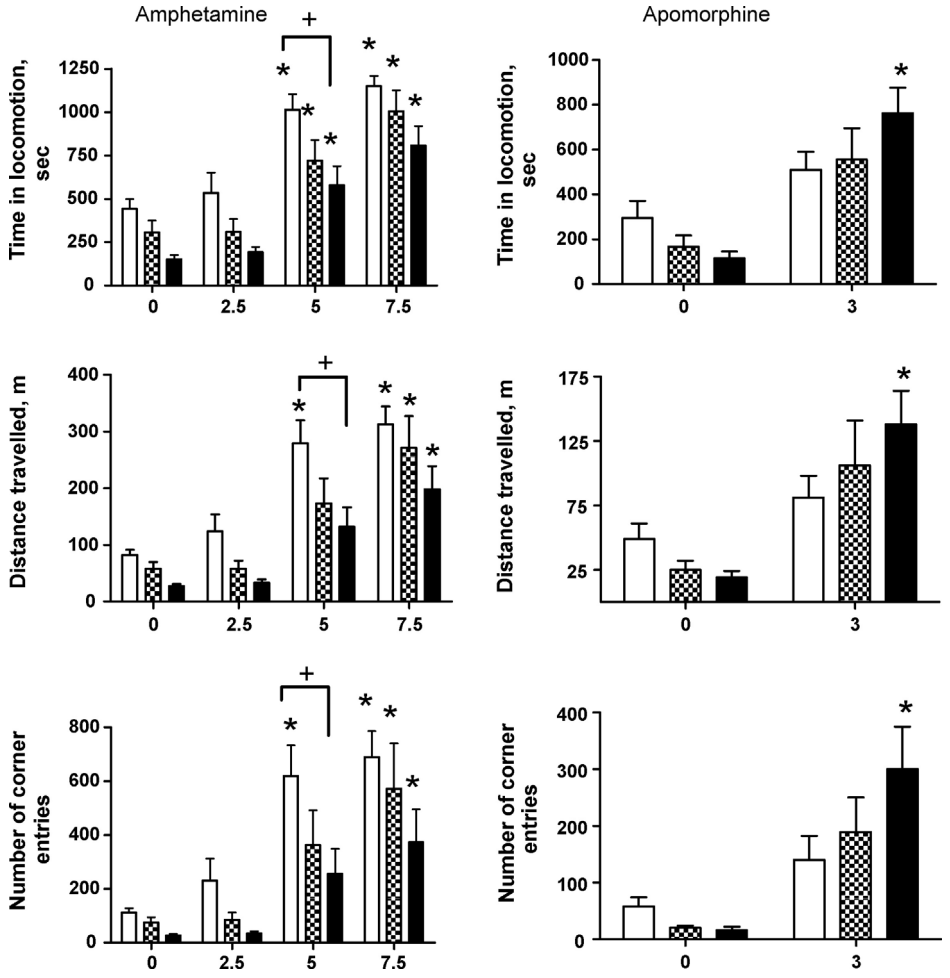


**Figure 10.** Differential sensitivity of female Wfs1-deficient mice to the locomotor stimulatory effects of amphetamine (2.5, 5 and 7.5 mg/kg) and apomorphine (3 mg/kg). White bars, wild-type mice; hatched bars, heterozygous Wfs1-deficient mice; black bars, homozygous Wfs1-deficient mice. *$p < 0.05$ (compared to saline-treated mice of respective genotype; Newman–Keuls test after significant repeated measures ANOVA); $^{+}p < 0.05$ (compared to respective group of wild-type animals). Number of animals in each group was 10 (Luuk *et al.,* 2009).

**Effect of short-term isolation on behaviour in light-dark box exploration test**

The exploratory activity of non-isolated wild-type and homozygous Wfs1-deficient mice was not different in the light-dark box exploration test (Fig. 11). By contrast, short-term isolation induced a significant suppression of exploratory behaviour only in Wfs1-deficient mice. Wfs1-deficient mice made less transitions between the two compartments ($F(1, 42) = 8.40$, $p < 0.01$ (genotype); $F(1, 42) = 0.96$, $p = 0.33$ (isolation, between-subjects); $F(1, 42) = 2.99$, $p = 0.09$ (genotype × isolation)) and spent less time in the light compartment as compared to wild-type littermates ($F(1, 42) = 9.32$, $p < 0.01$ (genotype); $F(1, 42) = 3.41$, $p = 0.07$ (isolation, between-subjects); $F(1, 42) = 0.92$, $p = 0.34$ (genotype × isolation)) (Luuk *et al.,* 2009).

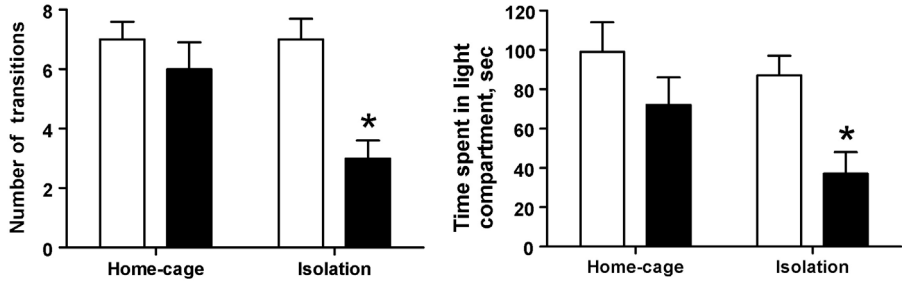


**Figure 11.** Anxiety-like behaviour in Wfs1-deficient mice in light-dark box exploration test. White bars, wild-type mice; black bars, homozygous Wfs1-deficient mice. *$p < 0.05$ (compared to isolated wild-type mice; Newman–Keuls test after significant two-way ANOVA). Number of animals in groups ranged from 11 to 12 (Luuk *et al.,* 2009).

**Effect of diazepam in elevated plus-maze**

Vehicle-treated homozygous Wfs1-deficient mice displayed lower exploratory activity when compared to vehicle-treated wild-types (Fig. 12). Specifically, the percentage of open arm entries and the number of head-dippings was significantly lower in Wfs1-deficient mice (Newman–Keuls test, $p < 0.05$). A similar, albeit not statistically significant, trend was observed for the number of open arm entries and time spent on open arms. Additionally, the number of incomplete attempts to enter the central platform, a measure of risk assessment behaviour (Nelovkov *et al.,* 2006), was significantly higher in homozygous Wfs1-deficient mice when compared to wild-type littermates. Exploratory activity of wild-type mice was not affected by the administration of diazepam (1 mg/kg). In Wfs1-deficient mice, the administration of diazepam caused a robust anxiolytic-like effect as evidenced by increased number of open arm entries ($F(1, 60) = 0.01$, $p = 0.99$ (genotype); $F(1, 60) = 6.19$, $p < 0.01$ (treatment, between-subjects); $F(1, 60) = 3.72$, $p < 0.05$ (genotype × treatment)), increased time spent on open arms ($F(1, 60) = 0.46$, $p = 0.50$ (genotype); $F(1, 60) = 6.18$, $p < 0.01$ (treatment, between-subjects); $F(1, 60) = 2.75$, $p = 0.10$ (genotype × treatment)), increased percentage of open arm entries from total arm entries

($F(1, 60) = 0.18$, $p = 0.67$ (genotype); $F(1, 60) = 6.08$, $p < 0.05$ (treatment, between-subjects); $F(1, 60) = 4.65$, $p < 0.05$ (drug × treatment)), increased number of head-dippings ($F(1, 60) = 9.16$, $p < 0.01$ (genotype); $F(1, 60) = 3.44$, $p = 0.06$ (treatment, between-subjects); $F(1, 60) = 2.30$, $p = 0.13$ (genotype × treatment)) and decreased number of incomplete attempts to enter the central platform ($F(1, 60) = 2.08$, $p = 0.15$ (genotype); $F(1, 60) = 5.31$, $p < 0.05$ (treatment, between-subjects); $F(1, 60) = 1.28$, $p = 0.26$ (genotype × treatment)) (Luuk *et al.,* 2009).

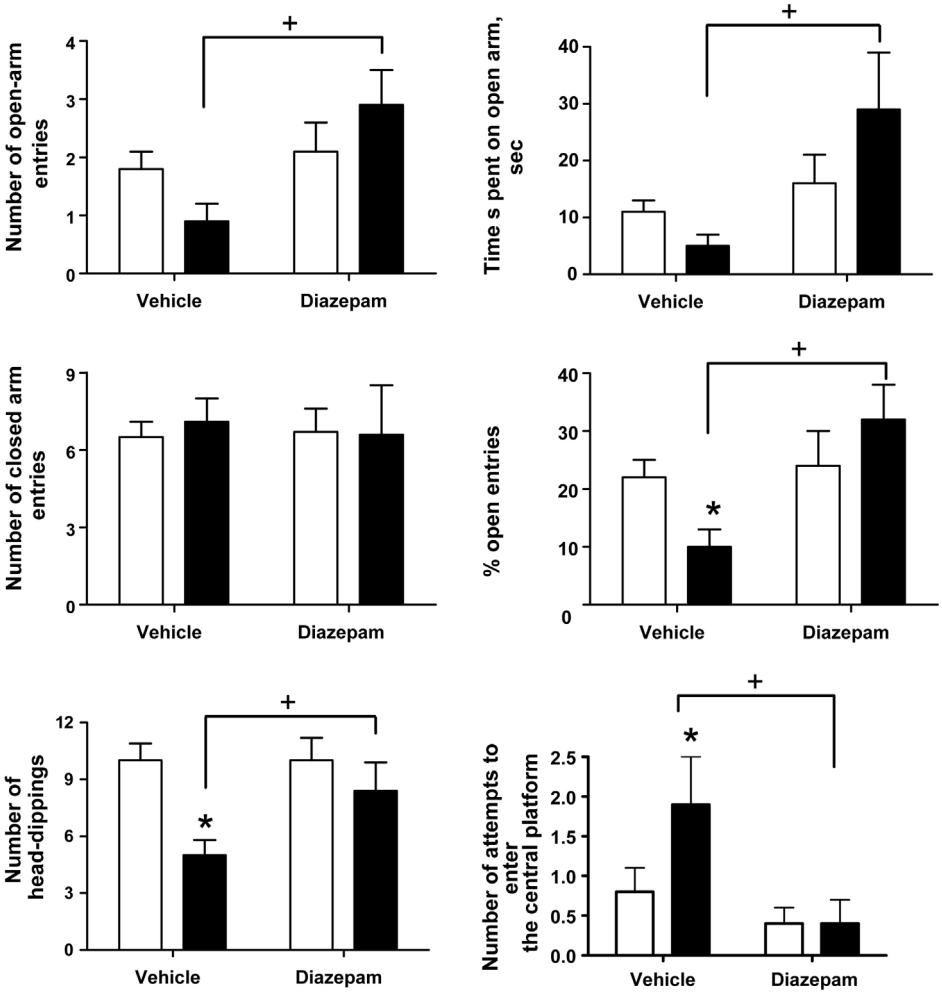


**Figure 12.** Anxiolytic effect of diazepam (1 mg/kg) on exploratory behaviour of female Wfs1-deficient mice in the elevated plus-maze test. White bars, wild-type mice; black bars, homozygous Wfs1-deficient mice. $*p < 0.05$ (compared to vehicle-treated wild-type mice; Newman–Keuls test after significant two-way ANOVA); $^{+}p < 0.05$ (compared to vehicle-treated Wfs1-deficient mice). Number of animals in each group was 16 (Luuk *et al.,* 2009).

**Fear conditioning test**

Homozygous Wfs1-deficient and wild-type mice exhibited comparably low freezing behaviour in the pre-conditioning test (no freezing in wild-type and two freezing episodes in Wfs1-deficient mice). Both genotypes exhibited normal contextual memory after conditioning by increased freezing in the absence of the conditioned stimulus (Fig. 13A). However, in the Wfs1-deficient mice, there was also a tendency towards the reduced number of rearings compared to wild-type littermates (Student's *t*-test, $p < 0.05$). When transferred to a new context, both, freezing behaviour and rearing were similar to that observed in the pre-conditioning session (Fig 13B). Testing of cued fear by presenting the conditioned stimulus in the novel context produced a significant increase in freezing behaviour ($F(1, 66) = 1.57$, $p = 0.21$ (genotype); $F(1, 66) = 152.1$, $p < 0.001$ (conditioned stimulus, within-subjects); $F(1, 66) = 0.42$, $p = 0.52$ (genotype × conditioned stimulus)) and a decrease in rearing ($F(1, 66) = 8.02$, $p < 0.01$ (genotype); $F(1, 66) = 30.6$, $p < 0.001$ (conditioned stimulus, within-subjects); $F(1, 66) = 10.3$, $p < 0.01$ (genotype × conditioned stimulus)) in both genotypes. Reduction in the number of rearings upon CS presentation was statistically significant only in wild-type mice ($p < 0.05$). While there was a tendency towards reduced rearing in Wfs1-deficient mice in the cued situation, floor-effect is a likely explanation of it not reaching statistical significance (Luuk *et al.,* 2009).

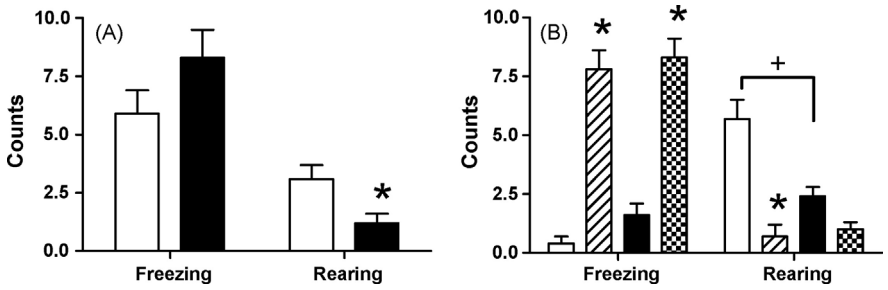


**Figure 13.** Contextual and cue-dependent responses in female Wfs1-deficient mice in fear conditioning test. (A) Contextual fear. White bars, wild-type mice; black bars, homozygous Wfs1-deficient mice. $*p < 0.05$ (compared to wild-type mice, Student's *t*-test). (B) Cued fear. White bars, wild-type mice in new context; striped bars, wild-type mice exposed to cued signal in new context; black bars, homozygous Wfs1-deficient mice in new context; hatched bars, homozygous Wfs1-deficient mice exposed to cued signal in new context. $*p < 0.05$ (compared to same mice in non-cued situation; Newman–Keuls test after significant repeated measures ANOVA); $^{+}p < 0.05$ (compared to wild-type mice in non-cued situation). Thirty-four wild-type and 33 Wfs1-deficient mice were used in this experiment (Luuk *et al.,* 2009).

**Hyponeophagia test**
Latency to start eating was significantly longer (Student's *t*-test, $p < 0.05$) in homozygous Wfs1-deficient mice (162 ± 8 s; $n = 19$) when compared to wild-type littermates (135 ± 10 s; $n = 19$) (Luuk *et al.,* 2009).

**Forced swimming test**
The number of mice was 19 for wild-type and 20 for Wfs1-deficient mice. Forced swimming did not reveal statistically significant differences between wild-type and homozygous Wfs1-deficient mice. For the following parameters the values (in seconds) for wild-type and Wfs1-deficient mice were, respectively: 39 ± 1.3 and 36 ± 2.4 (immobility), 5.2 ± 0.6 and 8.7 ± 2.4 (swimming), 3.9 ± 1.2 and 2.9 ± 1.3 (climbing) (Luuk *et al.,* 2009).

**Morris water maze test**
When compared to wild-type littermates, Wfs1-deficient mice displayed overall slower swimming speed ($F(1, 37) = 8.23$, $p < 0.01$ (genotype); $F(3, 111) = 6.0$, $p < 0.001$ (trial, within-subjects); $F(3, 111) = 0.19$, $p = 0.91$ (genotype × trial)) (Fig. 14). Hence, the distance covered in finding the escape platform was a more appropriate measure of the learning effect than escape latency. The distances to find the platform during the initial training were as follows. Day 1: 879 ± 55 cm (wild-type) vs. 749 ± 51 cm (Wfs1-deficient); day 2: 434 ± 46 cm (wild-type) vs. 475 ± 57 cm (Wfs1-deficient); day 3: 300 ± 49 cm (wild-type) vs. 367 ± 57 cm (Wfs1-deficient); day 4: 177 ± 23 cm (wild-type) vs. 300 ± 43 cm (Wfs1-deficient). There was a significant learning effect in terms of swimming distance in both genotypes (day 1 vs. day 4: $p < 0.001$), but no effect of genotype on the swimming distance ($F(1, 37) = 0.42$, $p = 0.52$ (genotype); $F(3, 111) = 58.0$, $p < 0.001$ (trial, within-subjects); $F(3, 111) = 2.73$, $p < 0.05$ (genotype × trial)). Additionally, both wild-type and Wfs1-deficient mice displayed significant learning effect by spending more time in the target quadrant in the probe trial on day 4 (Fig. 15A) ($F(1, 148) = 0.001$, $p = 0.99$ (genotype); $F(3, 148) = 87.3$, $p < 0.001$ (quadrant, within-subjects); $F(3, 148) = 2.83$, $p < 0.05$ (genotype × quadrant)). However, wild-type mice spent more time in the target quadrant than their Wfs1-deficient littermates ($p < 0.05$). A significant learning effect was observed in both genotypes also after reversal training as evidenced by preference for the new target quadrant in probe trial on day 6 (Fig. 15B). However, in reversal training, Wfs1-deficient mice covered longer distances when searching for the platform (Fig. 14) ($F(1, 37) = 11.4$, $p < 0.01$ (genotype); $F(3, 111) = 51.4$, $p < 0.001$ (trial, within-subjects); $F(3, 111) = 3.0$, $p < 0.05$ (genotype × trial)) (Luuk *et al.,* 2009).

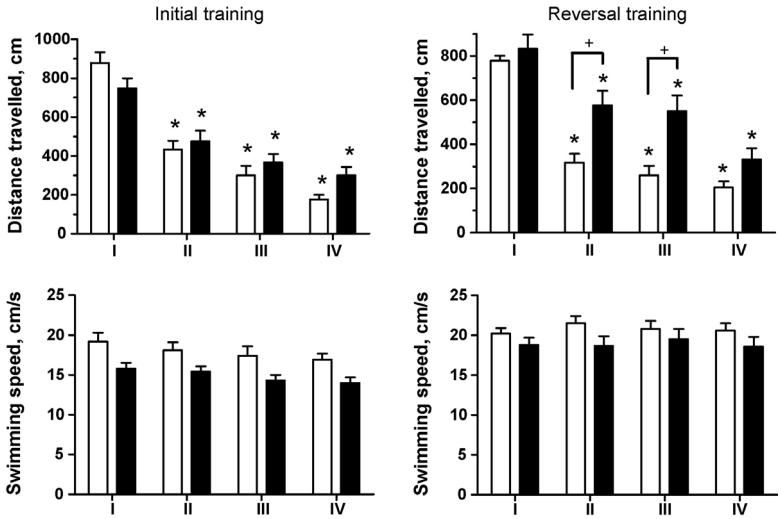


**Figure 14.** Morris water maze: distance travelled and swimming speed. White bars, wild-type mice; black bars, homozygous Wfs1-deficient mice. *$p < 0.05$ (compared to the first trial of respective genotype, Newman–Keuls test after significant repeated measures ANOVA); $^{+}p < 0.05$ (compared to respective group of wild-type animals). Nineteen wild-type and 20 homozygous Wfs1-deficient mice were used in the test (Luuk *et al.,* 2009).

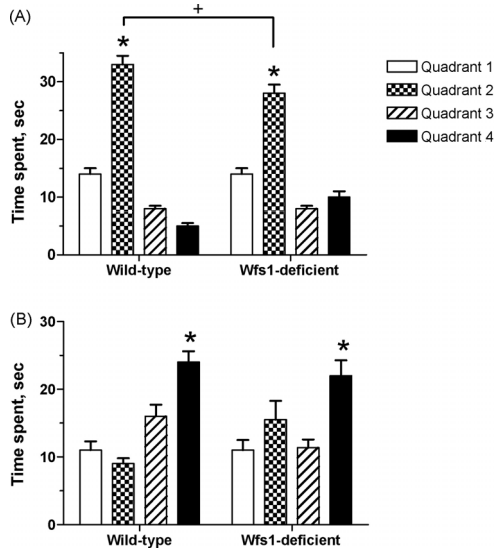


**Figure 15.** Spatial memory of Wfs1-deficient mice in Morris water maze: time spent in the four quadrants. (A) Probe trial after initial training (day 4). *$p < 0.05$ (compared to time spent in quadrants 1, 3 and 4; Newman–Keuls test after significant two-way ANOVA); $^{+}p < 0.05$ (compared to wild-type animals). (B) Probe trial after reversal training (day 6). *$p < 0.05$ (compared to time spent in quadrants 1, 2 and 3; Newman–Keuls test after significant two-way ANOVA). Nineteen wild-type and 20 homozygous Wfs1-deficient mice were used in the test (Luuk *et al.,* 2009).

**Active avoidance test**

Both, wild-type and homozygous Wfs1-deficient mice learned the task of active avoidance without significant differences (Fig. 16) ($F(1, 36) = 0.58$, $p = 0.45$ (genotype); $F(3, 108) = 12.2$, $p < 0.001$ (trial, within-subjects); $F(3, 108) = 0.05$, $p = 0.98$ (genotype × trial)) (Luuk *et al.,* 2009).

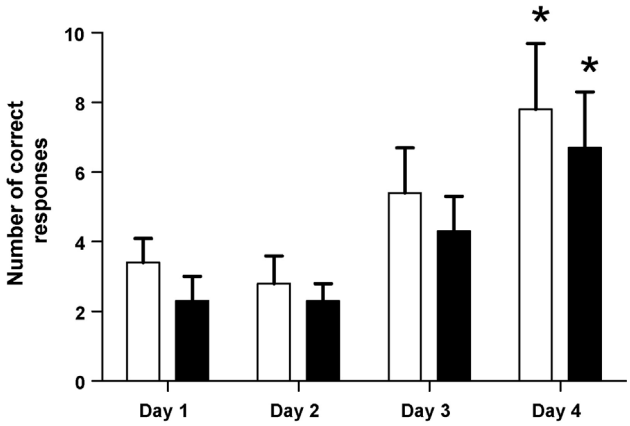


**Figure 16.** Conditioned responses of Wfs1-deficient mice in active avoidance test. White bars, wild-type mice; black bars, homozygous Wfs1-deficient mice. *$p < 0.05$ (compared to the first day of respective genotype, Newman–Keuls test after significant repeated measures ANOVA). Number of animals in each group was 19 (Luuk *et al.,* 2009).

## 6.2. Study 2. Reduced expression of alpha1 and alpha2 subunits of GABA(A) receptors in Wfs1-deficient mice

**Exploratory behavior of Wfs1 deficient mice in elevated plus-maze**

In the plus-maze test Wfs1-deficient mice displayed increased anxiety-like behavior in terms of increased open arm avoidance and affected risk assessment behaviors. Homozygous animals made significantly less open arm entries as compared to wild-type mice (Student's *t*-test, $p < 0.05$) (Fig. 17). Also, a similar tendency was observed for other conventional measures of anxiety: time spent on open arms ($p = 0.07$) and ratio between the open and total arm entries ($p = 0.07$). Additionally, the genotype differences were noticeable for the ethological measures of anxiety. Namely, Wfs1-deficient mice performed significantly more stretch-attend postures compared to their wild-type littermates ($p < 0.05$) (Fig. 17). The number of head-dippings was also reduced, but remained statistically not significant in genetically modified animals ($p = 0.09$) (Raud *et al.,* 2009).

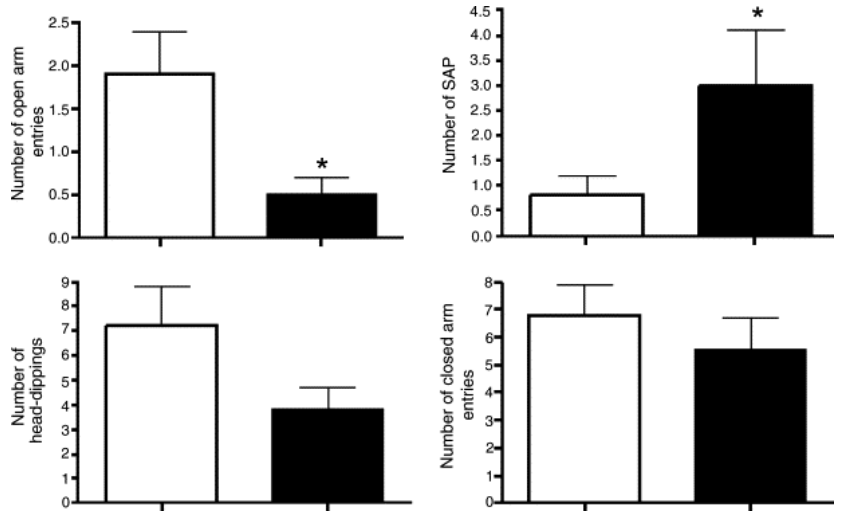


**Figure 17.** Exploratory behavior of Wfs1-deficient mice in the elevated plus-maze. White bars: wild-type mice; black bars: homozygous mice.*$p < 0.05$ (compared to wild-type mice, Student's *t*-test for independent samples) (Raud *et al.,* 2009).

**Gene expression studies of Gabra1, Gabra2, Gad1 and Gad2 genes**

Expression of Gabra1 gene was significantly higher if compared to Gabra2 gene in the forebrain structures. In the frontal cortex it was 2.7-fold in favor of the Gabra1 gene, whereas in the temporal lobe and hippocampus the respective differences were 1.8-fold and 1.5-fold. The only exception was the mesolimbic area where it was only 1.2-fold in favor of Gabra1 gene. It was also expected that the Gad1 gene would be significantly more abundant compared to the Gad2 gene in the forebrain structures. The respective differences were 15-fold and 13-fold in the frontal cortex and temporal lobe in favor of the Gad1 gene (Raud *et al.,* 2009).

We established significant genotype as well as genotype and elevated plus-maze exposure interactions for the Gabra1 and Gabra2 genes in the frontal cortex and temporal lobe. Genetic invalidation of the Wfs1 gene induced a remarkable reduction of the Gabra1 and Gabra2 genes in the temporal lobe and frontal cortex (Fig. 18). This effect was not established in the mesolimbic area and hippocampus. The exposure of wild-type mice to the elevated plus-maze also reduced the expression level of these genes in the temporal lobe and frontal cortex. However, only in the temporal lobe it was statistically significant. The exposure of Wfs1-deficient mice to the elevated plus-maze did not cause a further reduction in the expression of the Gabra1 and Gabra2 genes compared to the experimentally naïve mutant mice. Genetic invalidation of the Wfs1 gene or exposure of wild-type mice to the elevated plus-maze did not affect the expression of the Gad1 and Gad2 genes in the frontal cortex and temporal lobe (Fig. 19) (Raud *et al.,* 2009).

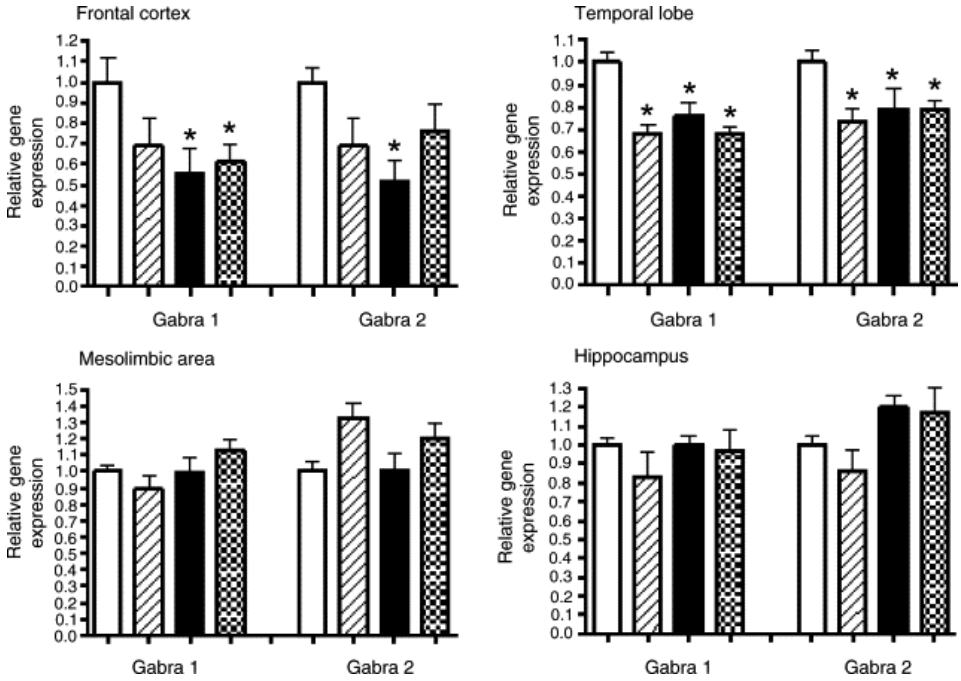


**Figure 18.** Effect of Wfs1 gene invalidation and plus-maze exposure on the expression of the Gabra1 and Gabra2 genes in the forebrain structures. White bars: wild-type mice; stripped bars: wild-type mice exposed to the plus-maze; black bars: homozygous mice; hatched bars: homozygous mice exposed to the plus-maze. Frontal cortex – Gabra1: $F1,28 = 7.09$, $p = 0.02$ (genotype); $F1,28 = 2.03$, $p = 0.17$ (plus-maze exposure); $F1,28 = 3.59$, $p = 0.07$ (genotype × plus-maze exposure). Gabra2: $F1,28 = 3.28$, $p = 0.03$ (genotype); $F1,28 = 0.03$, $p = 0.85$ (plus-maze exposure); $F1,28 = 6.02$, $p = 0.02$ (genotype × plus-maze exposure). Mesolimbic area – Gabar1: $F1,28 = 0.28$, $p = 0.60$ (genotype); $F1,28 = 1.10$, $p = 0.30$ (plus-maze exposure); $F1,28 = 1.27$, $p = 0.27$ (genotype × plus-maze exposure). Gabar2: $F1,28 = 6.80$, $p = 0.015$ (genotype); $F1,28 = 0.34$, $p = 0.57$ (plus-maze exposure); $F1,28 = 0.41$, $p = 0.53$ (genotype × plus-maze exposure). Temporal lobe – Gabar1: $F1,28 = 8.97$, $p = 0.0058$ (genotype); $F1,28 = 23.7$, $p = 0.00004$ (plus-maze exposure); $F1,28 = 9.42$, $p = 0.0058$ (genotype × plus-maze exposure). Gabra2: $F1,28 = 1.80$, $p = 0.19$ (genotype); $F1,28 = 5.57$, $p = 0.03$ (plus-maze exposure); $F1,28 = 5.43$, $p = 0.03$ (genotype × plus-maze exposure). Hippocampus – Gabar1: $F1,28 = 0.43$, $p = 0.52$ (genotype); $F1,28 = 1.18$, $p = 0.29$ (plus-maze exposure); $F1,28 = 0.63$, $p = 0.43$ (genotype × plus-maze exposure). Gabar2: $F1,28 = 8.01$, $p = 0.009$ (genotype); $F1,28 = 0.85$, $p = 0.37$ (plus-maze exposure); $F1,28 = 0.36$, $p = 0.55$ (genotype × plus-maze exposure) (Raud *et al.,* 2009).

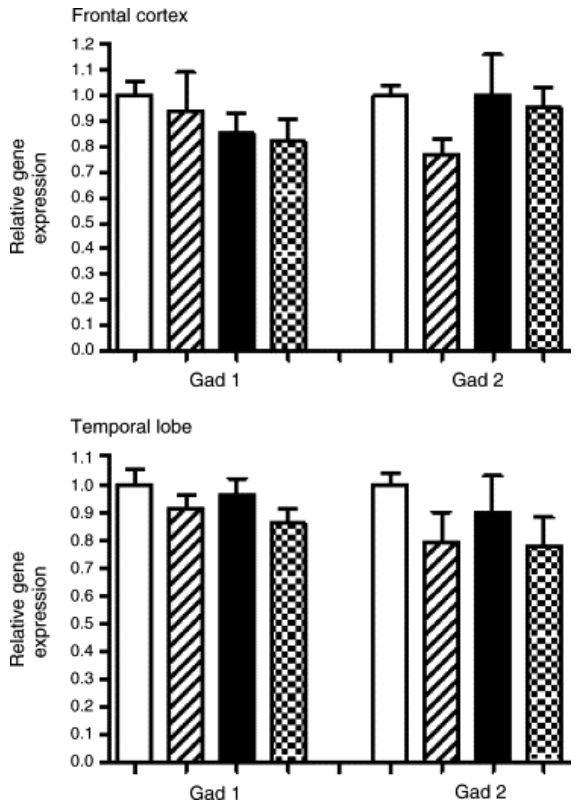


**Figure 19.** Effect of the Wfs1 gene invalidation and plus-maze exposure on the expression of the Gad1 and Gad2 genes in the frontal cortex and temporal lobe. White bars: wild-type mice; stripped bars: wild-type mice exposed to the plus-maze; black bars: homozygous mice; hatched bars–homozygous mice exposed to the plus-maze. Frontal cortex – Gad1: $F1,28 = 2.06$, $p = 0.16$ (genotype); $F1,28 = 0.82$, $p = 0.37$ (plus-maze exposure); $F1,28 = 0.05$, $p = 0.82$ (genotype × plus-maze exposure). Gad2: $F1,28 = 0.94$, $p = 0.34$ (genotype); $F1,28 = 2.53$, $p = 0.13$ (plus-maze exposure); $F1,28 = 0.66$, $p = 0.42$ (genotype × plus-maze exposure). Temporal lobe – Gad1: $F1,28 = 3.73$, $p = 0.07$ (genotype); $F1,28 = 0.92$, $p = 0.35$ (plus-maze exposure); $F1,28 = 0.003$, $p = 0.95$ (genotype × plus-maze exposure). Gad2: $F1,28 = 2.63$, $p = 0.12$ (genotype); $F1,28 = 0.32$, $p = 0.57$ (plus-maze exposure); $F1,28 = 0.21$, $p = 0.65$ (genotype × plus-maze exposure) (Raud *et al.,* 2009).

## 6.3. Study 3. Impaired activity of dopaminergic system in Wfs1 deficient mice

**Effect of exposure of mice to the motility boxes on dopamine levels in the dorsal and ventral striatum**

The levels of DA and its major metabolites were compared in mice exposed to the motility boxes for 30 min and in animals taken directly from their home-cages. Both female and male mice were used for this study. However, the statistical

analysis did not reveal any sex-dependent differences and, therefore, the data obtained from the female and male mice were pooled. As in the previous study (Luuk *et al.,* 2009), the locomotor activity of homozygous mice tended to be lower compared to the other genotypes. However, the application of one-way ANOVA did not reveal any statistically significant differences in motor activity between the genotypes (data not shown). Exposure of mice to the motility boxes caused a statistically significant change in the metabolism of DA in the dorsal and ventral striatum (Tables 5a, b). A genotype as well as exposure effect was established for homovanillic acid (HVA), a major metabolite of DA, in the dorsal striatum (genotype effect: $F_{2,51}=3.53$, $p<0.036$; exposure effect: $F_{1,51}=34.9$, $p<0.001$, genotype x exposure effect: $F_{2,51}=0,87$, $p=0.42$ Table 5a). Exposure to the motility boxes also changed the ratio between HVA and DA (DA turnover) ($F_{1,51}=57.0$, $p<0.001$) in the dorsal striatum (Fig. 20). *Post hoc* analysis (Tukey HSD test) established that exposure to the motility boxes induced a significant increase in the levels of HVA in wild-type and heterozygous mice, but not in homozygous animals. By contrast, the increase in DA turnover was significantly elevated in all genotypes (Fig. 20). In the ventral striatum, an exposure effect was established for HVA ($F_{1,46}=31.3$, $p<0.001$)(Table 5b). The exposure effect was also significant ($F_{1,46}=11.1$, $p<0.01$) for DA turnover in the ventral striatum (Fig. 20), but *post hoc* analysis did not establish any significant changes between the groups (Visnapuu *et al.,* 2013).

**Table 5.** Effect of exposure of Wfs1-deficient mice to the motility boxes compared to mice taken from the home-cage on the metabolism of dopamine in the dorsal and ventral striatum. Mean values ± SEM are presented in the table. Data are expressed as pmol/mg of tissue weight (Visnapuu *et al.,* 2013).

| | | Wild-type | Wild-type+ exposure | Hetero-zygous | Hetero-zygous+ exposure | Homo-zygous | Homo-zygous+ exposure |
|---|---|---|---|---|---|---|---|
| (A) Dorsal striatum | DA | 46.1±3.2 | 46.6±3.4 | 52.2±4.8 | 49.1±2.6 | 50.5±3.4 | 46.0±2.8 |
| | DOPAC | 6.5±1.0 | 7.9±0.7 | 7.6±1.0 | 9.1±0.8 | 7.4±0.8 | 6.8±1.2 |
| | HVA | 3.9±0.3 | 6.3±0.5* | 4.9±0.5 | 7.4±0.5* | 4.4±0.3 | 5.9±0.5 |
| | 3-MT | 2.6±0.2 | 3.5±0.4 | 3.2±0.4 | 3.3±0.2 | 3.3±0.3 | 2.8±0.3 |
| (B) Ventral striatum | DA | 26.5±3.0 | 30.5±4.5 | 33.7±3.9 | 35.0±4.0 | 25.8±2.3 | 29.6±2.7 |
| | DOPAC | 5.7±0.9 | 5.8±0.7 | 4.9±0.4 | 7.4±1.1 | 5.1±0.6 | 3.9±0.6 |
| | HVA | 3.7±0.4 | 5.8±0.5* | 4.1±0.2 | 6.5±0.7* | 3.6±0.2 | 5.0±0.3 |
| | 3-MT | 3.6±0.2 | 3.8±0.2 | 3.2±0.3 | 3.9±0.5 | 2.9±0.2 | 2.5±0.2 |

* – $p < 0.05$ compared to the respective group of naive mice (Tukey HSD test after significant two-way ANOVA). DA – Dopamine; DOPAC – 3,4-Dihydroxyphenylacetic acid; HVA – Homovanillic acid; 3-MT – 3-Methoxytyramine. There were 5 male and 5 female mice in each group (Visnapuu *et al.,* 2013).

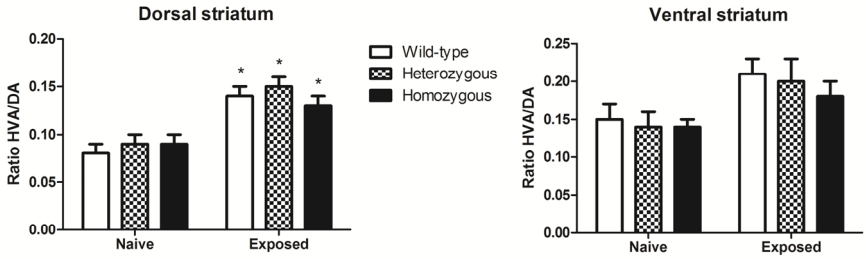


**Figure 20.** Effect of exposure to the motility boxes on DA turnover in the dorsal and ventral striatum. * – $p < 0.05$ compared to respective group of mice not exposed to the motility boxes (Tukey HSD test after significant two-way ANOVA). HVA – Homovanillic acid; DA – Dopamine. Data are expressed as ratio between the levels of HVA (pmol/mg) and DA (pmol/mg) in the respective brain structures. There were 5 male and 5 female mice in each group (Visnapuu *et al.,* 2013).

**Effect of amphetamine on locomotor activity and dopamine metabolism in the dorsal and ventral striatum**

30 minutes after treatment with amphetamine (5 mg/kg), animals were placed into the motility boxes and their locomotor activity was measured. As a result, a gene-dose effect on amphetamine-induced hyperlocomotion was established (Fig. 21). Altogether, similarly to our previous study (Luuk *et al.,* 2009, Study 1) amphetamine-induced locomotor stimulation was clearly stronger in wild-type mice compared to their heterozygous and homozygous littermates. Two-way ANOVA revealed significant effects of genotype and treatment on all measures of motor activity (genotype effects: time in locomotion $F_{2,53}=9.78$, $p<0.001$, distance travelled $F_{2,53}=8.82$, $p<0.001$, number of corner entries $F_{2,53}=5.57$, $p<0.01$; treatment effects: time in locomotion $F_{1,53}=54.0$, $p<0.001$, distance travelled $F_{1,53}=55.7$, $p<0.001$; number of corner entries $F_{1,53}=43.4$, $p<0.001$; genotype and treatment interactions: time in locomotion $F_{2,53}=3.62$, $p<0.05$, distance travelled $F_{2,53}=5.94$, $p<0.01$, number of corner entries $F_{2,53}=4.83$, $p<0.05$). According to *post hoc* analysis, the elevation of all measures of locomotor activity of amphetamine-treated wild-type and heterozygous mice was statistically significant compared to the respective saline-treated groups ($p<0.05$, Tukey HSD) (Fig. 21). There was a significant difference between amphetamine-treated wild-type and homozygous mice for all measures of locomotor activity ($p<0.05$, Tukey HSD). The difference in distance travelled after treatment with amphetamine was also significant when wild-type and heterozygous mice were compared (Visnapuu *et al.,* 2013).

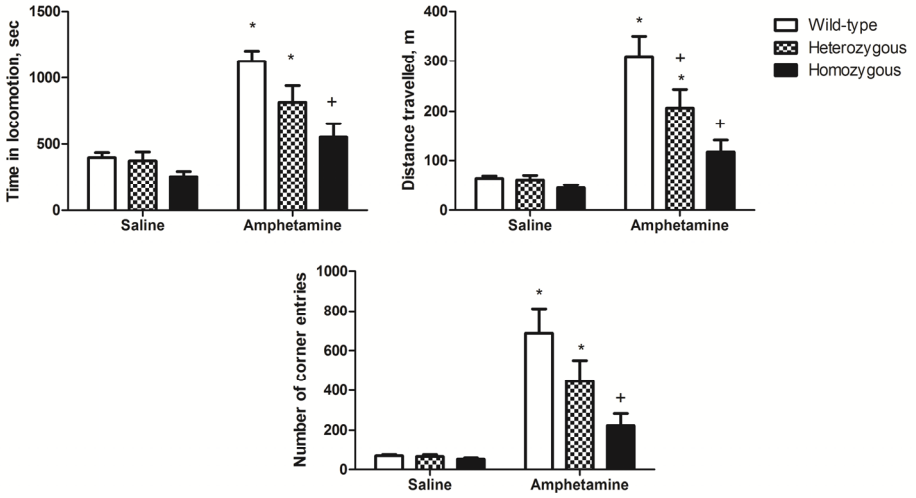


**Figure 21.** Effect of amphetamine (5 mg/kg) on the locomotor activity of Wfs1-deficient mice. Amphetamine-induced locomotor stimulation was clearly stronger in wild-type mice compared to their heterozygous and homozygous littermates. * – $p < 0.05$ compared to the respective group of saline treated mice (Tukey HSD test after significant two-way ANOVA); + – $p < 0.05$ (compared to amphetamine-treated wild-type mice).There were 5 male and 5 female mice in each group (Visnapuu *et al.,* 2013).

In the biochemical studies, treatment with amphetamine caused significant changes in DA metabolism in the dorsal and ventral striatum. In the dorsal striatum, genotype effect was significant for DA ($F_{2,53}=3.50$, $p<0.05$) and for its metabolite 3-MT ($F_{2,53}=5.99$, $p<0.01$) (Table 6A). Treatment effect was significant for DA ($F_{1,53}=19.2$, $p<0.001$), DOPAC ($F_{1,53}=44.7$, $p<0.001$), HVA ($F_{1,53}=24.0$, $p<0.001$), 3-MT ($F_{1,53}=11.5$, $p<0.01$) (Table 6A) and DA turnover ($F_{1,53}=68.3$, $p<0.001$) (Fig. 21). Genotype and treatment interaction was established for DA ($F_{2,53}=3.19$, $p<0.05$) and for DA turnover it was close to being significant ($F_{2,53}=2.74$, $p=0.07$). Amphetamine was found to increase the levels of DA and lower DOPAC and DA turnover in the dorsal striatum of wild-type mice when compared to saline-treated wildtype mice ($p<0.05$, Tukey HSD test) (Table 6A, Fig. 22). In heterozygous mice, amphetamine caused a significant reduction of levels of DOPAC and HVA, but also increased the level of 3- MT. Moreover, DA turnover was also reduced in these mice. We found a reduction of DOPAC in response to amphetamine in homozygous mice. Besides that, homozygous mice displayed significantly lower levels of DA and 3-MT in the dorsal striatum in response to amphetamine compared to their wild-type littermates (Visnapuu *et al.,* 2013).

In the ventral striatum, a genotype effect was established for DOPAC ($F_{2,49}=8.92$, $p<0.001$) and 3-MT ($F_{2,49}=12.5$, $p<0.001$) (Table 6B). Treatment effect was significant for DA ($F_{1,49}=4.68$, $p<0.05$), DOPAC ($F_{1,49}=9.16$, $p<0.01$), HVA ($F_{1,49}=8.99$, $p<0.01$), 3-MT ($F_{1,49}=9.41$, $p<0.01$) and DA turnover ($F_{1,49}=24.7$, $p<0.001$). The interaction of genotype with treatment was

close to statistical significance in the case of DA turnover ($F_{2,49}=2.80$, $p=0.07$). In wild-type mice, amphetamine induced a significant increase in the level of DA and reduced DA turnover (Table 6B, Fig. 22). In heterozygous mice, an increase in the level of 3-MT was evident. Also, the levels of DOPAC, HVA and the turnover of DA were reduced in heterozygous mice. The level of 3-MT in homozygous mice, after treatment with amphetamine, was significantly lower compared to heterozygous and wild-type animals. Amphetamine did not induce a significant reduction of DA turnover in the ventral striatum of homozygous mice. Altogether, the strongest differences between the genotypes were established for the elevation of DA levels under the influence of amphetamine in the dorsal and ventral striatum. There was a clear gene-dose effect similar to that established for amphetamine-induced hyperlocomotion (Fig. 22, Table 6A, 6B). Moreover, amphetamine was not able to induce a statistically significant suppression of DA turnover in the dorsal and ventral striatum of homozygous mice (Visnapuu *et al.,* 2013).

**Table 6.** Effect of amphetamine (5 mg/kg) on the metabolism of dopamine in the dorsal and ventral striatum of Wfs1-deficient mice (Visnapuu *et al.,* 2013).

| | | Wild-type+ saline | Wild-type+ amphetamine | Hetero-zygous+ saline | Hetero-zygous+ amphet-amine | Homo-zygous+ saline | Homo-zygous+ amphet-amine |
|---|---|---|---|---|---|---|---|
| (A) Dorsal striatum | DA | 46.6±3.4 | 69.3±6.1*$^{+}$ | 49.1±2.6 | 63.6±2.9 | 46.0±2.8 | 50.7±4.2 |
| | DOPAC | 7.9±0.7 | 4.5±0.5* | 9.1±0.8 | 3.7±0.6* | 6.8±1.2 | 3.0±0.6* |
| | HVA | 6.3±0.5 | 4.7±0.4 | 7.4±0.5 | 4.5±0.7* | 5.9±0.5 | 4.3±0.4 |
| | 3-MT | 3.5±0.4 | 4.4±0.5$^{+}$ | 3.3±0.2 | 5.3±0.6*$^{+}$ | 2.8±0.3 | 3.2±0.2 |
| (B) Ventral striatum | DA | 30.5±4.5 | 46.0±5.0*$^{+}$ | 35.0±4.0 | 42.3±4.0 | 29.6±2.7 | 31.7±3.3 |
| | DOPAC | 5.8±0.7 | 4.5±0.8 | 7.4±1.1 | 4.9±0.9* | 3.9±0.6 | 2.1±0.4 |
| | HVA | 5.8±0.5 | 4.1±0.6 | 6.5±0.7 | 4.4±0.4* | 5.0±0.3 | 4.9±0.7 |
| | 3-MT | 3.8±0.2 | 4.8±0.7$^{+}$ | 3.9±0.5 | 5.5±0.5*$^{+}$ | 2.5±0.2 | 3.0±0.3 |

Mean values ± SEM are presented in the table. Data are expressed as pmol/mg of tissue weight. * – $p < 0.05$ compared to the respective group of saline treated mice (Tukey HSD test after significant two-way ANOVA); $^{+}$ – $p < 0.05$ compared to homozygous mice treated with amphetamine. DA – Dopamine; DOPAC – 3,4-Dihydroxyphenylacetic acid; HVA – Homovanillic acid; 3-MT – 3-Methoxytyramine. There were 5 male and 5 female mice in each group (Visnapuu *et al.,* 2013).

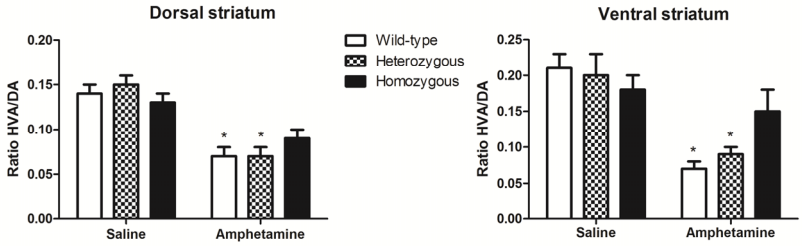


**Figure 22.** Effect of amphetamine (5 mg/kg) on the turnover of dopamine in the dorsal and ventral striatum. * – $p < 0.05$ compared to respective group of saline treated mice (Tukey HSD test after significant two-way ANOVA). HVA – Homovanillic acid; DA – Dopamine. Data are expressed as ratio between the levels of HVA (pmol/mg) and DA (pmol/mg) in the respective brain structures. There were 5 male and 5 female mice in each group (Visnapuu *et al.,* 2013).

## Effect of apomorphine on motor activity and dopamine levels in the dorsal and ventral striatum

In the case of treatment with apomorphine, a two-way ANOVA established that only treatment effect was significant for all measures of locomotor activity (time spent in locomotion $F_{1,54}=15.3$, $p<0.001$, distance travelled $F_{1,54}=11.3$, $p<0.01$ and the number of corner entries $F_{1,54}=16.7$, $p<0.001$). Administration of apomorphine (3 mg/kg) tended to increase the locomotor activity in wild-type mice, but this effect was not statistically significant (Fig. 23). Apomorphine-treated heterozygous mice displayed significantly longer distance travelled and a greater number of corner entries compared to saline-treated heterozygous mice. Homozygous Wfs1-deficient mice had a significant increase in time spent in locomotion compared to the respective saline-treated group ($p<0.05$, Tukey HSD) (Visnapuu *et al.,* 2013).

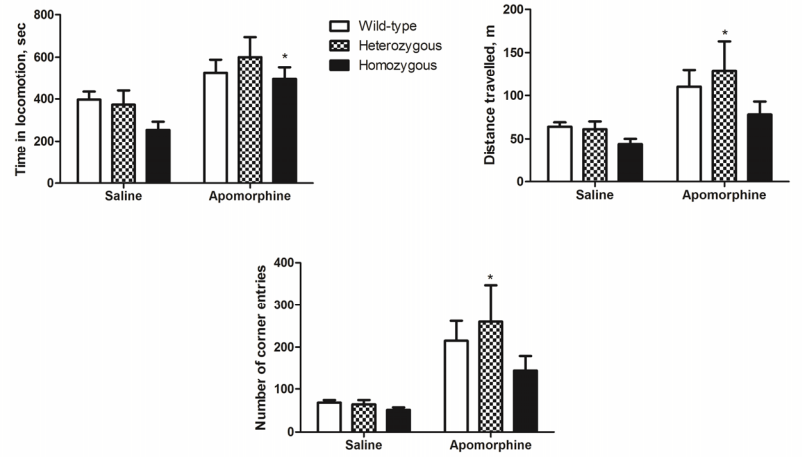


**Figure 23.** Apomorphine (3 mg/kg) induced changes in locomotor activity of Wfs1-deficient mice * – $p < 0.05$ compared to the respective group of saline treated mice (Tukey HSD test after significant two-way ANOVA). There were 5 male and 5 female mice in each group (Visnapuu *et al.,* 2013).

In the dorsal striatum, treatment effect of apomorphine was significant for DOPAC ($F_{1,52}=43.8$, $p<0.001$), HVA ($F_{1,52}=117.2$, $p<0.001$) (Table 7A) and DA turnover ($F_{1,52}=95.1$, $p<0.001$) (Fig. 24). Genotype and treatment interaction was significant for HVA ($F_{2,52}=3.26$, $p<0.05$). Apomorphine significantly reduced the levels of HVA, DOPAC, and DA turnover in the dorsal striatum of all genotypes (Visnapuu *et al.,* 2013).

In the ventral striatum, treatment effect of apomorphine was significant for DOPAC ($F_{1,50}=8.97$, $p<0.01$), HVA ($F_{1,50}=57.9$, $p<0.001$), 3-MT ($F_{1,50}=5.38$, $p<0.05$) (Table 7B), and DA turnover ($F_{1,50}=58.1$, $p<0.001$) (Fig. 24). Apomorphine caused a significant reduction of HVA and DA turnover in the ventral striatum of all genotypes. In heterozygous mice, the level of DOPAC was also reduced in the ventral striatum in response to apomorphine (Visnapuu *et al.,* 2013).

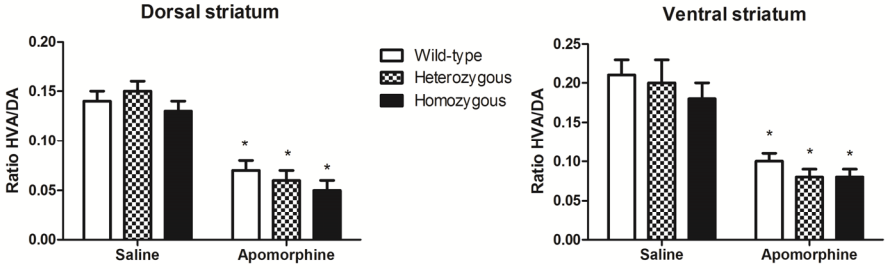


**Figure 24.** Effect of apomorphine (3 mg/kg) on the turnover of dopamine in the dorsal and ventral striatum. * – $p < 0.05$ compared to the respective group of saline treated mice (Tukey HSD test after significant two-way ANOVA). HVA – Homovanillic acid; DA – Dopamine. Data are expressed as ratio between the levels of HVA (pmol/mg) and DA (pmol/mg) in the respective brain structures. There were 5 male and 5 female mice in each group (Visnapuu *et al.,* 2013).

**Tables 7.** Effect of exposure to apomorphine (3 mg/kg) on the metabolism of dopamine in the dorsal and ventral striatum in Wfs1-deficient mice (Visnapuu *et al.,* 2013).

| | | Wild-type+ saline | Wild-type+ apo-morphine | Hetero-zygous+ saline | Hetero-zygous+ apo-morphine | Homo-zygous+ saline | Homo-zygous+ apo-morphine |
|---|---|---|---|---|---|---|---|
| (A) Dorsal striatum | DA | 46.6±3.4 | 49.9±3.3 | 49.1±2.6 | 43.3±3.1 | 46.0±2.8 | 52.7±3.4 |
| | DOPAC | 7.9±0.7 | 4.3±0.5* | 9.1±0.8 | 3.3±0.8* | 6.8±1.2 | 3.4±0.4* |
| | HVA | 6.3±0.5 | 3.3±0.5* | 7.4±0.5 | 2.4±0.3* | 5.9±0.5 | 2.5±0.2* |
| | 3-MT | 3.5±0.4 | 2.7±0.2 | 3.3±0.2 | 2.4±0.3 | 2.8±0.3 | 2.1±0.2 |
| (B) Ventral striatum | DA | 30.5±4.5 | 28.0±5.3 | 35.0±4.0 | 35.1±3.8 | 29.6±2.7 | 39.3±6.5 |
| | DOPAC | 5.8±0.7 | 4.8±1.0 | 7.4±1.1 | 3.9±0.4* | 3.9±0.6 | 3.1±0.4 |
| | HVA | 5.8±0.5 | 2.9±0.5* | 6.5±0.7 | 3.1±0.4* | 5.0±0.3 | 2.8±0.4* |
| | 3-MT | 3.8±0.2 | 3.0±0.5 | 3.9±0.5 | 3.1±0.5 | 2.5±0.2 | 1.9±0.2 |

Mean values ± SEM are presented in the table. Data are expressed as pmol/mg of tissue weight. * – $p < 0.05$ compared to the respective group of saline treated mice (Tukey HSD test after significant two-way ANOVA). DA – Dopamine; DOPAC – 3,4-Dihydroxyphenylacetic acid; HVA – Homovanillic acid; 3-MT – 3-Methoxytyramine. There were 5 male and 5 female mice in each group (Visnapuu *et al.,* 2013).

**Amphetamine-induced motor sensitization in Wfs1-deficient mice**

During the course of repeated treatments with amphetamine (2.5 mg/kg) its motor stimulating effect became steadily stronger in male wild-type animals. Repeated measures three-way ANOVA established the following main and interaction effects: time in locomotion [$F_{2,76}=10.2$, $p<0.001$ (genotype); $F_{5,76}=4.1$, $p<0.01$ (day); $F_{5,76}=13.9$, $p<0.001$ (day x treatment)], distance travelled [$F_{2,76}=6.33$, $p<0.01$ (genotype); $F_{1,76}=6.92$, $p<0.05$, (treatment); $F_{5,76}=9.74$, $p=0.09$ (day x treatment)] and number of corner entries [$F_{2,76}=3.29$, $p<0.05$ (genotype); $F_{1,76}=7.2$, $p<0.01$ (treatment), $F_{5,76}=5.28$, $p<0.01$ (day x treatment)]. *Post hoc* analysis (Tukey HSD test) revealed that in wild-type mice the effect of amphetamine on the 6th day was significantly increased compared to the administration of saline on the 6th day and to the treatment with amphetamine on the 1st day (Fig. 25). In heterozygous mice, there was some increase in the action of amphetamine, but this change was not statistically significant. Repeated treatments with amphetamine even tended to reduce the locomotor activity in homozygous mice (Fig. 25). Therefore, in the amphetamine sensitization study, a gene-dose effect was established in Wfs1-deficient mice as in the case of acute treatment with the higher dose of amphetamine (5 mg/kg) (Visnapuu *et al.,* 2013).

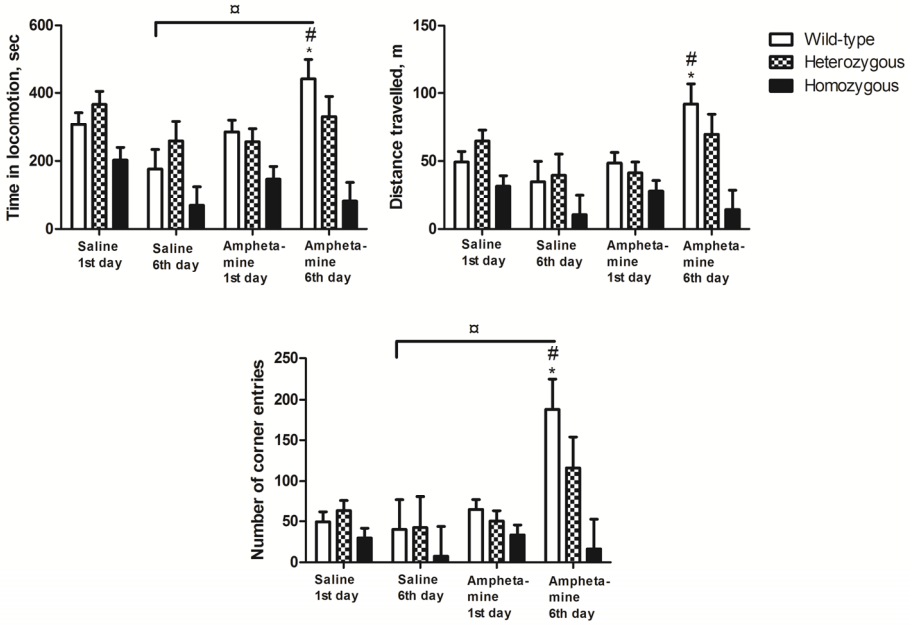


**Figure 25.** Sensitization of Wfs1-deficient mice to amphetamine-induced motor stimulation. * – $p < 0.05$ compared to amphetamine-treated wild-type mice on the first day; ¤ – $p < 0.05$ compared to saline-treated wild-type mice on the sixth day; # – $p < 0.05$ compared to amphetamine-treated homozygous mice on the 6th day. (Tukey HSD after significant three way repeated measures ANOVA.) The number of animals in each group was 13-14 (Visnapuu *et al.,* 2013).

**Expression of Dat (midbrain) and Drd2 (dorsal and ventral striatum) mRNA in Wfs1-deficient mice**

The expression level of Dat mRNA in the midbrain was significantly affected in male and female mice ($F_{2,20}=3.95$, $p<0.05$ and $F_{2,47}=4.82$, $p<0.05$, respectively). *Post hoc* analysis established a significantly lower level of Dat expression in homozygous animals of both sexes (Fig. 26A and 25B) (Visnapuu *et al.,* 2013).

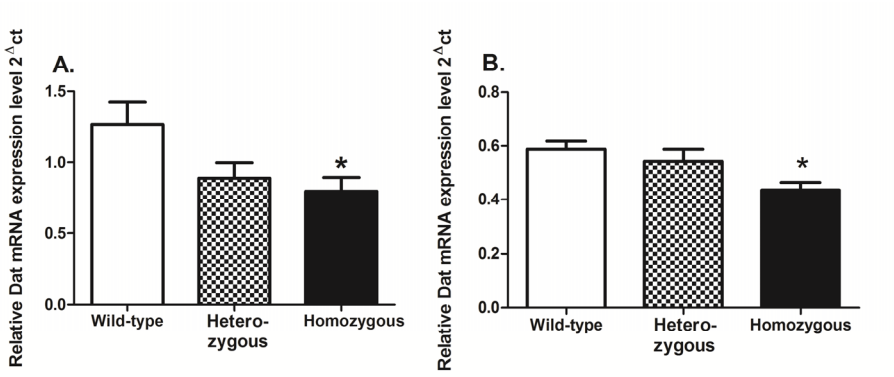


**Figure 26.** Expression of Dat gene in the mesencephalon of Wfs1-deficient mice: *A*. Dat mRNA in the mesencephalon of female mice; *B*. Dat mRNA in the mesencephalon of male mice * – $p < 0.05$ compared to wild-type animals (Tukey HSD after significant one-way ANOVA). The number of animals in each group was 16-17 in case of females and 7-8 in case of males (Visnapuu *et al.,* 2013).

The expression level of Drd2 gene in the ventral striatum of both male and female mice was not affected by the disruption of the Wfs1 gene (Fig. 26B and 26D). However, we found a significantly reduced Drd2 receptor level in the dorsal striatum of male homozygous mice compared to wild-type animals ($F_{2,15}=6.32$, $p<0.05$) (Fig. 26A), while in the female mice it was unaffected (Fig. 26C) (Visnapuu *et al.,* 2013).

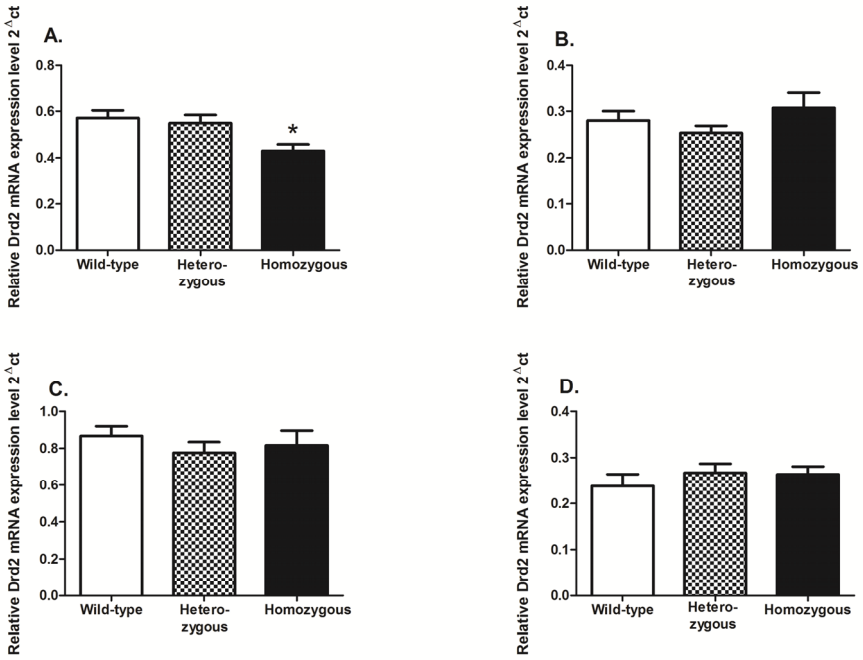


**Figure 27.** Expression of Drd2 gene in Wfs1-deficient mice. Relative Drd2 mRNA levels in the dorsal (*A*) and ventral striatum (*B*) of male mice and relative Drd2 levels in the dorsal (*C)* and ventral striatum (*D)* in female mice. * – $p < 0.05$ compared to wild-type animals (Tukey HSD after significant one-way ANOVA). The number of animals in each group was 16-17 in case of females and 7-8 in case of males (Visnapuu *et al.,* 2013).

**Expression of Dat protein in the dorsal and ventral striatum of Wfs1 deficient mice**

In the dorsal striatum Dat protein level tended to be lower both in the male and female homozygous mice. However, this difference was not statistically significant. (Fig. 28 A, C) (Visnapuu *et al.,* 2013).

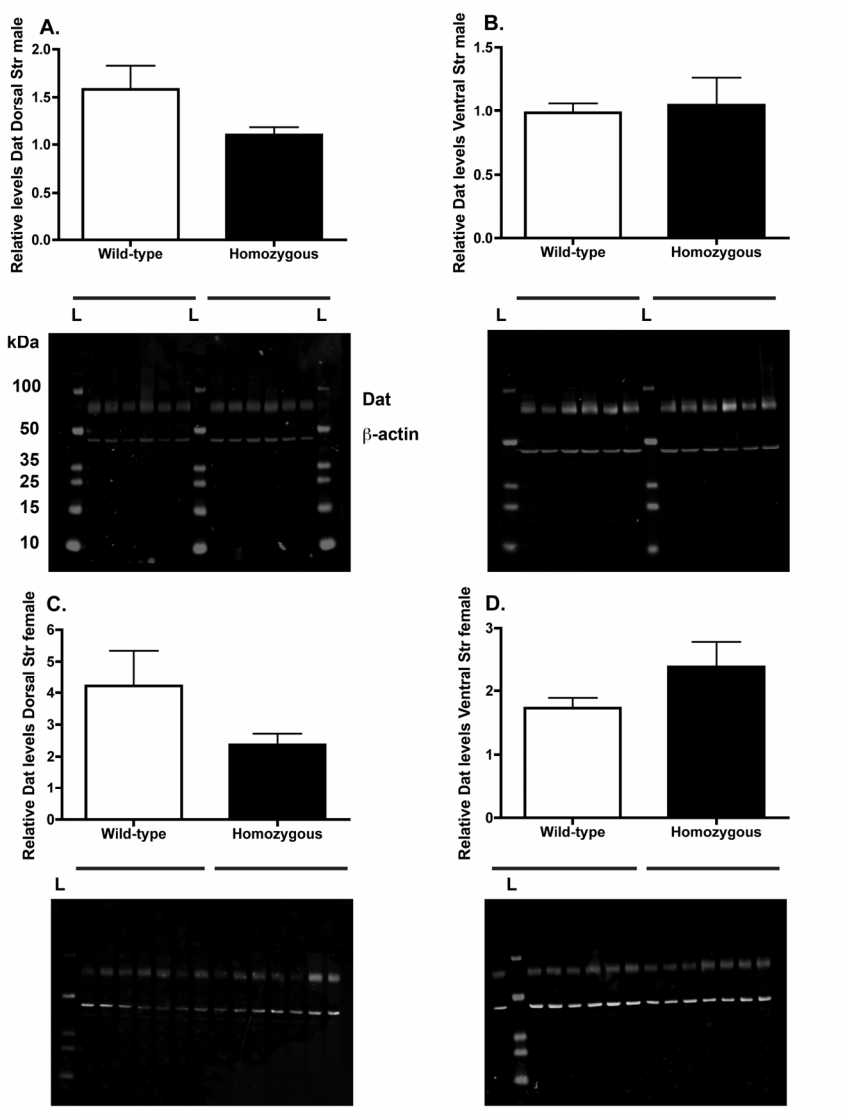


**Figure 28.** Expression of Dat protein in the dorsal and ventral striatum of Wfs1-deficient mice. Relative Dat protein levels in the dorsal (*A*) and ventral (*B*) striatum of male mice and relative Dat protein levels in the dorsal (*C)* and ventral (*D)* striatum of female mice. No significant difference ($p > 0.05$, Mann-Whitney U) was found in Dat protein levels between wild-type mice and Wfs1 homozygous mice. The number of animals was 6-7 in each group. Abbreviation: Protein ladder (L), str – striatum (Visnapuu *et al.,* 2013).

# 7. DISCUSSION

## 7.1. Wfs1-deficient mice display impaired behavioural adaptation in stressful environment

In Study 1 we provided a behavioural profile of female Wfs1-deficient F2 generation mice with mixed background [(129S6/SvEvTac × C57BL/6) × (129S6/SvEvTac × C57BL/6)]. To make the Wfs1 gene nonfunctional, exon 8, the principal coding exon, was disrupted which resulted in premature termination of the Wfs1 protein. Absence of Wfs1 C-terminal immunostaining in Wfs1-deficient mice has been demonstrated previously (Luuk *et al.,* 2008). Nonfunctionality of the Wfs1 gene was confirmed by intolerance to glucose challenge, a finding consistently reported in Wfs1-deficient mice by other groups employing different strategies of Wfs1 disruption (Ishihara *et al.,* 2004; Riggs *et al.,* 2005). For general phenotyping of Wfs1-deficient mice, we used only young adult female mice as they displayed milder metabolic disturbances than male mice and older mice (Luuk *et al.,* 2009).

The behaviour of Wfs1-deficient mice did not differ from that of wild-type littermates in terms of motor performance in the rota-rod and forced swimming tests, but several measures of general motor activity such as time spent in locomotion, distance travelled and the number of rearings in the motility box, and swimming speed in the Morris water maze test were consistently diminished in Wfs1-deficient mice. In most cases, however, these effects remained at tendency level. Also, no obvious disruptions in sensory functioning were observed in the behavioural tests used. Wfs1-deficient mice appeared normal in heat-induced pain perception, were able to differentiate between brightly and dimly lit environments, developed intact responses to electric footshocks and a 10 kHz conditioned auditory stimulus, and were able to navigate by means of visual contextual cues in Morris water maze. Wfs1-deficient mice displayed no gross learning deficits as evidenced by intact learned responses to conditioned fear-eliciting stimuli, as well as the existence of spatial memory in the Morris water maze test (both wild-type and knockout mice showed preference for the target quadrant after both initial and reversal training). However, during reversal training Wfs1-deficient mice covered significantly longer distances before finding the platform than their wild-type littermates. This subtle impairment in reversal learning may be indicative of dysfunction in the hippocampal and/or prefrontal circuits as in humans and monkeys, impairments in reversal learning have been associated with orbitofrontal cortex dysfunction (Jentsch *et al.,* 2002; Waltz and Gold, 2007). In mice, hippocampal CA1 and CA3 regions have been implicated specifically in reversal learning (Havekes *et al.,* 2006). Notably, both the prefrontal cortex and hippocampal CA1 region are enriched in Wfs1 protein and mRNA (Lein *et al.,* 2007; Luuk *et al.,* 2008). The metabolic profile of Wfs1-deficient female mice revealed lower body weight at 4 months of age by latest, and intolerance to intraperitoneal

administration of glucose. However, we observed no differences in baseline concentrations of blood glucose in non-fasted animals (Luuk *et al.,* 2009).

It is notable that in most cases altered behavioural responses were established when Wfs1-deficient mice were exposed to novel environments or stressful manipulations. In the hyponeophagia test, Wfs1-deficient mice displayed increased behavioural inhibition in a novel environment as their latency to start eating was significantly longer than in wild-type littermates. Also, the locomotor activity was significantly lower in Wfs1-deficient mice compared to wild-type mice only in the more aversive bright environment as opposed to dim environment. Similarly, short-term social isolation of Wfs1-deficient mice induced significant anxiety-like behaviour in the light-dark box exploration test when compared to non-isolated mice. In contrast, social isolation had no major impact on the behaviour of wild-type mice in the same test. The relatively higher sensitivity of Wfs1-deficient mice to environmental stressors was further supported by increased analgesic response after electric foot-shocks of intermediate intensity and a nearly three-fold higher plasma corticosterone concentration after handling and injection stress when compared to wild-type littermates (Luuk *et al.,* 2009).

Corticosterone measurements revealed no differences in baseline concentrations between wild-type and Wfs1-deficient mice. A higher level of anxiety in Wfs1-deficient mice was suggested by the administration of a moderate dose of diazepam (1 mg/kg) which had a robust anxiolytic-like effect on plus-maze behaviour in Wfs1-deficient mice but not in the wild-type mice. It is also of interest that some (as an average approximately 20–30%) of Wfs1-deficient mice produced spontaneous audible vocalizations which increased in loudness under stressful conditions and were suppressed by the administration of diazepam. Neuroanatomical evidence indicating an enrichment of Wfs1 expression in the central extended amygdala and paraventricular hypothalamic nucleus (Becker *et al.,* 2008; Luuk *et al.,* 2008) also supports the conclusion that the behaviour of Wfs1-deficient mice can best be described by increased anxiety response. Rather than being a single anatomical entity, the extended amygdala concept stands for a network of basal ganglionic nuclei with similar connectivity and neurochemical properties forming a functional-anatomical macrostructure involved in the etiology of such neuropsychiatric disorders as anxiety and depression (Alheid *et al.,* 1988; Heimer, 2003). The paraventricular hypothalamic nucleus, on the other hand, is involved in the endocrine regulation of stress response by controlling the activity of the hypothalamic–pituitary–adrenocortical (HPA) axis, secretion of neurohypophysial peptides such as vasopressin and oxytocin, and by regulating autonomic centres in brainstem and spinal cord (Herman *et al.,* 2008; Luuk *et al.,* 2009).

The psychomotor stimulant effects of amphetamine and apomorphine, the drugs that exert their effects through the dopamine system, were different in Wfs1-deficient mice compared to wild-type mice. At intermediate and high doses the stimulatory effect of amphetamine was significantly weaker in Wfs1-deficient mice, which is probably indicative of lower presynaptic release

potential for dopamine in the mesolimbic and nigrostriatal pathway. On the other hand, postsynaptic dopamine receptor agonist apomorphine induced significantly higher locomotor activation in Wfs1-deficient mice reflecting most likely postsynaptic upregulation of dopamine receptors in the ventral and dorsal striatum. As opposed to the present results, reduction in the effect of apomorphine has been demonstrated by Rowland *et al.* (Rowland *et al.,* 1985) in hypoinsulinaemic mice displaying otherwise a similar behavioural profile in terms of lower basal locomotor activity (Owens *et al.,* 2005; Sevak *et al.,* 2007) and resistance to the motor stimulatory properties of amphetamine and other related psychomotor stimulants (Marshall *et al.,* 1978; Merali *et al.,* 1988; Rowland *et al.,* 1985). Therefore, the behavioural phenotype of Wfs1-deficient mice is probably not reducible to just indirect effects of metabolic disturbances resulting from impaired stimulus-secretion coupling of insulin in the pancreatic beta-cells. In mice, Wfs1 protein is expressed at high levels in the ventral striatum (nucleus accumbens and olfactory tubercle) and, to a lesser degree, in the caudal part of dorsal striatum while being undetectable in the more voluminous rostral part and dopaminergic neurons residing in ventral tegmental area and compact part of substantia nigra (Luuk *et al.,* 2008). Hence, the nucleus accumbens is the most obvious candidate for producing disturbances in dopaminergic transmission in Wfs1-deficient mice. Although mostly known as the brain structure mediating the reinforcing actions of addictive drugs (Koob and Moal 2001) the nucleus accumbens has been additionally implicated in the regulation of anxiety and stress response (Barrot *et al.,* 2002; Barrot *et al.,* 2005). In addition to high Wfs1 expression in neuronal perikarya in the core and shell regions of nucleus accumbens, there is a prominent Wfs1-rich projection from the nucleus accumbens to the reticular part of substantia nigra and less dense projections of unknown origin to the compact part of substantia nigra and ventral tegmental area (Luuk *et al.,* 2008). Hypothetically, reduced activation of Wfs1-positive neurons in the nucleus accumbens leading to increased inhibitory tone on motor regions targeted by inhibitory efferents from the reticular part of substantia nigra could account for behavioural inhibition apparent in Wfs1-deficient mice. Such a situation is plausible as Wfs1-deficiency has been shown to reduce stimulus-secretion coupling in pancreatic beta-cells (Ishihara *et al.,* 2004; Takei *et al.,* 2006; Luuk *et al.,* 2009).

The behavioural phenotype of male Wfs1-deficient mice has been described by another research group who generated their mutant mice by means of a genetic ablation of the second exon of the Wfs1 gene, resulting in full knock-out mice (Kato *et al.*, 2008). In these mice, behavioural alterations are less apparent than in the present study. Homozygous Wfs1-deficient mice and their wild-type littermates differed significantly in terms of increased escape latencies during the conditioning phase in the passive avoidance test and in the first block of trials on day 3 of the active avoidance test, in terms of increased freezing during conditioning phase in the fear conditioning test, and in terms of increased latency to find the platform during training phase of the Morris water maze. These results support the idea that the Wfs1-deficient mice generated by

Kato *et al*., 2008 have subtle impairments in behavioural activation in demanding situations. Generally, however, the behavioural differences in these mice are quite modest compared to our Wfs1-deficient mouse line. Also, Kato *et al.*, 2008 reported no alterations in body weight or other phenotypic parameters. The markedly increased severity of phenotypic alterations in Wfs1 deficient mice in the present study could be due to several reasons. First, our Wfs1 targeting strategy spared amino acids 1–359 of Wfs1 protein whereas in the other knockout no N-terminal immunoreactivity of Wfs1 was retained (Ishihara *et al.,* 2004). Thus, in our mice, the functionality of Wfs1 protein is disrupted while its N-terminal cytoplasmic region is free to interact with its molecular partners possibly prohibiting the activation of compensatory pathways. Furthermore, Ishihara *et al.,* 2004 have demonstrated that the diabetogenic effect of Wfs1-deficiency is considerably weaker in C57BL/6 background when compared to F2 intercross bearing a more or less equal and random mix of 129S6/SvEvTac and C57BL/6 genetic material (Wolfer *et al.,* 2002). The results of our behavioural experiments indicate that the dependency of phenotypic severity on genetic background extends also to the central nervous system. This is not unexpected as the differential impact of 129S6-derived and C57BL/6 inbred genetic backgrounds on anxiety-like behaviour is well known (Abramov *et al.,* 2008; Holmes *et al.,* 2003; Hovatta *et al.,* 2005). Also, differences in experimental design may have contributed to the differences between the two Wfs1-deficient mouse lines. We have seen previously that long-term social isolation induces an anxiety-like state in female mice, whereas increased aggressiveness and exploratory drive are observed in males (Abramov *et al.,* 2004). Kato *et al*., 2008 studied male mice housed individually for several days, whereas in our experiments group-housed female mice were used. Lastly, the order of experiments can also have an impact on the observed phenotype because a number of behavioural tests are sensitive to previous experience and/or handling. For example, Kato *et al.* (2008) never performed plus-maze as the first test, however, it known that the plus-maze exposure is extremely sensitive to previous experience (Voikar *et al.,* 2007) and therefore we always perform it as the very first test of a behavioural battery and always with naive mice (Luuk *et al.,* 2009).

## 7.2. Relation between increased anxiety and reduced expression of alpha1 and alpha2 subunits of GABA(A) receptors in Wfs1-deficient mice

The results of the behavioural battery described above indicate that the genetic invalidation of Wfs1 induces an impaired adaptation response in mice in a novel and stressful environment in terms of increased anxiety in ethological models. Pre-treatment with diazepam (1 mg/kg), an anxiolytic drug acting via $GABA_A$ receptors, antagonized increased anxiety and stress-induced vocalizations in Wfs1-deficient mice (Luuk *et al.,* 2009). As GABA-ergic system plays a

significant role in the regulation of anxiety, and the α1 subunit of $GABA_A$ receptor is responsible for the sedative effects of diazepam and α2 subunit plays a role in its stimulating action (Olsen *et a.,* 2009; Wafford *et al.,* 2004), we analyzed the expression of Gabra1 and Gabra2 in the forebrain structures, showing high expression of Wfs1 protein (frontal cortex, temporal lobe, hippocampus and mesolimbic area) (Luuk *et at.,* 2008) of wild-type and Wfs1-deficient mice. Besides that the expression levels of Gad1 and Gad2 genes, the enzymes responsible for synthesis of GABA from glutamate, were measured in the same forebrain structures (Raud *et al.,* 2009).

The genetic invalidation of the Wfs1 gene increased not only anxiety in mice (Luuk *et al.,* 2009), but also affected the expression level of the Gabra1 and Gabra2 genes, playing a role in the pharmacological action of diazepam. Namely, Wfs1-deficient mice displayed reduced expression of these genes in the temporal lobe and frontal cortex. Both these structures have been shown to play an eminent role in the regulation of negative emotions, especially anxiety (Finn *et al.,* 2003). The down-regulation of two dominating subunits of $GABA_A$ receptors (Henschel *et al.,* 2008) probably reflects reduced GABA-ergic neuro-transmission in these particular brain regions. Reduced activity of the GABA-ergic system may also explain the increased sensitivity of Wfs1-deficient mice to the anxiolytic action of diazepam (Luuk *et al.,* 2009). On the other hand, the lack of changes in the expression of the Gad1 and Gad2 genes demonstrates that the invalidation of the Wfs1 gene does not have a major impact on the synthesis of GABA in the brain. Lyons *et al.,* 2001 have shown that the long-term exposure of brain neurons to GABA results in down-regulation of $GABA_A$ receptor number and uncoupling of GABA and benzodiazepine binding sites. They have revealed that the down-regulation of $GABA_A$ receptors occurs due to GABA-induced elevation of intracellular $Ca^{2+}$ levels. Therefore, in the light of finding that the Wfs1 protein modulates $Ca^{2+}$ levels in the intracellular space (Takei *et al.,* 2006), one could speculate that the elevated release of GABA due to heightened sensitivity to stress of Wfs1-deficient mice may be responsible for the down-regulation of $GABA_A$ receptor subunits. It is noteworthy that a similar down-regulation of the Gabra1 and Gabra2 genes in the temporal lobe and frontal cortex occurred in wild-type mice in response to exposure to the elevated plus-maze. This finding further underlines a possible role of stress in the down-regulation of $GABA_A$ receptor subunits established in Wfs1-deficient mice. Therefore, one can state that the present study is in favor of a relation between increased anxiety and reduced expression of subunits of $GABA_A$ receptors in the frontal cortex and temporal lobe in Wfs1-deficient mice (Raud *et al.,* 2009).

## 7.3. Evidence for impaired function of dopaminergic system in Wfs1-deficient mice

As in the above described study 1 the locomotor activity of saline-treated male homozygous Wfs1-deficient mice tended to be lower compared to their heterozygous and wild-type littermates. Moreover, 5 mg/kg of amphetamine, an indirect DA agonist, caused a gene-dose dependent increase of locomotor activity. Specifically, amphetamine-induced hyperlocomotion was the strongest among wild-type mice and weakest in homozygous animals. A diminished effect in Wfs1-deficient mice was also detected for sub-threshold dose (2.5 mg/kg) of amphetamine – repeated treatments with this dose of amphetamine caused significant motor sensitization in wild-type mice, but not in their heterozygous and homozygous littermates. By contrast, apomorphine (3 mg/kg), a direct DA agonist, caused a significant motor stimulation in heterozygous and homozygous Wfs1-deficient mice, but not in wild-type mice. The nature of these behavioral and pharmacological alterations was further explored by biochemical measurements of DA and its metabolites in the dorsal (involving the caudate putamen) and ventral (involving the nucleus accumbens and olfactory tubercle) striatum.

Exposure of saline-treated mice to the motility boxes revealed a significantly stronger increase in the tissue level of homovanillic acid (HVA), a major metabolite of DA, both in the dorsal and ventral striatum of wild-type and heterozygous mice compared to their homozygous littermates. This change probably reflects an increased utilization of DA in wild-type and heterozygous animals under the influence of exposure. The established finding is consistent with the above described locomotor activity experiment where the activity of homozygous mice tended to be lower compared to the other genotypes. Treatment with amphetamine (5 mg/kg) significantly increased tissue levels of DA in the dorsal and ventral striatum of wild-type mice, but not in their homozygous littermates. This finding probably reflects an impaired release of DA in Wfs1- deficient mice. This statement coincides with a recent study showing that depolarization-induced release of DA is severely blunted in Wfs1-deficient mice (Matto *et al.,* 2011). Besides that, the influence of amphetamine on DA turnover was significantly reduced in the dorsal and ventral striatum of homozygous animals compared to other genotypes. It is known that the administration of DA agonists reduces turnover of DA as a homeostatic mechanism (Bannon *et al.,* 1980; Garcia *et al.,* 2005). In the case of the disruptionof the Wfs1 gene, this biochemical action of amphetamine is diminished, because amphetamine is unable to augment tissue levels of DA in the dorsal and ventral striatum. However, treatment with apomorphine caused a similar reduction of DA utilization across all genotypes. Therefore, one could suggest that the disruption of Wfs1 gene does not affect the regulation of DA metabolism via DA receptors. To a certain extent, this hypothesis is supported by the finding that the expression of Drd2 gene (gene for DA D2 receptor) was not affected in the ventral striatum of both male and female Wfs1-deficient

animals. Although in the dorsal striatum of male Wfs1- deficient mice the expression of Drd2 gene was reduced, in female homozygous mice it remained unchanged (Visnapuu *et al.,* 2013).

In order to further clarify the reasons for reduced efficacy of amphetamine in Wfs1-deficient mice we measured gene and protein expression levels of DA transporter, a major target in the action of amphetamine (Sulzer *et al.,* 2005). First, the gene expression measurements revealed marked differences between genotypes. In both sexes, the expression level of Dat gene was significantly reduced in the midbrain of homozygous mice compared to wild-type animals. We aimed to substantiate gene expression findings with protein analyses. Although there were no significant statistical differences between genotypes, the level of Dat protein remained lower in the dorsal striatum of male and female homozygous mice compared to wild-type animals. This tendency was not seen in the ventral striatum. There might be two factors contributing to not having overlap between gene and protein analyses. First, the obtained protein expression data are too variable and it is likely that bigger group sizes would allow to establish an effect in the dorsal striatum. Secondly, it has been shown that mRNA and protein abundances are not always unidirectional or overlapping (Muller *et al.,* 2011; Pra – llner *et al.,* 2001; Visnapuu *et al.,* 2013).

In conclusion, it is likely that Wfs1 gene deficiency impairs the release of DA and therefore compromises the function of DA-ergic system. Although the precise mechanism remains to be elucidated, a possible reason for this manifestation could be the altered $Ca^{2+}$ signaling observed in Wfs1-deficient mice. For instance, Ishihara *et al.* (2004) have demonstrated a reduced $Ca^{2+}$-dependent stimulus-secretion coupling for insulin in Wfs1-deficient mice. Taking into account the above cited study by Matto *et al.* (2011), showing that depolarization-induced DA release is blunted in Wfs1-deficient mice, one could suggest a similar alteration of $Ca^{2+}$ signaling for DA transmission in the brain. However, this hypothesis needs to be verified by further studies. On the other hand, amphetamine-induced release of DA depends on the activity of DA transporter. Results of the present study introduce the possibility that the activity of DA transporter is reduced in Wfs1-deficient mice. Several lines of evidence coming from recent literature show that insulin signaling, via phosphatidylinositol 3-kinase (PI3K) and Akt, effectively modulates DA transporter activity (Garcia *et al.,* 2005; Wei *et al.,* 2007, Williams *et al.,* 2007). Indeed, the inhibition of insulin-mediated PI3K signaling reduces the cell surface expression of Dat protein, and DA uptake is reduced as a result (Williams *et al.,* 2007). Therefore, pharmacologically induced insulin deficiency (caused by steptozotocin) apparently down-regulates the level of DA transporter (Williams *et al.,* 2007), suppresses amphetamine-induced DA release (Owens *et al.,* 2012; Williams *et al.,* 2007) and hyperlocomotion in rodents (Owens *et al.,* 2005). However, there is a substantial difference between streptozotocin and Wfs1-deficiency induced changes in the DA-ergic system. Namely, Owens *et al.* (2012) have found that repeated treatment with amphetamine in rats reverses the reduced sensitivity to amphetamine induced by

streptozotocin, whereas in the present study the administration of sub-threshold dose of amphetamine (2.5 mg/kg) has been unable to affect the reduced sensitivity of Wfs1-deficient mice toward amphetamine. Accordingly, one could claim that altered $Ca^{2+}$ signaling is a possible reason for the observed alterations in the function of DA-ergic system in Wfs1-deficient mice and the reduced expression of DA transporter is likely a compensatory change to the inhibited DA release (Visnapuu *et al.,* 2013).

## 7.4. Relevance of Wfs1 deficient mice as an model of WS

The studies performed so far demonstrate that Wfs1 deficient mice can be taken as a relevant model of WS (Ishihara *et al.,* 2004; Riggs *et al.,* 2005). It is known that diabetes occurring in WS patients begins earlier and is more severe in male persons (Noormets *et al.,* 2011). The similar severity of metabolic disturbances is true concerning male Wfs1 deficient mice. The elevation of blood glucose during glucose tolerance test is much more serious and it lasted longer in male homozygous mice compared to their female littermates (Noormets *et al.,* 2011). Therefore, the male Wfs1 deficient mice have more pronounced growth retardation compared to female ones. This growth retardation is accompanied by the activation of growth hormone pathways and substantial elevation of IGF-1 levels in the plasma (Kõks *et al.,* 2009). On the other hand, the development of diabetic symptoms in the case of Wfs1 deficiency depends on the genetic background. C57/Bl6 background is apparently protective against the development of symptoms (Ishihara *et al.,* 2004). The dependence of outcomed of WFS1 gene mutations from the genetic background may be a possible reason why WS is very pleiomorphic disease, i.e. different patients display different symptoms. Therefore, one could suggest that the metabolic effects of Wfs deficiency should be studied either in 129Sv background or in F2-hybrids having the randomly mixed background from C57/Bl6 and 129Sv parents. In the case of behavioural phenotyping the mixed background is more preferrable, because the behavioural abilities of 129Sv mice are in some cases limited. We have found that the stress-related vocalizations are much louder and more frequent in F2 hybrids compared to 129Sv background. Altogether, based on these data it looks like that Wfs1 deficient mice to certain extent resemble to that of seen in the case of human disease – WS.

The roles of serotonin and noradrenaline in the mechanism and treatment of depression have been widely discussed and proven (Delgado *et al.,* 1990; Moret *et al.,* 2011; Neumeister *et al.*, 2004). However, it has become apparent that dopamine in the ventral and dorsal striatum, involved in the neural circuits of motivations and reward, plays a distinct role in the neurobiology of depression (Salamone *et al.,* 2012). Taking into account a possible role of dopamine in depression, one could suggest that the impaired function of dopamine system, established in Wfs1-deficient mice, may be extended to the patients suffering from mutations in wolframin1 gene to explain their depressive symptoms.

# 8. CONCLUDING REMARKS AND FUTURE PROSPECTS

The present study demonstrates that Wfs1 gene deficiency impairs the adaptation of mice in the novel and stressful environment Table 8). The subsequent studies revealed that the altered adaptation is probably related to the impaired function of GABA- and dopaminergic systems. There are clear indications for the reduced activity of GABAergic system in Wfs1 deficient mice. The expression of dominating (α1 and α2) subunits of $GABA_A$ receptors is reduced in the temporal lobe and frontal cortex of genetically modified mice. This is accompanied by the elevated anxiety and increased anxiolytic action of diazepam, an agonist of $GABA_A$ receptors (Table 8). The function of dopaminergic system is also impaired. Wfs1 gene deficiency impairs the release of DA and therefore compromises the function of DA-ergic system. Although the precise mechanism remains to be elucidated, a possible reason for this manifestation could be the altered $Ca^{2+}$ signaling observed in Wfs1-deficient mice (Table 8).

**Table 8.** The major findings of study

| Aim of the study | Findings and statements |
|---|---|
| 1. Behavioural phenotyping of Wfs1 deficient mice | Wfs1 deficient mice display the impaired adaptation in novel and stressful environment |
| 2. Characterization of GABA system in Wfs1 deficient mice | Increased anxiety established in Wfs1 deficinet mice is related to the impaired function of GABA system in the temporal lobe and frontal cortex |
| 3. Characterization of dopamine system in Wfs1 deficient mice | Altered adaptation established in Wfs1 deficient mice is related to the compromised function of dopamine system due to the impaired release of neurotransmitter |
| 4. Validity of Wfs1 deficient mice as a model of WS | Deficient regulation of glucose metabolism, occuring in Wfs1 deficient mice resembles to that seen in the patients suffering from WS. Therefore, Wfs1 deficient mice can be taken as a valid model of WS and compromised function of GABA and dopamine system, established in Wfs1 deficient mice can be extended to WS patients |

It is known that insulin mediates the release of adrenaline and noradrenaline from adrenergic terminals in neurons, inhibits adrenaline and noradrenaline reuptake in the neural synapse, modifies the kinetics of catecholamines, stimulates serotonin reuptake and regulates transport of DAT to the cell membrane (Duarte *et al.*, 2012). Insulin also controls the expression of NMDA

receptors by increasing the influx of $Ca^{2+}$ ions and thereby strengthening the synaptic communication between neurons (Duarte *et al.*, 2012). Therefore, insulin modulates the long-term activation of neurons and learning on a molecular level. Furthermore, insulin controls the extracellular concentration of glutamate, aspartate, taurine, GABA and $GABA_A$ receptors (Duarte *et al.*, 2012). Moreover, the brain regions rich in insulin (brain cortex, olfactory bulb, hippocampus, hypothalamus and amygdala) overlap in a great extent with the brain structures where the expression level of Wfs1 is the highest (Luuk *et al.*, 2008; Duarte *et al.*, 2012). Therefore, the Wfs1 gene may have a vital role in the production and release of insulin in the central nervous system and this could be the reason why WS patients develop such severe neurological impairments. Taken together, the relevance of insulin for development of behavioural phenotype established in Wfs1 deficient mice should be the target of following studies with these genetically modified animals. Moreover, the depressive symptoms are frequent in patients suffering from WS as well as in the heterozygous carriers of WFS1 gene mutations (Swift *et al*., 1998). Thus, the behavioural effects of various antidepressant drugs (imipramine, paroxetine) should be evaluated in Wfs1 deficient mice. Simultaneously, the alterations in the activity of serotonin- and noradrenergic neurotransmission should be studied in these mice. We expect to find the similar alterations as in a case of DA-ergic system.

# 9. CONCLUSIONS

1. The mice with a deleted exon 8 in the Wfs1 gene do not have any noticable sensory deficiencies. They have a normal pain sensitivity and they can successfully navigate in the Morris water maze. They have a normal learning curve in the active avoidance test. Also, their locomotor activity does not differ from that of their wild-type littermates in the motility box, Morris water maze and forced swimming test. However, Wfs1-deficient mice reacted with increased anxiety in stress-inducing environments as evidenced by longer latencies to eat in the hyponeophagia test, anxiety-like behaviour in the light-dark test in response to short-term social isolation and decreased exploratory activity in the open field test in intense lighting conditions. Also, their level of corticosterone after a stress-inducing intraperitoneal saline injection is three times higher than in their wild-type littermates and they display increased sensitivity to diazepam, $GABA_A$ receptor agonist, in the elevated plus maze test. Diazepam also eliminated audible vocalizations characteristic to Wfs1 deficient mice in a stressful situation. In addition, these mice have alterations in the dopaminergic system. They have markedly lower sensitivity to the locomotor stimulating effects of amphetamine, an undirect agonist of dopamine, and they display hypersensitivity to the locomotor stimulating effect of apomorphine, a direct agonist of dopamine, compared to their wild-type littermates. Altogether, the initial behavioural phenotyping of Wfs1 deficient mice reflected their impaired adaptation in the novel and stressful environment.

2. The mice with a deleted exon 8 in the Wfs1 gene have a lower expression level of the Gabra1 and Gabra2 genes in the temporal lobe and frontal cortex, in the brain structures playing a role in the regulation of negative emotions. These genes code the alpha1 and alpha2 subunits of the $GABA_A$ receptor, being the important mediators of the pharmacological effects of diazepam. This is probably a reason for the increased anxiolytic effect of diazepam in Wfs1 deficient mice. The similar change in expression of Gabra1 and Gabra2 gene in wild-type mice was established after their challenge to the elevated plus-maze, demonstrating a relation between anxiety and these subunits of $GABA_A$ receptors. The decrease in the expression of the Gabra1 and Gabra2 genes indicates an impairment in GABA-ergic neurotransmission in Wfs1-deficient mice. There is no differences in the expression levels of glutamate decarboxylase (Gad1 and Gad2) genes, indicating that Wfs1-deficiency does not induce profound changes in the GABA synthesis in mice.

3. Amphetamine (5 mg/kg), significantly increased the locomotor activity of wild-type and heterozygous mice, but had almost no effect on the activity level of homozygous mice with the deleted 8th exon in Wfs1 gene. In addition, repeated administration of low dose (2.5 mg/kg) of amphetamine failed to induce a motor sensitization in homozygous mice. Amphetamine (5 mg/kg) administration increased significantly the level of dopamine in the ventral and dorsal striatum in wild-type mice, but not in homozygous mutants. Also, amphetamine administration significantly decreased the dopamine turnover in

the ventral and dorsal striatum in wild-type and heterozygous mice, but not in homozygous mutants. Motor stimulation induced by apomorphine (3 mg/kg) was somewhat stronger in mutant mice compared to wild-type animals. However, treatment with apomorphine induced a similar decrease in dopamine turnover in all genotypes, which indicates that the deficiency of Wfs1 gene does not influence dopamine metabolism via dopamine receptors. This statement is also confirmed by results of the gene expression experiment where the gene expression of Drd2 receptors between genotypes was not different in the ventral striatum. However, both female and male Wfs1-deficient mice had a lower level of the dopamine transporter gene in the midbrain. This finding together with the blunted behavioral and biochemical effects of amphetamine is indicative for the impaired function of dopaminergic system in Wfs1 deficient mice.

4. Based on studies of glucose metabolism it is obvious that the mice with the deleted 8th exon of Wfs1 gene are the valid models of WS and related disorders. Therefore, the established disturbances in Wfs deficient mice occurring in the function of GABA- and dopaminergic system can be extended to understand the neuropsychiatric symptoms in WS patients.

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# 11. SUMMARY IN ESTONIAN

## Wolframi sündroomi mudel hiirtel: käitumuslik, biokeemiline ja psühhofarmakoloogiline iseloomustamine

Wolframi sündroom (WS, OMIM 222300) on haruldane autosomaalne retsessiivne neurodegeneratiivne häire, mida iseloomustavad varajases lapseeas algav suhkurtõbi, progresseeruv nägemisnärvi atroofia, magediabeet ja kurtus (Domenech *et al.,* 2006). WS iseloomustasid esmakordselt Saksa arstid Wolfram ja Wagener 1938.a (Wolfram and Wagner 1938). Wolframi sünroomi põhjustavad mutatsioonid Wolframiin 1 (WFS1) geenis (Strom *et al.,* 1998; Inoue *et al.,* 1998). Antud sündroomile on iseloomulikud terve rida neuroloogilisi ja psühhiaatrilisi sümptomeid, mille hulka kuuluvad raske depressioon, psühhoos, ülemäärane impulsiivsus ja agressiivsus (Barrett *et al.*, 1995; Swift *et al.*, 1991; Swift *et al.*, 1990). Wfs1 on seotud hirmu ja ärevusega hiirtel ja rottidel (Kesner *et al.*, 2007; Koks *et al.*, 2002) ja tema polümorfismid on tõenäoliselt seotud suurenenud meeleoluhäirete riskiga (Koido *et al.*, 2005).

WfS1 geen asub inimese 16 kromosoomis lookuses 4p16.1 (Strom *et al.,* 1998; Inoue *et al.,* 1998). Paljud uuringud on seda kromosoomi piirkonda seostanud bipolaarse häirega (Ewald *et al.,* 1998, 2002; Detera-Wadleigh *et al.,* 1999), bipolaarse häire psühhootilise vormi (Als *et al.,* 2004; Cheng *et al.,* 2006) ning suitsidaalse käitumisega (Cheng *et al.,* 2006). Nende uuringute alusel võib oletada, et WFS1 geeni mutatsioonid omavad teatud rolli bipolaarsete häirete ja teiste depressiivsete seisundite patofüsioloogias (Kato *et al.,* 2008).

Inimestel, kes kannavad erinevaid WFS1 geeni mutatsioone, kuid ei oma WS ilminguid, on 26 korda suurem risk sattuda psühhiaatriahaiglasse, enamasti depressiooni tõttu (Swift *et al.,* 1998). On näidatud WFS1 geeni mutatsioonide esinemist mitmete neuropsühhiaatriliste häirete puhul (skisofreenia, bipolaarne häire, depressioon, suitsidaalne käitumine) (Ohtsuki *et al.,* 2000; Martorell *et al.,* 2003; Torres *et al*., 2001; Crawford *et al.,* 2002; Evans *et al.,* 2000), kuid oluline on siin rõhutada, et ükski neist patsientidest ei omanud WS iseloomulikke ilminguid. Vaatamata sellele, et WFS1 geeni mutatsioonid ei ole tõenäoliselt eriti sagedaseks vaimuhaiguste tekke põhjuseks, on oluline ikkagi selgitada võimalikke neurobioloogilisi mehhanisme, mille kaudu WFS1 puudulikkus viib WS patisentidel ning heterosügootstel mutatsiooni kandjatel vaimsete häirete välja kujunemiseni (Kato *et al.,* 2008).

Käesoleval ajal ei ole veel päris selge, mis moodi WFS1 geeni puudulikkus põhjustab neuropsühhiaatriliste kõrvalekallete ilmnemist. Kuid võttes arvesse WS puhul esinevad psühhiaatrilised sümptomid (Barrett *et al.*, 1995; Swift *et al.*, 1991; Swift *et al.*, 1990), ning ka Wfs1 geeni ja valgu lokalisatsiooni ajus (Luuk *et al.,* 2008), võib arvata, et ühetedeks põhjustajateks võivad olla kõrvalekalded GABA- ja dopamiinergilise süsteemi talitluses. Depressiooni ja ärevushäirete vahel esinev silmapaistev komorbiidsus viitab nende haiguste etioloogilisele sarnasusele (Murphy *et al.,* 2004; Gamez *et al.,* 2007). Pidurdusmediaatori γ-aminovõihappe (GABA) kontsentratsiooni vähenemine ajus ja

virgatsaine toimes põhiliste $GABA_A$ retseptorite funktsiooni muutus või ekspressiooni vähenemine on seotud nii depressiooni kui ka ärevushäirete väljakujunemisega (Luscher *et al.,* 2011). Samuti on leitud, et dopamiinergilise süsteemia alafunktsioon, esmajoones prefrontaalses koores, on seotud depressiooni ja skisofreenia negatiivsete sümptomitega (apaatia, huvi kadumine, anhedoonia jne.) (Davis *et al.,* 1991). Vastupidiselt, dopamiinergilise süsteemi ülefunktsiooni, iseäranis ventraalses ja dorsaalses striatumis, on seostatud skisofreenia positiivsete sümptomidega: hallutsinatsioonid, luulumõtted, psühhomotoorne rahutus ja kontrollimatu käitumine (Davis *et al.,* 1991). Nimetatud kõrvalekalded GABA- ja dopamiinergilise süsteemi talitluses on vägagi sarnansed nendele häiretele, mida on kirjeldatud WS põdevatel patsientidel (Swift *et al.,* 1998).

Käesoleva doktoritöö keskseks eesmärgiks oli WS hiire mudeli loomine. Selleks valmistati mutantne hiireliin kustutades Wfs1 geenist 8nda eksoni ja asendades selle beeta galaktosidaasi ekspresiooni kassetiga (NLS-LacZ). Kaheksas ekson valiti, sest meie eelduseks oli C-terminaali „funktsiooni kaoga" Wfs1 puudulikkusega hiire loomine, kuna WS patsientidel enamik mutatsioone asub just selles eksonis. Selline lähenemisviis aitas luua WS sarnase seisundi, et uurida sündroomi poolt esilekutsutud patoloogilisi muutusi.

Katseloomade iseloomustamiseks kasutati käitumuslikke (ärevus, motoorne koordinatsioon, motoorne aktiivsus, õppimine ja mälu, valutundlikkus, katseloomade reaktsioon stressile jne.), biokeemilisi (dopamiini ja tema peamisete metaboliitide sisalduse määramine katseloomade ajus, geeni ja valgu ekspressiooni uurimine ajustruktuurides, glükoosi tolerantsuse test, kortikosterooni taseme määramine vastusena stressile) ja psühhofarmakoloogilisi (GABAA retseptorite agonist diasepaam, dopamiini agonistid amfetamiin ja apomorfiin) uurimismeetodeid. Katsete tulemuste usaldusväärtust kontrolliti statistiliste meetoditega, kasutades selleks Statistica for Windows 10 arvutiprogrammi.

Uuringute käigus jõuti järgmiste tulemusteni. Wfs1 geeni kaheksanda eksoni puudulikkusega hiirtel ei olnud nähtavaid sensoorseid häireid. Neil oli normaalne valutundlikkus ja nad suutsid orienteeruda väliste märkide järgi Morrise vesipuuris. Nende õppimine aktiivse vältimise testis ei erinenud metsik-tüüpi pesakonna kaaslaste omast. Samuti ei erinenud nende käitumine motoorika testis, Morrise vesipuuris ja sundujumise testis. Wfs1 puudulikkusega hiired reageerisid suurenenud ärevusega stressi esilekutsuvates keskkondades. Nii oli pikenenud nende söömise alustamise latents hüponeofaagia testis, vähenenud oli nende uudistamisaktiivsus heleda-tumeda puuri testis peale lühiajalist isolatsiooni ja nad olid vähem aktiivsed avarvälja testis suurema keskkonna valgustatuse korral. Samuti suurenes neil tunduvalt enam, võrreldes metsik-tüüpi hiirtega, korikosterooni tase ringlevas veres peale füsioloogilise lahuse akuutset süstimist. Wfs1 puudulikkusega hiirtel oli suurenenud tundlikkus GABAA retseptorite agonisti diasepaami ärevusvastase toime suhtes. Diasepaam kõrvaldas ka Wfs1 puudulikksuega hiirtele iseloomulikud häälitsemised stressogeenses situatsioonis. Lisaks kõigele muule esinesid nendel hiirtel kõrvalekalded dopamiinergilise süsteemi funktsioonis. Nende tundlikkus amfetamiini,

kaudse dopamiini agonisti, motoorikat stimuleeriva toime suhtes oli vähenenud, kuid nende tundlikkus oli suurenenud apomorfiini (dopamiini otsese agonisti) toime suhtes võrreldes metsik-tüüpi pesakonnakaaslastega. Kokkuvõttes, esialgne käitumuslik fenotüpeerimine peegeldab Wfs1 puudulikkusega hiirte häirunud võimet kohaneda uudses ja stressogeenses keskkonnas.

Wfs1 geeni kaheksanda eksoni puudulikusega hiirtel oli vähenenud Gabra1 ja Gabra2 geenide eskpressioon oimusagaras ja otsmikukoores ehk ajustruktuurides, mis on seotud negatiivsete emotsioonide regulatsiooniga. Need geenid kodeerivad $GABA_A$ retseptori alfa1 ja alfa2 alaühiku valke, mis on olulised diasepaami farmakoloogiliste toimete vahendajad. Gabra 1 ja Gabra 2 geenide ekspressiooni langus on ilmselt seotud Wfs1 puudulikkusega hiirtel esineva ülitundlikkusega diasepaami ärevusvastase toime suhtes. Sarnane Gabra1 ja Gabra2 geenieksрessiooni langus esines metsik-tüüpi hiirtel peale nende eksponeerimist pluss puuris (ärevuse mudel), mis viitab, et on olemas seos $GABA_A$ retseptorite alatüüpide ekspressiooni languse ja ärevuse vahel. Kokkuvõttes, Gabra1 and Gabra2 geenide ekspressiooni langus viitab GABA-ergilise närviülekande häirumisele Wfs1 puudulikkusega hiirte. Samas ei ole erinevusi glutamaat dekarboksülaasi (Gad1 ja Gad2) geenide ekspressioonis, mille alusel võib väita, et GABA biosüntees ei ole Wfs1 geeni puudulikkuse mõjul häiritud.

Amfetamiin (5 mg/kg) suurendas oluliselt liikumisaktiivsust metsik-tüüpi ja heterosügootsetel hiirtel, kuid mitte homosügootsetel mutantsetel hiirtel. Lisaks sellele, amfetamiini madalate annuste (2,5 mg/kg) korduv manustamine ei kutsunud esile motoorset sensitisatsiooni homosügootsetel hiirtel, võrreldes metsik tüüpi pesakonna kaaslastega. Amfetamiini (5 mg/kg) manustamine suurendas märkimisväärselt dopamiini taset metsik-tüüpi hiirte dorsaalses ja ventraalses striatumis, aga mitte homosügootsetel mutantidel. Samuti vähendas amfetamiini manustamine dopamiini ringkäiku metsik-tüüpi ja heterosügootsete hiirte dorsaalses ja ventraalses striatumis, kuid see ei leidnud aset homosügootsetel mutantidel. Apomorfiinist (3 mg/kg) tingitud motoorika stimulatsioon oli mõnevõrra tugevam mutantsetel hiirtel võrreldes metsik-tüüpi hiirtega. Kuid apomorfiin põhjustas kõikidel fenotüüpidel ühesugust dopamiini ringkäiku vähenemist, mille alusel võib väita, et Wfs1 geeni puudulikkus ei mõjusta dopamiini metabolismi dopamiini retseptorite vahendusel. Seda väidet kinnitavad ka geeni ekspressiooni tulemused, kus dopamiini retseptori (Drd2) ekspressioon oli ühesugune kõikide genotüüpide ventraalses striatumis. Siiski oli nii emastel kui ka isastel Wfs1 puudulikusega hiirtel vähenenud dopamiini transporteri geeni ekspressioon keskajus. See leid koos amfetamiini käitumuslike ja biokeemiliste toimete vähenemisega viitab dopamiinergilise süsteemi funktsiooni olulisele häirumisele Wfs1 puudulikkusega hiirtel.

Põhinedes glükoosi metabolismi uuringute tulemustele ja nende sarnasusele WS on selge, et Wfs1 geeni kaheksanda eksoni puudulikkusega hiir on valiidseks mudeliks WS südnroomile ja ka teistele WFS1 geenimutatsioonidest tingitud häiretele. Sellepärast võib käesolevas uuringus leitud muutusi dopamiini- ja GABA-ergilise süsteemi aktiivsuses Wfs1 puudulikkusega hiirtel laiendada WS patsientidele, et mõista nendel esinevaid neuropsühhiaatrilisi sümptomeid.

# 12. ACKNOWLEDGEMENTS

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# ORIGINAL PUBLICATIONS

# CURRICULUM VITAE

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**Education:**

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| 1996–1999 | Haapsalu Grammar shcool |
| 1999–2003 | University of Tartu, Bsc Genetechnology |
| 2003–2005 | University of Tartu, Msc Developmental Biology |
| 2005–2013 | University of Tartu, Phd studies in Developmental Biology |

**Career:**

| | |
|---|---|
| 2003–2005 | OÜ Visgenyx, Technican, transgenic technology (microinjections) |
| 2004–2005 | OÜ Naxo, Technican, transgenic technology (microinjections) |
| 2005– | University of Tartu, Institute of Technology, Head of the laboratory of transgenic technology |
| 2008– | Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences; Researcher (0.50) |
| 2008– | OÜ KPA Scientific, Member of board |
| 2012– | OÜ Icosagen Toxicology Services, Member of board |
| 2013– | Estonian Ministry of Agriculture, Chairman of the Animal experiments commitee |

**Awards:**

| | |
|---|---|
| 2006 | Nets prize for the business plan of running the laboratory of transgenic tehnology |
| 2010 | 3 place in Estonian business plan contest called “brain storm”. Hepatitis Virus C infectable small animal models |
| 2011 | The U.S. Embassy, the Baltic American Freedom Foundation (BAFF), and the American Chamber of Commerce in Estonia (AmCham) selected Mario Plaas company KPA Scientific OÜ to the six finalists for the 2011 Estonian American Innovation Award |

**Practical courses:**

| | |
|---|---|
| 2003 | Hands on ES cell and DNA microinjection into mouse embryo (EMBL, Heidelberg, Germany) |
| 2006 | Competence course on laboratory animal science (Helsinki University, Finland) |
| 2012 | Hands on DNA microinjection training into rat embryo (University of Michigan, USA) |

**Research:**
Main fields are a) the function of wfs1 protein – molecular and systems biology approach. b) development of new HCV infectable small animal models. 14 publications have appeared in international peer-review journals.

**List of publications:**


1. Visnapuu, T., **Plaas, M**., Reimets, R., Raud, S., Terasmaa, A., Kõks, S., Sütt, S., Luuk, H., Hundahl, A, C., Eskla, L, K., Altpere, A., Alttoa, A., Harro, J., Vasar, E. Evidence for impaired function of dopaminergic system in Wfs1-deficient mice. *Behav Brain Res*., *in press*
2. Ord, T., Ord, D., Kuuse, S., Plaas, M., Ord, T. Trib3 is regulated by IL-3 and affects bone marrow-derived mast cell survival and function. Cell Immunol. 2012 Nov;280(1):68–75.
3. Kõks, S., Soomets, U., Plaas, M., Terasmaa, A., Noormets, K., Tillmann, V., Vasar, E., Fernandes, C., Schalkwyk, L, C. Hypothalamic gene expression profile indicates a reduction in G protein signaling in the Wfs1 mutant mice. Physiol Genomics, 2011 Dec 16;43(24):1351–8.
4. Innos, J., Philips, M. A., Leidmaa, E., Heinla, I., Raud, S., Reemann, P., Plaas, M., Nurk, K., Kurrikoff, K., Matto, V., Visnapuu, T., Mardi, P., Kõks, S., Vasar, E. Lower anxiety and a decrease in agonistic behaviour in Lsamp-deficient mice. Behav Brain Res, 2011 Feb 2;217(1):21–31.
5. Philips, M. A., Abramov, U., Lilleväli, K., Luuk, H., Kurrikoff, K., Raud, S., Plaas, M., Innos, J., Puussaar, T., Kõks, S., Vasar, E. Myg1-deficient mice display alterations in stress-induced responses and reduction of sex-dependent behavioural differences. Behav Brain Res. 2010 Feb 11;207(1):182–95
6. Raud, S., Sütt, S., Luuk, H., **Plaas, M**., Innos, J., Kõks, S., Vasar, E. Relation between increased anxiety and reduced expression of alpha1 and alpha2 subunits of GABA(A) receptors in Wfs1-deficient mice. Neurosci Lett. 2009 Aug 28;460(2):138–42.
7. Sütt, S., Raud, S., Abramov, U., Innos, J., Luuk, H., Plaas, M., Kõks, S., Zilmer, K., Mahlapuu, R., Zilmer, M., Vasar, E. Relation of exploratory behaviour to plasma corticosterone and Wfs1 gene expression in Wistar rats. J Psychopharmacol. 2010 Jun;24(6):905–13
8. Kõks, S., Soomets, U., Paya-Cano, L. J., Fernandes, C., Luuk, H., Plaas, M., Terasmaa, A., Tillmann, V., Noormets, K., Vasar, E., Schalkwyk, C, L. Wfs1 gene deletion causes growth retardation in mice and interferes with the growth hormone pathway. Physiol. Genomics 37:249–259, 2009.
9. Luuk, H., Plaas, M., Raud, S., Innos, J., Sütt, S., Lasner, H., Abramov, U., Kurrikoff, K., Kõks, S., Vasar, E. Wfs1-deficient mice display impaired behavioural adaptation in stressful environment. *Behav Brain Res*. 2009 Mar 17;198(2):334–45.
10. Plaas, M., Karis, A., Innos, J., Rebane, E., Baekelandt, V., Vaarmann, A., Luuk, H., Vasar, E., Koks, S. Alpha-synuclein A30P point-mutation

generates age-dependent nigrostriatal deficiency in mice. J Physiol Pharmacol. 2008 Jun;59(2):205–16.
11. Luuk, H., Koks, S., Plaas, M., Hannibal, J., Rehfeld, J. F., Vasar, E. 2008. Distribution of Wfs1 protein in the central nervous system of the mouse and its relation to clinical symptoms of the Wolfram syndrome. *J Comp Neuro*l. 2008 Aug 20;509(6):642–60.
12. Raud, S., Sütt, S., Plaas, M., Luuk, H., Innos, J., Philips, M. A., Kõks, S., Vasar, E. Cat odor exposure induces distinct changes in the exploratory behavior and Wfs1 gene expression in C57Bl/6 and 129Sv mice. Neurosci Lett. 2007 Oct 16;426(2):87–90
13. Tõnissoo, T., Kõks, S., Meier, R., Raud, S., Plaas, M., Vasar, E., Karis, A. Heterozygous mice with Ric-8 mutation exhibit impaired spatial memory and decreased anxiety. Behav Brain Res. 2006 Feb 15;167(1):42–8
14. Tõnissoo, T., Meier, R., Talts, K., Plaas, M., Karis, A. Expression of ric-8 (synembryn) gene in the nervous system of developing and adult mouse. Gene Expr Patterns. 2003 Oct;3(5):591–4.

**Inventions:**

Patent application: Transgenic animal model for modelling pathological anxiety, a method for identifying compounds for treatment of disease or disorders caused by pathological anxiety and a method for using wfs1 protein as a target for identifying effective compounds against pathological anxiety. Owner: University of Tartu. Authors; Eero Vasar, Sulev Kõks, Hendrik Luuk, Sirli Raud, Mario Plaas. Priority number; P200600039. Priority date; 12.12.2006

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**Teenistuskäik:**

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| 2003–2005 | OÜ Visgenyx, Transgeense tehnoloogia spetsialist (mikroinjektsioonid) |
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**Tunnustused:**

| | |
|---|---|
| 2006 | Nets ettevõtlus konkursi auhind biomeditsiini valdkonnas |
| 2010 | 3 koht Eesti suurimal ettevõtluskonkursil “Ajujaht” Uued humaniseeritud transgeensed hiired Hepatiidi viirus C ravimite ja ravimeetodite testimiseks |
| 2011 | U.S. Saatkond, Baltic American Freedom Foundation (BAFF) ja American Chamber of Commerce in Estonia (AmCham) valisid osaühingu KPA Scientific 6 finalisti hulka (Eesti ettevõtted), kes teevad innovatsiooni Ameerika ühendriikidega |

**Erialane enesetäiendus:**

| | |
|---|---|
| 2003 | Hiire transgeense tehnoloogia kursus (mikroinjektsioonid) EMBL Heidelbergis (Saksamaa) |
| 2006 | Loomkatse loa kursus (Helsinki Ülikool, Soome) |
| 2012 | Roti transgeense tehnoloogia kursus (mikroinjektsioonid) (Michigani Ülikool, Ameerika Ühendriigid) |

**Teadustegevus:**
Täna on põhilisteks teadusarendus suundadeks a) Wolframin 1 geeni funktsiooni ning WS sündroomi kandva transgeense hiire uurimine b) Uute humaniseeritud transgeensete hiirte arendamine, mis oleksid nakkusvõimelised Hepatiidi Viirus C suhtes. Eelrefereeritud ajakirjades olen avaldanud 14 teaduspublikatsiooni.

**Publikatsioonid:**


1. Visnapuu, T., **Plaas, M**., Reimets, R., Raud, S., Terasmaa, A., Kõks, S., Sütt, S., Luuk, H., Hundahl, A, C., Eskla, L, K., Altpere, A., Alttoa, A., Harro, J., Vasar, E. Evidence for impaired function of dopaminergic system in Wfs1-deficient mice. *Behav Brain Res*., *in press*
2. Ord, T., Ord, D., Kuuse, S., Plaas, M., Ord, T. Trib3 is regulated by IL-3 and affects bone marrow-derived mast cell survival and function. Cell Immunol. 2012 Nov;280(1):68–75.
3. Kõks, S., Soomets, U., Plaas, M., Terasmaa, A., Noormets, K., Tillmann, V., Vasar, E., Fernandes, C., Schalkwyk, L, C. Hypothalamic gene expression profile indicates a reduction in G protein signaling in the Wfs1 mutant mice. Physiol Genomics, 2011 Dec 16;43(24):1351–8.
4. Innos, J., Philips, M. A., Leidmaa, E., Heinla, I., Raud, S., Reemann, P., Plaas, M., Nurk, K., Kurrikoff, K., Matto, V., Visnapuu, T., Mardi, P., Kõks, S., Vasar, E. Lower anxiety and a decrease in agonistic behaviour in Lsamp-deficient mice. Behav Brain Res, 2011 Feb 2;217(1):21–31.
5. Philips, M. A., Abramov, U., Lilleväli, K., Luuk, H., Kurrikoff, K., Raud, S., Plaas, M., Innos, J., Puussaar, T., Kõks, S., Vasar, E. Myg1-deficient mice display alterations in stress-induced responses and reduction of sex-dependent behavioural differences. Behav Brain Res. 2010 Feb 11;207(1): 182–95
6. Raud, S., Sütt, S., Luuk, H., **Plaas, M**., Innos, J., Kõks, S., Vasar, E. Relation between increased anxiety and reduced expression of alpha1 and alpha2 subunits of GABA(A) receptors in Wfs1-deficient mice. Neurosci Lett. 2009 Aug 28;460(2):138–42.
7. Sütt, S., Raud, S., Abramov, U., Innos, J., Luuk, H., Plaas, M., Kõks, S., Zilmer, K., Mahlapuu, R., Zilmer, M., Vasar, E. Relation of exploratory behaviour to plasma corticosterone and Wfs1 gene expression in Wistar rats. J Psychopharmacol. 2010 Jun;24(6):905–13
8. Kõks, S., Soomets, U., Paya-Cano, L. J., Fernandes, C., Luuk, H., Plaas, M., Terasmaa, A., Tillmann, V., Noormets, K., Vasar, E., Schalkwyk, C. L. Wfs1 gene deletion causes growth retardation in mice and interferes with the growth hormone pathway. Physiol. Genomics 37:249–259, 2009.
9. Luuk, H., Plaas, M., Raud, S., Innos, J., Sütt, S., Lasner, H., Abramov, U., Kurrikoff, K., Kõks, S., Vasar, E. Wfs1-deficient mice display impaired behavioural adaptation in stressful environment. *Behav Brain Res*. 2009 Mar 17;198(2):334–45.

10. Plaas, M., Karis, A., Innos, J., Rebane, E., Baekelandt, V., Vaarmann, A., Luuk, H., Vasar, E., Koks, S. Alpha-synuclein A30P point-mutation generates age-dependent nigrostriatal deficiency in mice. J Physiol Pharmacol. 2008 Jun;59(2):205–16.
11. Luuk, H., Koks, S., Plaas, M., Hannibal, J., Rehfeld, J. F., Vasar, E. 2008. Distribution of Wfs1 protein in the central nervous system of the mouse and its relation to clinical symptoms of the Wolfram syndrome. *J Comp Neuro*l. 2008 Aug 20;509(6):642–60.
12. Raud, S., Sütt, S., Plaas, M., Luuk, H., Innos, J., Philips, M. A., Kõks, S., Vasar, E. Cat odor exposure induces distinct changes in the exploratory behavior and Wfs1 gene expression in C57Bl/6 and 129Sv mice. Neurosci Lett. 2007 Oct 16;426(2):87–90
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**Leiutised:**

Patent application: Transgenic animal model for modelling pathological anxiety, a method for identifying compounds for treatment of disease or disorders caused by pathological anxiety and a method for using wfs1 protein as a target for identifying effective compounds against pathological anxiety. Owner: University of Tartu. Authors; Eero Vasar, Sulev Kõks, Hendrik Luuk, Sirli Raud, Mario Plaas. Priority number; P200600039. Priority date; 12.12.2006

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