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III – The author participated designing the study, performed some of the behavioral experiments, carried out statistical analysis and participated in writing the manuscript.

ABBREVIATIONS

129	–	129S6/SvEv strain or, more generally, all 129 strains
3-MT	–	3-methoxytyramine
5-HIAA	–	5-hydroxyindoleacetic acid
5-HT	–	5-hydroxytryptamine or serotonin
5-HTP	–	5-hydroxytryptophan
AADC	–	aromatic amino acid decarboxylase
AC	–	adenylate cyclase
ANOVA	–	analysis of variance
B6	–	C57BL/6 (strain; a substrain to C57)
BNST	–	bed nucleus of the stria terminalis
C57	–	C57BL strains
CA1	–	cornu ammonis 1 of hippocampus
CA3	–	cornu ammonis 3 of hippocampus
cDNA	–	complementary DNA
COMT	–	catechol-O-methyl transferase
CPu	–	caudate nucleus and putamen (dorsal striatum)
DA	–	dopamine
DAT	–	DA transporter
DBH	–	DA beta-hydroxylase
D1	–	DA type 1 (receptor)
D2	–	DA type 2 (receptor)
D2Lh	–	long variant of the D2 receptor
D2Sh	–	short variant of the D2 receptor
EP	–	entopeduncular nucleus
DIDMOAD	–	diabetes insipidus, diabetes mellitus, optic nerve atrophy, deafness
Drd1	–	gene encoding the D1 receptor
Drd2	–	gene encoding the D2 receptor
DNA	–	deoxyribonucleic acid
DOPAC	–	3,4-dihydroxyphenylacetic acid
E	–	embryonic day
ER	–	endoplasmic reticulum
FST	–	forced swim test
GABA	–	gamma-aminobutyric acid
$GABA_A$-R	–	GABA-A receptor
GABA-T	–	GABA transaminase
HPLC	–	high performance liquid chromatography
Hprt1	–	hypoxanthine phosphoribosyltransferase 1
HVA	–	homovanillic acid
LacZNeo	–	beta-galactosidase/neomycin fusion gene
LORR	–	loss of righting reflex
MAO	–	monoamine oxidase

mRNA	–	messenger ribonucleic acid
MSN	–	medium spiny neuron
NA	–	noradrenaline
NAT	–	NA transporter
NMDA	–	N-methyl-D-aspartate receptor
NMN	–	normetanephrine
P	–	postnatal day
qRT-PCR	–	quantitative real-time polymerase chain reaction
SEM	–	standard error of the mean
SERT	–	5-HT transporter
siRNA	–	small interfering RNA
SN	–	substantia nigra
SNc	–	compact part of the substantia nigra
SNr	–	reticular part of the substantia nigra
SSADH	–	succinate-semialdehyde dehydrogenase
SSRI	–	selective 5-HT reuptake inhibitor
STN	–	subthalamic nucleus
TCA	–	tricyclic antidepressant
TPH	–	tryptophane hydroxylase
TST	–	tail suspension test
UPR	–	unfolded protein response
VTA	–	ventral tegmental area
Wfs1	–	Wolfram Syndrome 1 gene in species other than humans
WFS1	–	Wolfram Syndrome 1 gene in humans
Wfs1 –/–	–	homozygous mutants lacking both functional alleles of the Wfs1 gene
Wfs1 +/–	–	heterozygous mutants having only one functional allele of the Wfs1 gene
Wfs1 +/+	–	wild-type littermates of Wfs1-deficient mice
WS	–	Wolfram syndrome

INTRODUCTION

Wolframin (WFS1 protein) is transcribed from the WFS1 gene. It is an endoplasmic reticulum (ER) membrane protein important for preserving intracellular calcium homeostasis (Osman et al., 2003, Takei et al., 2006) and insulin secretion (Fonseca et al., 2012). Wfs1 is also essential for maintaining the unfolded protein response (UPR) in ER stress and lack of WFS1 leads to uncontrolled UPR and apoptosis (Fonseca et al., 2010). WFS1 expression has been detected in the heart, lung, liver, pancreas and brain. It seems that pancreatic beta cells and neurons are most vulnerable to WFS1 deficiency. In the pancreas, WFS1 is located to secretory granules of pancreatic β-cells (Hatanaka et al., 2011) and WFS1 deficiency leads to apoptosis of β-cells and diabetes (Fonseca et al., 2010). Homozygous mutations and some forms of compound heterozygosity in the WFS1 gene in humans cause Wolfram syndrome (WS), a very low prevalence disease (estimated 1/700 000 to 1/550 000), first described in 1938 by Wolfram and Wagener (Wolfram & Wagener, 1938, Inoue et al., 1998, Strom et al., 1998, Cano et al., 2007). Starting in early adolescence, WS patients in addition to DIDMOAD (diabetes insipidus, mellitus, optic atrophy and deafness) have a high susceptibility to psychiatric disorders, such as depression, psychosis, impulsivity and aggression (Barrett et al., 1995, Swift et al., 1990, Swift et al., 1991, Swift & Swift, 2005). WFS1 heterozygotes have 26 times higher psychiatric hospitalization primarily due to severe depression (Swift et al., 1998). WFS1 carriers have been shown to suffer from generalized anxiety and suicide attempts (Swift et al., 1998). It is also important to note that some WFS1 polymorphisms are associated with increased risk for mood disorders (Koido et al., 2005).

These findings raise the question why the lack of wolframin results in psychiatric symptoms. Detailed Wfs1 expression has not been studied in the human brain, the most comprehensive research to date regarding the neuroanatomical localization of Wfs1 have been conducted in mice (Luuk et al., 2008, Tekko et al., 2014). By looking at the ontogenetic expression of Wfs1, Tekko et al (2014) showed that wolframin is first detected in the mouse brain on embryonic day 15.5 in the central amygdala and caudate-putamen (CPu). Later, Wfs1 expression widens to encompass additional brain regions associated with the extended amygdala, limbic system and basal ganglia concepts, which constitute important brain networks responsible for experiencing emotions, anxiety, motivations and the smooth execution of movements (Luuk et al 2008). Luuk et al (2008) studied expression of wolframin protein in adult mouse brain, Wfs1-positive nerve fibers were detected in the substantia nigra (SN), ventral tegmental area (VTA) and medial forebrain bundle and Wfs1-positive neurons were shown in the nucleus accumbens and posterior CPu. Moreover, strong Wfs1 expression was found in the central amygdala and CA1 region of the hippocampus (Luuk et al., 2008). In support to the neuroanatomical findings, behavioral animal studies have associated Wfs1 to anxiety (Luuk et al., 2009,

Kesner et al., 2009, Koks et al., 2002) and also point to a role of Wfs1 in the dopaminergic system (Luuk et al., 2009).

Together, the previous results lead us to suspect that alterations in monoaminergic (dopamine – DA, serotonin – 5-HT, noradrenaline – NA) and gamma-aminobutyric (GABA-ergic) systems are responsible for the symptoms seen in Wfs1 deficiency. Monoamines are known to be involved in a wide range of brain functions and disturbances in these systems lead to various neurologic and psychiatric conditions. For example low striatal DA levels due to loss of DA-ergic neurons in the compact part of the SN cause serious motor system deficiencies in the form of Parkinson's disease. Higher levels of depression (around 40%), likely caused by loss of DA and NA neurons, are also seen in Parkinson's patients (Cummings, 1992, Cummings & Masterman, 1999, Remy et al., 2005). Some DA agonists are effective antidepressants (Willner, 1997). By contrast, pathologically elevated DA signaling in DA pathways is considered the cause for psychotic disorders and is treated with DA receptor antagonists (Howes & Kapur, 2009). Lower levels of central 5-HT are found in patients with mood disorders (Edwards & Anderson, 1999). Similarly, drugs elevating brain 5-HT and NA levels are effective antidepressants. Therefore, monoamine systems are the main drug targets in mood disorders. GABA is the main inhibitory neurotransmitter in the brain and, specifically, GABA receptor A ($GABA_A$-R) subtypes have been implicated in anxiety, since they are the molecular target for benzodiazepines which are highly effective anxiolytic agonists of these receptors (Petroff, 2002, Hines et al., 2012).

The aim of this dissertation was to psychopharmacologically study the monoaminergic and GABA-ergic systems in Wfs1-deficient mice in order to detect accompanying biochemical alterations in the brain and assess this mouse strain as a potential model for depressive and anxiety disorders. For this, we used Wfs1-deficient mice that are missing exon 8 in the Wfs1 gene (Luuk et al., 2009). It is the largest exon of the Wfs1 gene and in WS has the majority of the mutations (Khanim et al., 2001). Replacement of this exon with a LacZ cassette did not abolish wolframin production, but rendered the protein dysfunctional. In the first paper, several methods were utilized to measure various aspects of the DA-ergic system in Wfs1-deficient mice after administration of either direct or indirect DA agonists. The second paper concerns 5-HT-ergic and NA-ergic signaling where emphasis was put on behavioral despair methods, which are sensitive to 5-HT-ergic and NA-ergic antidepressants. In the third paper, GABA-related behavior was studied along with the expression of $GABA_A$ receptor subtype encoding genes in response alcohol administration.

REVIEW OF LITERATURE

1. Characterization of the WFS1 gene and protein

Wolfram syndrome 1 (WFS1) gene is located on chromosome 4p16.1 in humans and 5qB3 in mice (Inoue et al., 1998, Strom et al., 1998). Wfs1 gene has 8 exons and spans approximately 33.4 kb of genomic DNA. Wfs1 gene code is used to generate the Wfs1 protein, wolframin. Translation of wolframin starts from the second exon. Wolframin is an endoglycosidase H-sensitive endoplasmic reticulum (ER) membrane glycoprotein, which consists of 890 amino acids and weighs approximately 100 kDa (Takeda et al., 2001). The carboxy-terminal domain is in the ER lumen and the amino-terminal lumen lies in the cytoplasm (Hofmann et al., 2003, Inoue et al., 1998, Strom et al., 1998). Wolframin is a hydrophobic and tetrameric protein with 9 transmembrane segments (Hofmann et al., 2003, Rigoli et al., 2011). Presence of wolframin in the ER suggests roles in membrane protein biosynthesis and modification (Yamaguchi et al., 2004, Yamada et al., 2006, Zatyka et al., 2008). Wolframin has been found to participate in calcium homeostasis (Osman et al., 2003) and protect against ER stress response, since lack of Wfs1 leads to UPR and cell death (Fonseca et al., 2010). Strong expression of wolframin is found in pancreatic beta cells (Hofmann et al., 2003, Fonseca et al., 2010) and loss of wolframin leads to apoptosis and progressive beta cell loss (Ishihara et al., 2004, Riggs et al., 2005, Yamada et al., 2006).

WFS1 can be found in multiple tissues, but the precise localization has not been established for most organs. According to the Human Protein Atlas (www.proteinatlas.org), highest levels of WFS1 RNA are found in the cerebral cortex, lung, heart muscle, thyroid gland, spleen, adipose tissue, smooth muscle, ovaries, fallopian tubes, endometrium, prostate, testis, and urinary bladder. Expression of wolframin protein is strongest in the brain, pancreas, kidney, testis, placenta and parathyroid gland (Human Protein Atlas). Similarly, strong expression levels of Wfs1 protein in the mouse are found in the brain and heart, intermediate levels are found in the pancreas, skeletal muscle and liver and low amount is detected in the kidney and spleen (Hofmann et al., 2003).

2. Wolfram syndrome and WFS1 mutations in psychiatric disorders

Homozygous mutations and some forms of compound heterozygosity in the WFS1 gene cause Wolfram syndrome (WS, OMIM 222300), an autosomal recessive disorder characterized by diabetes insipidus, juvenile-onset non-autoimmune diabetes mellitus, optic atrophy and deafness (Inoue et al., 1998, Strom et al., 1998, Cano et al., 2007). Individuals display variability in the clinical manifestations of WS, but the minimal diagnostic signs are considered

early onset diabetes mellitus and optic atrophy (83 percent of cases) (Barrett et al., 1995). WS has also been associated to deafness, urinary tract atony, gastrointestinal disorders (dysmotility), hydronephrosis, primary gonadal atrophy in males, mental retardation, neuropathy, epilepsy, glaucoma, cerebellar ataxia, nystagmus, dysphagia and sleep apnea (Swift et al., 1990, Swift et al., 1991, Bitoun, 1994, Barrett et al., 1995, Minton et al., 2003, Chaussenot et al., 2011). It has been found that the prevalence of WS is 1 in 100,000 in a North American population and 1 in 770,000 in the UK (Fraser & Gunn, 1977, Barrett et al., 1995). The prevalence of WS carriers is 0.28% in the United Kingdom and 1% in the United States (Barrett et al., 1995, Swift et al., 1991).

WS is a progressive degenerative disease. Degeneration has been shown to start in early childhood with profound cerebellar and brainstem alterations and is fully evident by the age of 15 (Chaussenot et al., 2011, Hershey et al., 2012). Imaging studies suggest atrophy in the brainstem, cerebellum, optic nerve, hypothalamus and hippocampus (Leiva-Santana et al., 1993, Galluzzi et al., 1999, Shannon et al., 1999, Hadidy et al., 2004). WS patients have a mean life expectancy of 30 years and usually die of neurological complications (central respiratory failure caused by brainstem atrophy) and kidney failure (result of urinary tract atony) (Barrett et al., 1995, Kinsley et al., 1995).

It has been found that around 60% of WS patients manifest psychiatric symptoms such as depression, psychosis, impulsivity, and aggression (Swift et al., 1990). A recent study by Bischoff et al. (2015) found that individuals with genetically confirmed WFS had no impairment in cognitive functions or self-reported psychiatric domain whereas by the clinical evidence these people did have psychiatric problems mainly in the form of anxiety and hypersomnolence. Heterozygous carriers of WFS1 mutations have increased psychiatric hospitalization mainly due to depression (Swift & Swift, 2005). It has therefore been suggested that mutations in the WFS1 gene play a key role in the susceptibility to mood disorders (Swift et al., 1998, Koido et al., 2005, Swift & Swift, 2005). Animal studies support the role of Wfs1 in psychiatric symptoms (Koks et al., 2002, Luuk et al., 2009, Kesner et al., 2009). Although WFS1 mutations are prevalent in many clinically important emotional and neurologic disorders, WFS1 has not been mapped in the human brain. Instead, thorough expression studies of Wfs1 have been conducted in the mouse brain (Luuk et al., 2008, Tekko et al., 2014).

3. Neuroanatomical localization of Wfs1

Developmentally, Wfs1 expression in the mouse forebrain first appears in the posterior CPu (dorsal striatum) and central amygdala from embryonic (E) day 15.5 (Tekko et al., 2014). At the same time, weak Wfs1 expression is detected in the medial CPu. At E18.5 the signal becomes stronger also in the anterior part of the CPu. In the ventral striatum (nucleus accumbens), Wfs1 expression

appears at E16.5 and reaches its adult level at approximately postnatal (P) day 20 (Tekko et al., 2014). Additionally, Wfs1 expression becomes detectable in many other brain nuclei, eg the cornu ammonis (CA) and subiculum regions of the hippocampus at E18.5, which show remarkable increase in Wfs1 expression immediately after birth at P0. At P2 expression still encompassed the whole CA region including the subiculum and dentate gyrus. However, selective CA1 expression is achieved in the subsequent days while the CA3, subiculum and dentate gyrus expression faded. At P20, the adult (P60) level of Wfs1 expression selectively in the CA1 and parasubiculum was reached (Tekko et al., 2014).

In the adult mouse brain, strong Wfs1 expression remains in the posterior caudate nucleus and capsular division of the central amygdaloid nucleus, being expressed also in the nucleus accumbens, dorsal part of the lateral bed nucleus of stria terminalis (BNST) and CA1 pyramidal cell layer region of the hippocampus (Luuk et al., 2008). In the midbrain, Wfs1-positive fibers are found in the reticular SN (Luuk et al., 2008). These fibers are most dense in the dorsomedial and dorsolateral reticular SN. Low levels of Wfs1-positive fibers are also detectable in the compact part of SN (Luuk et al., 2008).

The expression pattern of Wfs1 in the mouse brain corresponds to the limbic system, extended amygdala and basal ganglia concepts. These systems are connected to each other and some of their regions are overlapping. The limbic system is composed of different subcortical nuclei responsible for the regulation and experience of anxiety, emotions, motivation, and formation of long-term memories (Rolls, 2015). It consists of the amygdala, hippocampus, anterior nuclei of thalamus, fornix, mammillary bodies, septum pellucidum, cingulate gyrus, parahippocampal gyrus, and midbrain limbic areas although the concept of a single limbic system seems to be outmoded (Rolls, 2015). The Wfs1-rich central amygdaloid nucleus is considered a crucial region for experiencing fear (Kalin et al., 2004). The extended amygdala with the BNST mediates fear, anxiety and reward (Jennings et al., 2013) and is connected to the VTA. For example, photostimulation of gamma-aminobutyric (GABA-ergic) projections of the BNST result in rewarding and anxiolytic behavior. In vivo photostimulation of BNST glutamatergic projections to the VTA induce anxiogenic behavior (Jennings et al., 2013).

The basal ganglia are collection of subcortical nuclei responsible for reward behavior and the execution of skilled movements. They are made up of the caudate nucleus, nucleus accumbens, putamen, subthalamic nucleus (STN), globus pallidus (GP) and SN. The varied roles of the basal ganglia become evident from the pathologies, which stem from damage and metabolic alterations to these regions, such as hemiballismus, dystonia, addiction to psychoactive drugs, Parkinson's disease and Huntington's disease (Albin et al., 1989).

4. Wfs1-deficient mice as models of WS and emotional disorders

In order to elucidate the function of Wfs1, multiple Wfs1-deficient mouse lines have been generated by using different strategies (Ishihara et al., 2004, Riggs et al., 2005, Luuk et al., 2009). Riggs et al 2005 made a conditional beta-cell specific knock-out mouse line (129 SVJ background) targeting Wfs1 exon 8 that showed impaired glucose-stimulated insulin secretion, asymmetry and disruption of islet architecture without loss of beta cell mass at 12 weeks of age. At 24 weeks of age, reduction in beta cell mass was observed along with overt diabetes (Riggs et al., 2005). Homozygous Wfs1-deficient mice also had a significantly lower body mass at six months.

Ishihara et al made a full knock-out by deleting the second exon of the Wfs1 gene. These mice, in a 129/SVEV x C57BL6 F2 background, generally displayed a similar diabetic phenotype (decreased insulin secretion in the glucose tolerance test and increased glucose levels) as in Wfs1-deficient made by Riggs et al (2005). It became evident that in a C57 background, diabetes did not develop. Kato et al (2008) studied the behavior of mice in a B6 background and found few differences between the Wfs1-deficient homozygotes and wild-type mice. The mutant animals have significantly shorter escape latency in passive and active avoidance tests and increased freezing in the training phase of the fear conditioning test (Kato et al., 2008).

Mice used in this study were F2 hybrids [(129S6/SvEvTac × C57BL/6) × (129S6/SvEvTac × C57BL/6)]. Young adult mutant animals (2–3 months) in this background have glucose intolerance, although the baseline levels of blood glucose of Wfs1-deficient mice are not different from their wild-type littermates (Raud et al., 2009). The mice display significantly more anxiety-like behavior in the elevated plus maze test and are more responsive to the $GABA_A$-R agonist diazepam (Luuk et al., 2009). Wfs1-deficient homozygous animals also exhibited a three-fold increase in plasma corticosterone levels (Luuk et al., 2009). Interestingly, the homozygous Wfs1-deficient mice display characteristic vocalizations when under stress-inducing conditions (Luuk et al 2009). Injections of the direct DA agonist apomorphine caused a stronger motor response whereas the indirect agonist amphetamine induced significantly less motor stimulation in Wfs1-deficient mice (Luuk et al., 2009).

In summary, findings from clinical research conducted on humans, along with neuroanatomical and preliminary psychopharmacological studies of Wfs1-deficient mice point to the fact that Wfs1-deficiency may lead to disturbances in central monoamine and GABA systems.

5. Etiologies of emotional disorders

5.1. Monoamine hypothesis

Central monoamines are a group of neurotransmitters, which participate in mediating a wide range of brain functions. Monoamines derive from aromatic aminoacids tyrosine, phenylalanine and tryptophan. They are categorized into two groups, the catecholamines DA, adrenaline and NA, and the indoleamine 5-HT. According to the monoamine hypothesis, mood disorders are caused by a functional deficiency of monoamines, mostly NA-ergic and/or 5-HT-ergic (Hirschfeld, 2000). It has become clear that DA is also involved in regulating mood (Dailly et al., 2004). Therefore, monoamines are targeted by drugs used for treating mood disorders, although these drugs can be effective also when treating anxiety (Zohar & Westenberg, 2000).

5.1.1. Dopamine

5.1.1.1 Dopamine pathways of behavior and emotion regulation

DA is a neurotransmitter implicated in many different brain functions and clinical disorders. Three of the DA pathways (nigrostriatal, mesolimbic, and mesocortical) have their cell bodies in the ventral midbrain and one, the tuberoinfundibular pathway, in the hypothalamus. The nigrostriatal and mesolimbic systems dominate in behavior and emotional regulation.

The *nigrostriatal DA* pathway is important in the control of movement. Alterations in DA neurotransmission in this pathway thus usually result in either in the lack of (Parkinson's disease) or exaggerated movements. DA agonists elevate DA neurotransmission in the nigrostriatal pathway to increase behavioral activation in mice.

Cell bodies of the nigrostriatal neurons lie in the compact part of the SN and their axon terminals are situated in the CPu, forming the presynaptic DA neurons (Anden et al., 1964). DA-ergic neurons make synapses with the postsynaptic GABA-ergic medium spiny neurons (MSN), which express two different receptor types, the DA D1-like (D1 and D5) and the DA D2-like (D2, D3, D4) receptors with the exception of presynaptic D2 autoreceptors. The D1 receptor subtypes activate adenylate cyclase (AC), and the D2 subtypes inhibit AC (Missale et al., 1998). D1 receptors, encoded by the Drd1 gene, are only postsynaptically expressed. The D1-like receptors are primarily expressed in the direct pathway of movement originating from the basal ganglia. The direct pathway (also called striato-nigral/striato-entopenduncular) is composed of GABA-ergic neurons, which send axons to entopenduncular nucleus (EP) and to the reticular SN.

The D2-like receptors are situated both pre- and postsynaptically (Khan et al., 1998). These two sites have different isoforms of the D2 receptor protein, which are encoded by the same gene (Drd2) but are the result of alternative

splicing. The presynaptic D2 receptors are of the short form (D2Sh) and the postsynaptic variant is of the long form (D2Lh). Presynaptic D2 neurons are autoinhibitory and act to antagonize DA release (Bowyer and Weiner, 1987, Mercuri et al., 1997) and lower excitability at axon terminals (Bunney et al., 1973, Tepper et al., 1984). Blockage of D2 autoreceptors augments DA release (Fasano et al., 2008). The postsynaptic D2 receptors are expressed in the indirect DA pathway. The indirect pathway, otherwise known as the striato-pallidal pathway, sends projections to the STN. From the STN, glutamatergic neurons are sent to the EP and SNr.

The dorsal striatum is also innervated by other neurotransmitter systems, which modulate DA-ergic transmission. For example 5-HT (from raphe nuclei) and NA (from locus coeruleus and lateral tegmental area) neurons give rise to projections ending in the striatum (Mori et al., 1985). It has been shown that 5-HT binding to the 5-HT2C receptors can inhibit DA release in the striatum (Alex et al., 2005).

The *mesolimbic DA* pathway is considered the most important reward pathway in the brain (Robison & Nestler, 2011). It is also strongly implicated in schizophrenia and depression (Heimer et al., 1997, Epstein et al., 1999, Malone et al., 2009). Changes related to drug addiction occur mainly in this pathway. Neuronal bodies of the mesolimbic system originate in the VTA and their axons traverse the lateral hypothalamus to innervate the nucleus accumbens (ventral striatum) in the basal forebrain. As in the dorsal striatum, the ventral striatum consists mostly of GABA-ergic MSN-s which are postsynaptic and express either D1 or D2 receptors (Robison & Nestler, 2011). The nucleus accumbens has efferents to multiple brain regions, eg to the ventral pallidum, lateral hypothalamus, SN, VTA, lateral hypothalamus, CPu, the BNST, septum, preoptic area, lateral habenula, lateral septum, retrorubral nucleus and central gray (Nauta et al., 1978, Groenewegen & Russchen, 1984, Usuda et al., 1998).

5.1.1.2 Dopamine metabolism and pharmacology

Dopamine is synthesized in two steps. First, L-tyrosine is hydroxylated by tyrosine hydroxylase to DOPA. Then, DOPA is decarboxylated to DA by aromatic amino acid decarboxylase (AADC) (Meiser et al., 2013). DA is metabolized to 3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA). These reactions are carried out by monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT).

Physiologically, synaptic DA levels are set by tonic and phasic firing of midbrain DA neurons (Grace, 1991, Wanat et al., 2009). Greater levels of DA can be achieved by administering drugs acting as DA releasers. Two classes of DA agonist drugs are used to achieve motor activation, the direct DA agonists (eg apomorphine), which act on DA receptors to produce an effect similar to DA binding to its receptors and the indirect DA agonists (eg amphetamine), which enhance DA release by reversing the direction of the pumping

mechanism of the presynaptically located DA transporter (DAT) (Millan et al., 2002, Vaughan & Foster, 2013). The DAT protein is physiologically responsible for the uptake or clearance of excess DA from the synaptic cleft (Vaughan & Foster, 2013). Strong expression of DAT is found on DA-ergic terminals in the dorsal and ventral striatum.

One dose or many administrations of a dose of amphetamine in rodents causes an augmented psychomotor response or sensitization which can last several weeks. The mechanism underlying sensitization is under debate but it has been found to reverse, for example, insulin deficiency-induced reduction in sensitivity to amphetamine (Owens et al 2012).

5.1.2. Serotonin and noradrenaline

5-HT synthesis happens in two steps. The amino acid L-tryptophan is converted by tryptophan hydroxylase (TPH) to 5-hydroxytryptophan (5-HTP). Then, 5-HTP is decarboxylated by AADC to form 5-HT. By contrast, NA is synthesized from DA by DA beta-hydroxylase (DBH). The main metabolite of 5-HT is 5-HIAA (synthesized by MAO) and the metabolite of NA is normetanephrine (NMN) (synthesized by COMT).

Depending on their mechanisms of action, different classes of 5-HT-ergic antidepressants are known. The SSRI-s (eg paroxetine) act specifically on the 5-HT-ergic system by blocking the 5-HT transporter (SERT), the main molecule responsible for clearing 5-HT from the synaptic cleft (Murphy et al., 2004). Therefore, the SSRI-s raise serotonin levels and consequently produce an anti-depressive effect. 5-HT is also important in the stress response (Chaouloff et al., 1999). Currently, 7 families of 5-HT receptors, which mediate both excitatory and inhibitory transmission, are known. They are designated 5-HT_1-5-HT_7 (Frazer & Hensler, 1999). All but the 5-HT_3 are G-protein coupled receptors that activate intracellular messengers. 5-HT_3 receptor is a ligand-gated ion channel. 5-HT neuron bodies lie in the raphe nuclei which are in 9 clusters and located in the midbrain, pons and medulla. 5-HT-ergic neurons innervate, among others, the spinal cord, SN, hypothalamus, amygdala, septum, neocortex, hippocampus, striatum and habenula.

It has been found that tricyclic antidepressants (TCA-s) (eg imipramine) also target NA system in the brain. The TCA-s increase 5-HT and NA levels by blocking monoamine transporters with almost 4 times higher affinity of imipramine to NAT compared to SERT (Shank et al., 1988, Bolden-Watson & Richelson, 1993, Owens et al., 1997, Richelson, 2001). NA is involved in a variety of neurophysiologic processes and has for a long time been a target for antidepressive drugs in the treatment of mood disorders. NA-ergic neurons originate in the locus coeruleus and lateral tegmental area, innervating a large number of different brain areas (Racagni & Brunello, 1999). Key areas innervated by NA-ergic neurons are the amygdala, cingulate, gyrus, hippo-campus, hypothalamus, cortex, striatum, and thalamus.

5.2. Gamma-aminobutyric system

GABA is the main inhibitory neurotransmitter in the brain and spinal cord, present in up to 44% of all neurons and distributed over most brain areas. GABA can, due to the ubiquitous presence, influence practically all aspects of brain function. GABA is formed from glutamate by glutamic acid decarboxylase (GAD) and is metabolized to succinate by GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) (Petroff, 2002).

There are two general classes of GABA receptors (GABA-R-s) - $GABA_A$ and $GABA_B$. $GABA_A$-R-s are heteropentameric ligand-gated ion channels and $GABA_B$-R-s are G protein-coupled metabotropic receptors. $GABA_A$ receptor is the main mediator of inhibitory neurotransmission in the brain and also the target of multiple drugs, such as anxiolytics, anticonvulsants, the sedative hypnotic benzodiazepines and alcohol. Pharmacologically, anxiety is generally treated with drugs that are $GABA_A$-R agonists. A widely used anxiolytic drug is diazepam. At least 15 subunits of the $GABA_A$-R are known and they show great heterogeneity in distribution (Fritschy & Mohler 1995). When molecules bind with the extracellular part of $GABA_A$-R, a chloride-specific pore is opened. This hyperpolarizes the cell, thus inhibiting further action potentials (Petroff, 2002).

The role of $GABA_A$-R in anxiety is known, but it is under debate which of the $GABA_A$-R subtypes mediate this action. There are four $GABA_A$-R subtypes which are sensitive to anxiolytics, named α1, α2, α3, or α5. The α2 is more expressed in the limbic system and α3 in the reticular formation; accordingly, studies tend to show that the α2 subunit is responsible for the anxiolytic effect of diazepam in animals (Low et al., 2000).

6. Concluding remarks

It is evident that mutations in the gene encoding wolframin protein result in emotional disorders. First, data from human studies suggest that mutations in WFS1 have a role in anxiety and mood disorders. Second, although no data exists on the neuroanatomical localization of WFS1 in the human brain, studies of the mouse brain have located Wfs1 in regions involved in reward, movement, emotions and anxiety – functions, which are largely mediated by monoamine and GABA neurotransmitters. Third, psychopharmacological results have shown a possible DA release deficit and increased anxiety.

Therefore, the aim of this dissertation was to study the GABA-ergic and monoaminergic systems in a Wfs1-deficient mouse line by using behavioral, biochemical and genetic methods. We hypothesize that Wfs1 deficiency in mice induces behavioral symptoms reflective of human emotional disorders and biochemical alterations to associated neurotransmitter systems.

AIMS OF THE STUDY

According to the above presented analysis of literature the present dissertation aims to answer to the following questions:

Dopamine

1. Does Wfs1-deficiency induce behavioral symptoms indicative of a DA-ergic deficiency?
2. How is DA metabolism affected by Wfs1 deficiency?

Serotonin and noradrenaline

1. Do the Wfs1-deficient mice display behavioral despair symptoms, reflective of depression, and altered behavioral response to SSRI-s and TCA-s?
2. Are there biochemical differences to striatal 5-HT and NA systems between Wfs1-deficient and wild-type animals in response to a stressful open field exposure?

Gamma-aminobutyric acid

1. How does ethanol administration affect the anxiety-like phenotype of Wfs1-deficient mice?
2. Does Wfs1 deficiency in mice cause differences to ethanol-induced expression of genes encoding the $GABA_A$ receptor subunits?

In general

Does this Wfs1-deficient mouse model represent a usable choice when screening anxiolytic and antidepressant drugs and testing novel drug administration schemes in the treatment of WS?

MATERIALS AND METHODS

1. Animals (Papers I, II, III)

The majority of the mutations inducing Wolfram syndrome occur in exon 8 (Cano et al., 2007, Hansen et al., 2005, Hardy et al., 1999). To mimic the human condition in the Wfs1-deficient mouse model the 8th exon was disrupted (Luuk et al., 2008). Breeding and genotyping were conducted in the Department of Physiology, University of Tartu. The papers were based on studies on young adult (age 2–4 months) male and female Wfs1-deficient mice. According to our previous data the animals at this age range do not display elevated basal glucose levels, i.e. they do not have overt diabetes (Luuk et al., 2009) However, they do have impaired response in the glucose tolerance test. We used F2 hybrids [(129S6/SvEvTac × C57BL/6) × (129S6/SvEvTac × C57BL/6)] for the experiments, because the congenic footprint effect does not allow a pure background by backcrossing. It has been argued that a robust behavioral phenotype, caused by a mixed genetic background, obviates the need for back-crosses (Schalkwyk et al., 2007). All the genotypes (wild-type, heterozygous and homozygous Wfs1-deficient) were housed in their respective home cages. The mice were housed in groups of eight under a 12 hour light/dark cycle with lights on at 7:00 a.m. The behavioral studies were performed between 9:00 a.m. and 6:00 p.m. In the studies only littermates were used and compared. This approach was applied to overcome the possible extensive variability coming from the random background of F2 hybrids. All genotypes were represented by an equal or close to equal number of animals. Heterozygous animals were not used in the western blotting experiment in paper I and in the gene expression study in paper III. The number of mice used in each experiment is given under the corresponding figure and table. The animals had free access to food and water except during testing. In papers I and II, naive batches of mice were used for each experiment.

In paper III, the first batch of mice was used for the elevated plus-maze test and 7 days later for the locomotor activity test. The second batch of mice was used for the righting reflex test. To reduce the number of animals, mice receiving ethanol in the righting reflex test were used after a washout period of 7 days for measuring the sedative/ hypnotic effect of pentobarbital and ketamine. The third batch of mice was used for the rotarod test. For ethanol metabolism studies and gene expression studies, the fourth and fifth batches of mice were used, respectively. Wfs1-deficient mice were always used in parallel with wild-type and heterozygous mice and the animals were randomly divided into the experimental groups.

2. Drugs (I, II, III)

In paper I, amphetamine (amphetamine sulfate, Sigma-Aldrich, St Louis, MO, USA), an indirect agonist of DA, was injected intraperitoneally (i.p.) 30 min prior to testing. Apomorphine (apomorphine hydrochloride, Sigma-Aldrich, St Louis, MO, USA), a direct DA receptor agonist, was given subcutaneously (s.c.) 15 min before the experiment. Both compounds were diluted in 0.9% NaCl (B. Braun Melsungen AG, Germany). In the experiments measuring the stimulation of locomotor activity and DA metabolism, we used 5 mg/kg of amphetamine and 3 mg/kg of apomorphine. The mentioned doses of drugs were selected according to preliminary experiments where 5 mg/kg of amphetamine and 3 mg/kg of apomorphine caused a statistically significant increase in the locomotor activity of mice (Luuk et al., 2009). In the sensitization study, animals were treated with 2.5 mg/kg of amphetamine. This is a sub-threshold dose of amphetamine because it did not cause behavioral stereotypy or locomotor activation if given as a single dose (Luuk et al., 2009).

In paper II, control group animals in the TST and FST received an injection of saline (0.9% NaCl solution) (B. Braun Melsungen AG, Germany). Imipramine hydrochloride and paroxetine hydrochloride hemihydrate (both purchased from Sigma-Aldrich, St Louis, MO, USA) were dissolved in saline. Imipramine was administered at doses of 10, 20 and 30 mg/kg and paroxetine at doses of 5, 10, 20 and 30 mg/kg. All drugs were injected at a volume of 100 µl / 10 g 40 minutes before using the animal in the TST or FST. Effect of paroxetine was studied only in TST since this test was more sensitive for establishing the antidepressant-like effect of drugs compared to FST (Liu and Gershenfeld, 2001).

In paper III, three doses of ethanol (0.5, 1 and 2 g/kg) were used in the elevated plus-maze and locomotor activity tests. In the rotarod test, only ethanol at the dose of 2 g/kg was used. Ethanol [5% (v/v) for 0.5 and 1 g/kg or 20% (v/v) for 2 g/kg] was injected 20 min prior to testing. In the LORR test, ethanol (4 g/kg), pentobarbital sodium salt (Sigma/Aldrich, 45 mg/kg) and ketamine hydrochloride (Vetoquinol Biowet Sp. Z.o.o., 150 mg/kg) were used. For the study of ethanol metabolism, mice received ethanol [2 or 4 g/kg 20% (v/v)] 30 min before blood concentration measurements. For gene expression studies, animals were injected with ethanol (2 g/kg) 30 or 60 min before decapitation. All agents were diluted in 0.9% NaCl solution (B. Braun Melsungen AG, Germany) and injected intraperitoneally at a volume of 100 ml/10 g.

3. Behavioral experiments (I, II, III)

3.1. Locomotor activity tests (I, II, III)

3.1.1. Administration of amphetamine and apomorphine (I)

In paper I, locomotor activity of mice was automatically registered for 30 min in photoelectric plexiglas motility boxes (448 mm × 448 mm × 450 mm, TSE, Technical and Scientific Equipment GmBH, Germany). 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 DA agonists, otherwise being masked with a high exploratory activity of animals. The floors of motility boxes were cleaned thoroughly with 5% alcohol and dried after each animal. 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).

Immediately after the behavioral testing, animals were taken to another room and decapitated for brain tissue dissection (for further details see paragraph 4 of Materials and methods). 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.

3.1.2. Locomotor activity of drug-naive mice in the motility boxes (II)

In paper II, one group of animals were taken for decapitation straight from their home cages and the other, also a drug-naive group were taken after a locomotion measurement box challenge. (For further details see paragraph 4 of Materials and methods.)

3.1.3. Administration of ethanol (III)

In paper III, the animals were placed singly into the same motility boxes as in papers I and II, for 30 min. The distance travelled (m), time in locomotion (s) and the number of rearings were registered.

3.2. Behavioral despair tests (II)

3.2.1 Tail suspension test (II)

The TST has been extensively validated with a wide range of antidepressants (Porsolt et al 1987). Most of the antidepressants maximally reduce the duration of immobility in the TST with doses less than those required for the FST (Liu & Gershenfeld, 2001). This test has been used alongside FST because hyper-activity may be a confounding issue in the FST. Mice were suspended from the edge of a shelf 58 cm above a tabletop by adhesive tape, placed approximately 1 cm from the tip of the tail. Animals were allowed to hang for 6 min and the duration of immobility was scored during the last 4 min from videotapes by an observer blind to the treatment protocol. Mice were considered immobile only when they hung passively and completely motionless.

3.2.2. Forced swim test (II)

The FST was performed as described by (Porsolt et al., 1977). Briefly, a glass cylinder 12 cm in diameter was filled with 18 cm water at 25 °C. The animal was gently put in the water, and the behavior recorded during 6 min. Subsequently, the immobility time was counted for the last 4 min of the test by an observer blind to the treatment protocol.

3.3. Elevated plus-maze (III)

The plus-maze consisted of two opposite open (17.5 cm × 5 cm) arms without sidewalls and two enclosed arms of the same size with 14 cm high sidewalls and an end wall. The entire plus-maze apparatus was elevated to a height of 30 cm and placed in a brightly lit room (illumination level: around 500 lx in the open arms). Standard 5 min test duration was employed (Lister, 1987) and the maze was cleaned thoroughly with 5% alcohol and dried between the subjects. The following parameters were observed: (1) percentage (%) of time spent in the open arms, (2) % of open arm entries (3) number of unprotected head dippings (4) number of closed arm entries. % of time spent in the open arms and % of open arm entries are spatiotemporal measures of anxiety whereas the number of unprotected head dippings is an ethological measure which can be considered “risk assessment” behavior (Rodgers and Johnson, 1995).

3.4. Motor coordination in the rotarod test (III)

Rotarod is one of the standard tests to measure coordination, balance and motor skill learning. The learning effect appears as the elongated falling latency with the trial numbers (Shiotsuki et al., 2010). This test also enables to evaluate sedation (Soderpalm et al., 1989, Steiner et al., 2011). The equipment consisted of a motor-driven drum (3 cm in diameter) rotating at fixed speed (9 rpm). Five minutes before the first trial on the rotarod, mice were habituated to stay on the drum for one minute. In later trials, habituation was not used. The second and third trials were conducted after 2 h and 24 h, respectively. The effect of ethanol on motor coordination was measured on the fourth trial (after 48 h). The time of maximal performance for each trial was set at 120 s. The animal was placed on the rotating drum and the latency (s) to the first fall from the drum was registered manually. Immediately after the fall, the mouse was put back on drum and the total number of falls was counted.

3.5. Loss of righting reflex test (III)

The mice were given an intraperitoneal injection of 4 g/kg of ethanol (20%, v/v), pentobarbital (45 mg/kg) or ketamine (150 mg/kg), placed in supine position in a V-shaped cardboard trough and tested for the ability to right itself. It was considered that the animal had lost the righting reflex if it could not right itself on all four paws three times within 30 s and regained the righting reflex if it could fully right itself three times within 30 s. The onset of drug-induced sedation (the onset of LORR) and the duration of LORR [time (min) between the loss of righting reflex and the regain of righting reflex] were measured.

4. Tissue monoamine content measurements by HPLC (I, II)

In paper I, 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 the DA agonists the animals were placed into the above described motility boxes (TSE, Germany) 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 CPu) 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 & Paxinos, 1997). DA and its metabolites were assayed by high performance liquid chromatography (HPLC) with electrochemical detection. We measured the tissue levels of DA (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 end-metabolite HVA and DA itself (HVA/DA).

In paper II, 5-HT and NA were measured in two groups: mice exposed for 30 min to the brightly lit motility boxes (exposure group) and mice taken directly from their home-cages (naïve group). The animals were immediately decapitated after completing the experiment in the motility boxes or after taking them out from their home-cage. Again, the animals were transported to a separate room for the decapitation. The dorsal (encompassing the nucleus caudatus and putamen) striatum and the ventral (encompassing the nucleus accumbens and tuberculum olfactorium) striatum were dissected according to coordinates by Franklin and Paxinos (1997). The dissected tissues were promptly frozen in liquid nitrogen. Tissue levels of 5-HT and NA (in pmol/mg) were determined using HPLC with electrochemical detection. Additionally, their respective metabolites 5-hydroxyindoleacetic acid (5-HIAA) and normetanephrine (NMN) were assayed.

The following homogenization and HPLC protocol was used in papers I and II. 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 octylsulphonate 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.

5. Gene expression analysis by qRT-PCR (I, II, III)

5.1. RNA isolation and cDNA synthesis

These studies – with the exception of paper III, details given below – were done on drug-naive groups of animals taken directly from their home-cages. Mice were decapitated immediately after taking them out from their home-cage and bringing them into the room where the decapitations took place. The gene expression studies in wild-type, heterozygous and homozygous animals were conducted in parallel. 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 oligo-

nucleotides and SuperScript™ III Reverse Transcriptase (Invitrogen, USA). In paper **I** the measurements were performed on the midbrain (DAT) and the dorsal and ventral striatum (Drd2) of the respective mice. In paper II the midbrain (SERT) and pons (SERT and NAT) were dissected and frozen in liquid nitrogen.

In paper III, the acute effect of ethanol (2 g/kg) was investigated on *Gabra1*, *Gabra2* and *Gabra3* mRNA in wild-type and homozygous Wfs1-deficient mice. The animals were decapitated in a separate room 30 and 60 min after ethanol or 30 min after vehicle injection (ethanol 30, ethanol 60 and vehicle group, respectively). The frontal cortex (including the prefrontal cortex) and temporal lobe were dissected according to the coordinates provided in the mouse brain atlas (Franklin & Paxinos, 1997). For dissection of the temporal lobe, the anterior coronal cut was performed on the level of anterior border of hypothalamus and the second cut was done 2.5 mm behind that line. The temporal lobe was bilaterally dissected from the lower lateral corner of this slice, containing the piriform cortex and, basolateral, central and medial nuclei of amygdala. The dissected brain structures were quickly frozen in liquid nitrogen.

5.2. qRT-PCR

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 measured in papers I–III, see Table 1.

Table 1. The Taqman assays and probes used in each paper

Gene symbol	Paper	Assay ID or sequence	Gene ID
Slc6a3 (DAT)	I	Mm00438396_m1	NM_010020
Drd2 (D2)	I	Mm00438545_m1	NM_010077.2
Slc6a2 (*NAT*)	II	Mm00436661_m1	NM_009209.3
Slc6a4 (*SERT*)	II	Mm00439391_m1	NM_010484.2
Gabra1	III	Mm00439046_m	NM_010250
Gabra2	III	Mm00433435_m1	NM_008066
Gabra3	III	Mm01294271_m1	NM_008067
Hprt1 for	I, II, III	5'-GCAGTACAGCCCCAAAATGG-3'	
Hprt1 rev	I, II, III	5'- AACAAAGTCTGGCCTGTATCCAA-3'	NM_013556
Hprt1 probe (VIC_TAMRA)	I, II, III	5'-VIC-AAGCTTGCTGGTGAAAAGG-ACCTCTCG TAMRA-3'	

DAT – DA transporter gene; *Slc6a3* – solute carrier family 6 (neurotransmitter transporter, DA), member 3; *Drd2* – DA D_2 receptor gene; *Slc6a2* – solute carrier family 6 (neurotransmitter transporter, NA), member 2; *Slc6a4* – solute carrier family 6 (neurotransmitter transporter, 5-HT), member 4; *Hprt1* – hypoxanthine phosphoribosyltransferase 1 gene.

6. Western blotting (I)

The protein measurements were performed on separate groups of animals taken directly from their home-cages. Brains were removed and the dorsal 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 previously (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 a primary antibody (Table 2)**.** After primary antibody incubation the membranes were washed 6 times in Milli-Q water and incubated with the respective secondary antibodies (Table 3) for one hour at RT. After secondary antibody 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.

Table 2. The primary antibodies

Protein	Paper	Species	Company	Cat #	Dilution
DAT	I	Rat	Santa Cruz Biot.	sc-32258	1:1K
ß-actin	I, III	Rabbit	Cell Signaling	4970	1:10K

DAT – DA transporter.

Table 3. The secondary antibodies

Species	Anti	Company	Dilution	Conjugant	Cat #
Donkey	Rabbit	Jackson ImmunoResearch	1:40K	Alexa-790	711-655-152
Donkey	Rat	Jackson ImmunoResearch	1:40K	Alexa-680	712-625-150

7. Statistical analyses (I, II, III)

Mean values and S.E.M. are presented in all the figures and tables. All data were analyzed using Statistica version 8.0 (StatSoft, Inc., USA). Results were considered statistically significant when $p<0.05$.

In paper I, one-way analysis of variance (ANOVA) was applied in the gene expression experiments and Mann-Whitney U test was used for the protein expression studies. Two-way ANOVA (genotype and treatment as independent measures) was performed in the experiments measuring the locomotor activity and brain DA levels. A three-way repeated measures ANOVA (genotype × treatment × day) was used in the experiment measuring sensitization to amphetamine. Tukey HSD *post-hoc* analysis was used when applicable after significant ANOVA.

In paper II, the results of the behavioral and gene expression studies are expressed as mean values ± SEM. Since there were no significant sex differences in the results of any of the performed experiments, and to raise the statistical power of the study, data from male and female animals were pooled. The results of the TST and FST were analysed using two-way ANOVA (genotype × treatment). One-way ANOVA was applied for the statistical analysis of gene expression data. The results of monoamine and their metabolite assays were analysed using two-way ANOVA (genotype × exposure). *Post-hoc* comparisons were performed using Scheffe or Tukey HSD tests.

In paper III, the results are expressed as mean values ± S.E.M. The results of the elevated plus-maze and locomotor activity tests were analyzed using two-way independent groups ANOVA (genotype × ethanol treatment). Data from the rotarod measurements were analyzed using repeated measures ANOVA with 1 within-subjects variable (trial) and two-way ANOVA (genotype × ethanol treatment). The results of the LORR test were analyzed with one-way ANOVA. Two-way ANOVA was applied to analyze gene expression studies (genotype × ethanol treatment). *Post-hoc* comparisons were performed by means of Newman-Keuls test.

8. Ethics permit (I, II, III)

The permission for the studies was given by the Estonian National Board of Animal Experiments (No. 13 from 16.09.2009) in accordance with the European Communities Directive of November 1986 (86/609/EEC)

RESULTS

I. Paper I

I.1. DA measurement in the dorsal and ventral striatum

I.1.1. Effect of exposure of mice to the motility boxes

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 (Table 4, A and 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 × exposure effect: $F_{2,51}=0,87$, $p=0.42$ Table 4A). 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 (Figure 1). *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 (Figure 1). In the ventral striatum, an exposure effect was established for HVA ($F_{1,46}=31.3$, $p<0.001$)(Table 4B). The exposure effect was also significant ($F_{1,46}=11.1$, $p<0.01$) for DA turnover in the ventral striatum (Figure 1), but *post-hoc* analysis did not establish any significant changes between the groups.

Table 4. Effect of exposure of Wfs1-deficient mice to the motility boxes compared to mice taken from the home cage on the metabolism of DA in the dorsal and ventral striatum.

		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

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 naive mice (Tukey HSD test after significant two-way ANOVA). DOPAC – 3,4-Dihydroxyphenylacetic acid; HVA – Homovanillic acid; 3-MT – 3-Methoxytyramine. There were 5 male and 5 female mice in each group.

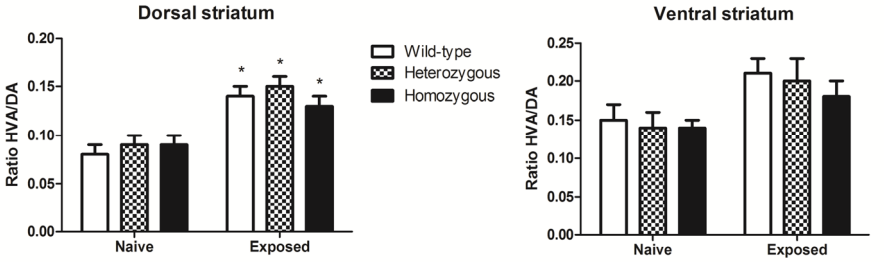

Figure 1. Effect of exposure to the motility boxes on DA turnover in the dorsal and ventral striatum. * – $p<0.05$ compared to respective group of mice not exposed to the motility boxes (Tukey HSD test after significant two-way ANOVA). Data are expressed as ratio between the levels of HVA (pmol/mg) and DA (pmol/mg) in the respective brain structures. There were 5 male and 5 female mice in each group.

1.1.2. Effect of amphetamine on locomotor activity and DA metabolism

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 (Figure 2). Altogether, similarly to our previous study (Luuk et al., 2009), 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) (Figure 2). 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.

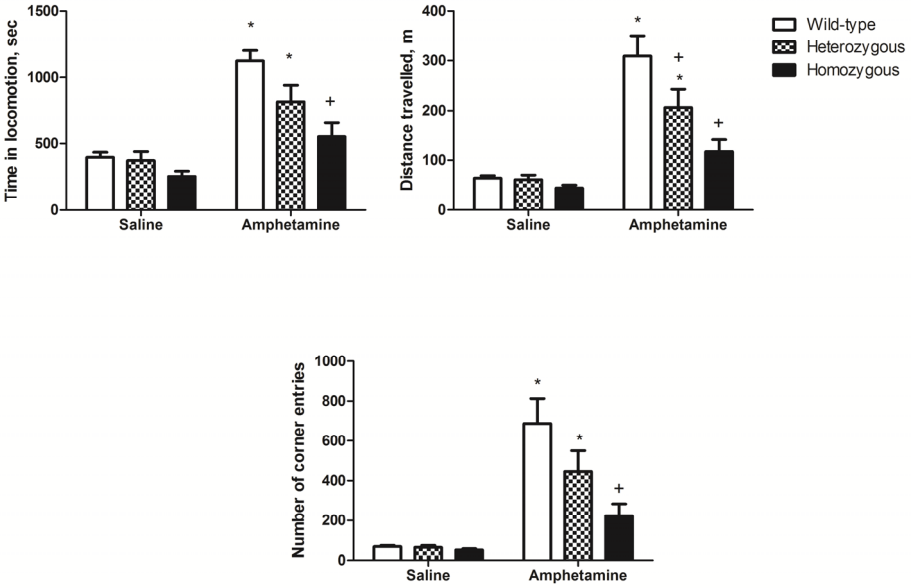

Figure 2. Effect of amphetamine (5 mg/kg) on the locomotor activity. * – $p<0.05$ compared to the respective group of saline treated mice (Tukey HSD test after significant two-way ANOVA); $^{+}$ – $p<0.05$ (compared to amphetamine-treated wild-type mice). There were 5 male and 5 female mice in each group.

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 5A). 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 5A) and DA turnover ($F_{1,53}=68.3$, $p<0.001$) (Figure 3). 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 wild-type mice ($p<0.05$, Tukey HSD test) (Table 5A, Figure 3). In heterozygous mice, amphetamine caused a significant reduction DOPAC and HVA levels, 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.

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 5B). 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 5B, Figure 3). 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 (Figure 2 and Table 5A and 5B). Moreover, amphetamine was not able to induce a statistically significant suppression of DA turnover in the dorsal and ventral striatum of homozygous mice.

Table 5. Effect of amphetamine (5 mg/kg) on the metabolism of DA in the dorsal and ventral striatum of Wfs1-deficient mice.

		Wild-type+ saline	Wild-type+ amphet-amine	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. DOPAC – 3,4-Dihydroxyphenylacetic acid; HVA – Homovanillic acid; 3-MT – 3-Methoxytyramine. There were 5 male and 5 female mice in each group.

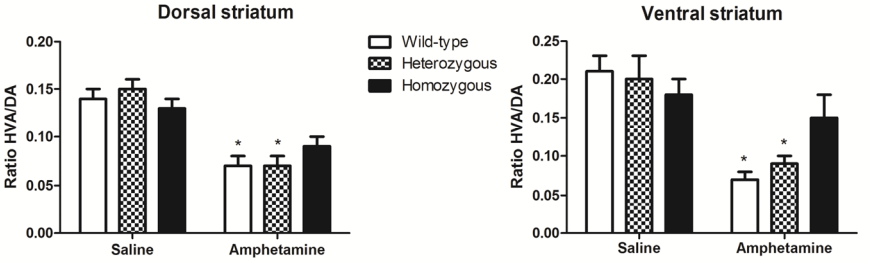

Figure 3. Effect of amphetamine (5 mg/kg) on the turnover of DA in the dorsal and ventral striatum. * – $p<0.05$ compared to respective group of saline treated mice (Tukey HSD test after significant two-way ANOVA). Data are expressed as ratio between the levels of HVA (pmol/mg) and DA (pmol/mg) in the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

1.1.3. Effect of apomorphine on locomotor activity and DA metabolism

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 (Figure 4). 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).

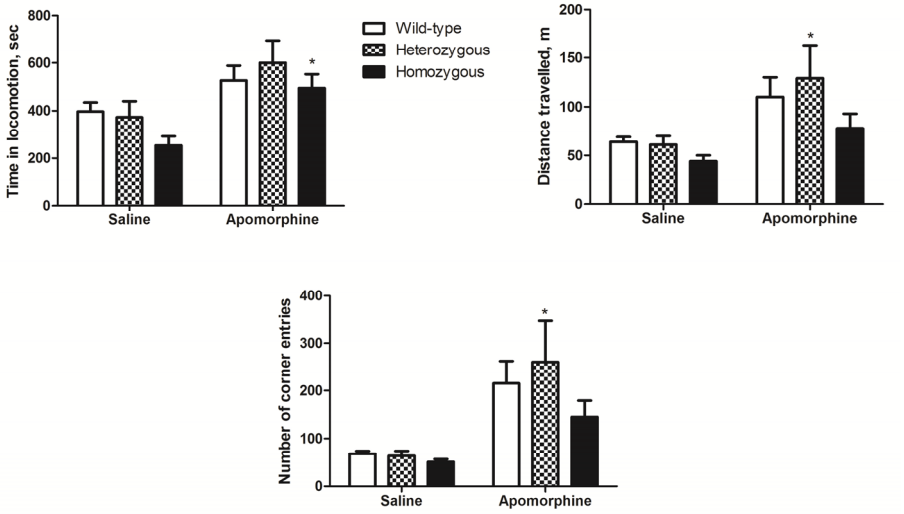

Figure 4. Apomorphine (3 mg/kg) induced changes in locomotor activity of Wfs1-deficient mice. * – $p<0.05$ compared to the respective group of saline treated mice (Tukey HSD test after significant two-way ANOVA). There were 5 male and 5 female mice in each group.

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 6A) and DA turnover ($F_{1,52}=95.1$, $p<0.001$) (Figure 5). 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.

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 6B), and DA turnover ($F_{1,50}=58.1$, $p<0.001$) (Figure 5). 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.

Table 6. Effect of exposure to apomorphine (3 mg/kg) on the metabolism of DA in the dorsal and ventral striatum in Wfs1-deficient mice

		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). DOPAC – 3,4-Dihydroxyphenylacetic acid; HVA – Homovanillic acid; 3-MT – 3-Methoxytyramine. There were 5 male and 5 female mice in each group.

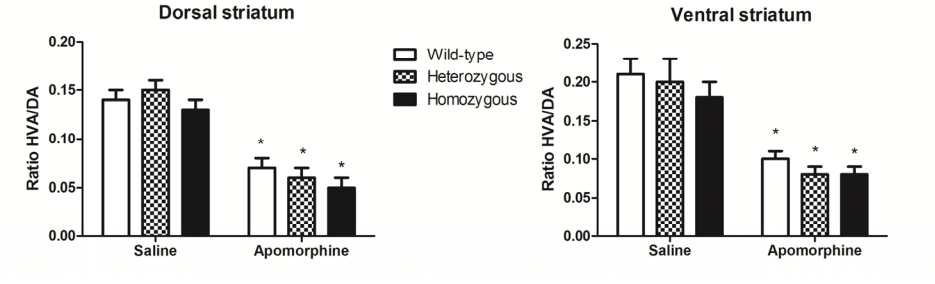

Figure 5. Effect of apomorphine (3 mg/kg) on the turnover of DA in the dorsal and ventral striatum. * – $p<0.05$ compared to the respective group of saline treated mice (Tukey HSD test after significant two-way ANOVA). Data are expressed as ratio between the levels of HVA (pmol/mg) and DA (pmol/mg) in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

1.2. Amphetamine-induced motor sensitization

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 × treatment)], distance

travelled [F2,76=6.33, p<0.01 (genotype); F1,76=6.92, p<0.05, (treatment); F5,76=9.74, p=0.09 (day × treatment)] and number of corner entries [F2,76=3.29, p<0.05 (genotype); F1,76=7.2, p<0.01 (treatment), F5,76=5.28, p<0.01 (day × 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 (Figure 6). In heterozygous mice, there was some increase in the action of amphetamine, but this change was not statistically significant. Repeated treatments with amphetamine tended to reduce the locomotor activity in homozygous mice (Figure 6). 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).

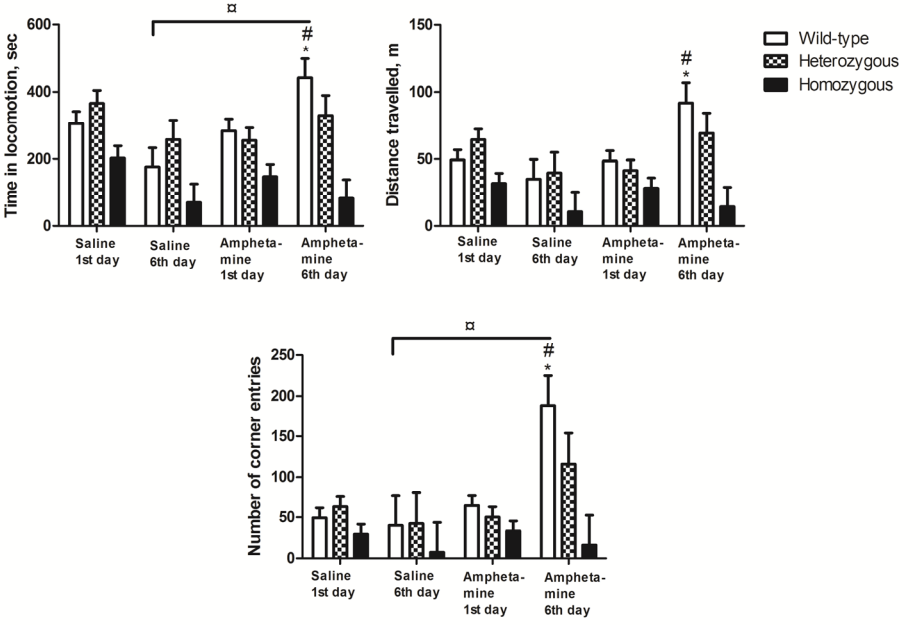

Figure 6. Sensitization of Wfs1-deficient mice to amphetamine-induced motor stimulation. * – p<0.05 compared to amphetamine-treated wild-type mice on the first day; ¤ – p<0.05 compared to saline-treated wild-type mice on the sixth day; # – p<0.05 compared to amphetamine-treated homozygous mice on the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

1.3. Expression of DAT (midbrain) and Drd2 (striatum) mRNA

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 (Figures *7A* and *7B*).

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 (Figures *8B* and *8D*). However, we found a significantly reduced Drd2 receptor level in the dorsal striatum of male homozygous mice compared to wild-type animals ($F_{2,15}=6.32$, $p<0.05$) (Figure *8A*), while in the female mice it was unaffected (Figure *8C*).

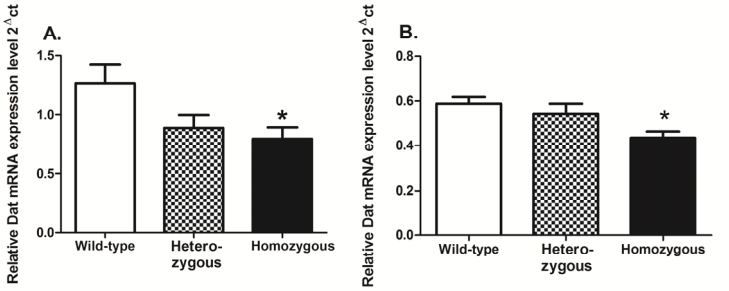

Figure 7. Expression of DAT gene in the midbrain. *A*. DAT mRNA in the midbrain of female mice; *B*. DAT mRNA in the midbrain of male mice. * – $p<0.05$ compared to wild-type animals (Tukey HSD after significant one-way ANOVA). The number of animals in each group was 16–17 in case of females and 7–8 in case of males.

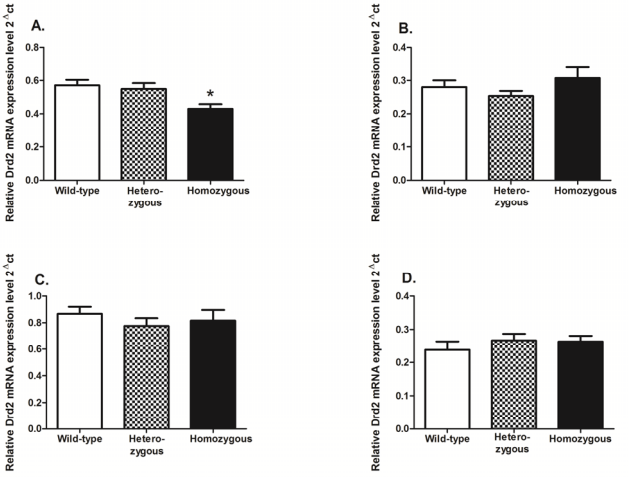

Figure 8. Expression of Drd2 gene in the dorsal and ventral striatum. Relative Drd2 mRNA levels in the dorsal (*A*) and ventral striatum (*B*) of male mice and relative Drd2 levels in the dorsal (*C)* and ventral striatum (*D)* in female mice. * – $p<0.05$ compared to wild-type animals (Tukey HSD after 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.

1.4. Expression of DAT protein in the dorsal and ventral striatum

There was no significant effect of genotype on DAT protein expression in either male or female mice, in the dorsal and ventral striatum (Mann-Whitney U-test, $p>0.05$).

2. Paper II

2.1. Tail suspension test (imipramine and paroxetine)

There was no difference in the basal immobility levels between genotypes. It was found that in homozygous Wfs1-deficient mice, imipramine induced a significant decrease in immobility time at doses of 10 mg/kg and 20 mg/kg as compared to vehicle treated homozygous mice (Figure 9). In both heterozygous and wild-type animals, only 20 mg/kg of imipramine was effective at significantly lowering immobility time compared to the respective vehicle-treated group of the same genotype.

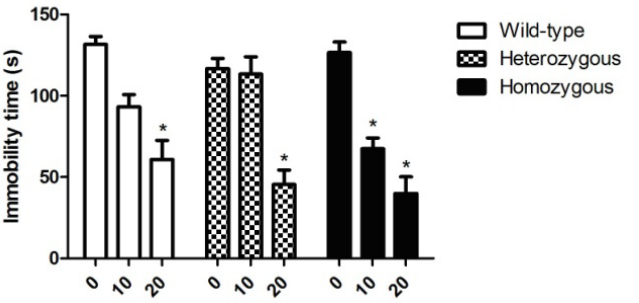

Figure 9. Effect of imipramine on the immobility time of Wfs1-deficient mice in the TST. * – $p<0.01$ compared to vehicle-treated mice of the same genotype (Scheffe *post-hoc* test after significant two-way ANOVA). Altogether, 70 wild-type, 67 heterozygous and 71 homozygous mice were used. Mice were randomly divided between respective study groups. Genotype ($F_{2,199}=4.2$, $p<0.05$); treatment ($F_{2,199}=84.1$, $p<0.01$); genotype × treatment ($F_{4,199}=3.5$, $p<0.01$).

In the study where the effect of paroxetine was investigated, the basal immobility levels of all genotypes showed no statistically significant difference. In homozygous Wfs1-deficient mice, paroxetine induced a significant decrease in immobility time already at a dose of 5 mg/kg compared to vehicle group from the same genotype (Figure 10). For heterozygous animals, a significant difference in immobility time between vehicle-treated and drug-treated mice was established at doses of 20 and 30 mg/kg of paroxetine. In wild-type mice, only the highest dose (30 mg/kg) led to a significant reduction in immobility time compared to vehicle group from the same genotype (Figure 11).

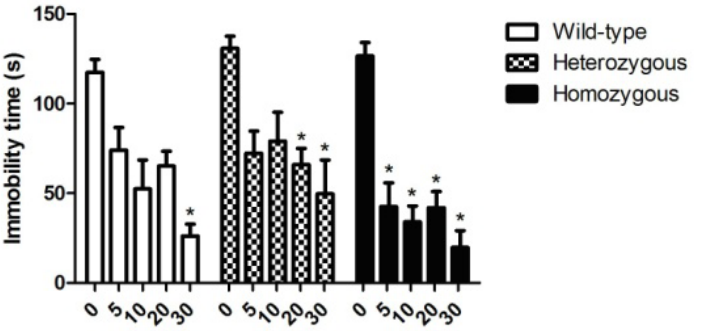

Figure 10. Effect of paroxetine on the immobility time of Wfs1-deficient mice in the TST. * – p<0.01 compared to vehicle-treated mice of the same genotype (Scheffe *post-hoc* test after significant two-way ANOVA). Altogether, 93 wild-type, 95 heterozygous and 91 homozygous mice were used. Mice were randomly divided between respective study groups. Genotype ($F_{2,264}=7.1$, $p<0.01$); treatment ($F_{4,264}=41.4$, $p<0.01$); genotype × treatment ($F_{8,264}=1.02$, $p=0.42$).

2.2. Forced swimming test (imipramine)

In contrast to the TST, effective doses of imipramine were somewhat higher in this test. These results are in accordance with previous findings (Liu and Gershenfeld, 2001). As in the TST, basal immobility levels were similar across genotypes. For homozygous mice, a remarkable reduction in immobility behavior was observed at 20 and 30 mg/kg doses compared to vehicle-treated mice from the same genotype (Figure 11). Compared to vehicle received heterozygous mice, heterozygous animals treated with 20 mg/kg of imipramine exhibited significant decrease in immobility time whereas the highest dose (30 mg/kg) had no statistical effect. Finally, wild-type animals were sensitive only to the highest dose of imipramine as compared to drug-naïve wild-type mice (Figure 11).

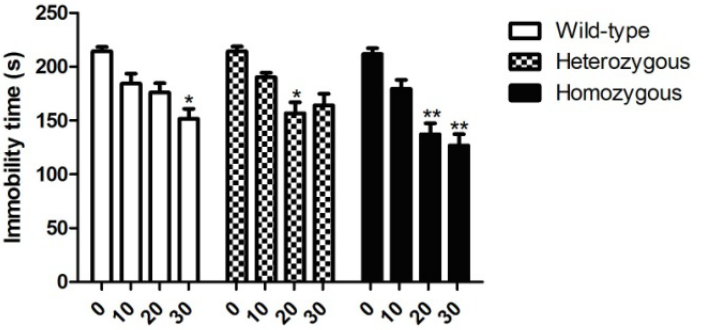

Figure 11. Effect of imipramine on the immobility time of Wfs1-deficient mice in the FST. * – p <0.05, ** – p<0.01 compared to vehicle-treated mice of the same genotype (Scheffe *post-hoc* test after significant two-way ANOVA). Altogether, 77 wild-type, 80 heterozygous and 79 homozygous mice were used. Mice were randomly divided between respective study groups. Genotype ($F_{2,224}=5.9$, $p<0.01$); treatment ($F_{3,212}=37.1$, $p<0.01$); genotype × treatment ($F_{6,224}=1.7$, $p=0.13$).

2.3. Gene expression studies

In the pons, the expression of SERT was significantly lower in homozygous mice compared to their wild-type littermates (Figure 12A). The level of SERT mRNA in the midbrain was not changed in Wfs1-deficient mice (Figure 12C). The expression of NAT was lower in Wfs1-deficient mice in the pons compared to wild-type mice, but this difference did not reach statistical significance (Figure 12B). Furthermore, the levels of NAT mRNA in the midbrain were below detection limit and, consequently, these data are not presented.

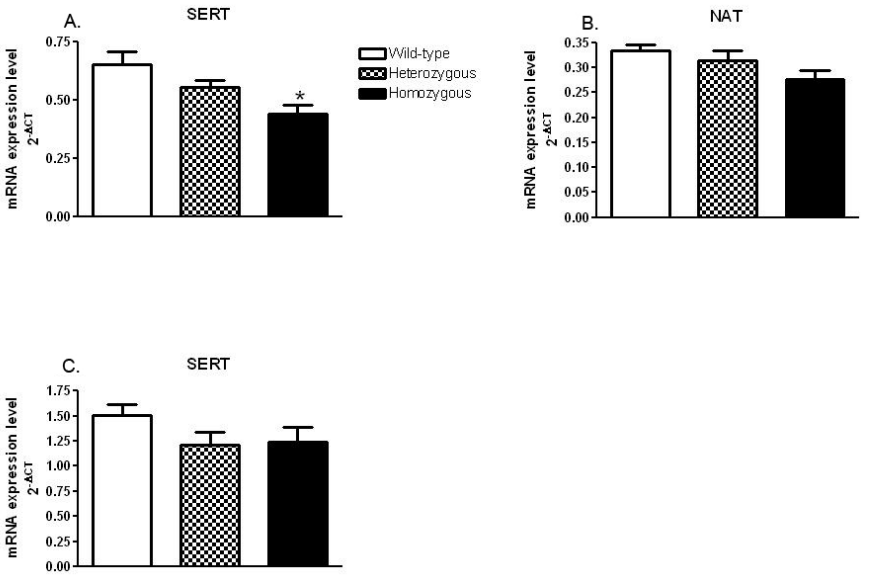

Figure 12. Effect of Wfs1 gene invalidation on the expression of monoamine transporter genes in the pons (A and B) and midbrain (C). * – $p<0.01$ compared to wild-type mice (Tukey HSD test after significant one-way ANOVA). The number of mice in each group was 12–16. SERT (pons): genotype ($F_{2,41}=6.49$, $p<0.01$); NAT (pons): genotype ($F_{2,40}=2.57$, $p=0.09$). SERT (midbrain); genotype ($F_{2,39}=1.76$, $p=0.19$).

2.4. 5-HT and NA measurements

There was no statistically significant difference in the locomotor activity of the three genotypes (data not shown). However, comparison between mice exposed to the motility boxes and experimentally naïve mice showed that exposure to the brightly lit motility boxes caused activation of the 5-HT-ergic, but not the NA-ergic, system in the dorsal and ventral striatum (Table 7). In the ventral striatum 5-HT and 5-HIAA levels were significantly increased in wild-type mice exposed to the motility boxes as compared to naïve wild-type littermates. The behavioral challenge failed to alter the levels of 5-HT and 5-HIAA in Wfs1-deficient homozygous mice, compared to naïve Wfs1-deficient homozygous

mice (Table 7A). In heterozygous mice, this stressful challenge caused a rise in the levels of NA and 5-HIAA as compared to naïve heterozygous animals.

In the dorsal striatum, 5-HIAA levels were significantly increased in wild-type and heterozygous mice exposed to the motility boxes as compared to their respective naïve littermates. For homozygous animals, no difference was established in the level of 5-HIAA between the experimental and control groups (Table 7B). In wild-type mice, the level of 5-HT was not significantly increased ($p=0.09$), whereas in homozygous mice, the concentration of 5-HT was reduced. Consequently, a simultaneous rise in wild-type mice and a decline in homozygous animals was the reason for a significant genotype × exposure interaction in two-way ANOVA analysis of 5-HT levels.

Table 7. Effect of exposure of Wfs1-deficient mice to the motility boxes on the levels of monoamines and their metabolism in the ventral and dorsal striatum.

		Wild-type	Wild-type+ exposure	Heterozygous	Heterozygous + exposure	Homo-zygous	Homo-zygous+ exposure
Ventral striatum (A)	NA	1.9±0.3	2.4±0.4	1.7±0.2	3.7±0.8*	2.7±0.4	1.8±0.3
	NMN	1.0±0.1	0.8±0.1	0.8±0.1	1.0±0.1	0.8±0.1	0.8±0.1
	5-HT	2.8±0.2	3.9±0.4*	3.2±0.1	3.3±0.2	3.2±0.1	3.4±0.2
	5-HIAA	2.6±0.2	3.9±0.2*	2.7±0.2	3.7±0.2*	2.7±0.1	3.3±0.2
Dorsal striatum (B)	NA	2.4±0.3	2.8±0.6	2.2±0.2	2.3±0.2	3.1±0.7	2.7±0.2
	NMN	0.7±0.1	0.8±0.1	0.8±0.1	0.9±0.1	0.8±0.1	0.7±0.1
	5-HT	4.9±0.1	6.1±0.4	5.2±0.1	5.8±0.3	6.0±0.4	5.3±0.2
	5-HIAA	1.8±0.1	2.8±0.2*	2.0±0.2	3.3±0.2**	2.1±0.2	2.8±0.2

$p<0.05$, **– $p<0.01$ compared to naïve mice from the same genotype (Tukey HSD test after significant two-way ANOVA). There were 8–10 mice in each group. 5-HT (ventral striatum): exposure effect ($F_{1,46}=5.62$, $p<0.05$), 5-HIAA (ventral striatum): exposure effect ($F_{1,46}=34.4$, $p<0.01$); NA (ventral striatum): genotype × exposure ($F_{2,46}=4.98$, $p<0.01$); 5-HIAA (dorsal striatum): exposure effect ($F_{1,51}=41.6$, $p<0.01$); 5-HT (dorsal striatum): genotype × exposure effect ($F_{2,51}=4.85$, $p<0.05$).

3. Paper III

3.1. Elevated plus-maze

There was no difference in the basal exploratory activity between genotypes. It was found that in homozygous Wfs1-deficient mice, ethanol induced a significant increase in open arm exploratory behavior (Figure 13).

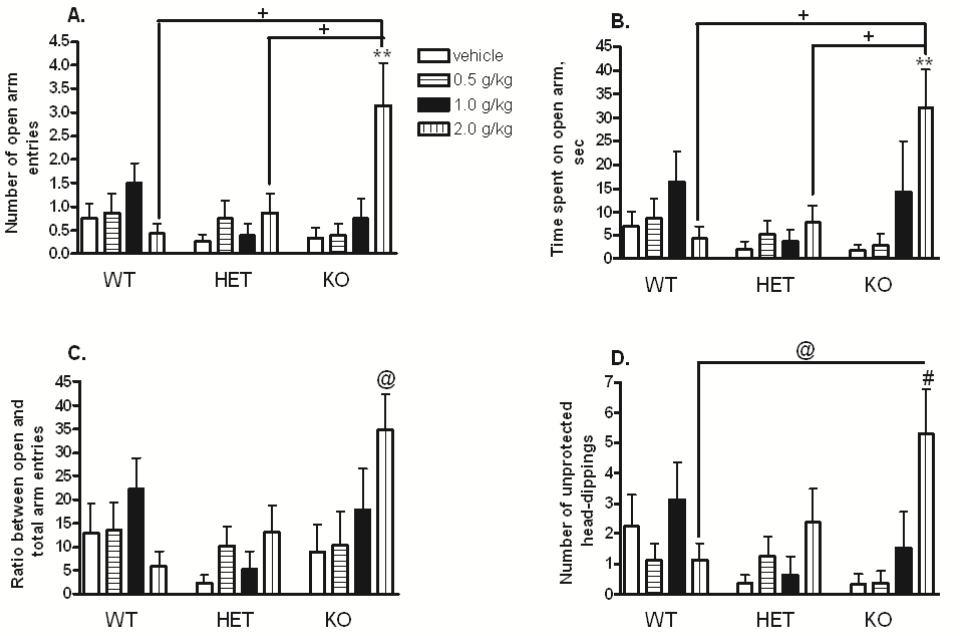

Figure 13. The effect of ethanol (0.5–2.0 g/kg) on the exploratory behavior of Wfs1-deficient mice in the elevated plus-maze test. n=7–8 per group. ** – $p<0.01$ compared to vehicle-treated homozygous mice; + – $p<0.01$ compared to homozygous mice treated with ethanol at a dose of 2 g/kg; @ – $p<0.05$ compared to wild-type mice treated with ethanol at a dose of 2 kg/kg. WT – wild-type mice, HET – heterozygous mice, KO – homozygous mice.

3.2. Motor coordination in the rotarod test

3.2.1. Latency to the first fall from the drum

There was a significant effect of trial ($F2,88=9.2$, $p<0.01$), but not genotype ($F2,44=0.1$, $p=0.86$) and trial × genotype ($F4,88=1.6$, $p=0.16$). Wfs1-deficient mice showed substantial improvement in motor skill performance over trials as they displayed longer latency to fall from the rotarod on the third trial compared to the first trial (Figure 14A). In wild-type and heterozygous mice, no remarkable change in this parameter was seen over trials.

A significant effect of ethanol treatment ($F1,44=35,3$, $p<0.01$), but not genotype ($F2,44=1.7$, $p=0.17$) or genotype × treatment ($F2,44=0.02$, $p=0.98$) was demonstrated. Treatment with ethanol (2 g/kg) significantly decreased the latency to fall from the rotarod in all the genotypes (Figure 14C).

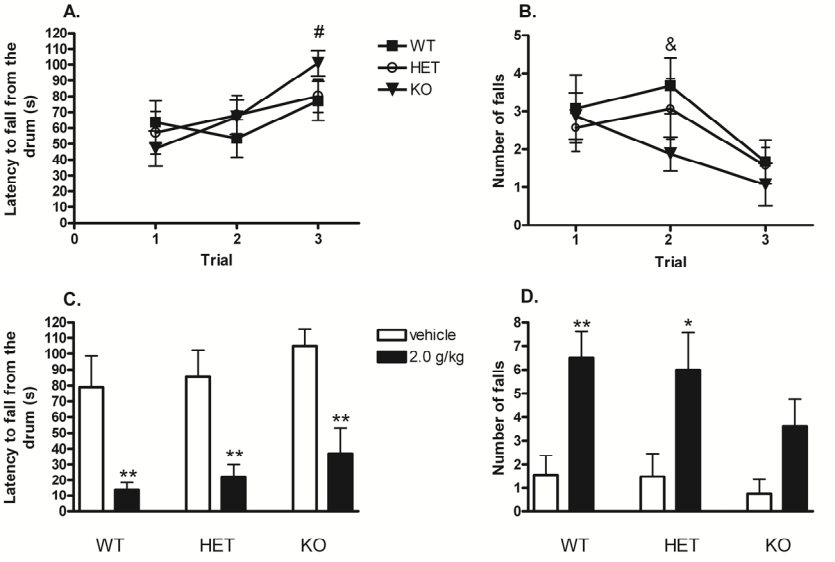

Figure 14. The effect of trial (A, B) and ethanol (2.0 g/kg) (C, D) on motor coordination of Wfs1-deficient mice in the rotarod test. n=7–8 per group. # – $p<0.01$ compared to the first measurement in homozygous mice; and – $p<0.05$ compared to the third measurement in wild-type mice; ** – $p<0.01$, * – $p<0.05$ compared to the respective vehicle-treated group. WT – wild-type mice, HET – heterozygous mice, KO – homozygous mice.

3.2.2. The number of falls

Again, there was a significant effect of trial ($F_{2,88}=9.5$, $p<0.01$), but not genotype ($F_{2,44}=0.7$, $p=0.52$) and trial × genotype ($F_{4,88}=1.0$, $p=0.39$). Wfs1-deficient mice showed a

gradual decrease in the number of falls over trials, but this change did not reach the level of statistically significant difference (Figure 14B). In heterozygous mice, no significant improvement in performance was detected over trials. Wild-type mice fell substantially less on the third trial compared to their second trial due to the high number of falls on the second trial ($p<0.05$) (Figure 14B).

There was a significant effect of ethanol treatment ($F_{1,44}=21.4$, $p<0.01$), but no genotype ($F_{2,44}=1.7$, $p=0.20$) or genotype × treatment ($F_{2,44}=0.5$, $p=0.61$) effects. Treatment with ethanol (2 g/kg) caused less incoordination in Wfs1-deficient mice as it failed to induce significant increase in the number of falls from the drum (Figure 14D).

3.3. Loss of righting reflex (LORR)

3.3.1. Ethanol

One-way ANOVA revealed a significant genotype effect for both onset of LORR ($F_{2,19}=11.9$, $p<0.01$) and duration of LORR ($F_{2,19}=3.4$, $p<0.05$). In Wfs1-deficient mice, ethanol (4 g/kg) significantly increased both the latency to LORR and the duration of LORR as compared to wild-type mice (Figure 15A). Compared to ethanol-treated heterozygous mice, homozygous mice also demonstrated a longer latency to LORR.

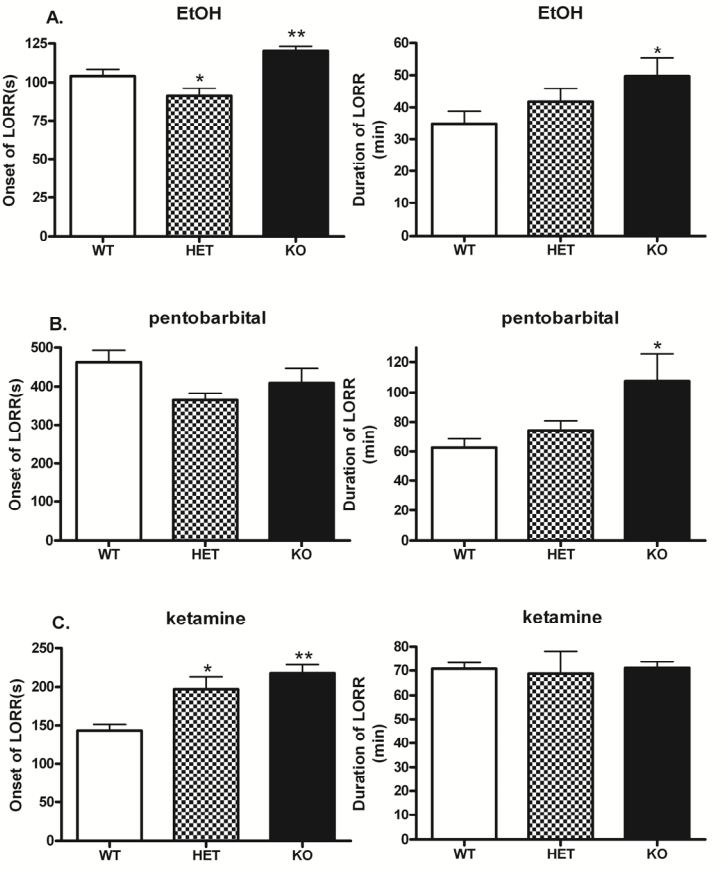

Figure 15. The onset and duration of loss of righting reflex (LORR) in Wfs1-deficient mice after administration of ethanol (4.0 g/kg) (A), pentobarbital (45 mg/kg) (B) and ketamine (150 mg/kg) (C). n=7–8 per group.** – $p<0.01$; * – $p<0.05$ compared to wild-type mice; and – $p<0.01$ compared to heterozygous mice. WT – wild-type mice, HET – heterozygous mice, KO – homozygous mice.

3.3.2. Pentobarbital

There was a significant genotype effect for the duration of LORR ($F_{2,19}=4.1$, $p<0.05$), but not for the onset of LORR ($F_{2,19}=1.9$, $p=0.16$). *Post-hoc* comparison revealed that pentobarbital (45 mg/ kg) induced a similar onset of LORR in all genotypes. As for the duration of LORR, Wfs1-deficient mice treated with this $GABA_A$ agonist slept significantly longer compared to wild-type animals (Figure 15B).

3.3.3. Ketamine

One-way ANOVA established a significant genotype effect for the onset of LORR ($F_{2,19}=7.9$, $p<0.01$), but not for the duration of LORR ($F_{2,15}=0.04$, $p=0.96$). In Wfs1-deficient mice, ketamine (150 mg/kg) significantly delayed the latency to LORR compared to wild-type mice (Figure 15C). In heterozygous mice, ketamine increased the latency to LORR compared to wild-type mice. No remarkable differences in the duration of LORR were established between the genotypes.

3.4. Gene expression studies

Two-way ANOVA did not establish any significant effects for Gabra1 and Gabra3 genes (data not shown). *Post-hoc* analysis revealed that 60 min after the injection ethanol-treated homozygous mice displayed a remarkable increase in the levels of *Gabra2* in the temporal lobe as compared to the Wfs1-deficient vehicle group (Figure 16A). Two-way ANOVA established a significant effect of ethanol treatment ($F_{2,39}=6.9$, $p<0.01$), but not genotype ($F_{1,39}=0.2$, $p=0.64$) and genotype × ethanol treatment ($F_{2,39}=0.96$, $p=0.39$).

In the frontal cortex, homozygous mice had remarkably higher levels of Gabra2 mRNA 30 min after ethanol injection compared to the respective group of wild-type mice ($p<0.01$) (Figure 16B). However, at 60 min post injection of ethanol, the levels of Gabra2 in homozygous mice had returned to the level of vehicle-treated mice. With two-way ANOVA we found significant effects of ethanol treatment ($F_{2,39}=4.9$, $p<0.01$) and genotype × ethanol treatment ($F_{2,39}=3.8$, $p<0.05$), but not genotype ($F_{2,39}=2.7$, $p=0.10$).

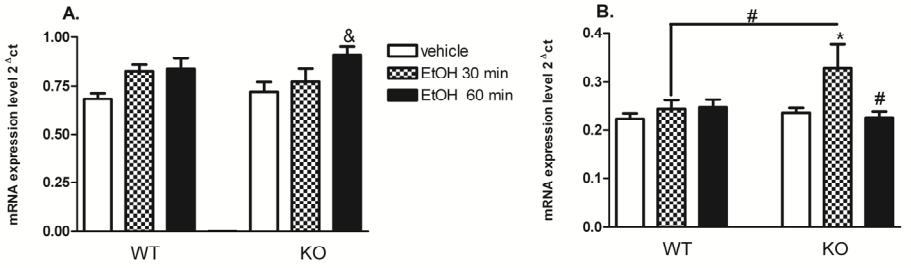

Figure 16. The effect of acute ethanol administration after 30 or 60 min on the expression of Gabra2 subunit of $GABA_A$ receptor in the temporal lobe (A) and frontal cortex (B). n=6–8 per group. * – $p<0.05$ compared to vehicle-treated homozygous mice; # – $p<0.01$ compared to ethanol 30 min group of homozygous mice; and – $p<0.05$ compared to vehicle-treated homozygous mice. & – $p < 0.05$ compared to vehicle-treated homozygous mice WT – wild-type mice, HET – heterozygous mice, KO – homozygous mice.

DISCUSSION

WS patients and people with mutations in the WFS1 gene exhibit a higher incidence of mood disorders with comorbid anxiety. We have in the three articles comprising the dissertation studied the possibility that Wfs1 deficiency in mice induces behavioral symptoms reflective of the human neuropsychiatric states and biochemical alterations to associated brain systems. Therefore, in the first and second articles, we sought to measure the behavioral and biochemical response of Wfs1-deficient mice to DA-ergic, 5-HT-ergic and NA-ergic agonists. In the third article, the anxiety of Wfs1-deficient mice was studied with an emphasis on the central GABA system, targeted by anxiolytic drugs. Although in humans, carriers (heterozygotes) have increased susceptibility to psychiatric diseases, the effect of Wfs1 heterozygosity in mice was not clearly evident in the studies. Therefore, only the results from homozygous Wfs1-deficient mice are discussed in relation to wild-type animals.

1. Dopamine

There was a tendency for lower basal locomotor activity in Wfs1-deficient homozygous mice but this difference was statistically non-significant. Exposure of vehicle-treated mice to the automated 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 Wfs1-deficient heterozygous mice compared to their homozygous littermates. This change is likely related to increased DA utilization in wild-type and heterozygous animals under the influence of motility box exposure and could reflect the moderately lower activity of homozygous mice compared to the other genotypes.

As shown before (Luuk et al., 2009), homozygous Wfs1-deficient mice had significantly lower locomotor activity in response to the acute administration of amphetamine and we found this phenotype to persist even with repetitive administrations of a subthreshold dose (2.5 mg/kg) of amphetamine. 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. 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). Although the tissue monoamine content measurement by HPLC does not directly reflect DA release, it is reasonable to speculate that DA release is impaired since amphetamine is a substrate for the DA transporter (DAT), which reverses DAT to release DA from the presynaptic neurons and thereby increases locomotor activity (Jones et al., 1998). By default, DAT is responsible for clearing DA

from the synaptic cleft (Sulzer et al., 2005). In support to this, Matto et al (2011) have showed blunted potassium-induced release of striatal DA in homozygous Wfs1-deficient mice (Matto et al., 2011). We next measured DAT levels in order to clarify the reasons for reduced potency of amphetamine in Wfs1-deficient mice. The gene expression measurements revealed marked differences between genotypes. In both sexes, the expression level of the DAT gene was significantly reduced in the ventral 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 was not reflected in the ventral striatum.

To test the intactness of the DA receptors, we injected apomorphine (3 mg/kg), which caused a significant motor stimulation in heterozygous and homozygous Wfs1-deficient mice, but not in wild-type animals. Apomorphine is a non-selective DA agonist that directly stimulates the DA D1 and D2 receptors, having more preference for the D2 receptors (Millan et al., 2002). Treatment with apomorphine caused a similar reduction of DA utilization across all genotypes. The expression of Drd2 gene (gene for DA D_2 receptor) was not affected in the ventral striatum of male and female Wfs1-deficient animals. In the dorsal striatum of male Wfs1-deficient mice the expression of Drd2 gene was reduced but in female homozygous mice it remained unchanged. The data thus indicate that the disruption of Wfs1 gene does not affect the regulation of DA signaling via DA D1 receptors. In the literature, this claim receives support as the D1 receptors are also considered to be responsible for mediating locomotor activity (Xu et al 1994).

According to the clinical studies a significant number of WS patients suffer from depression (Swift et al, 1990). It is generally accepted that 5-HT and NA play a role in the etiology and treatment of depression (Delgado et al., 1990, Neumeister et al., 2004, Moret & Briley, 2011). However, it has become apparent that DA in the striatum, involved in the neural circuits of movement, motivations and reward, plays a distinct role in the neurobiology of depression (Dailly et al., 2004, Salamone et al., 2012). The role of DA in depression is evident in Parkinson's disease patients who reportedly have a 40% higher risk of depression (Cummings, 1992). Taking into account the role of DA in depression, it can be hypothesized that the impaired function of DA-ergic system established in Wfs1-deficient mice might be extended to WS patients in order to help explain their depressive symptoms.

2. Serotonin and noradrenaline

The 5-HT-ergic and NA-ergic monoamine systems have classically been associated with mood disorders. In the second article we studied whether there are alterations in the 5-HT-ergic and NA-ergic systems owing to Wfs1 deficiency. The behavioral despair paradigm has been used for modeling depression-like behavior in rodents and encompasses two behavioral experiments, the tail suspension test (TST) and forced swim test (FST), both of which show sensitivity to antidepressant drugs. We utilized two different kinds of anti-depressants, the selective serotonin reuptake inhibitor (SSRI) paroxetine and tricyclic antidepressant (TCA) imipramine. Paroxetine exerts its effect by blocking the 5-HT transporter (SERT) and imipramine blocks, in addition to SERT, also the NA transporter (NAT). These two transporters are similar in function to the DAT, which clears excess DA from the synaptic cleft. By blocking SERT and NAT, more 5-HT and NA will be left in the cleft, thereby mediating an antidepressant-like behavioral effect in rodents. This means that mice, who have been administered an antidepressant, will spend less time immobile while suspended from their tails or swimming. Statistical comparison of basal immobility times did not show significant differences between the three genotypes in either of the two tests. Similarly, Kato et al 2008, using a different model of Wfs1-deficient mice, did also not report marked deviations in the basal activity levels in the TST and FST (Kato et al., 2008). Our results demonstrate that the doses of paroxetine and imipramine, necessary for significantly reducing immobility time compared to drug-naive animals of the same genotype, are lower in homozygous mice than in their wild-type littermates. The effectiveness of paroxetine was higher than that of imipramine, thus one could suggest that SERT has a more prominent role in the elevated behavioral response of homozygous mice. Next, we measured gene expression levels of SERT and NAT in experimentally naïve mice. Homozygous mice exhibited significantly lower expression of SERT mRNA in the pons as compared to their wild-type littermates. Moreover, the expressions of SERT in the midbrain and NAT in the pons tended to be lower, but these differences were not statistically significant. Based on these findings, it can be speculated that the activity of SERT, but not NAT, is lower in Wfs1-deficient mice which may contribute to altered response to imipramine and paroxetine in these mice. Further studies measuring SERT and NAT protein expression need to be done to confirm these changes in Wfs1-deficient mice.

The following step was to evaluate the effect of a stressful (brightly lit) motility box challenge on the metabolism of 5-HT and NA in the dorsal and ventral striatum as a large body of research have shown that 5-HT in different brain areas might provide adaptation to stress (Chaouloff et al., 1999). Stress induces elevated levels of the 5-HT in the hypothalamus (Shimizu et al, 1992) and elevates 5-HT metabolism in the nucleus accumbens, medial prefrontal cortex and amygdala (Inoue et al., 1994). In addition, 5-HT-ergic pathways

innervate the striatum (Sur et al., 1996) and stress can have a robust effect by increasing 5-HT levels in the striatum (Kirby et al., 1995). Exposure to a brightly lit environment induced notable changes to the levels of 5-HIAA, a metabolite of 5-HT. A statistically significant elevation of 5-HIAA levels was established in the dorsal and ventral striatum of heterozygous and wild-type mice. However, in the homozygous mice, the elevation of 5-HIAA was blunted. By contrast, we did not find any changes in the levels of NMN, a metabolite of NA, demonstrating a stronger role of 5-HT in the stress coping mechanism of mice. In stress-challenged wild-type mice, the levels of 5-HT tended to be elevated in the dorsal striatum but this effect was statistically significant only in the ventral striatum. 5-HT levels did not significantly change in Wfs1-deficient animals. Therefore, the present study reveals stronger activation of 5-HT system in wild-type mice compared to Wfs1-deficient mice. Moreover, this finding, together with the reduced expression of SERT, can be indicative of the compromised function of 5-HT system and stress response in animals lacking the Wfs1 gene.

3. GABA

In the third article, the GABA-ergic phenotype of Wfs1-deficient mice was studied. To test if $GABA_A$-receptor ($GABA_A$-R) is affected we administered ethanol, a $GABA_A$-R agonist. It was found that the anxiolytic-like effect of ethanol was increased in Wfs1-deficient homozygous mice since they displayed lower levels of behaviors associated with anxiety in the elevated plus maze (EPM) test. Ethanol did not induce any significant anxiolytic-like effects in wild-type and heterozygous mice. Anxiety-related effect of ethanol was distinguished from its locomotor-enhancing properties by observing that it did not cause a significant increase in horizontal activity in the motility box test. All the genotypes had similar blood ethanol kinetics following the administration of ethanol (2 or 4 g/kg) (data not shown). It can therefore be excluded the effect of ethanol on activity behavior was caused by altered ethanol metabolism. We had previously found a similar behavioral response in Wfs1-deficient to diazepam, also a $GABA_A$-R agonist, confirming the present pharmacological findings (Luuk et al 2009).

In the rotarod test, ethanol (2 g/kg) impaired motor coordination in all genotypes, however, impairment of performance was less obvious in Wfs1-deficient mice since ethanol failed to induce a substantial increase in the number of falls from the rotarod in these mice. The results suggest that Wfs1 deficiency decreases sensitivity to the sedative/ataxic effect of ethanol. Loss of righting reflex (LORR) test revealed genotype differences in the sedative response to ethanol as the onset of LORR was delayed in Wfs1-deficient mice. At the same time, a significant increase in the duration of LORR seen in mice lacking Wfs1 gene points to a stronger hypnotic effect of ethanol in these mice.

In the case of pentobarbital (45mg/kg), a $GABA_A$-R modulator, all the genotypes demonstrated a similar onset of LORR, whereas Wfs1-deficient mice slept for a longer time than wild-type mice. Ketamine, an antagonist of NMDA receptors, increased the latency of LORR in Wfs1-deficient mice, but did not cause significant differences in the duration of LORR between the genotypes. These results indicate that decreased sensitivity to the sedative effect of ethanol in mice lacking Wfs1 gene may be related to the altered function of both $GABA_A$ and NMDA receptors. At the same time enhanced sensitivity to the hypnotic effect of ethanol in mice lacking Wfs1 gene is related to the altered function of $GABA_A$ receptor rather than NMDA receptors.

It has been suggested that among the subunits of $GABA_A$-Rs, the α2 (Gabra2) subunit might be responsible for the hypnotic action of ethanol because mice lacking the α2 gene display shorter ethanol-induced LORR (Boehm et al., 2004). The exact brain region responsible for the regulation of ethanol-induced LORR is not clearly known, but because temporal lobe structures (eg amygdala and entorhinal cortex) regulate sleep-wake cycle (Leung et al., 2014), one may expect that changes there could reflect mediating mechanisms for ethanol-induced LORR.

Gene expression studies revealed that the anxiolytic dose of ethanol (2 g/kg) had no effect on the levels of Gabra1 and Gabra3 mRNA following ethanol treatment. At the same time, a significant increase in Gabra2 mRNA was detected in the frontal cortex of Wfs1-deficient mice at 30 min after ethanol injection as compared to the respective vehicle-treated mice. Comparison of ethanol exposed groups at 30 min after ethanol injection showed that Wfs1-deficient mice displayed significantly higher levels of Gabra2 mRNA than wild-type mice. The temporal lobe displayed the same dynamic of Gabra2 gene expression 60 min after ethanol treatment as was seen in the frontal cortex. Differences in ethanol responsive gene expression networks may contribute to altered ethanol-induced behavioral phenotype in Wfs1-deficient mice. Both brain areas used in the gene expression studies are relevant for the regulation of negative emotions, including anxiety (Martijena et al., 2002, Panksepp, 1998, Leistedt & Linkowski, 2013). Therefore, the increased level of Gabra2 mRNA following ethanol treatment in these brain areas may explain the increased sensitivity to anti-anxiety effect of ethanol in Wfs1-deficient mice.

Results from human research also appear to support the finding that Gabra2 gene mediates the stimulating effect of ethanol. Several genetic studies have shown the involvement of GABRA2 in increased stimulation and euphoria following ethanol administration (Arias et al., 2014) and in the pathogenesis of alcohol dependence (Uhart et al., 2013, Arias et al., 2014, Li et al., 2014). On the other hand transgenic mice lacking the Gabra2 subunit displayed a tendency for increased exploratory activity in response to ethanol administration indicating that the Gabra2 subunit is not required for anti-anxiety effect of ethanol (Boehm et al., 2004). Moreover, it cannot be entirely excluded that the increased anxiolytic effect of ethanol is also mediated by alterations in the 5-HT-ergic

system, which is also targeted by ethanol and has been shown to be affected in homozygous Wfs1-deficient mice (Paper II).

In mice, the magnitude of anxiogenic effect of alcohol depends on many factors, such as the genetic background, the dose of ethanol and the nature of the behavioral task (Durcan & Lister, 1988, Cao et al., 1993, Stewart et al., 1993). Differences in mouse background used for producing transgenic animals most likely explain discrepancies between these and our studies. Indeed, it has been established that 129 substrains have extensive genetic variability (Simpson et al., 1997) and, therefore, the different substrains may not be equally sensitive to all effects of ethanol. It should be noted that only female mice were used in the current study. Sex differences in anxiety behavior following acute ethanol administration have been reported before (Abramov et al., 2006).

Finally**,** it has to be taken into account that the monoamines share a complex reciprocal relationship with GABA (Brambilla et al., 2003). Administering GABA agonists or GABA transaminase inhibitors results in reduced DA neuron firing and D2 receptor binding (Dewey et al. 1992, Schiffer et al., 2000). Also, GABA agonists tend to reduce 5-HT synthesis (Nishikawa & Scatton, 1983, Nishikawa & Scatton, 1985). On the other hand, some studies find that GABA agonists can probably also stimulate the DA- and 5-HT-ergic systems, depending on different experimental applications (Garbutt & van Kammen, 1983, Francois-Bellan et al., 1988). Similarly, depending on the type of manipulation to the monoaminergic system, GABA levels can be elevated or lowered (Harsing & Zigmond, 1997, Seamans et al., 2001). Together, the relationship between monoamines and GABA would need to be elucidated further in Wfs1-deficient animals to ascertain the precise alterations to the interplay of these neurotransmitter systems.

4. Concluding remarks and future prospects

This dissertation is based on studies conducted on the monoaminergic and GABA-ergic systems of Wfs1-deficient mice. These systems are strongly implicated in the etiologies of mood and anxiety disorders and therefore also targeted by drugs used in the treatment of depression and anxiety.

Mice deficient in Wfs1 are less sensitive to the locomotion-enhancing effects of indirect DA agonist amphetamine and more sensitive to the post-synaptic DA agonist apomorphine. Moreover, the release and metabolism of DA is altered. In general, the DA-ergic system seems to be impaired in Wfs1-deficient mice. Although these findings are apparent when comparing Wfs1-deficient and wild-type animals, other causes that result in similar DA system alterations and behavioral phenotype, such as insulin or calcium levels, have been identified in the past. The behavioral phenotype we have observed in Wfs1-deficient animals only partly mimics that of insulin deficient animals. Further experiments (for example with insulin replacement) would need to be

performed to confirm whether this impairment is caused by the insulin deficiency that inevitably accompanies Wfs1 deficiency and has been shown to disrupt the DA-ergic system in diabetic animal models. In addition, it is necessary to conduct experiments to measure and manipulate calcium levels in the brains of Wfs1-deficient animals to ascertain the true role of Wfs1 deficiency in the impaired DA-ergic phenotype.

Wfs1 deficiency produces distinct changes to the 5-HT-ergic system. We did not see any notable effects of Wfs1 deficiency on the NA-ergic system. There were no differences to the baseline levels of immobility parameters in the behavioral despair tests, therefore these animals cannot be considered "depressed". Instead, Wfs1-deficient homozygous mice are more sensitive to SSRI and TCA drugs paroxetine and imipramine in the behavioral despair tests and this phenotype makes them suitable for studying both classes of anti-depressants. The other method we employed here was a stressful environment which normally causes an elevation of 5-HT levels in various emotion- and stress-related areas. The levels of striatal 5-HT were blunted or even lower in exposed Wfs1-deficient animals which could be interpreted as an indicator of a disturbed molecular coping mechanism.

It has been observed previously that Wfs1-deficient animals are more anxious and responsive to the $GABA_A$ receptor agonist diazepam which is a highly effective anxiolytic drug. In addition, some of the Wfs1-deficient animals have consistently displayed characteristic vocalizations under stress. Here, the animals were more responsive to ethanol in the elevated plus maze. It is likely that the behavioral anxiety phenotype was not observed because of different experimental conditions. This discrepancy warrants further clarification. The fact that Wfs1-deficient mice are behaviorally more sensitive to both GABA receptor agonists would make them candidates for anxiolytic screening.

Altogether, these results indicate this to be a mouse model that has bio-chemical alterations in brain systems implicated in mediating movements, emotions and anxiety. These Wfs1-deficient mice could be considered a potentially useful model for the screening of antidepressants and anxiolytics.

CONCLUSIONS

In the three articles we studied the possibility that Wfs1 deficiency in mice induces behavioral symptoms reflective of human psychiatric states and alterations to central DA-ergic, 5-HT-ergic, NA-ergic and GABA-ergic systems.

Dopamine

1. In the first article, we found that Wfs1-deficient mice have altered behavioral responses to DA agonists and biochemical impairments in the DA-ergic system. The presynaptically acting drug amphetamine had a lower locomotion-inducing effect in these animals, both when given acutely and chronically. Apomorphine, which is a direct DA receptor agonist, induced a higher behavioral response in Wfs1-deficient mice.
2. It was found that DA levels did not rise in response to amphetamine administration and the turnover of DA was not inhibited in Wfs1-deficient animals. On the other hand, apomorphine caused a significant reduction in DA turnover across the genotypes.
   In summary, DA metabolism was affected by Wfs1-deficiency and this might be a cue about the causes of emotional disorders seen with WFS1 mutations in humans.

Serotonin and noradrenaline

1. In the second article we found that Wfs1-deficient animals have altered response to antidepressant drugs in behavioral despair tests and a blunted 5-HT reaction in a stressful environment. The animals did not have baseline differences in the time spent immobile in the TST or spent swimming (FST). By contrast, Wfs1-deficient mice had a heightened response to antidepressants in both behavioral despair tests. The SSRI antidepressant paroxetine was more effective at a lower dose than the TCA imipramine, possibly reflecting a stronger 5-HT-ergic alteration in homozygous Wfs1-deficient animals.
2. Further, we subjected Wfs1-deficient animals and their wild-type littermates to a stressful open field environment to measure the biochemical response of the mice. Stressful conditions are known to cause elevation in central 5-HT levels. We found that 5-HT levels did not rise in response to this experimental manipulation in Wfs1-deficient mice. Differently from Wfs1-deficient animals, this rise was detected in wild-type mice.
   It can therefore be concluded that the adaptation to stress might be impaired in Wfs1-deficient animals due to 5-HT-ergic alterations.

Gamma-aminobutyric acid

1. In the third article, it became evident that Wfs1-deficient mice were more responsive than wild-type animals to ethanol in the elevated plus maze test and also found $GABA_A$ receptor subtype expression differences. Intriguingly, the baseline levels of anxiety were not different between the genotypes.

2. Because $GABA_A$-R is known to mediate ethanol-induced anxiolytic effect, we measured the gene expression levels of $GABA_A$-R subtypes in Wfs1-deficient animals. Ethanol upregulated the expression of $GABA_A$ subtype α2 encoding gene *Gabra2*. This subtype of $GABA_A$ receptor has been implicated in mediating the anxiolytic effect of $GABA_A$ agonists. The study suggests that both anxiolytic-like and sedative/hypnotic properties of ethanol are changed in Wfs1-deficient mice. The increased level of *Gabra2* mRNA expression after ethanol administration in brain areas considered responsible for experiencing anxiety could partly explain the greater anxiolytic-like effect of ethanol.

In general, Wfs1 deficency induces distinct changes to the DA-ergic, 5-HT-ergic and GABA-ergic systems. We did not detect notable changes to the NA-ergic system. Taken together, the results of the pharmacological studies show that this Wfs1-deficient mouse model is a potentially viable option for translational medicine in the field of anxiolytic and antidepressant screening and to test new drug administration schemes in the treatment of WS.

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EESTI-KEELNE KOKKUVÕTE

Wfs1 geeni puudulikkusega hiire monoamiinergilise ja GABA-ergilise süsteemi farmakoloogiline ja käitumuslik iseloomustus

WFS1 geenilt toodetakse volframiini (WFS1 valk). Homosügootsed mutatsioonid ja mõned liitheterosügootsuse variandid WFS1 geenis põhjustavad väga haruldast ja rasket Wolframi sündroomi (WS), mida iseloomustavad suhkurdiabeedi, magediabeedi, optilise närvi atroofia ja kurtuse esinemine. WS patsientidel ja WFS1 heterosügootidel, kel WS-i pole, esineb sageli psühhiaatrilisi häireid nagu depressioon ja ärevus. Lisaks on teatud WFS1 geeni polümorfismid seotud psühhiaatriliste häiretega. Kuigi WFS1 geeni puudulikkus on seotud psühhiaatriliste sümptomitega, pole teada, millised muutused ajus sel puhul tekivad. Samuti on volframiini täpne ekspressioon teada ainult hiire ajus. Volframiin lokaliseerub ajus piirkondades, mis on olulised tunnete, hirmu ja käitumise regulatsioonis (mh amügdalas, hipokampuses, dorsaalses ja ventraalses striaatumis ning stria terminalise sängituumas). Uurimaks volframiini puudulikkusest tulenevaid käitumuslikke ja biokeemilisi muutusi, loodi Wfs1-puudulik hiireliin. Esialgsed uuringud näitasid, et see hiireliin on metsiktüüpi hiirtega võrreldes oluliselt ärevam ja tal võib esineda kõrvalekaldeid dopamiinergilise ja gamma-aminovõihappe (GABA) süsteemi töös.

Käesolevas töös uurisime laiemalt, kui suurel määral Wfs1-puudulike hiirte käitumuslik fenotüüp sarnaneb WSF1 mutatsioonidest tulenevatele psühhiaatrilistele nähtudele ja millised muutused esinevad neil aju monoamiinergilistes (dopamiin, serotoniin, noradrenaliin) ja GABA süsteemides. Tsentraalsed monoamiinid on olulised käitumise ja meeleolu regulatsioonis. GABA on peamine inhibitoorne neurotransmitter ajus, ja $GABA_A$ retseptorid omavad olulist rolli ärevushäirete ravis, olles anksiolüütikumide sihtmärgiks.

Esimeses artiklis uurisime dopamiinergilise süsteemi kõrvalekaldeid. Leidsime, et Wfs1-puudulike homosügootsete hiirte reaktsioon kaudse dopamiini agonistina toimiva amfetamiini käitumuslikku aktiivsust tõstvale toimele on oluliselt väiksem kui metsiktüüpi hiirtel. Teisalt on Wfs1-puudulikud hiired käitumuslikult tundlikumad apomorfiinile, mis võib olla dopamiini retseptorite ülesregulatsioonist tulenev, sest see ravim toimib dopamiiniretseptori otsesese agonistina. Lisaks, amfetamiini manustamine tõstis dopamiini tasemeid metsiktüüpi ja heterosügootsete loomade striaatumis, kuid olulist tõusu ei esinenud Wfs1 homosügootsetel hiirtel. Peale selle oli amfetamiini mõju dopamiini ringkäigule Wfs1-puudulikel hiirtel erinev metsiktüüpi hiirtest. Wfs1-puudulikel hiirtel esines ventraalses keskajus dopamiini transporterit (DAT) kodeeriva geeni oluliselt madalam ekspressioon. Kuigi geenieksressioonilt oli DAT madalam, ei leidnud me sellele erinevusele kinnitust striaatumis valguekspressiooni kujul.

Teises artiklis vaatlesime serotonergiliste ja noradrenergiliste süsteemide muutusi Wfs1-puudulikes hiirtes, kasutades närilistele stressitekitavalt mõjuvat avarväljakatset ja nö käitumusliku ahastuse loomkatsemudeleid – sabast riputamise ning sunnitud ujumise katseid. Käitumuslik ahastus peegeldab looma akuutset „depressiooni" ja on üldiselt tundlik antidepressantide manustamisele. Sabast riputamise ja sunnitud ujumise katseid kasutatakse meeleoluhäirete ravis oluliste ravimite skriinimiseks. Käitumusliku ahastuse katsetes kasutasime selektiivset serotoniini tagasihaarde inhibiitorit paroksetiini ja tritsüklilist antidepressanti imipramiini, mis mõjub noradrenaliini transporteri inhibiitorina. Wfs1-puudulikel hiirtel esines suurem tundlikkus nende ravimite võimele vähendada liikumatust, kusjuures käitumuslikult tugevamalt mõjusid pigem väiksed paroksetiini doosid. Avarväljale eksponeerimine tõi esile pigem serotonergilised muutused Wfs1-puudulikel homosügootsetel hiirtel: serotoniini tase ei tõusnud Wfs1-puudulikel hiirtel striaatumis vastusena stressitekitavasse keskkonda paigutamisele (dorsaalses striaatumis esines isegi langus). Samas esines serotoniini taseme tõus metsiktüüpi hiirtel. Need tulemused peegeldavad stressile adapteerumisel olulise neurotransmittersüsteemi kõrvalekallet tulenevalt Wfs1 puudulikkusest.

Kolmandas artiklis keskendusime Wfs1-puudulike hiirte ärevusfenotüübi ja gamma-aminovõihappe (GABA) süsteemide uurimisele. Ilmnes, et etanoolil oli Wfs1-puudulikele hiirtele suurem anksiolüütiline toime ülestõstetud plusspuuris. Etanool ei halvendanud rotarod testis homosügootsetel Wfs1-puudulikel hiirtel motoorset koordinatsiooni samaväärselt teiste genotüüpidega, mistõttu võib järeldada, et Wfs1 puudulikkus vähendab tundlikkust etanooli sedatiivsele ja ataksilisele mõjule. Nn püstumise kadumise testis (Loss of righting reflex, LORR) oli LORRi algus Wfs1-puudulikel hiirtel hilisem peale etanooli manustamist. Samal ajal oli LORRi kestvus pikem, mis peegeldab etanooli suurenenud hüpnootitilist efekti neis hiirtes. LORRi algus oli peale pentobarbitaali ($GABA_A$ modulaator) manustamist kõigil genotüüpidel sarnane, kuid kestvus oli jällegi Wfs1-puudulikel hiirtel pikem. Ketamiini (NMDA antagonist) manustamise järgselt polnud genotüüpide vahelisi erinevusi LORRi kestvuses. Lisaks sellele tõusis nende hiirte $GABA_A$ retseptori α2 alaühikut kodeeriva geeni *Gabra2* ekspressioon vastusena etanooli manustamisele. $GABA_A$ retseptori α2 alaühikut peetakse oluliseks anksiolüütikumide toime vahendamises.

Kokkuvõtvalt uurisime Wfs1-puudulikel hiirtel monoamiinergilist ja GABAergilist fenotüüpi. Tulemused näitavad, et kõigis neis süsteemides (va noradrenergilises) on kõrvalekaldeid ja need võivad peegeldada WFS1 mutatsioonidega inimeste neuropsühhiaatriliste häirete põhjuseid. Käitumuslikult on see hiireliin ärevam ja samas tundlikum ärevus- ja meeleoluhäirete raviks kasutatavatele ravimitele. See Wfs1-puudulik loommudel omab potentsiaali olla olulise tähtsusega siirdemeditsiinilistes uuringutes, et tuvastada efektiivsemad ravimid ja raviskeemid WS-ga kaasnevate häirete ravis.

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