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Relation between increased anxiety and reduced expression of alpha1 and alpha2 subunits of $GABA_A$ receptors in Wfs1-deficient mice

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ABSTRACT

Mutations in the coding region of the WFS1 gene cause Wolfram syndrome, a rare multisystem neurodegenerative disorder of autosomal recessive inheritance. In clinical studies a relation between mutations in the Wfs1 gene and increased susceptibility for mood disorders has been established. According to our previous studies, mice lacking Wfs1 gene displayed increased anxiety in stressful environment. As the GABA-ergic system plays a significant role in the regulation of anxiety, we analyzed the expression of GABA-related genes in the forebrain structures of wild-type and Wfs1-deficient mice. Experimentally naïve Wfs1-deficient animals displayed a significant down-regulation of α1 (Gabra1) and α2 (Gabra2) subunits of $GABA_A$ receptors in the temporal lobe and frontal cortex. Exposure of wild-type mice to the elevated plus-maze decreased levels of Gabra1 and Gabra2 genes in the temporal lobe. A similar tendency was also established in the frontal cortex of wild-type animals exposed to behavioral test. In Wfs1-deficient mice the elevated plus-maze exposure did not induce further changes in the expression of Gabra1 and Gabra2 genes. By contrast, the expression of Gad1 and Gad2 genes, enzymes responsible for the synthesis of GABA, was not significantly affected by the exposure of mice to the elevated plus-maze or by the invalidation of Wfs1 gene. Altogether, the present study demonstrates that increased anxiety of Wfs1-deficient mice is probably linked to reduced expression of Gabra1 and Gabra2 genes in the frontal cortex and temporal lobe.

Wolfram syndrome (DIDMOAD) is a rare multisystem neurodegenerative disorder of autosomal recessive inheritance characterized by early-onset diabetes mellitus, progressive optic atrophy, diabetes insipidus, and deafness [5,13,22]. It has been suggested that mutations in wolframin (WFS1) gene in patients are associated with increased susceptibility for mood disorders [14,15]. Anatomically, Wfs1 mRNA and protein are represented particularly high concentrations in brain regions related to the emotional behaviour and motivations like amygdaloid area, nucleus accumbens, hippocampus CA1 region, and superficial layer of allocortex [7,17]. Luuk et al. [8] have found that the genetic invalidation of Wfs1 impairs the adaptation of mice in a novel and stressful environment in terms of increased anxiety in ethological models. Pre-treatment of mutant mice with diazepam (1 mg/kg), an anxiolytic drug acting via $GABA_A$ receptors, antagonized increased anxiety and stress-induced vocalizations in Wfs1-deficient mice [8]. $GABA_A$ receptors are formed by the co-assembly of five subunits belonging to different families (α_{1-6}, β_{1-3}, γ_{1-3}, δ, π and θ) [1] and [12], which are heterogeneously distributed throughout the brain [21]. In addition to GABA, a variety of allosteric ligands such as neurosteroids, barbiturates, ethanol, convulsants, anaesthetics and benzodiazepines, can modulate GABA-gated conductance changes through binding to distinct sites in $GABA_A$ receptor [6]. In particular, the best characterized binding site is the benzodiazepine site, localized at the alpha/gamma subunit interface [12], in which α subunits are the main determinants of benzodiazepine receptor ligand action selectivity [10]. Based on the findings that α1 subunit is responsible for the sedative effects of diazepam and α2 subunit plays a role in its stimulating action [11,20], α1 and α2 subunit genes (Gabra1 and Gabra2, respectively) were selected for the present study. In order to reveal a relevance of stress for gene expression, a part of mice were challenged to the plus-maze model of anxiety. The expression of Gabra1 and Gabra2 genes was studied in the frontal cortex, temporal lobe, hippocampus and mesolimbic area. All these brain areas show high concentrations of Wfs1 protein and mRNA [7]. To determine the overall impact of Wfs1 deficiency for GABA-ergic system, the expression of Gad1 and Gad2 genes, enzymes responsible for the synthesis of GABA, were studied in the temporal lobe and frontal cortex.

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Wfs1-deficient mice were generated by invalidating the 8th exon of the Wfs1 gene (for detail see [7]). Breeding and genotype analysis of mice were carried out in the Department of Physiology, University of Tartu. The gene expression studies were performed in female F2 hybrids [(129S6/SvEvTac × C57BL/6) × (129S6/SvEvTac × C57BL/6)] 2–3 months old at the time of testing. Mice were housed in groups of eight at 20 ± 2 °C under a 12-h/12-h light/dark cycle (lights on at 07:00 h). Tap water and food pellets were available ad libitum. The mean body weight of Wfs1-deficient mice was significantly lower as compared to wild-type littermates (21.8 ± 0.6 in wild-type, 19.3 ± 0.5 in homozygous). The permission (No. 39, 7 October 2005) for the present study was given by the Estonian National Board of Animal Experiments in accordance with the European Communities Directive of 24 November 1986 (86/609/EEC).

The behavioral experiments lasted from 10 a.m. to 3 p.m. and were performed in two separate rooms. In the first room the elevated plus-maze experiment was performed and in the second room animals were decapitated for dissection of brain structures. The elevated plus-maze test was carried out as described previously [8]. Briefly, plus-maze consisted of two opposite open arms without sidewalls and two enclosed arms of the same size with 14-cm-high sidewalls and an end wall. The arms extended from a common central square (5 cm × 5 cm) and were perpendicular to each other, making the shape of a plus sign. The entire plus-maze apparatus was elevated to a height of 30 cm and placed in a dim room (illumination level ~20 lx). In order to encourage open arm exploration, a slightly raised edge (0.25 cm) was put around the perimeter of the open arm, providing a grip for the animals. The open arms were divided into three equal parts by lines. Before testing in the elevated plus-maze, animals were kept in isolation for 15 min. According to our previous experience, short-term isolation of mice increases their exploratory activity. During a 5-min observation session the following measures were taken: (1) the number of closed and open arm entries, (2) time spent in exploring the open arms and (3) the number of head-dipping and stretch-attend postures. Subsequently, the ratio between open and total arm entries was calculated. Testing began by placing an animal on the centre of the elevated plus-maze facing the closed arm. An arm entry was counted only when all four limbs of a mouse were within a given arm.

In gene expression studies, experimentally naïve animals and animals exposed to the elevated plus-maze, belonging to both genotypes, were used in parallel. Mice were decapitated immediately after the elevated plus-maze exposure or taking them out from the home-cage. Brains were quickly dissected into four parts (the frontal cortex, mesolimbic area [including the nucleus accumbens and olfactory tubercle], hippocampus and temporal lobe [including the amygdala]) and frozen in liquid nitrogen. The brain dissection was performed according to the coordinates presented in the mouse brain atlas [3]. The quantitative real-time PCR (qRT-PCR) experiment for Gabra1 and Gabra2 expression studies was performed using SYBR Green. SYBR® Green technology was chosen, because these primers were previously designed and optimized for this method in our other study [16].

The following primers were used:

forward 5′-TGTACACCATGAGGTTGACCGT-3′,
reverse 5′-GAAGTCTTCCAAGTGCATTGGG-3′ (Gabra1);
forward 5′-ATGGTCTCTGCTGCTTGTTCTTCT-3′,
reverse 5′-AGCACCAACCTGACTGGGTC-3′ (Gabra2).
forward 5′-GCAGTACAGCCCCAAAATGG-3′
reverse 5′-AACAAAGTCTGGCCTGTATCCAA-3′ (Hprt).

However, we did not have such experience for Gad1 and Gad2 genes. Therefore, commercially available and proven oligonucleotides, based on the alternative technology TaqMan®, were purchased from the company Applied Biosystems. For Gad1 and Gad2 gene expression, Taqman assays Mm00725661_s1 and Mm01329282_m1 (Applied Biosystems) were used, respectively. In order to select the most stably expressed reference gene we used pairwise comparison approach developed by Vandesompele et al. [19]. This approach top ranks the candidates with the highest degree of similarity of the expression profile across the sample set. In the present study three candidate normalization genes, HPRT1, GAPDH, B2M (β_2 -microglobulin), were used as they are considered the most common used housekeeping genes expressed in brain tissues. The amplification efficiency of the reference genes was as follows:

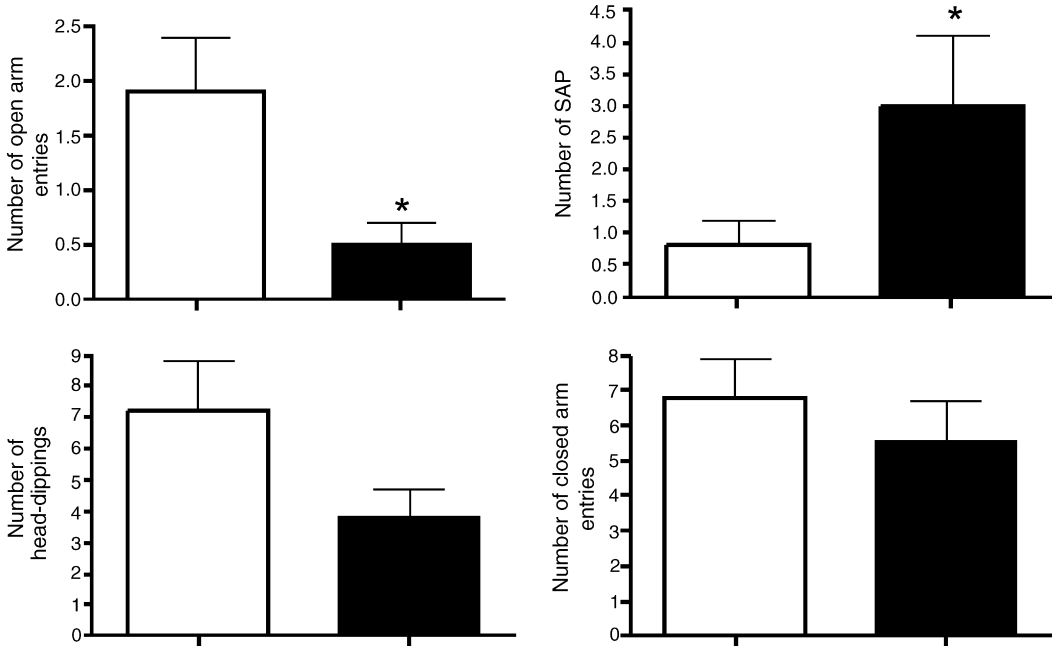

Fig. 1. Exploratory behavior of Wfs1-deficient mice in the elevated plus-maze. White bars: wild-type mice; black bars: homozygous mice.*$p < 0.05$ (compared to wild-type mice, Student's *t*-test for independent samples).

HPRT1 96%, GAPDH 67% and B2M 92%. The intra-assay coefficient of variability for B2 M, GAPDH and HPRT1 genes was 0.62%, 0.11% and 0.06%, respectively. The inter-assay variability for B2 M, GAPDH and HPRT1 was 3.4%, 2.3% and 2%, respectively. According to the internal gene-stability measure (M) of the candidate gene the most stable genes were HPRT1 ($M = 0.013$) and GAPDH ($M = 0.014$). The M value for B2 M was 0.022. However, since the amplification efficiency of HPRT1 was higher compared to GAPDH and also it was equal to the amplification efficiency on target genes Gabra1 and Gabra2 (data not shown), we chose HPRT1 as the housekeeper for the real-time PCR analysis. Another argument for selecting HPRT1 was the fact that [C_t] for GAPDH resulted in higher value between the target and reference gene and this makes following comparison between groups not so effective as in case of HPRT1, because a higher value between the target and reference gene gives smaller differences in ratio calculation.

For the Hprt gene, a housekeeping gene, the following primers were used: 5′-GCAGTACAGCCCCAAAATGG-3′ (forward) and 5′-AACAAAGTCTGGCCTGTATCCAA-3′ (reverse). The probe for Hprt was 5′-VIC-AAGCTTGCTGGTGAAAAGGACCTCTCG TAMRA-3′.

Since we wanted to show how genetic and/or behavioral manipulations affect the gene expression as compared to the control group (naïve wild-type mice), it seemed to be more appropriate to present results as fold changes rather that determining the precise initial copy number of gene of interest, which is relevant, for example, in virology for determination of concentration of a gene of interest in different tissues. Also, the use of relative values helps to compare the results from experiments performed on different days, because sometimes the results coming from different experimental sets may be so variable that the application of ANOVA does not allow obtain the statistically reliable results.

The results are expressed as mean values ± S.E.M. The plus-maze studies were analyzed by means of Student's t-test for independent samples. As our goal was to compare the effect of the plus-maze exposure and genetic manipulation on the expression of GABA-related genes, the only acceptable way to test the validity of this approach was application of two-way ANOVA (genotype and plus-maze exposure as independent variables) using Statistica for Windows software (Statsoft, USA). *Post hoc* comparisons between the individual groups in gene expression studies were performed with Tukey HSD test.

In the plus-maze test Wfs1-deficient mice displayed increased anxiety-like behavior in terms of increased open arm avoidance and affected risk assessment behaviors. Homozygous animals made significantly less open arm entries as compared to wild-type mice (Student's t-test, $p < 0.05$) (Fig. 1). Also, a similar tendency was observed for other conventional measures of anxiety: time spent on open arms ($p = 0.07$) and ratio between the open and total arm entries ($p = 0.07$). Additionally, the genotype differences were noticeable for the ethological measures of anxiety. Namely, Wfs1-deficient mice performed significantly more stretch-attend postures compared to their wild-type littermates ($p < 0.05$) (Fig. 1). The number of head-dippings was also reduced, but remained statistically not significant in genetically modified animals ($p = 0.09$).

As expected, the expression of Gabra1 gene was significantly higher if compared to Gabra2 gene in the forebrain structures. In the frontal cortex it was 2.7-fold in favor of the Gabra1 gene, whereas in the temporal lobe and hippocampus the respective differences

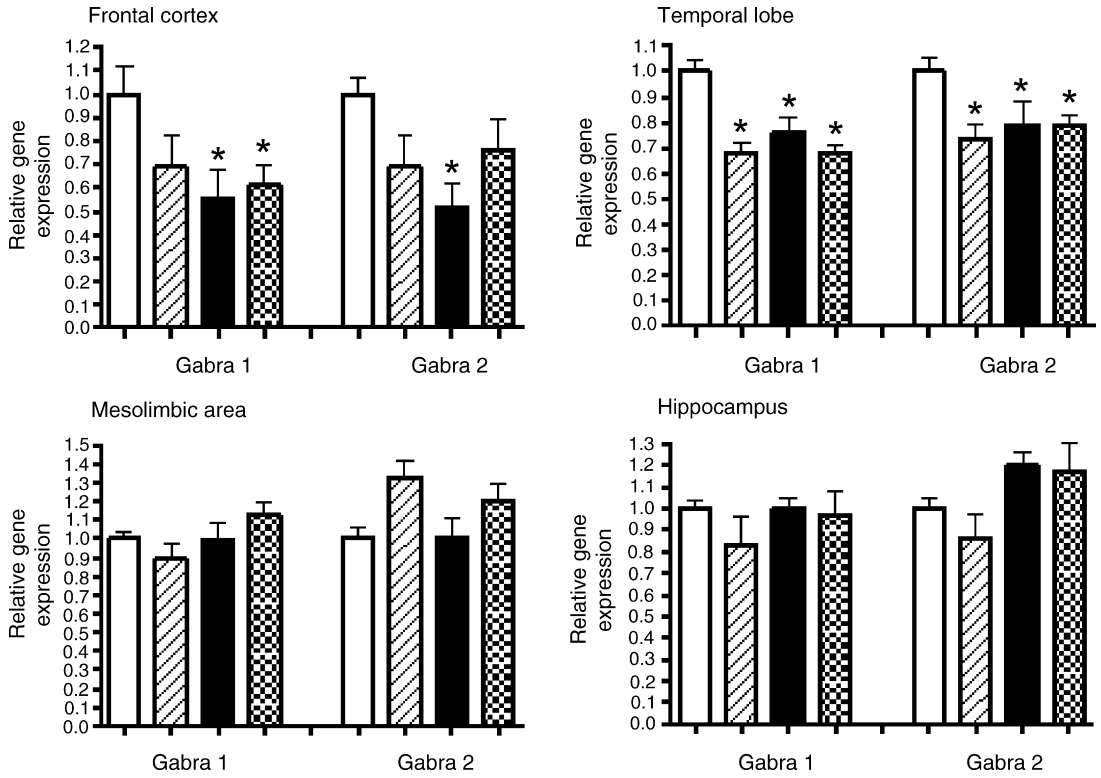

Fig. 2. Effect of Wfs1 gene invalidation and plus-maze exposure on the expression of the Gabra1 and Gabra2 genes in the forebrain structures. White bars: wild-type mice; stripped bars: wild-type mice exposed to the plus-maze; black bars: homozygous mice; hatched bars: homozygous mice exposed to the plus-maze. Frontal cortex—Gabra1: $F1,28 = 7.09$, $p = 0.02$ (genotype); $F1,28 = 2.03$, $p = 0.17$ (plus-maze exposure); $F1,28 = 3.59$, $p = 0.07$ (genotype × plus-maze exposure). Gabra2: $F1,28 = 3.28$, $p = 0.03$ (genotype); $F1,28 = 0.03$, $p = 0.85$ (plus-maze exposure); $F1,28 = 6.02$, $p = 0.02$ (genotype × plus-maze exposure). Mesolimbic area—Gabar1: $F1,28 = 0.28$, $p = 0.60$ (genotype); $F1,28 = 1.10$, $p = 0.30$ (plus-maze exposure); $F1,28 = 1.27$, $p = 0.27$ (genotype × plus-maze exposure). Gabar2: $F1,28 = 6.80$, $p = 0.015$ (genotype); $F1,28 = 0.34$, $p = 0.57$ (plus-maze exposure); $F1,28 = 0.41$, $p = 0.53$ (genotype × plus-maze exposure). Temporal lobe—Gabar1: $F1,28 = 8.97$, $p = 0.0058$ (genotype); $F1,28 = 23.7$, $p = 0.00004$ (plus-maze exposure); $F1,28 = 9.42$, $p = 0.0058$ (genotype × plus-maze exposure). Gabra2: $F1,28 = 1.80$, $p = 0.19$ (genotype); $F1,28 = 5.57$, $p = 0.03$ (plus-maze exposure); $F1,28 = 5.43$, $p = 0.03$ (genotype × plus-maze exposure). Hippocampus—Gabar1: $F1,28 = 0.43$, $p = 0.52$ (genotype); $F1,28 = 1.18$, $p = 0.29$ (plus-maze exposure); $F1,28 = 0.63$, $p = 0.43$ (genotype × plus-maze exposure). Gabar2: $F1,28 = 8.01$, $p = 0.009$ (genotype); $F1,28 = 0.85$, $p = 0.37$ (plus-maze exposure); $F1,28 = 0.36$, $p = 0.55$ (genotype × plus-maze exposure).

were 1.8-fold and 1.5-fold. The only exception was the mesolimbic area where the expression was 1.2-fold in favor of Gabra1 gene. It was also expected that the Gad1 gene would be significantly more abundant compared to the Gad2 gene in the forebrain structures. The respective differences were 15-fold and 13-fold in the frontal cortex and temporal lobe in favor of the Gad1 gene.

We established significant genotype as well as genotype and elevated plus-maze exposure interactions for the Gabra1 and Gabra2 genes in the frontal cortex and temporal lobe. Genetic invalidation of the Wfs1 gene induced a remarkable reduction of the Gabra1 and Gabra2 genes in the temporal lobe and frontal cortex (Fig. 2). This effect was not established in the mesolimbic area and hippocampus. The exposure of wild-type mice to the elevated plus-maze also reduced the expression level of these genes in the temporal lobe and frontal cortex. However, only in the temporal lobe it was statistically significant. The exposure of Wfs1-deficient mice to the elevated plus-maze did not cause a further reduction in the expression of the Gabra1 and Gabra2 genes compared to the experimentally naïve mutant mice. Genetic invalidation of the Wfs1 gene or exposure of wild-type mice to the elevated plus-maze did not affect the expression of the Gad1 and Gad2 genes in the frontal cortex and temporal lobe (Fig. 3).

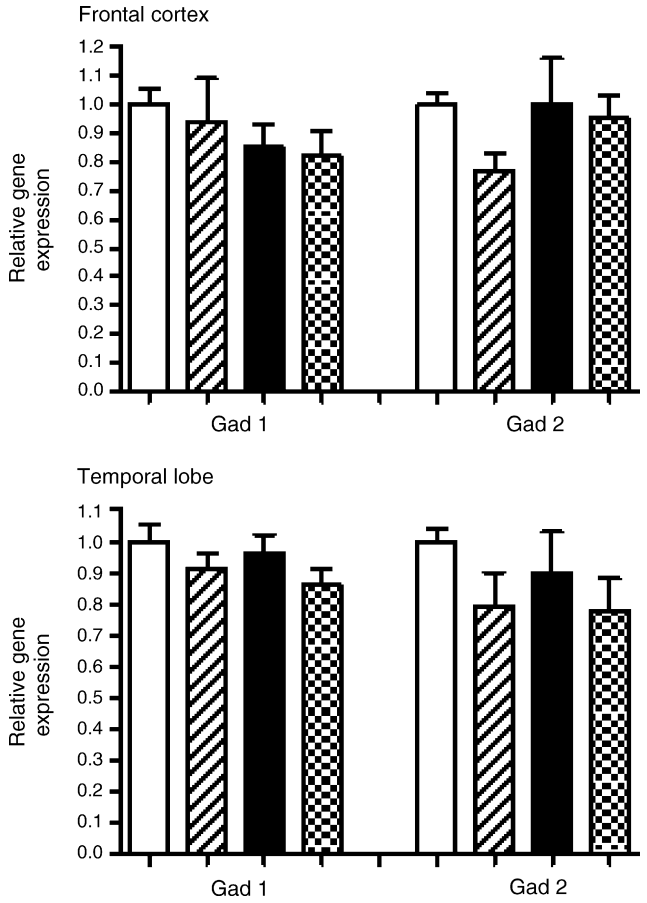

Fig. 3. Effect of the Wfs1 gene invalidation and plus-maze exposure on the expression of the Gad1 and Gad2 genes in the frontal cortex and temporal lobe. White bars: wild-type mice; stripped bars: wild-type mice exposed to the plus-maze; black bars: homozygous mice; hatched bars–homozygous mice exposed to the plus-maze. Frontal cortex—Gad1: $F1,28 = 2.06$, $p = 0.16$ (genotype); $F1,28 = 0.82$, $p = 0.37$ (plus-maze exposure); $F1,28 = 0.05$, $p = 0.82$ (genotype × plus-maze exposure). Gad2: $F1,28 = 0.94$, $p = 0.34$ (genotype); $F1,28 = 2.53$, $p = 0.13$ (plus-maze exposure); $F1,28 = 0.66$, $p = 0.42$ (genotype × plus-maze exposure). Temporal lobe—Gad1: $F1,28 = 3.73$, $p = 0.07$ (genotype); $F1,28 = 0.92$, $p = 0.35$ (plus-maze exposure); $F1,28 = 0.003$, $p = 0.95$ (genotype × plus-maze exposure). Gad2: $F1,28 = 2.63$, $p = 0.12$ (genotype); $F1,28 = 0.32$, $p = 0.57$ (plus-maze exposure); $F1,28 = 0.21$, $p = 0.65$ (genotype × plus-maze exposure).

The present study demonstrates that genetic invalidation of the Wfs1 gene increases not only anxiety in mice [8], but also affects the expression level of the Gabra1 and Gabra2 genes, playing a role in the pharmacological action of diazepam. Namely, Wfs1-deficient mice displayed reduced expression of these genes in the temporal lobe and frontal cortex. Both these structures have been shown to play an eminent role in the regulation of negative emotions, especially anxiety [2]. The down-regulation of two dominating subunits of $GABA_A$ receptors [4] probably reflects reduced GABA-ergic neurotransmission in these particular brain regions. Reduced activity of the GABA-ergic system may also explain the increased sensitivity of Wfs1-deficient mice to the anxiolytic action of diazepam [8]. On the other hand, the lack of changes in the expression of the Gad1 and Gad2 genes demonstrates that the invalidation of the Wfs1 gene does not have a major impact on the synthesis of GABA in the brain. Lyons et al. [9] have shown that long-term exposure of brain neurons to GABA results in down-regulation of $GABA_A$ receptor number and uncoupling of GABA and benzodiazepine binding sites. They have revealed that the down-regulation of $GABA_A$ receptors occurs due to GABA-induced elevation of intracellular Ca^{2+} levels. Therefore, in the light of finding that the Wfs1 protein modulates Ca^{2+} levels in the intracellular space [18], one could speculate that the elevated release of GABA due to heightened sensitivity to stress of Wfs1-deficient mice may be responsible for the down-regulation of $GABA_A$ receptor subunits. It is noteworthy that a similar down-regulation of the Gabra1 and Gabra2 genes in the temporal lobe and frontal cortex occurred in wild-type mice in response to exposure to the elevated plus-maze. This finding further underlines a possible role of stress in the down-regulation of $GABA_A$ receptor subunits established in Wfs1-deficient mice. Therefore, the present study is in favor of a relation between increased anxiety and reduced expression of subunits of $GABA_A$ receptors in the frontal cortex and temporal lobe in Wfs1-deficient mice.

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