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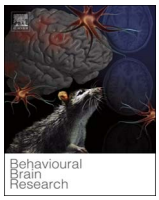

Research report

Mild stress induces brain region-specific alterations of selective ER stress markers' mRNA expression in Wfs1-deficient mice

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ABSTRACT

In this work, the effect of mild stress (elevated plus maze test, EPM) on the expression of endoplasmic reticulum (ER) stress markers in different brain areas of wild type (WT) and Wfs1-deficient (Wfs1KO) mice was investigated. The following ER stress markers were studied: activating transcription factor 6α (Atf6α), protein kinase-like ER kinase (Perk), X-box binding protein 1 (Xbp1) and its spliced form (Xbp1s), 78-kilodalton glucose regulated protein (Grp78), 94-kilodalton glucose regulated protein (Grp94), C/EBP homologous protein (Chop). Wfs1KO and WT mice, not exposed to EPM, had similar patterns of ER stress markers in the studied brain areas. The exploratory activity of Wfs1KO mice in the EPM was inhibited compared to WT mice, probably reflecting increased anxiety in genetically modified mice. In response to the EPM, activation of inositol-requiring transmembrane kinase and endonuclease 1α (Ire1α) ER stress pathway was seen in both genotypes, but in different brain areas. Such a brain region-specific Ire1α activation was linked with dominant behavioural trends in these mice as more anxious, neophobic Wfs1KO mice had increased ER stress markers expression in the temporal lobe, the brain region related to anxiety, and more curious WT mice had ER stress markers increased in the ventral striatum which is related to the exploratory drive. The molecular mechanism triggering respective changes in ER stress markers in these brain regions is likely related to altered levels of monoamine neurotransmitters (serotonin, dopamine) in Wfs1KO mice.

Endoplasmic reticulum (ER) coordinates synthesis, folding, and posttranslational modification of proteins, cytoplasmic and mitochondrial metabolism, Ca^{2+} storage and cell death. Accumulation of unfolded proteins causes ER stress [1], which activates different ER stress pathways: activating transcription factor 6α (Atf6α), inositol-requiring transmembrane kinase and endonuclease 1α (Ire1α) and protein kinase-like ER kinase (Perk). When activated, each of these factors can initiate unfolded protein response (UPR), either leading to the recovery of ER homeostasis or triggering apoptosis [1,2]. ATF6 pathway leads to the production of chaperones Grp78 and Grp94 and the activation of X-box binding protein 1 (Xbp1) while Perk phosphorylates translation initiation factor elF2α leading to the inhibition of translation as well as the activation of C/EBP homologous protein (Chop). Ire1α pathway regulates Xbp1 splicing. Spliced Xbp1 (Xbp1s) induces UPR target genes encoding factors involved in ER protein folding and degradation (Fig. 1) [1].

Wolfram syndrome (WS) is caused by mutations in the *WFS1* gene and characterized by diabetes mellitus, diabetes insipidus, optic nerve atrophy and deafness. Often anxiety and depression are seen in these patients [3]. Elevated expression of Chop, Grp78, Grp94 and Xbp1 has been shown in diabetes, neurodegeneration and depression [4,5]. Besides pathological aspect of ER stress it is known that psychological stress can also induce ER stress activation [6]. In most of the previous animal studies the expression of ER stress markers has been investigated in depression models which are caused by moderate or severe stressors [6,7]. These studies have revealed increased expression of majority of ER stress markers studied. The effect of mild stress on ER stress activation is not yet known. The aim of the present study was to establish the effect of mild stress on the expression of ER stress related genes (Grp78, Grp94, Xbp1, Perk, Chop, Atf6α) in the ventral striatum, temporal lobe and hippocampus of wild-type (WT) and Wfs1-deficient (Wfs1KO) mice. Elevated plus-maze test (EPM), a test to study neurochemical and genetic mechanisms underlying anxiety-like behaviour [8], was used to induce a mild stress. In response to stress, altered expression of ER stress markers were expected in Wfs1KO mice, because previous studies have revealed their lower motivational drive/higher avoidance behaviour accompanied with increased sensitivity to anxiolytics and antidepressants [9,10], and altered activity of anxiety- and

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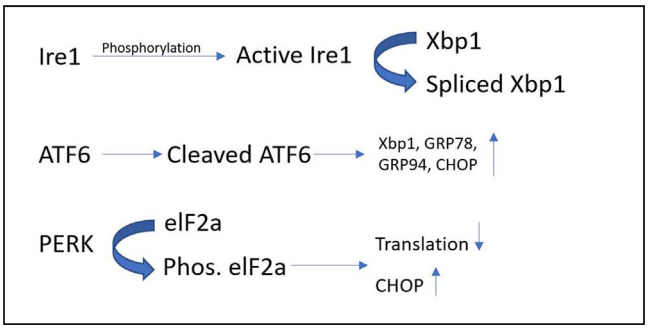

Fig. 1. Schematic representation of UPR response pathways.

depression-related neurotransmitters [10,11].

Wfs1KO mice were generated by invalidating the 8th exon of the Wfs1 gene. The experiments were performed in 2–3 months old female WT and Wfs1KO F2 hybrid mice. Number of animals was 6–9 in each group. Female mice were chosen for this experiment, since Wfs1 deficiency affects their health condition less than in males [9]. The mean body weight of Wfs1KO mice was significantly lower compared to WT littermates (18.6 ± 0.5 and 21.7 ± 0.6, respectively). The animals were housed in respective home cages under a 12 h light/dark cycle (lights on at 07:00). Tap water and food pellets were available *ad libitum*, except during testing. Permission for the present study was given by the Estonian National Board of Animal Experiments (No. 88, 25th of August 2011) in accordance with the European Communities Directive of September 2010 (2010/63/EU).

EPM employs elements of neophobia, exploratory drive and approach/avoidance conflict [12]. According to the Directive 2010/63/EU it is categorized as mild procedure (http://ec.europa.eu/environment/chemicals/lab_animals/pdf/guidance/directive/en.pdf). The plus-maze was constructed and test performed as described before [13]. The following measures were registered by the observer: (1) % of open arm (OA) entries [number of OA entries/number of total arm entries x 100]; (2) % on OA [time on OA/time on total arm x 100]; (3) number of unprotected head-dippings (counted only when animal was on OA); (4) risk assessment behaviour (sum of the number of stretch-attend postures and the number of attempts to enter the central platform located between open and closed arms), (5) the number of closed arm entries. For gene expression studies, experimentally naïve animals and animals exposed to the EPM, belonging to both genotypes, were used in parallel. Mice were decapitated immediately after behavioural testing or taking them out from the home-cage. To avoid the negative impact of this procedure on other animals, a separate room from the testing room was used. The brain was rapidly removed from the skull, dissected and snap-frozen in liquid nitrogen. The brain dissection was performed according to the coordinates presented in the mouse brain atlas [14].

Total RNA extraction, cDNA synthesis and qRT-PCR were performed as described before [13]. Results were normalized to Hprt1 and presented according to Livak and Schmittgen [15]. The assays and the sequences of primers and probes are given in Table S1 (Supplementary material).

The results are expressed as mean values ± S.E.M. In the case of EPM, nonparametric data were analyzed by using Mann-Whitney's *U* test. For gene expression studies, two-way ANOVA (genotype and EPM exposure as independent variables) was applied. Post hoc comparisons were performed by means of Tukey's HSD test. Statistical analyses were done using the Statistica V10 (Statsoft Inc., Oklahoma, USA).

In the EPM, Wfs1KO mice displayed more anxiety-like behaviour compared to WT mice. This was reflected by significantly decreased% of OA entries ($Z = 1.97$, $p = 0.05$), time spent on OA ($Z = 2.11$, $p = 0.03$) and frequency of unprotected head-dippings ($Z = 1.98$, $p = 0.05$; Fig. 2A) without significant increase in general activity (number of closed arm entries ($Z = 0.15$, $p = 0.88$; Fig. 2C). Additionally, Wfs1KO mice demonstrated increased risk assessment behaviours compared to WT littermates ($Z = -2.49$, $p = 0.01$; B).

We did not detect any significant changes in ER stress related genes in the studied brain areas of naïve Wfs1KO mice compared to respective WT littermates. In response to EPM exposure, WT mice had significantly increased expression of Xbp1 s mRNA in the ventral striatum ($F(1, 20) = 10,95$; $p = 0.002$) (Fig. 3A), Grp78 ($F(1,32) = 8397$; $p = 0.029$) (Fig. 4B) and Xbp1s ($F(1,31) = 11,02$; $p = 0.009$) in hippocampus (Fig. 3C), whereas Wfs1KO exposed to the EPM displayed higher levels of Xbp1 s ($F(1,20) = 11,06$; $p = 0.039$) (Fig. 3B) and Grp94 ($F(1,20) = 4,945$; $p = 0.029$) (Fig. 4A) mRNA in the temporal lobe and the ventral striatum, respectively. Comparison of EPM-exposed groups showed that in the ventral striatum, WT mice had significantly higher expression of Chop compared to Wfs1KO mice ($F(1,20) = 7169$; $p = 0.020$) (Fig. 4A).

Although, two-way ANOVA showed significant effects of genotype (in hippocampus) and EPM exposure (in ventral striatum and temporal lobe) in PERK expression, these changes did not reach statistically significant level in post hoc analysis (Fig. 4 and Supplementary material Fig. S2).

No genotype- or exposure-related differences were observed in the expression of Grp78 in ventral striatum (Fig. 4A), the expression of Xbp1t (Fig. 3B), Grp78, Grp94 and Chop in temporal lobe (Supplementary material Fig. S2) and the expression of Grp94 (Fig. 4B) and Xbp1t (Fig. 3C) in hippocampus. There were no statistically significant differences in mRNA expression of Atf6α in any studied brain structures (Supplementary material Fig. S1).

Activity on OA reflects animal's innate motivation to explore novel environment [12] and risk assessment behaviour can be interpreted as cautious exploration (neophobia) induced by potentially dangerous novel environment [16]. In terms of behaviour-brain relationships, neophobic and/or low exploratory behaviour, like it was seen in Wfs1KO mice, is regulated by the amygdala [17]. Increased exploratory drive, as it was observed in WT mice, is regulated by the ventral striatum [18]. Both reward- and goal-directed as well as emotion-related functions are related to the hippocampus [19].

The EPM test induces mild and short-term stress and this could explain why changes were detected only in some ER stress markers. This is different from other studies where more severe stressors induced an increase in the expression of most of the ER stress related genes in different brain regions [6,7]. Mild stressor, on the contrary, allows to investigate the induction of earlier and more sensitive ER stress markers. Among them, Xbp1s mRNA was significantly increased in both genotypes after exposure to the EPM, whereas higher expression of Xbp1s was found in brain regions responsible for dominant behavioural trend of the genotype, i.e. in more anxious Wfs1KO mice in the temporal lobe and in more curious WT mice in the ventral striatum. Huang et al. also found in their study that protein levels of Grp78 and Chop were higher in the amygdala of mice that were susceptible to defeat stress, but not in unsusceptible mice [20].

It is proposed that ER stress activation by psychological stressors is triggered by oxidative stress and oxidative stress could be activated by neurotransmitters. For example, oxidative stress can be triggered by auto-oxidation of dopamine (DA) which generates H_2O_2 [21] or by tryptophan, precursor of serotonin (5-HT), which induces lipid peroxidation and decreases antioxidant capacities [22]. Our previous studies have shown significantly impaired function of dopaminergic and serotonergic systems in naïve Wfs1KO mice [10,11]. Whereas, mild stress caused increased levels of DA and 5-HT in the striatum of WT mice, but not in Wfs1KO mice, which explains the elevation of Xbp1s in the ventral striatum of WT mice. Extending these causal connections to the temporal lobe, contrary dynamics of neurotransmitters between genotypes would be expected, i.e. the EPM should induce higher levels of neurotransmitters in Wfs1KO mice leading to higher expression of Xbp1s mRNA, whereas in WT mice the level of neurotransmitters and