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# Deletion of the Wolfram syndrome-related gene Wfs1 results in increased sensitivity to ethanol in female mice

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ABSTRACT

Wolfram syndrome, induced by mutation in WFS1 gene, increases risk of developing mood disorders in humans. In mice, Wfs1 deficiency cause higher anxiety-like behaviour and increased response to anxiolytic-like effect of diazepam, a $GABA_A$ receptor agonist. As GABAergic system is also target for ethanol, we analysed its anxiolytic-like and sedative properties in Wfs1-deficient mice using elevated plus-maze test and tests measuring locomotor activity and coordination, respectively. Additionally loss of righting reflex test was conducted to study sedative/hypnotic properties of ethanol, ketamine and pentobarbital. To evaluate pharmacokinetics of ethanol in mice enzymatic colour test was used. Finally, gene expression of alpha subunits of $GABA_A$ receptors following ethanol treatment was studied by real-time-PCR. Compared to wild-types, Wfs1-deficient mice were more sensitive to ethanol-induced anxiolytic-like effect, but less responsive to impairment of motor coordination. Ethanol and pentobarbital, but not ketamine, caused longer duration of hypnosis in Wfs1-deficient mice. The expression of Gabra2 subunit at 30 minutes after ethanol injection was significantly increased in the frontal cortex of Wfs1-deficient mice as compared to respective vehicle-treated mice. For the temporal lobe, similar change in Gabra2 mRNA occurred at 60 minutes after ethanol treatment in Wfs1-deficient mice. No changes were detected in Gabra1 and Gabra3 mRNA following ethanol treatment. Taken together, increased anxiolytic-like effect of ethanol in Wfs1-deficient mice is probably related to altered Gabra2 gene expression. Increased anti-anxiety effect of $GABA_A$ receptor agonists in the present work and earlier studies (Luuk et al., 2009) further suggests importance of Wfs1 gene in the regulation of emotional behaviour.



## 1. Introduction

Mutations in WFS1 gene cause Wolfram syndrome (WS, MIM222300), a severe multisystem disorder with progressive neurodegeneration and diabetes mellitus. Patients with WS have often been diagnosed with psychiatric disorders. Therefore, it has been suggested that WFS1 gene might play a role in the regulation of emotions (Koido et al., 2005; Swift and Swift, 2005; Swift et al., 1990). In order to study this hypothesis, Wfs1-deficient mice were generated (Luuk et al., 2008). Indeed, a link between emotionality and Wfs1 protein was found as stress increased avoidance behaviour and induced higher plasma corticosterone levels in mice lacking Wfs1 gene (Luuk et al., 2009). In addition, Wfs1-deficient mice were more sensitive to the anxiolytic-like effect of diazepam, a gamma-aminobutyric acid (GABA) positive allosteric modulator at $GABA_A$ receptor, and experimentally naive mice displayed altered expression of alpha 1 and 2 subunits of $GABA_A$ receptor (Luuk et al., 2009; Raud et al., 2009). Alpha 1 and alpha 2 subunits are responsible for benzodiazepine-induced sedative and

*Abbreviations:* WS, Wolfram syndrome; GABA, gamma-aminobutyric acid; LORR, loss of righting reflex; NMDA, N-methyl-D-aspartate; qRT-PCR, quantitative real-time PCR.

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stimulating properties, respectively (Löw et al., 2000; McKernan et al., 2000; Rudolph and Möhler, 2004). It has been reported that alpha 3 subunit of $GABA_A$ receptor may also participate in the regulation of anxiety as nonselective and selective alpha 3 $GABA_A$ receptor modulators exhibit anti-anxiety effect in mice (Morris et al., 2006; Navarro et al., 2006) and rats (Atack et al., 2005, 2006).

Several studies have confirmed that $GABA_A$ receptor is also one of the targets for action of ethanol in the central nervous system (Criswell and Breese, 2005; Grobin et al., 1998: Koob, 2006; Kumar et al., 2009; Nutt and Malizia, 2001; Weiner and Valenzuela, 2006). At low concentration, ethanol affects GABAergic neurotransmission by increased release of GABA (Roberto et al., 2004) and release of substances which are active at the $GABA_A$ receptors (Barbaccia et al., 1999). At high concentration, ethanol has a direct effect on $GABA_A$ receptor (Mihic et al., 1997; Weiner and Valenzuela, 2006) and, therefore, may affect the expression of the subunits of $GABA_A$ receptor responsible for certain behavioural effects of ethanol.

So far the specific roles of each subunit of $GABA_A$ receptor in ethanol-induced behavioural modifications are not fully clear. For example, in one study the hypnotic effect of ethanol was found to be modulated by alpha 2 $GABA_A$ receptors (Boehm et al., 2004), while in other studies (Blednov et al., 2011; Dixon et al., 2012) this finding was not supported. Concerning regulation of anxiety, studies with knockout mice lacking different subunits of $GABA_A$ receptor (e.g. alpha 1, alpha 2, alpha 4, alpha 5, delta, gamma 2L, rho 1) have failed to reveal specific subunit responsible for anxiolytic effect of ethanol (Boehm et al., 2004; Blednov et al., 2014; Chandra et al., 2008; Homanics et al., 1999; Kralic et al., 2003; Mihalek et al., 2001). Unexpected results were obtained with alpha 1 knock-in mice, demonstrating significantly increased ethanol-induced anxiolytic effect and explained by the compensatory changes in cortical alpha 2 or 3 subunits of $GABA_A$ receptor (Borghese et al., 2006; Werner et al., 2006).

The aim of the present study was to explore the effects of ethanol on exploratory and motor behaviour, righting reflex, metabolism and $GABA_A$ subunits gene expression of Wfs1-deficient mice. For that purpose the following methods were applied:

1. The elevated plus-maze test was used to study the anxiolytic-like property of ethanol. In order to examine whether exploratory activity in this test could be influenced by the effect of ethanol on the motor activity in animals, the motility test was also applied. In addition, the sedative/ataxic effect of ethanol was evaluated by means of the rotarod test, measuring coordination.
2. The loss of righting reflex (LORR) test was used to determine the sedative/hypnotic action of ethanol. The influences of ethanol on neurons include both the potentiation of inhibitory $GABA_A$ receptors (Harris et al., 1995; Wallner et al., 2003) and antagonism of the excitatory N-methyl-D-aspartate (NMDA) receptors (Lovinger et al., 1989, 1990). In order to clarify whether ethanol-induced LORR behaviour is mediated via $GABA_A$ or NMDA receptors, pentobarbital, a $GABA_A$ receptor modulator, and ketamine, an antagonist of NMDA receptors were applied.
3. The enzymatic colour test was used for ethanol metabolism studies. This was done to ensure that changes in the action of ethanol were not due to its modified metabolism in Wfs1-deficient mice.
4. Finally, quantitative real-time PCR (qRT-PCR) analysis was used to measure the gene expression level of alpha1-3 subunits (Gabra1-3) of $GABA_A$ receptors in the frontal cortex and temporal lobe of naive and ethanol-exposed mice. The frontal cortex and temporal lobe were chosen because these brain areas have high concentrations of Wfs1 mRNA and Wfs1-deficient mice display reduced expression of Gabra1 and 2 genes in these structures (Luuk et al., 2008; Raud et al., 2009).

## 2. Materials and methods

### 2.1. Animals

Experiments were performed in 3–4 months old female wild-type, heterozygous and Wfs1-deficient F2 hybrid mice ([129S6/SvEvTac × C57BL/6] × [129S6/SvEvTac × C57BL/6)] Luuk et al., 2008). Breeding and genotyping were conducted in the Department of Physiology, Institute of Biomedicine and Translational Medicine, University of Tartu. The animals were kept eight per cage at 21 ± 2 °C in a room illuminated artificially from 7 am to 7 pm. Tap water and food pellets were available *ad libitum*. The permission (No. 88, 25th of August, 2011) 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 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. The behavioural experiments were carried out between 10:00 and 17:00. Wfs1-deficient mice were always used in parallel with wild-type and heterozygous mice and the animals were randomly divided into the experimental groups.

### 2.2. Treatment

In the elevated plus-maze test and locomotor activity test, three doses of ethanol (0.5, 1 and 2 g/kg) were used. 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 μl/10 g.

### 2.3. Behavioural studies

#### 2.3.1. Elevated plus-maze test

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 spatio-temporal measures of anxiety whereas the number of unprotected head dippings is an ethological measure which can be considered "risk assessment" behaviour (Rodgers and Johnson, 1995). The number of closed arm entries reflects motor activity of rodents (File, 1992).

#### 2.3.2. Locomotor activity in motility test

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

#### 2.3.3. Motor coordination in rotarod test

Rotarod is one of the standard tests to measure coordination, balance and motor skill learning. The learning effect appears as the elongated failing latency with the trial numbers (Shiotsuki et al., 2010). This test also enables to evaluate sedation (Söderpalm et al., 1989; Tang et al., 1995; Steiner et al., 2011). The equipment consisted of a motor-driven drum (3 cm in diameter) rotating at 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 (Kõks et al., 2001).

#### 2.3.4. Loss of righting reflex (LORR)

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