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Research report

Pharmacological treatment of fragile X syndrome with GABAergic drugs in a knockout mouse model

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ABSTRACT

Molecular and electrophysiological studies have provided evidence for a general downregulation of the GABAergic system in the *Fmr1* knockout mouse. $GABA_A$ receptors are the main inhibitory receptors in the brain and the $GABA_A$ receptor was proposed as a novel target for treatment of the fragile X syndrome, the most frequent form of intellectual disability. This study examined the functionality of the $GABA_A$ receptor in rotarod and elevated plus maze tests with fragile X mice treated with $GABA_A$ receptor agonists, the benzodiazepine diazepam and the neuroactive steroid alphaxalone. In addition, the effect of $GABA_A$ receptor activation on the audiogenic seizure activity was determined. We proved that the $GABA_A$ receptor is still sensitive to GABAergic drugs as the sedative effect of diazepam resulted in a decreased latency time on the rotarod and alphaxalone had a clear anxiolytic effect in the elevated plus maze, decreasing the frequency of entries, the total time spent and the path length in the closed arms. We also observed that treatment with ganaxolone could rescue audiogenic seizures in *Fmr1* knockout mice. These findings support the hypothesis that the $GABA_A$ receptor is a potential therapeutic target for fragile X syndrome.

1. Introduction

Fragile X syndrome is the most common form of intellectual disability. Besides cognitive impairment, patients suffer from several behavioural problems including hyperactivity, sleep problems and autistic-like behaviour [1]. Epileptic seizures are also commonly observed in patients [2]. The syndrome is caused by a dynamic expansion of a CGG triplet located within the 5′ untranslated region of the *fragile X mental retardation 1* (*FMR1*) gene [3]. Due to the dynamic mutation, the CGG repeat and the surrounding CpG island located in the promoter region of the gene become hypermethylated, leading to transcriptional silencing of *FMR1* and consequently absence of the *FMR1* protein product, FMRP [4]. FMRP is an RNA-binding protein that interacts with various neuronal mRNAs and is involved in the regulation of mRNA translation, transport and stability [5–9]. Absence of FMRP might lead to deregulation of many neuronal mRNAs eventually cumulating in the fragile X phenotype.

One of the main pathways affected in the fragile X syndrome is the $GABA_A$ receptor pathway. We have found an altered expression of several components of the GABAergic system in the *Fmr1* knockout mouse, including 8 subunits of the $GABA_A$ receptor ($\alpha_{1,\,3\text{ and }4}$, $\beta_{1\text{ and }2}$, $\gamma_{1\text{ and }2}$ and δ), proteins and enzymes involved in synthesis (*Gad 1*), transport (*Gat 1* and *Gat 4*) and degradation (*Ssadh*) of GABA and in the clustering and targeting of the $GABA_A$ receptors at the post-synaptic membrane (*Gephyrin*) [10,11]. Underexpression was also found for all three $GABA_A$ receptor subunits (*Grd*, *Rdl* and *Lcch3*) in the fragile X fly [10]. Other groups demonstrated decreased protein levels of several $GABA_A$ receptor subunits and abnormal GABA-mediated transmission in the fragile X mouse [12–15]. The combination of all these molecular and electrophysiological findings together with the fact that $GABA_A$ receptors are implicated in anxiety, depression, learning and memory, epilepsy and insomnia, all presenting in the fragile X syndrome, led us to propose the $GABA_A$ receptor as a novel target for treatment of the fragile X syndrome [16].

As the GABAergic system is compromised in the fragile X syndrome, it cannot *a priori* be excluded that the sensitivity of the receptor for $GABA_A$ receptor agonists is reduced in fragile X patients. In order to investigate the therapeutic potential of this type of drugs, we first wanted to investigate whether the $GABA_A$ receptors in the knockout mouse are amendable to treatment. By administering equal doses of GABAergic drugs to *Fmr1* knockout

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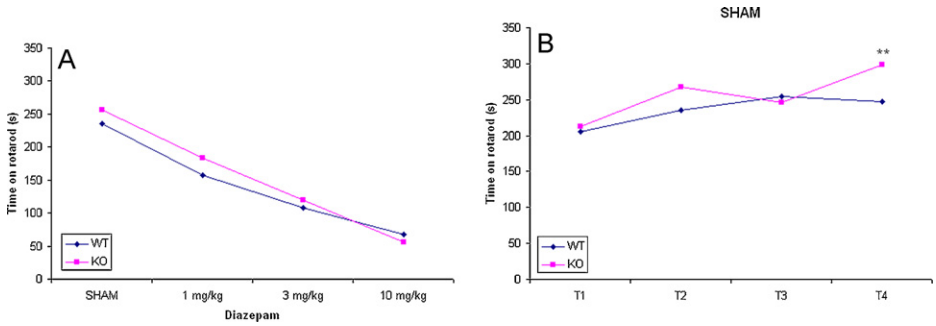

Fig. 1. Motor-coordination was tested in the rotarod assay. Time spent on the rotarod is presented for wild-type (n = 15–17/dose) and *Fmr1* knockout mice (n = 15/dose). (A) Mean total effect of diazepam on rotarod performance per concentration diazepam. (B) Performance over all trials of SHAM-treated mice. $^{**}p < 0.01$.

and control animals and comparing the performance in selected tests, we can determine the potential difference in drug sensitivity between both genotypes.

For our experiments, we selected two types of drugs that act on two different prevalent subtypes of the receptor. Diazepam, a classical benzodiazepine, binds $GABA_A$ receptors containing a β, γ_2 and either an α_1, α_2, α_3 or α_5 subunit [17]. Diazepam enhances the affinity of this most frequently present subtype of the receptor for GABA, resulting in an increased inhibition and thus a sedative effect. Neuroactive steroids are an entirely different class of agonists. Examples are the endogenous neurosteroid allopregnanolone, a metabolite of the steroid hormone progesterone and the synthetic drugs alphaxalone and ganaxolone [18]. These compounds bind predominantly to the δ-containing extrasynaptic $GABA_A$ receptor subtypes and regulate anxiety, stress and neuronal excitability by increasing both channel-open frequency and open duration [19,20]. At high concentrations, neurosteroids can even directly activate $GABA_A$ receptor channels [21]. Ganaxolone has a similar pharmacological activity as alphaxalone but due to its 3β-methyl substituent, ganaxolone is orally active and lacks hormonal side effects [22]. It was especially developed for its improved bioavailability and potential anxiolytic and anticonvulsant activity.

As sedation and anxiety are modulated through the $GABA_A$ receptor, we performed a motor-coordination test (rotarod) and an anxiety-related test (elevated plus maze) to determine the functionality of the $GABA_A$ receptor in fragile X mice. With a rotarod test, the motor-coordination and balance of a mouse is tested by placing the mouse on a rotating rod with accelerating speed. The elevated plus maze is commonly used to assess anxiety-like behaviour in mice models. The task is based on the naturalistic conflict between the tendency of mice to explore a novel environment and the aversive properties of a brightly lit, open area [23]. When anxious, the natural tendency of rodents is to prefer enclosed dark spaces over open brightly lit spaces. In addition, as several studies have shown that the $GABA_A$ receptor is implicated in epilepsy [24], we investigated the effect of GABAergic drugs on the audiogenic seizure phenotype. We did find that the $GABA_A$ receptor is a suitable target for treatment of at least some behavioural symptoms of the fragile X syndrome.

2. Materials and methods

2.1. Animals

Male *Fmr1* knockout mice and their control littermates (C57BL/6J background) were housed, bred and genotyped as described previously [10]. All experiments were carried out in compliance to the European Community Council Directive (86/609/EEC) and approved by the Animal Ethics Committee of the University of Antwerp.

2.2. Rotarod

We used an automated accelerating rotarod (Ugo Basile, Comerio, Varese, Italy; accelerating model 7650 for mice). Mice, 10 weeks old, were placed on a rotating drum and the latency to fall from the rotarod was recorded. Mice were given two practice trials (4 revolutions per minute (rpm), max 2 min) and four accelerated test trials (4–40 rpm, max 5 min), with 1 min between each trial. Mice were injected with diazepam (Roche, 1 mg/kg, 3 mg/kg or 10 mg/kg) or PBS (SHAM), 30 min prior to the test. Each group contained 15–17 mice. The results of the rotarod were analysed with a two-way ANOVA and a two-way repeated measures ANOVA.

2.3. Elevated plus maze

The elevated plus maze (EPM) is a cross-shaped maze, with two open arms and two closed arms, about a metre above the floor. It was constructed as described [23]. A stock solution of alphaxalone was made in a 22.5% 2-hydroxypropyl-β-cyclodextrin (HBC, Sigma–Aldrich; cat# 56332) aqueous solution. The solubility of lipophilic drugs increases linearly with the concentration of HBC and the product is non-toxic in rabbits and mice. AP (Steraloids Inc. Ltd., London, UK; cat# P3500-000) or an equal amount of solvent (SHAM) was injected 10 min before testing (n = 16/group for wild-type mice, n = 10–15/group for knockout mice; 15 mg/kg, i.p.).

At the start of the 10-min observation (EthoVision 3.1; Noldus Information Technology, Wageningen, The Netherlands), mice (10 weeks old) were placed on the central platform, facing one of the closed arms (preferably left). The parameters measured in the test were total path length, velocity and rearing in total EPM and frequency, time spent, path length, velocity and rearing in different parts of the EPM. Data were analysed using a two-way ANOVA and individual groups were compared by using a post hoc t-test or a Mann–Whitney rank sum test.

2.4. Audiogenic seizures

Mice were tested in an empty, transparent plastic box (28 cm × 18 cm × 17 cm) covered by a plate with a fire siren mounted on it. The box was placed into a sound-attenuating chamber equipped with a glass door for observation and the tests were recorded. After a 2-min habituation period, animals were exposed to a 120 dB noise (fire siren) until seizures were initiated or with a maximum of 5 min. Mice were tested between 02:00 p.m. and 05:00 p.m. for possible circadian variation.

Seizures were scored by the time of occurrence (test day) and by severity: no response = 0, wild running = 1, clonic seizures = 2, tonic seizures = 3 and respiratory arrest = 4. An intraperitoneal injection of diazepam (3 mg/kg) or ganaxolone (10 mg/kg) or an equal volume of vehicle (22.2% 2-hydroxypropyl-β-cyclodextrin) was administered 10 min (ganaxolone) or 30 min (diazepam and SHAM) before seizure testing. Mice were tested on 3 days with 21, 24 and 25 days of age and were injected on day 2 and 3 with drug or vehicle. Numbers of mice in each group were as follows: n = 11, 9, 12 wild-type mice and n = 18, 12, 11 knockout mice for SHAM, diazepam and ganaxolone treatment, respectively. Fisher's exact test (two-tailed) was used to analyse the percentage of seizures.

3. Results

3.1. $GABA_A$ receptors in fragile X mice are still sensitive to benzodiazepines

To investigate potential differences in the drug sensitivity of the most common $GABA_A$ receptor subtypes, we performed a rotarod test where we compared the performance of fragile X mice with control littermates using different concentrations of diazepam. We found a significant effect for treatment for both