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## Research Report

# Decreased expression of the GABA<sub>A</sub> receptor in fragile X syndrome

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### ABSTRACT

After our initial discovery of under expression of the GABA<sub>A</sub> receptor  $\delta$  subunit in a genome wide screening for differentially expressed mRNAs in brain of fragile X mice, a validated model for fragile X mental retardation syndrome, we analyzed expression of the 17 remaining subunits of the GABA<sub>A</sub> receptor using real-time PCR. We confirmed nearly 50% under expression of the  $\delta$  subunit and found a significant 35%–50% reduction in expression of 7 additional subunit mRNAs, namely  $\alpha_1$ ,  $\alpha_3$ , and  $\alpha_4$ ,  $\beta_1$  and  $\beta_2$  and  $\gamma_1$  and  $\gamma_2$ , in fragile X mice compared to wild-type littermates. In concordance with previous results, under expression was found in cortex, but not in cerebellum. Moreover, decreased expression of specific GABA<sub>A</sub> receptor subunits in fragile X syndrome seems to be an evolutionary conserved hallmark since in the fragile X fly (*Drosophila melanogaster*) model we also found almost 50% under expression of all 3 subunits which make up the invertebrate GABA receptor, namely Grd, Rdl and Lcch3. In addition, we demonstrated a direct correlation between the amount of dFmrp and the expression of the GABA receptor subunits Rdl and Grd. Our results add evidence to previous observations of an altered GABAergic system in fragile X syndrome. Because GABA<sub>A</sub> receptors are the major inhibitory receptors in brain, involved in anxiety, depression, insomnia, learning and memory and epilepsy, processes also disturbed in fragile X patients, the well described GABA<sub>A</sub> receptor pharmacology might open new powerful opportunities for treatment of the behavioral and epileptic phenotype associated with fragile X syndrome.

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## 1. Introduction

Fragile X syndrome is the most common form of inherited mental retardation, with a prevalence of 1 to 4000 in males

and 1 to 6000 in females [reviewed by Bardoni et al. (2006), Gantois et al. (2004), O'Donnell and Warren (2002)]. Patients are characterized by mild to severe impairment of the higher cognitive functions and display various physical

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abnormalities, e.g., macroorchidism (enlarged testes) and craniofacial anomalies such as a typical long face, prominent jaws and elongated ears (Hagerman, 2002). Associated behavioral problems include hyperactivity and autistic-like features. In addition, 20% of the patients suffer from epileptic seizures. The syndrome is usually caused by a dynamic mutation of a CGG repeat in the 5' untranslated region of the fragile X mental retardation gene 1 (FMR1) (Verkerk et al., 1991). Elongation of this repeat above a threshold of 200 copies induces hypermethylation of the CpG islands in the promoter region and concomitant transcriptional silencing, preventing synthesis of the FMR1 gene product FMRP (Pieretti et al., 1991).

FMRP is an RNA-binding protein with particular high expression in neurons and gonads. The protein aggregates with multiple mRNAs and proteins to form a messenger ribonucleic protein complex (mRNP), which is transported out of the nucleus through its nuclear export signal (Jin and Warren, 2003). Once in the cytoplasm, the complex can associate with members of the RNA-induced silencing complex (RISC) before associating with ribosomes. The FMRP-mRNP complex can be transported through dendrites to actively translating polyribosomes near the synapses, where it may play a role in local protein synthesis as a translational inhibitor (Laggerbauer et al., 2001; Li et al., 2001; Zalfa et al., 2003). Major mechanisms by which FMRP is thought to exert its repressing activity are through the RNA interference pathway or by acting as a nucleic acid chaperone (Bardoni et al., 2006; Gabus et al., 2004; Jin et al., 2004).

Interruption of the murine *Fmr1* gene generated a mouse model for fragile X syndrome (Bakker et al., 1994). Fragile X knockout mice show mild cognitive deficits, hyperactivity, macroorchidism and increased sensitivity to epileptic seizures, features comparable with symptoms observed in fragile X patients (Bakker and Oostra, 2003; Kooy, 2003). Pathological studies revealed the presence of long tortuous, immature dendritic spines being denser along dendrites, as observed in patients (Braun and Segal, 2000; Comery et al., 1997; Irwin et al., 2002; Nimchinski et al., 2001). FMRP may therefore affect synaptic development and maturation in the central nervous system. The invertebrate homologue of *Fmr1* in fruit flies, namely 'Drosophila melanogaster fragile X mental retardation gene 1' (*dFmr1*), exhibits high neuronal expression levels. The associated gene product *dFmrp* displays considerable amino acid sequence identity/similarity with the vertebrate FMRP, especially within the functional domains. It possesses similar RNA-binding capacity as well as the ability to interact with human FMR1 (Wan et al., 2000). *dFmr1* deficient fly models have been generated (Dockendorff et al., 2002; Michel et al., 2004; Morales et al., 2002; Zhang and Broadie, 2005). *dFmrp* is required for normal neurite expansion, guidance and branching. Loss of *dFmrp* causes behavioral defects like abnormal eclosion and circadian rhythm behavior and anomalies in the morphology of several central nervous system neuronal populations.

Despite increased insights in the function of FMRP in the cell, the central question why absence of FMRP causes mental retardation and additional symptoms in fragile X patients remains to be elucidated. In a previous genome wide expression profiling study, our group found differential expression in neurons of specific brain parts from fragile X knockout mice limited to 3 cDNAs only, including the  $\delta$  subunit of the GABA<sub>A</sub>

receptor (Gantois et al., 2006). To further investigate a possible role of decreased expression of this ion channel in fragile X syndrome, we determined the relative expression of all GABA<sub>A</sub> receptor subunits in the mouse and fly model using real-time PCR. We found evidence that under expression of multiple subunits of the GABA<sub>A</sub> receptor is an evolutionary conserved hallmark of fragile X syndrome. As GABA<sub>A</sub> receptors are the main inhibitory receptors in brain, involved in processes also disturbed in fragile X patients such as anxiety, depression, epilepsy, insomnia and learning and memory (Mihalek et al., 1999), we believe that new powerful therapeutic opportunities for treatment of behavioral problems associated with fragile X syndrome might arise from these observations.

## 2. Results

### 2.1. *Mus musculus*

Cortical and cerebellar tissue of adult fragile X male mice and their control littermates was isolated and reverse transcribed as indicated in the experimental procedure. These regions were selected because in the initial genome wide study under expression of the  $\delta$  subunit of the GABA<sub>A</sub> receptor was found in cortex but not in cerebellum. GABA<sub>A</sub> receptors display an extensive structural heterogeneity based on the differential assembly of a family of at least 18 different subunits ( $\alpha_{1-6}$ ,  $\beta_{1-3}$ ,  $\gamma_{1-3}$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$ ,  $\pi$  and  $\rho_{1-2}$ ) into distinct pentameric receptor complexes. Assays-on-demands® (ABI) were selected for every single known subunit of the GABA<sub>A</sub> receptor and for 3 reference genes (*Gapdh*, *Hmbs* and *Hprt*). Vandesompele et al. (2002) recommend the minimal use of 3 internal control genes to calculate the RT-PCR normalization factor to control for variables such as the amount of starting material, enzymatic efficiencies, and differences between tissues or cells in overall transcriptional activity. After performing the real-time PCR experiments, we calculated the coefficient of variance (CV) and the *M*-value for every single reference gene as described (Vandesompele et al., 2002) to analyze the stability of the internal control genes (Table 1). The measured values were amply within the norm.

For every subunit, we calculated the relative expression (RE), i.e. the ratio of the geometric means of the normalized expression values of the controls vs. the knockouts, e.g., RE ( $\delta$ ) = 58% means that the expression of the GABA<sub>A</sub> receptor  $\delta$  subunit in cortex of knockout mice is only 58% of the expression seen in cortex of control littermates, which means a reduction of 42% (Table 2). This value corresponds to our initial observations where we found a significant reduction of 45% for the expression of the  $\delta$  subunit in cortex but not in cerebellum (Gantois et al., 2006). In addition, 7 other subunits, namely  $\alpha_1$ ,  $\alpha_3$  and  $\alpha_4$ ,  $\beta_1$  and  $\beta_2$  and  $\gamma_1$  and  $\gamma_2$ , were significantly under expressed in the knockout mice. Differential expression was observed in cortex, but not in cerebellum (Table 2).

### 2.2. *Drosophila melanogaster*

Additionally, we measured the relative expression of the GABA receptor subunits in the *D. melanogaster* fragile X model (Morales et al., 2002). Ionotropic GABA receptors are found

**Table 1 – Coefficient of variance**

Brain part						
<i>Mus musculus</i>						
Reference gene	Cortex (C vs. KO)		Cerebellum (C vs. KO)			
	CV (%) <sup>a</sup>	M (geNorm) <sup>b</sup>	CV (%)	M (geNorm)		
<i>Gapdh</i>	25.13	1.3093	21.01	0.5924		
<i>Hmbs</i>	25.73	1.3120	19.00	0.5008		
<i>Hprt</i>	26.70	0.6288	29.92	0.6828		
Mean	25.85	1.0834	22.85	0.5920		
<i>Drosophila melanogaster</i>						
Reference gene	Or vs. wt		Or vs. 1r		Or vs. 2r	
	CV (%)	M (geNorm)	CV (%)	M (geNorm)	CV (%)	M (geNorm)
<i>Dsh</i>	30.05	0.7554	20.96	0.5971	23.93	0.6834
<i>Rpl32</i>	23.12	0.7554	20.72	0.5971	23.83	0.6834
Mean	26.58	0.7554	20.84	0.5971	23.88	0.6834

Or, 1r and 2r: Fly strains containing respectively none (Or), 1 (1r) and 2 (2r) *dFmr1* copies.

<sup>a</sup> The coefficient of variance represents the variation of the normalized relative quantities of a reference gene across all samples. Ideally, the variation after normalization is nihil. Hence, lower CV values denote higher stability.

<sup>b</sup> The M value is the gene expression stability as calculated by geNorm (qBase). The lower the M-value, the more stably expressed is the reference gene.

throughout the nervous system of various insect species. To date, 3 receptor subunit classes have been cloned in *D. melanogaster* with a high sequence identity to vertebrate ionotropic GABA receptors, namely *Rdl* (resistant to dieltrin), *Grd* (GABA and glycine-like receptor of *Drosophila*) and *Lcch3* (ligand-gated chloride channel homologue 3) (Hosie et al., 1997). Using real-time PCR, we compared the expression of *Rdl*, *Grd* and *Lcch3* in wild-type strains with *dFmr1*<sup>-/-</sup> mutant fruit flies (Table 2). Because reference genes for use in real-time PCR experiments are not well documented in *Drosophila*, we tested the stability of 4 different reference genes: *Dsh*, *Rpl32*, *Tf1b* and *Nadh*. Based on the coefficients of variance and M-values (geNorm) (Vandesompele et al., 2002), we selected the two most stable genes *Dsh* and *Rpl32* to normalize the real-time results (Table 1). Our results revealed significant reduction of 40%–50% in expression of all 3 subunits responsible for the assembly of the GABA receptor in *dFmr1*<sup>-/-</sup> mutants compared with the wild-type strain (Table 2).

To find out whether the expression of the GABA receptor subunits is directly regulated by FMRP, we additionally determined the expression levels in rescue strains containing 1 or 2 *dFmr1* copies, randomly inserted in the genome of the *dFmr1*<sup>-/-</sup> null mutant. We observed a significant rise in the normalized RNA amount for *Grd* (1-way ANOVA, *P*=0.016) and *Rdl* (*P*=0.009) in function of the number of *dFmr1* copies, which indicates a direct correlation between the expression of *dFmr1* and the amount of GABA receptor subunit mRNA in these two subunits (Fig. 1).

### 3. Discussion

Ionotropic receptors for the neurotransmitter  $\gamma$ -aminobutyric acid (GABA) are widespread mediators of rapid neurotransmission in the nervous systems of both vertebrates and

invertebrates. In mammals, 30–50% of all synapses in the central nervous system are GABAergic (Paredes and Agmo, 1992). GABA<sub>A</sub> receptors mediate fast synaptic inhibition in brain and spinal cord because their associated channels are permeable to Cl<sup>-</sup> ions; the flow of the negatively charged ions inhibits postsynaptic cells since the reversal potential for Cl<sup>-</sup> is more negative than the threshold for neuronal firing. Like other types of ionotropic receptors, mammalian GABA<sub>A</sub> receptors are pentamers assembled from a combination of individual subunits from 8 families with multiple isoforms:  $\alpha_{1-6}$ ,  $\beta_{1-4}$ ,  $\gamma_{1-4}$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$ ,  $\pi$  and  $\rho_{1-2}$ . The expression pattern of individual subunits generates a high diversity of GABA<sub>A</sub> receptor subtypes in a spatio-temporal dependent manner with a major functional and pharmacological diversity between GABA<sub>A</sub> receptor subtypes mutually (Barnard et al., 1998; Kneussel, 2002; Korpi et al., 2002). GABA<sub>A</sub> receptors are modulated by many drugs, including ethanol, benzodiazepines, various anesthetics and neuroactive steroids.

*D. melanogaster* has a much simpler GABA receptor system, consisting of three genes only, namely *Rdl* (resistant to dieltrin), *Grd* (GABA and glycine-like receptor of *Drosophila*) and *Lcch3* (ligand-gated chloride channel homologue 3) (Hosie et al., 1997). As in their vertebrate counterparts, the binding of GABA to the receptor causes a fast, temporary opening of anion-selective ion channels with an inhibitory response as a consequence. The subunits encoded by *Rdl* display 30%–38% homology with vertebrate GABA receptor subunits, about the same percentage of identity as seen between the different classes of vertebrate subunits (Ffrench-Constant et al., 1991). *Grd* displays 33–44% identity with vertebrate GABA<sub>A</sub> and glycine receptor  $\alpha$  subunits (Harvey et al., 1994), and *Lcch3* shows 47% identity with the vertebrate GABA<sub>A</sub> receptor  $\beta$  subunit isoforms (Henderson et al., 1993) (Fig. 2). *Lcch3* can function as a subunit with *Rdl* in Cl<sup>-</sup> channels (Zhang et al., 1995) and with *Grd* in cation

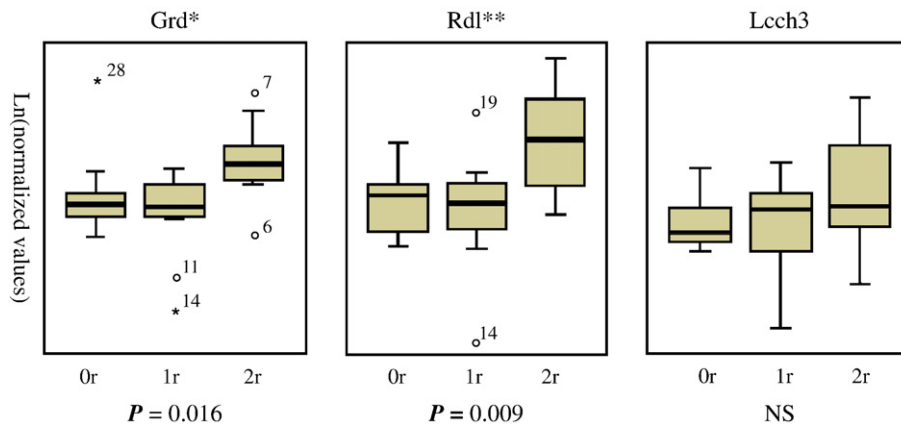
**Table 2 – Relative expression**

Mus musculus					
Subunit GABA <sub>A</sub> R	Cortex			Cerebellum	
	RE (KO)		Significance	RE (KO)	Significance
<b>α<sub>1</sub></b>	65%		p < 0.05	96%	NS
α <sub>2</sub>	90%		NS	97%	NS
<b>α<sub>3</sub></b>	58%		p < 0.01	97%	NS
<b>α<sub>4</sub><sup>a</sup></b>	77%		p < 0.05		LE
α <sub>5</sub>	83%		NS	104%	NS
α <sub>6<sup>b</sup></sub>		LE		67%	NS
<b>β<sub>1</sub></b>	67%		p < 0.05	98%	NS
<b>β<sub>2</sub></b>	55%		p < 0.01	86%	NS
β <sub>3</sub>	83%		NS	104%	NS
<b>δ</b>	58%		p < 0.01	83%	NS
<b>γ<sub>1</sub></b>	64%		p < 0.01	92%	NS
<b>γ<sub>2</sub></b>	61%		p < 0.01	97%	NS
γ <sub>3</sub>	87%		NS	113%	NS
π		LE			LE
ε		LE			LE
ρ <sub>1</sub>		LE			LE
ρ <sub>2</sub>		LE			LE
θ		LE			LE
Drosophila melanogaster					
Subunit GABAR	Wt vs. 0r				
	RE ( <i>dFmr1</i> <sup>-/-</sup> mutant)		Significance		
Grd	58%		p < 0.05		
Rdl	60%		p < 0.01		
Lcch3	51%		p < 0.01		

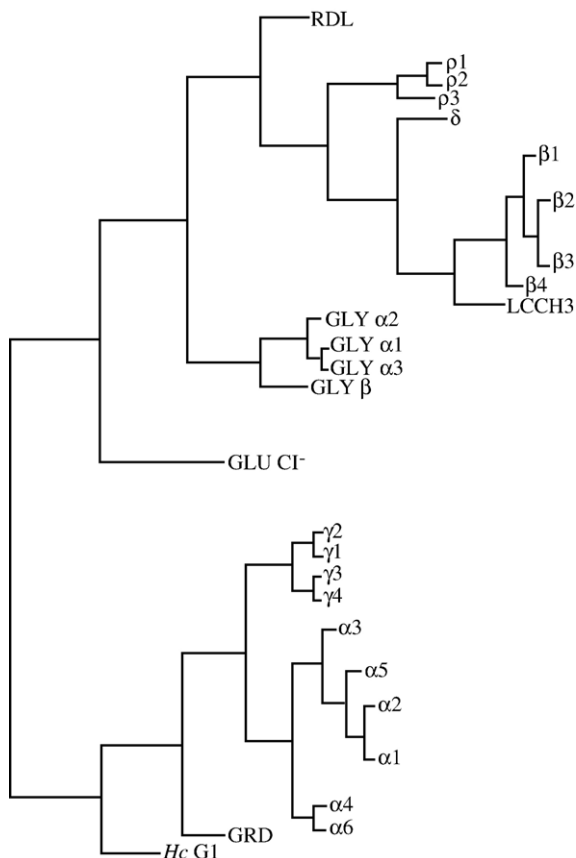
NS: not significant, LE: expression too low to detect reliable differences between samples.  
 The given percentages indicate the expression of the specific GABA<sub>A</sub> receptor subunits left in brain of respectively the mouse and fly model for fragile X syndrome. The subunits in bold are significantly under expressed.  
<sup>a</sup> Cortical subunit  
<sup>b</sup> Cerebellar subunit

channels (Gisselmann et al., 2004). It is the subunit composition which determines the picrotoxin and bicuculline sensitivity of the receptor.

Using real-time PCR, we found significant under expression of 8 out of 18 known subunits of the GABA<sub>A</sub> receptor, namely α<sub>1</sub>, α<sub>3</sub> and α<sub>4</sub>, β<sub>1</sub> and β<sub>2</sub> and γ<sub>1</sub> and γ<sub>2</sub> and δ, in cortex



**Fig. 1 – Boxplots showing the significant rise in the normalized RNA amount for Grd and Rdl (and not Lcch3) in function of the number of copies of *dFmr1* randomly inserted in the *dFmr1*<sup>-/-</sup> null mutant (rescue strains), which indicates a direct correlation between the amount of *dFmrp* and the expression of the GABA receptor subunits. The visible dots are outliers. 0r, 1r and 2r: fly strains containing respectively none (0r), 1 (1r) and 2 (2r) *dFmr1* copies. The significance was tested using one-way ANOVA.**



**Fig. 2** – A dendrogram illustrating the relative similarity of the known insect GABA receptor subunits to those of other ligand-gated anion channels. The PILEUP algorithm (Genetics Computer Group, Madison, WI, USA) was used to group all the known isoforms of these subunits on the basis of similarity in their amino acid sequences. Vertebrate GABA receptor subunits are marked as  $\alpha$ ,  $\beta$ , etc., GLY refers to GlyR subunits while Glu Cl<sup>-</sup> and Hc G1 refer to glutamate-gated chloride channels and a putative GABAR or GlyR subunits from *Haemonchus contortus*. Reprinted from *Trends in Neurosciences*, 20, Hosie et al., *Molecular biology of insect neuronal GABA receptors*, 578–583, Copyright (1997), with permission from Elsevier.

of fragile X mice, a validated model for fragile X syndrome, compared to their control littermates. The under expression of the  $\delta$  subunit is the most significant, thereby offering a possible explanation why only this subunit was picked up in our initial genome wide expression profiling study. Our results suggest under expression of both the most frequent subtype of the GABA<sub>A</sub> receptor,  $\alpha_1\beta_2\gamma_2$ , that makes up 60% of the GABA<sub>A</sub> receptors in brain and which is sensitive to benzodiazepines (e.g., Diazepam), and the  $\delta$ -containing subtype of the GABA<sub>A</sub> receptor,  $\alpha_4\beta_n\delta$ , which is sensitive to neuroactive steroids such as alfaxalone, a synthetic analogue of allopregnanolone (a natural occurring metabolite of progesterone).

In addition, we found a nearly 50% reduction in expression of all 3 GABA receptor subunits, Grd, Rdl and Lcch3, in the fragile X fruit fly compared with wild-type strains, suggesting

that under expression of specific subunits of the GABA<sub>A</sub> receptor is an evolutionary conserved hallmark of fragile X syndrome. Moreover, mRNA expression of subunits Rdl and Grd was shown to be dependent on the number of *dFmr1* copies, indicating a direct correlation between the amount of dFmrp and the expression of the GABA receptor.

We can only speculate the cause of the under expression of the GABA<sub>A</sub> receptor subunits in the fragile X animal models. Miyashiro et al. (2003) demonstrated a direct binding between FMRP and the mRNA of the  $\delta$  subunit of the GABA<sub>A</sub> receptor using the Antibody Positioned RNA Amplification (APRA) technique, suggesting a direct effect of FMRP on transport and/or localization of GABA<sub>A</sub> receptor subunits. As FMRP plays an important role in transport and translation of mRNA, it could be speculated that in the absence of FMRP RNAs, normally bound to FMRP, are misregulated and/or degraded.

Although we demonstrate under expression of various subunits at the mRNA level, results by others indicate that the protein level is also affected. Firstly, El Idrissi et al. (2005) have detected a reduced expression of the  $\beta$  subunit of the GABA<sub>A</sub> receptor on protein level in cortex, hippocampus, diencephalon and brainstem of fragile X mice using Western blot analysis. Nevertheless, additional protein expression studies are necessary to validate our results on protein level. Secondly, Gruss and Braun (2004) found that the ratio between inhibitory (taurine and GABA) and excitatory (aspartate and glutamate) amino acids in fragile X mouse brainstem, hippocampus and caudal cortex was decreased. Thirdly, electrophysiological recordings suggest a decreased GABAergic system efficiency in fragile X knock out mice that in turn may interfere with cholinergic mechanisms (D'Antuono et al., 2003). Moreover, preliminary results of our group predict mRNA under expression of glutamic acid decarboxylase (GAD), the limiting enzyme responsible for GABA synthesis in the presynaptic terminal of inhibiting synapses, in brain of fragile X mutant fruit flies (D'Hulst, Hassan and Kooy, unpublished results).

In conclusion, we present evidence that decreased expression of the GABA<sub>A</sub> receptor is an evolutionary conserved hallmark of fragile X syndrome. As GABA<sub>A</sub> receptors are involved in anxiety, depression, epilepsy, insomnia and learning and memory (Mihalek et al., 1999), processes also disturbed in patients (Table 3), we might have identified a new target for rational drug therapy of the behavioral abnormalities and epilepsy associated with fragile X syndrome.

## 4. Experimental procedures

### 4.1. Animal and tissue preparation

#### 4.1.1. *M. musculus*

Male C57BL/6J wild-type mice, purchased from Charles River (Wilmington, MA, USA), were crossed with females heterozygous for the *Fmr1* mutation and backcrossed for at least 20 generations in the same genetic background. After DNA isolation from mouse tails, genotypes were determined by polymerase chain reaction (PCR) as described (Bakker et al., 1994). Male *Fmr1* knockout mice and male control littermates with an average age of 8–12 weeks were used. Mixed genotype

**Table 3 – Fragile X symptoms that could be due to decreased expression of the GABA<sub>A</sub> receptor**

Symptoms	% of affected males	References
<i>Neurological abnormalities</i>		
Epileptic seizures	20–25%	Musumeci et al., 2000
Deviant EEG		
–Medium to high-voltage unilateral or bilateral spikes in the temporal lobe	58%	Musumeci et al., 1999
–Rolandic spikes	17%	Musumeci et al., 1999
Sleeping problems	NA	Gould et al., 2000
<i>Behavioral problems</i>		
Autistic features	60–92.5%	Hagerman, 2002
Hyperactivity	67%	Hagerman, 2002
Anxiety	73.5%	Hagerman, 2002
NA: not available.		

groups of approximately 5 littermates were housed in standard mouse cages under conventional laboratory conditions (food and water ad libitum, constant room temperature and humidity, 12:12h light–dark cycle). After cervical dislocation, the brain was immediately removed; frontal cortex and cerebellum were dissected and frozen in liquid nitrogen. All experiments were carried out in compliance to the European Communities Council Directive (86/609/EEC) and approved by the Animal Ethics Committee of the University of Antwerp.

#### 4.1.2. *D. melanogaster*

White Canton-S flies were used as wild-type controls (lab stock). Fragile X deficient flies were selected from a stock of the genotype *w;P{dfmr1}/+;dfmr13/TM6,Tb,Sb* (a kind gift from T. Jongens) allowing for the selection of *dfmr1*<sup>3</sup> mutants (0r) or *dfmr1*<sup>3</sup> mutants possessing 1 (1r) or 2 (2r) copies of a genomic rescue insertion (Dockendorff et al., 2002). Table 4 details the crossing scheme used to obtain the appropriate genotype. All mating schemes were performed on standard fly food at controlled ambient temperatures.

**Table 4 – Crossing scheme *Drosophila melanogaster***

<i>wt/rescue;dfmr1<sup>-</sup>/TM6 X wt/rescue;dfmr1<sup>-</sup>/TM6</i>			
Frequency	Genotype	Code	Phenotype
1/3	<i>rescue/wt; dfmr1<sup>-</sup>/TM6</i>		Short bristles
1/6	<i>rescue/rescue; dfmr1<sup>-</sup>/TM6</i>		Short bristles
1/6	<i>rescue/wt; dfmr1<sup>-</sup>/dfmr1<sup>-</sup></i>	1r	Long, normal bristles, orange eyes
1/12	<i>rescue/rescue; dfmr1<sup>-</sup>/dfmr1<sup>-</sup></i>	2r	Long, normal bristles, red eyes
1/12	<i>wt/wt; dfmr1<sup>-</sup>/dfmr1<sup>-</sup></i>	0r	Long, normal bristles, white eyes
1/6	<i>wt/wt; dfmr1<sup>-</sup>/TM6</i>		Short bristles

The rescue allele and the *dfmr1<sup>-</sup>* allele both are located on the 2nd chromosome. TM6 is a balancer chromosome that makes it possible to determine the genotype by looking at the phenotype. In this crossing the wild-type strain is not available; TM6/TM6 is lethal.

## 4.2. RNA

### 4.2.1. *M. musculus*

After homogenizing the different brain parts of 11 knockouts and 11 control littermates (with beads of 0.5mm in the Mini Beadbeater, Biospec products, Bartleville, OK, USA), total cellular RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After RNase-free DNase treatment (Ambion, Austin, TX, USA), RNA quality was tested using spectrophotometry with an optical density ratio 260/280 between 1.8 and 2 as requirement.

### 4.2.2. *D. melanogaster*

Total cellular RNA (per strain (wt, 0r, 1r and 2r) 10 pools of 20 fly heads) was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After RNase-free DNase treatment of the RNA samples, RNA quality was checked with the automated gel electrophoresis Experion system from Biorad (Hercules, CA, USA); 28S/18S ratios between 1 and 2 were used as requirement.

## 4.3. Real-time PCR (qPCR)

### 4.3.1. *M. musculus*

mRNA expression was examined by an optimized two-step real-time quantitative PCR assay (RT-PCR). In the reverse

**Table 5 – Assays real-time PCR**

Gene	Assay ID
<i>M. musculus</i>	
Reference genes	
<i>Hprt</i>	Mm00446968_m1
<i>Gapdh</i>	Mm99999915_g1
<i>Hmbs</i>	Mm006660261_g1
Subunits of GABA <sub>A</sub> R	
δ	Mm00433476_m1
α <sub>1</sub>	Mm00439040_m1
α <sub>2</sub>	Mm00433435_m1
α <sub>3</sub>	Mm00433440_m1
α <sub>4</sub>	Mm00802631_m1
α <sub>5</sub>	Mm00621092_m1
α <sub>6</sub>	Mm00433456_m1
β <sub>1</sub>	Mm00433461_m1
β <sub>2</sub>	Mm00433467_m1
β <sub>3</sub>	Mm00433473_m1
ε	Mm00489932_m1
γ <sub>1</sub>	Mm00439047_m1
γ <sub>2</sub>	Mm00433489_m1
γ <sub>3</sub>	Mm00433494_m1
θ	Mm00445057_m1
ρ <sub>1</sub>	Mm00433499_m1
ρ <sub>2</sub>	Mm00433507_m1
π	Mm00524604_m1
<i>D. melanogaster</i>	
Reference genes	
<i>Dsh</i>	Dm02371846_s1
<i>Rpl32</i>	Dm02151827_g1
Subunits of GABAR	
<i>Rdl</i>	Dm01822422_m1
<i>Grd</i>	Dm01823018_m1
<i>Lch3</i>	Dm01799500_g1

transcription step (RT), cDNA was reverse transcribed from total RNA samples using random hexamer primers from the Superscript™ III First-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). Genomic contamination of the generated cDNA was checked with 2 primers located in exons overspanning an intron. First strand cDNA was diluted in TE<sup>-4</sup> buffer until a final maximum concentration of 120ng/μl. In the PCR step, PCR products were synthesized from cDNA samples using the qPCR MasterMix plus w/o UNG (Eurogentec, Seraing, Belgium). As detection method, we used Assays-on-demand (ABI, Foster City, CA, USA) containing a forward and a reverse primer, and a TaqMan MGB probe (6-FAM dye-labeled) built on Applied Biosystems 5' nuclease chemistry and developed specifically for detection of the gene of interest (Table 5). In the majority of the cases, the probes crossed exon-exon junctions excluding the possibility of amplification of genomic DNA. The PCR mixtures were run on an ABI 7000 sequence detection system. The cycling conditions were as follows: 2min 50 °C, 10min 95 °C and 40 cycles at 95 °C for 15s and 60 °C for 1min. Per brain region, we compared 11 control samples with 11 knockouts. We maximized the number of samples and minimized the number of genes per run. Every 96 well plate contained a no template control and all the samples were spotted in duplex. The results of the Sequence Detection Software (Applied Biosystems) were exported as tab delimited files and imported into the relative quantification software qBase (Hellemans et al., in preparation; <http://medgen.ugent.be/qbase/>) for further analysis. This program performs a raw data quality control and calculates the normalized quantities of the genes of interest and reports the reference gene quality values (mean coefficient of variation of the normalized reference genes quantities and the geNorm stability value M, Table 1). Here the transcription levels were normalized by the geometric mean of 3 stably expressed reference genes (*Gapdh*, *Hmbs* and *Hprt*). A Mann-Whitney *U* non-parametrical test was used to check the statistical significance ( $p < 0.05$ ) of the obtained results (SPSS Inc., Version 12.0, Chicago, IL, USA).

#### 4.3.2. *D. melanogaster*

Setup and analysis of the real-time experiments were analogous to those described for the mouse model. The transcription levels were normalized by the geometric mean of 2 stably expressed reference genes (*Rpl32* and *Dsh*). Per strain (wt, 0r, 1r en 2r), we screened 10 independent cDNA samples. A Mann-Whitney *U* non-parametrical test was used to check the statistical significance ( $p < 0.05$ ) of the difference between wt and 0r. Additionally, we used a 1-way ANOVA to compare group differences between 0r, 1r and 2r (SPSS 12.0).

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