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Research Report

Expression of the GABAergic system in animal models for fragile X syndrome and fragile X associated tremor/ataxia syndrome (FXTAS)

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ABSTRACT

After our initial discovery of reduced expression of several subunits of the $GABA_A$ receptor in two different animal models for fragile X syndrome, a frequent form of inherited mental retardation, we analyzed further components of the GABAergic pathway. Interestingly, we found a down regulation of many additional elements of the GABA signalling system, strengthening our hypothesis of involvement of the GABAergic pathway in the pathophysiology of fragile X syndrome. This is of special interest with regard to new therapeutic opportunities for treatment of this disorder. Remarkably, under expression was predominantly observed in cortex, although some elements of the GABAergic system that are expressed presynaptically or in the glial cells were also down regulated in the cerebellum. Additionally, we assessed the GABAergic system in expanded CGG-repeat mice, a model for fragile X associated tremor/ataxia syndrome (FXTAS). This late onset neurodegenerative disorder occurs in carriers of the fragile X premutation (55–200 CGG repeats) and is completely distinct (from both clinical and molecular pathogenic perspectives) from the neurodevelopmental disorder fragile X syndrome. Here we found upregulation of many components of the GABAergic system in cerebellum, but not in cortex. This finding is consistent with the cerebellar phenotype of FXTAS patients and has implications for the mechanism causative of differential gene expression.

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1. Introduction

1.1. Fragile X syndrome and FXTAS

Loss of the fragile X mental retardation gene (*FMR1*) causes a neurodevelopmental and behavioural disorder called fragile X syndrome, which affects 1/2500 individuals (Verkerk et al., 1991; Hagerman, 2008). Patients present with mental retardation, autistic features, anxiety, hyperactivity and mood instability and display various physical abnormalities, such as enlarged testes (macroorchidism) and craniofacial anomalies (Hagerman, 2002). In contrast, over expression of *FMR1* mRNA causes fragile X associated tremor/ataxia syndrome (FXTAS). This late-onset neurodegenerative disorder is characterized by progressive intention tremor and gait ataxia, with more variable associated features including parkinsonism, dysautonomia, peripheral neuropathy, and dementia (Jacquemont et al., 2003). Thus, the same *FMR1* gene presents two opposing faces: a childhood-onset disorder (fragile X syndrome), caused by the absence of gene activity and a late-onset neurodegenerative syndrome (FXTAS), caused by toxic RNA gain of function (Jacquemont et al., 2007).

1.2. Mechanism

The 5′ untranslated region of the *FMR1* gene contains a CGG repeat sequence that is stably transmitted in the normal range (5–44/55 repeats). Expansion of this repeat is associated with changes in the amount of *FMR1* mRNA and protein with varied phenotypes [reviewed by Jacquemont et al., 2007]. Full mutation alleles (>200 repeats) are associated with gene methylation and transcriptional silencing, which lead to the absence of the *FMR1* gene product FMRP and cause fragile X syndrome. Premutation alleles (55–200 repeats) are unstable and may expand to full mutation alleles when transmitted maternally. The prevalence of the premutation has been estimated to be 1/813 males and 1/259 females in one study (Rousseau et al., 1995), but recent studies challenge this view (Hagerman, 2008). The pathogenic basis of FXTAS is (over) expression of this 'toxic' expanded CGG repeat *FMR1* RNA which leads to neural cell dysregulation, formation of intranuclear inclusions in neurons and astrocytes, and disruption of the nuclear lamin architecture (Greco et al., 2002; Garcia Arocena et al., 2005).

1.3. Animal models

Interruption of the murine *fmr1* gene generated a mouse model for fragile X syndrome (Bakker et al., 1994). Fragile X knockout (KO) mice show mild cognitive deficits, hyperactivity, macroorchidism, immature dendritic spines and increased sensitivity to epileptic seizures, features comparable with symptoms observed in fragile X patients (Bakker and Oostra, 2003; Kooy, 2003). The invertebrate homologue of *fmr1* in fruit flies, namely *Drosophila melanogaster fragile X mental retardation gene 1* (*dfmr1*), 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 (Morales et al., 2002; Dockendorff et al., 2002; Michel et al., 2004; Zhang and Broadie, 2005) and loss of dfmrp causes behavioural defects like abnormal eclosion and circadian rhythm behaviour and anomalies in the morphology of several central nervous system neuronal populations.

To better understand the timing and mechanism involved in the *FMR1* CGG repeat instability and methylation, a mouse model was generated in which the endogenous mouse CGG repeat was replaced by a human CGG repeat carrying 98 CGG units, which is in the premutation range (Bontekoe et al., 2001). The inheritance of the CGG repeat is only moderately unstable, upon both maternal and paternal transmission, indicating differences between the behaviour of the *fmr1* premutation CGG expanded-repeat in mouse and in human transmissions. The model displays biochemical, phenotypic and neuropathological characteristics of FXTAS (Willemsen et al., 2003). As in humans, the expanded CGG repeat model shows 2–3.5 fold elevated *fmr1* mRNA levels in brain tissue compared with control. Protein levels are mildly decreased. Immunohistochemical studies provide significant evidence for the presence of ubiquitin-positive intranuclear inclusions in neurons of this mouse model. Brouwer et al. (2007) reported further repeat instability up to 230 CGG repeats. In humans, this would be considered as a full mutation and result in hypermethylation and gene silencing. However, mice carrying long repeats do not show any signs of abnormal methylation, suggesting that modelling the fragile X full mutation requires additional repeats or other genetic manipulation.

1.4. Involvement of the GABAergic system in FXS

As genes that are over or under expressed might indicate which pathways are involved in fragile X syndrome, our group previously performed expression analyses on different brain regions of *fmr1* KO mice compared to wild type (WT) littermates (Gantois et al., 2006; D'Hulst et al., 2006). We found decreased expression of 8 out of 18 known subunits of the $GABA_A$ receptor in fragile X mouse cortex, but not in cerebellum. Additionally, we found under expression of all three subunits which make up the GABA receptor in the fragile X fruit fly model. Thus, we consider down regulation of the $GABA_A$ receptor as an evolutionary conserved hallmark of fragile X syndrome. As $GABA_A$ receptors are involved in hyperactivity, anxiety, epilepsy and learning and memory (Mihalek et al., 1999), we hypothesized that a dysfunction of the $GABA_A$ receptor has neurophysiologic and functional consequences that might relate to the behavioural and neurological phenotype associated with fragile X syndrome, and as such might be a novel target for treatment of the disorder (D'Hulst and Kooy, 2007).

Using animal models for fragile X syndrome and FXTAS, we analyzed the expression of genes encoding enzymes and proteins important in the synthesis, transport and degradation of GABA or in the clustering of $GABA_A$ receptors at the postsynaptic terminal. This study gives us a broader image of the involvement of the GABAergic system in both syndromes.

2. Results

2.1. *fmr1* KO mice

Cortical and cerebellar tissue of adult fragile X male mice and control littermates was dissected, RNA was isolated and reverse transcribed as indicated in the experimental procedure. These regions were selected because in our initial study (D'Hulst et al., 2006) under expression of different subunits of the $GABA_A$ receptor was found in cortex but not in cerebellum. Assays-on-demands® (ABI, Foster city, CA, USA) were selected for several important genes of the GABA signalling pathway including the genes encoding the limiting GABA synthesizing enzyme glutamic acid decarboxylase (*gad* 1–2); the GABA transporters (*gat1–4*); succinate semialdehyde dehydrogenase (ssadh), an enzyme important in the degradation of GABA, and gephyrin, a protein important in the clustering and targeting of $GABA_A$ receptors to the postsynaptic membrane (Fig. 1).

Three internal control genes were used to calculate the real time-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 (Vandesompele et al., 2002). We used *gapdh*, *hmbs* and *hprt* as reference genes, which we have shown to be stable in our previous experiments. 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 in Vandesompele et al. (2002) to analyze the stability of the internal control genes. The measured values were amply within the norm (Supplementary data, Table 1).

For each gene the relative expression was calculated as described in D'Hulst et al. (2006) (Fig. 2). We observed a down regulation of the mRNA expression for *gad1* (↓24%), *gat1*(↓26%)

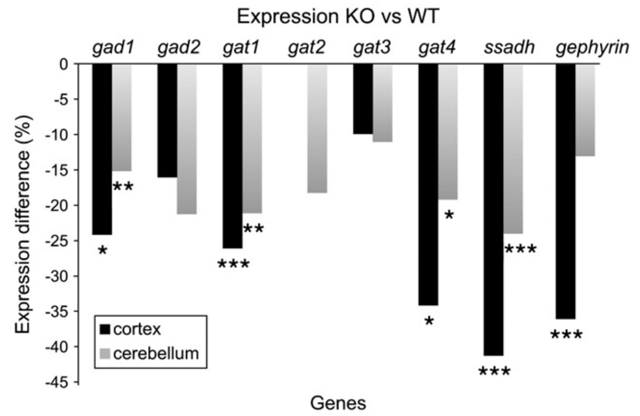

Fig. 2 – Difference in expression between wild types (WT) and *fmr1* KO mice. The genes *gad1*, *gat1* and 4, and *ssadh* are significantly lower expressed in cortex *and* cerebellum of the KO mice compared with their wild type littermates. Expression of gad2 in cortex was completely aberrant and data are not shown. *Gephyrin* is only significantly under expressed in cortex of KO mice. ***$P<0.001$; **$P<0.01$ and *$P<0.05$.

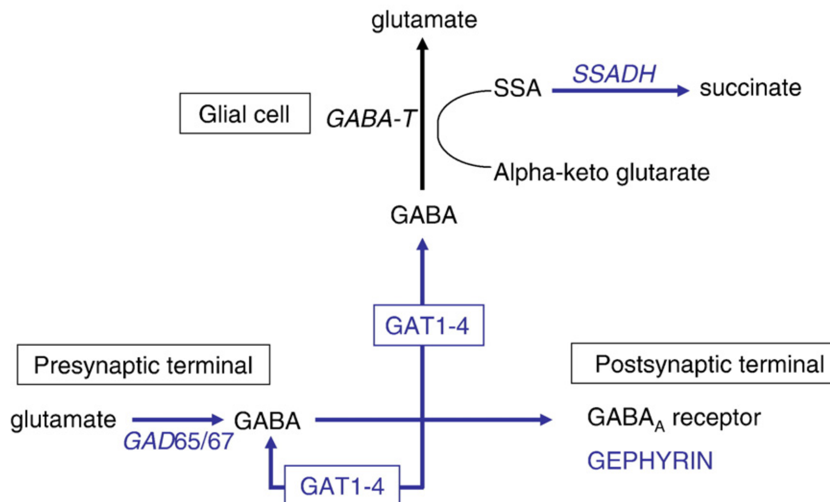

Fig. 1 – Schematic representation of the enzymes and proteins (in blue) analyzed in this study and their function in the GABA metabolism. For an elaborate image of the GABA metabolism and the GABAergic system, please refer to D'Hulst and Kooy (2007). GABA, the major inhibitory neurotransmitter in brain, is synthesized in the pre-synaptic terminal through decarboxylation of glutamate by *glutamic acid decarboxylase* (*GAD*), a rate-limiting enzyme which exists in two isoforms GAD67 and GAD65, encoded by *GAD1* and *GAD2*, respectively. GABA can activate metabotropic $GABA_B$ receptors or ligand-gated ion channels, $GABA_A$ and $GABA_C$ receptors. GEPHYRIN is an important protein in clustering and targeting of $GABA_A$ receptors to the postsynaptic terminal (Essrich et al., 1998). Alternatively, GABA can be transported by GABA-transporters (GAT1–4) back to the presynaptic terminal (recycling) or to the glial cells for degradation. One of the enzymatic steps of the degradation of GABA includes the dehydrogenation of succinyl semialdehyde (SSA) to succinate by SSA dehydrogenase (SSADH).

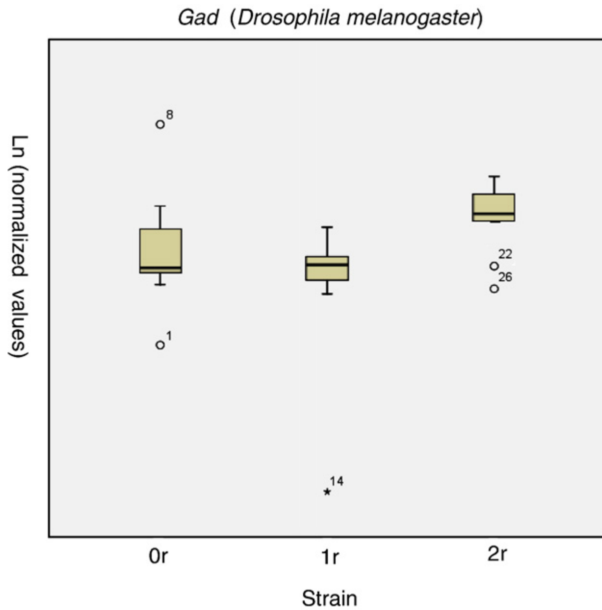

Fig. 3 – Boxplots showing the significant rise in the normalized RNA amount of gad in function of the number of copies of dfmr1 randomly inserted in the *dfmr1*$^{-/-}$ null mutant (rescue strains), which indicated a direct correlation between the amount of dfmrp and the expression of *gad* in the fly. 0r, 1r and 2r: rescue strains respectively containing none, one or two *dfmr1* copies. The significance was tested using one-way ANOVA.

and 4(↓34%) and *ssadh* (↓41%) in cortex and in cerebellum (↓15%, ↓21%, ↓19% and ↓24%, respectively) and for *gephyrin* (↓36%) in cortex only. *Gad2*, *gat2* and 3 didn't show a significant change.

2.2. *Dfmr1 deficient flies*

To verify the evolutionary conservation of decreased expression of genes of the GABAergic pathway in fragile X syndrome, we additionally measured the relative expression of the single isoform of the limiting GABA synthesizing enzyme gad in *Drosophila melanogaster* fragile X model (Morales et al., 2002). Using real time PCR we compared the expression of *gad* in wild type strains with *dfmr1*$^{-/-}$ mutant fruit flies using the same reference genes (*dsh* and *rpl32*) as described in D'Hulst et al. (2006) (Supplementary data, Table 1). Our results revealed a significant ($P<0.05$, Mann–Whitney U test) reduction of 35% in expression of *gad* in *dfmr1*$^{-/-}$ mutants compared with the wild-type strain, completely consistent with our findings in the vertebrate fragile X animal model.

To test whether the expression of the limiting GABA synthesizing enzyme *gad* might be directly regulated by dfmrp, we additionally determined the expression levels in rescue strains containing 1 or 2 *dfmr1* copies, randomly inserted in the *dfmr1*$^{-/-}$ null mutant (called 1r and 2r respectively). We observed a significant rise in the normalized RNA amount for *gad* (1-way ANOVA, $P=0.016$) in function of the number of *dfmr1* copies, indicating a direct correlation between dfmrp expression levels and the amount of GABA receptor subunit mRNA in these two subunits (Fig. 3).

2.3. *CGG repeat mice*

Using real time PCR we screened the expression of all the genes, which we have shown to be differentially expressed in the *fmr1* KO mouse model, in the premutation mouse model (201 CGG repeats). Experiments were carried out and analyzed precisely as described above for fmr1 KO mice. We found significant over expression of $\alpha_{1,\ 3\ \text{and}\ 4}$, β_{1-2}, γ_2, *gad1*, *gephyrin*

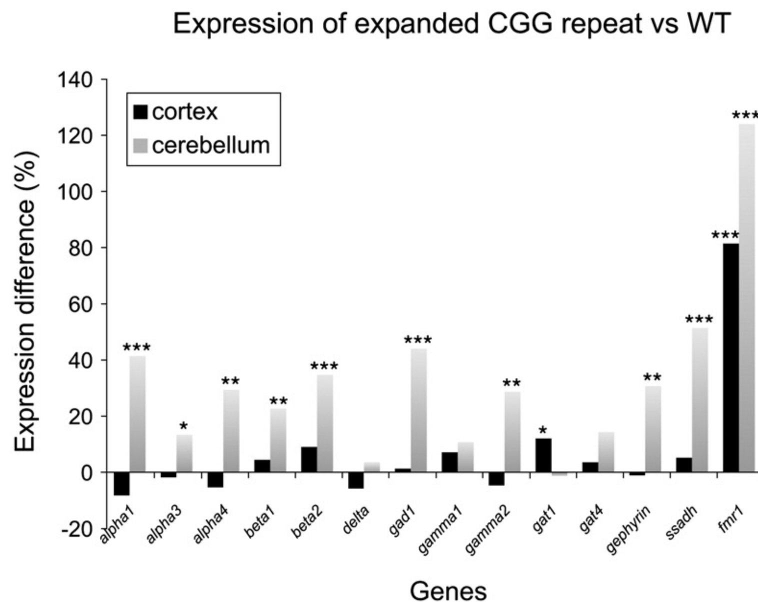

Fig. 4 – Difference in expression between wild types (WT) and CGG repeat mice. The genes $\alpha_{1,\ 3\ \text{and}\ 4}$, β_{1-2}, γ_2, *gad1*, *gephyrin*, *ssadh* and *fmr1* are significantly higher expressed in cerebellum of the CGG repeat mice compared with their wild type littermates. The expression difference in cortex is only significant for *fmr1* and *gat1*. ***$P<0.001$; **$P<0.01$ and *$P<0.05$.

and *ssadh* in the cerebellum, but not in the cortex (Fig. 4). Additionally we determined the expression of *fmr1* in both brain regions. *Fmr1* transcripts were significantly higher expressed in cortex (80%) and cerebellum (124%) compared to wild-types, in line with previous findings (Brouwer et al., 2007). Two-way ANOVA analysis shows that the difference in elevated expression levels between cortex and cerebellum (80% vs. 124%, respectively) is not significant ($P = 0.218$). Thus the difference in expression of our genes of interest between cortex and cerebellum is not due to the difference in *fmr1* expression seen in these regions.

3. Discussion

Using real-time PCR, we investigated the expression of several important genes of the GABA metabolism in brain of fragile X mice and we found significant under expression of *gad1*, *gat1* and 4, *ssadh* and *gephyrin* compared to wild-type animals. From our results it seems that there is no compensation in GABA synthesis or transport for the decreased expression of certain subunits of the $GABA_A$ receptor in fragile X mice, reported in our previous study (D'Hulst et al., 2006). It is remarkable that a decreased expression of several $GABA_A$ receptor subunits and *gephyrin* is observed in cortex only, whereas mRNAs encoding proteins and enzymes involved in transport, synthesis and degradation of GABA seem to be under expressed in cerebellum too. One plausible explanation for this discrepancy is that the location of expression of the analyzed genes might influence the expression differences we see. $GABA_A$ receptors and gephyrin are both located post-synaptically, whereas the other proteins investigated are located presynaptically or in the glial cells.

Another notable finding is that only one isoform of *gad* is under expressed in fragile X cortex and cerebellum. Gad, the rate-limiting GABA synthesizing enzyme, has two isoforms, gad65 and gad67, named for their molecular mass of 65 and 67 kDa, respectively, which are encoded by two independent genes, *gad2* and *gad1* (Erlander et al., 1991; Erlander and Tobin, 1991). It has been suggested that gad67 provides basal levels of GABA, whereas gad65 may supply GABA in situations of sudden demand (Namchuk et al., 1997). Gad67-deficient animals are born with cleft palate and die within the first days of life (Asada et al., 1996). Gad65-deficient animals appear to be normal with regard to GABA-levels, behaviour, locomotion, reproduction, and glucose homeostasis, but develop epilepsy (Kash et al., 1997). Thus, gad65 appears to synthesize a dynamic buffer of inhibitory neurotransmitter to provide a rapid response to certain stimuli like fear or stress, thereby fine-tuning complex mammalian neural networks. From our results we can conclude that fragile X mice have problems with the supply of basal GABA pools, due to under expression of *gad1* (encoding gad67). Because the only isoform of gad in *Drosophila melanogaster* is also under expressed in *dfmr1*$^{-/-}$ mutants, we can confirm our statement (D'Hulst et al., 2006) that dysfunction of the GABAergic system is an evolutionary conserved hallmark of fragile X syndrome.

We see a reduction in expression of 2 out of 4 GABA transporters present in the mouse brain. Gat 1–4 belong to the solute carrier family 6 (slc6) (Hoglund et al., 2005). The SLC6 family of proteins acts as specific transporters for neurotransmitters, amino acids, and osmolytes like betaine, taurine and creatinine. Neurotransmitters may function in a variety of processes, including termination of neurotransmission, detoxification, protection from reactive substances, nutrition and modulation of receptor activity (Jursky et al., 1994). It seems that each of the neurotransmitter transporters is present in specific places in the brain and is expressed in a different way in very specific areas. There has been confusion regarding the nomenclature, in particular the naming of orthologuous proteins in different species. In humans we find 3 different GABA transporters: SLC6A1 (GAT1), SLC6A13 (GAT2) and SLCA11 (GAT3). In mice however, we see four different proteins: slc6a1 (gat1), slc6a12 (gat2), slc6a13 (gat3) and slc6a11 (gat4); thus, GAT2 in humans is orthologuous to gat3 in mice and GAT3 to gat4. Gat2 correlates with the human and rat betaine/GABA transporter (BGT-1) and is also responsible for the cellular accumulation of betaine.

Our findings are in line with other recent results that implicate involvement of the GABAergic system in fragile X syndrome. In addition to differential expression at the mRNA level, under expression of the α_5, β_n and δ subunits – the only subunits measured – at the protein level was reported in specific brain regions of the fragile X mouse (Curia et al., 2008; El Idrissi et al., 2005). Dictenberg et al. (2008) demonstrated a reduced stimulus-induced presence of $GABA_A$ receptor δ subunit mRNA ($GABA_AR$ δ mRNA) in dendrites. Neurophysiologic and immunofluorescence experiments showed that the absence of fmrp is associated with apparently normal striatal glutamate-mediated transmission, but abnormal GABA transmission (Centonze et al., 2008). Most recently, patch clamp recordings from subicular pyramidal cells showed that tonic GABA currents were down regulated in neurons derived from KO mice in comparison with those derived from control mice, whereas no significant differences were observed in phasic currents, in line with the evidence implicating tonic GABA inhibition in learning and memory (Curia et al., 2008). Involvement of the $GABA_B$ receptor was recently suggested by Zupan and Toth (2008). Moreover, a recent drug screen in the fragile X fly model in which 2000 FDA approved compounds were tested, showed that 3 out of 9 compounds that were able to rescue lethality of the mutant flies when reared on peculiar food, were implicated in the GABAergic pathway (Chang et al., 2008).

Cerebellar RNA over expression of several $GABA_A$ receptor subunits (α_1, α_3, α_4, β_1, β_2 and γ_2) and proteins involved in the GABA metabolism (*gad1*, *ssadh* and *gephyrin*) in a CGG repeat mouse, a model for FXTAS, is a completely new finding. We found no differential expression in the cortex, suggesting that a modest amount of fmrp in the cell is sufficient for correct cellular function. Differential expression of these genes in cerebellum is consistent with the cerebellar phenotype of FXTAS patients. It so happens that the cerebellum is implicated in the precise timing for coordinated, smooth movements of the skeletal muscular system, processes disturbed in FXTAS patients. It was already suggested that patients with the cerebellar symptoms like ataxia with imbalance and the tendency to fall are due to dysfunction of the vestibulo-cerebellum and the vestibular system (Willemsen et al., 2003). Other cerebellar symptoms in the patients, especially the

intention tremor, are usually attributed to the cerebrocerebellum, the largest part of the cerebellum, receiving input from cortical motor areas by way of the pontine nuclei. It needs to be stressed though, that we have not investigated a possible dysregulation of other genes in addition to the GABAergic system in the expanded CGG repeat mice.

Nonetheless, it is surprising that we observe over expression of our genes of interest in the expanded CGG repeat mice and under expression of the same genes in the fragile X mouse model. Up till now, we can only speculate on the mechanisms responsible for this discrepancy. *Fmr1* KO mice do not generate fmrp. As FMRP is known to play an important role in transport, translation and stability of bound mRNAs (Jin and Warren, 2000; Zalfa et al., 2007), we hypothesize that, in the absence of FMRP, RNAs normally bound to FMRP are misregulated and/or degraded (D'Hulst et al., 2006), leading to the under expression of the specific genes observed in this study. The fact that $GABA_AR$ δ mRNA binds fmrp, (Miyashiro et al., 2003) and the deficient stimulus-induced localisation of $GABA_AR$ δ mRNA in KO dendrites (Dictenberg et al., 2008), both support this hypothesis. The CGG repeat model, on the other hand, shows increased *fmr1* mRNA levels and slightly reduced fmrp expression (Willemsen et al., 2003; Brouwer et al., 2007). We have shown a 2–3.5 fold over expression of *fmr1* mRNA in our mice at 10 weeks of age, in complete concordance with the findings of Willemsen et al. (2003), who demonstrated that these elevated *fmr1* message levels were already present in brain homogenates of pups at the age of 1 week. Because no differential expression of the GABA-related genes is observed in cerebellum of the fragile X KO mouse model, it is unlikely that a reduction of fmrp levels is responsible for our findings in the expanded CGG repeat mouse model. We could theorize that the cerebellar over expression of certain genes involved in the GABAergic system is, as a consequence, due to an RNA effect. The exact mechanism remains however elusive and further studies are clearly necessary to elucidate the transcriptional dysregulation of the over expressed genes.

It is remarkable that the differences in expression here reported are observed in expanded CGG repeat mice at 10 weeks of age. In parallel with the neurodegenerative character of the disease, mice at this age don't show ubiquitin-positive intranuclear inclusions nor behavioural abnormalities (Van Dam et al., 2005), implicating that the FXTAS pathology is not present yet. However, an upregulation of the GABAergic system might be a marker for the disease, and is in line with the findings of diminished brain activation in the amygdala and several brain areas that mediate social cognition in younger permutation carriers (Hessl et al., 2007). A dysfunction of the GABAergic system could also explain the association of premutation alleles of the *FMR1* gene with autism spectrum disorder in childhood (Tassone et al., 2000). Hence, investigation of the GABAergic system in the limbic system of expanded CGG repeat mice might be a very interesting future strategy.

In conclusion, given the fact that fragile X syndrome, a childhood onset disorder, and FXTAS, a late-onset neurodegenerative disease, are two completely different syndromes, we could assume that two totally diverse mechanisms are responsible for the differential expression of the GABAergic system observed in animal models for both syndromes.

4. Experimental procedures

4.1. *Animal models*

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.

4.2. *Fragile X mice (Mus musculus)*

Male fragile X mice and control littermates were obtained by backcrossing females heterozygous for the *fmr1* knockout mutation, inbred in the C57BL/6J background ($N>20$), with C57BL/6J wildtype males (Charles River Laboratories, Brussels, Belgium). Genotyping of the litters was performed as previously described in D'Hulst et al. (2006). 10 male KO mice were compared to 10 male WT littermates.

4.3. *Fragile X flies (Drosophila melanogaster)*

Flies were obtained as described in D'Hulst et al. (2006). To test whether the expression of the limiting GABA synthesizing enzyme (gad) is directly regulated by dfmrp we measured the expression of gad in:

0r = $dfmr1^{-/-}$ flies
1r = $dfmr1^{-/-}$ with 1 copy of $dfmr1^{-/-}$ randomly inserted
2r = $dfmr1^{-/-}$ with 2 copy of $dfmr1^{-/-}$ randomly inserted.

1r and 2r are so-called rescue strains. We used a one-way ANOVA test to examine whether the expression of *gad* is significantly different between different groups (0r, 1r and 2r).

4.4. *Expanded CGG repeat mice (Mus musculus)*

CGG repeat mice were transferred from the colony in Rotterdam (Prof. Dr. B. Oostra's lab) in a mixed C57BL/6 X FVB background to the Antwerp Animal Facility, where the mice were backcrossed for two further generations with C57BL/6J mice. Repeat lengths were determined as described in Brouwer et al. (2007). Our mice carried a repeat length of approximately 203 CGG trinucleotides. 10 male expanded CGG repeat mice were compared to 10 male WT littermates.

4.5. *Real time PCR*

Animal and tissue preparation, RNA isolation, cDNA synthesis, real-time PCR and analysis was performed as previously described (D'Hulst et al., 2006), except for a few changes: brains were homogenised using *Dispomix* (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and the quality of the RNA samples was tested using the automated gel electrophoresis *Experion* system from Biorad (Hercules, CA, USA); 28S/18S ratios between 1 and 2 were used as requirement. Table 2 (Supplementary data) shows the assay IDs of the ABI™ Taqman probes used in this study. Real time PCR was performed on a Lightcycler 480 (Roche Applied science). Cycling conditions were as follows: 10 min

95 °C; 10 s 95 °C; 30 s 60 °C (50 cycles); followed by a cooling step of 10 s 40 °C.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2008.11.075.

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