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Reduced EAAT1 and mGluR5 expression in the cerebellum of *FMR1* premutation carriers with FXTAS

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Abstract

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A premutation (PM) expansion (55–200 CGG) in the fragile X mental retardation gene 1 (*FMR1*) causes elevated mRNA and reduced *FMR1* protein (FMRP). Young PM carriers can develop characteristic physical features and mild cognitive disabilities. In addition, individuals with PM, particularly male carriers, are at high risk to develop Fragile X-associated tremor/ataxia syndrome (FXTAS) with aging. Human post-mortem FXTAS brains show extensive white matter disease in the cerebellum and the presence of intranuclear inclusions throughout the brain, although their etiological significance is unknown. In the current work, expression levels of the metabotropic glutamate (Glu) receptor mGluR5 and the Glu transporter EAAT1, examined by RT-PCR and WB analyses, were found to be reduced in the post-mortem cerebellum of PM carriers with FXTAS compared to age matched controls, with higher CGG repeat number having greater reductions in both proteins. These data suggests a dysregulation of Glu signaling in PM carriers, which would likely contribute to the development and severity of FXTAS.

Keywords

FMR1; FMRP; premutation; Fragile X Tremor/Ataxia Syndrome; FXTAS; Glu transporters; EAAT1; EAAT2; mGluR5

2. Introduction

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late onset neurodegenerative disorder. Tremor and ataxia accompanied by noticeable cognitive problems are usually the first clinical presentation of FXTAS. Progressive neuropathological involvement is manifested with signs of parkinsonism, psychiatric disturbances such as depression and anxiety with some patients developing dementia and severe disability (Leehey and Hagerman 2012). Carriers with the Fragile X Mental Retardation 1 (*FMR1*) gene expansion (55–200 CGG repeats, premutation, PM) in the 5’ UTR of the promoter are at risk of developing FXTAS (Hagerman and Hagerman 2001, Hagerman et al. 2008, Hagerman et al. 2001). The penetrance of the PM phenotype and manifestation of FXTAS is more severe in men than in women especially those individuals with higher than 70 CGG repeat alleles (Leehey et al. 2008). In the United States alone it has been estimated that at least 1 in 130–250 females and 1 in 260–800 males are PM carriers (reviewed in (Tassone et al. 2012)) and at least 40% of male PM carriers over age 50 (Jacquemont et al. 2004) and 8 % of female PM carriers over age 40 (Coffee et al. 2009) will develop FXTAS.

The *FMR1* protein, FMRP, is an RNA binding protein that functions as a translational repressor modulating the expression of important proteins involved in the formation of dendritic spine morphology, pruning of synaptic contacts and overall synapse maturation (Irwin et al. 2000, Bagni et al. 2012). The inefficient translation of FMRP in PM carriers, particularly in those carrying an allele in the upper premutation range, can therefore have a direct impact on overall synaptic connectivity, plasticity and brain function (Sidorov et al. 2013). Excessive transcription and accumulation of *FMR1* mRNA leads to toxicity (Tassone et al. 2004b) and to sequestration of CGG binding proteins including DGCR8, a key player in miRNA biogenesis (Sellier et al. 2013) and Sam 68, a regulator of alternative mRNA splicing (Sellier et al. 2010). In FXTAS, the formation of ubiquitin positive intranuclear

inclusions in a subset of neurons and astrocytes, along with white matter disease, are thought to contribute to neurodegeneration (Greco et al. 2002, Tassone et al. 2004a, Greco et al. 2006, Garcia-Arocena et al. 2010). However, the cellular mechanisms determining the onset and progression of FXTAS in PM carriers are not presently understood,

Glutamate (Glu) transport is a mechanism necessary for reestablishing basal levels of synaptic activity after neuronal stimulation by removal of Glu from the perisynaptic cleft. The transporters are coupled to subunits of the Na^+-K^+ ATPase pump requiring the ionic flux of Na^+ and K^+ currents, a Ca^{2+} dependent mechanism, for the uptake of Glu into cells against its concentration gradient (Rose et al 2009). Clearance of Glu prevents a potential constitutive firing of Glu binding receptors and formation of free radicals (Danbolt 2001). Disruption of this mechanism can lead to overstimulation of Glu receptors, resulting in excitotoxicity (Nakagawa and Kaneko 2013).

The family of excitatory amino acid transporters includes 5 different subtypes also known as solute carrier family 1 members, namely EAAC1/EAAT3 (Kanai and Hediger 1992), EAAT1/SLC1a3/GLAST (Storck et al. 1992, Tanaka 1993b, Tanaka 1993a), EAAT2/SLC1a2/GLT1 (Pines et al. 1992), EAAT4 (Fairman et al. 1995) and EAAT5 (Arriza et al. 1997). Glu transporters are primarily localized in glial cells although their expression has also been detected in neurons. EAAT1 and EAAT2 are the principal means by which Glu is recycled in the central nervous system and it is estimated that EAAT2 clears over 90% of Glu alone (Danbolt 2001); however, in the cerebellum, EAAT1 is the main Glu transporter (Danbolt et al. 1998, Rose et al. 2009). Immunoblotting of young rat cerebellar extracts showed that GLAST (the orthologue of EAAT1) is at least 6 times more abundant than GLT1 (the orthologue of EAAT2) (Danbolt et al. 1998, Lehre and Danbolt 1998). In addition, comparison between rat cerebellar and forebrain membranes, and astrocytic cultures, demonstrated greater GLAST expression in the cerebellum and astrocytes, whereas GLT1 expression was more pronounced in forebrain (Rose et al. 2009). GLAST distribution within the cerebellum has been mainly observed in Bergmann glia cells for both mice and primates (Williams et al. 2005). Consistently, immunohistochemical staining for GLAST in P2 rat cerebellar cortex shows prominent expression in the Bergman glia and granule cell layer (Rose et al. 2009). Bergmann glia and the expression of GLAST in these cells are crucial for proper signaling of the surrounding synapses, namely the parallel fiber-Purkinje cell synapses. Parallel fibers are axons of granule cells establishing synapses with the Purkinje cell dendritic arborization (Lopez-Bayghen et al. 2007). During synaptic activity the parallel fiber-Purkinje cell synapses utilize Glu as a neurotransmitter affecting synaptic transmission between Glu receptors and transporters expressed in the surrounding Bergmann glia and astroglia cells (Lopez-Bayghen et al. 2007). Thus, optimal Glu transporter expression, and especially GLAST protein expression in Bergmann glia cells in the cerebellum, is critical for modulation of synaptic plasticity.

Recent evidence points to abnormalities in glutamatergic signaling in the *fmr1* CGG knock-in (KI) mouse model of PM (PreCGG) (Cao et al. 2012) and in the iPSC derived human PM neurons from human fibroblast (Liu et al. 2012). Alterations in Glu uptake kinetics and augmented group I metabotropic Glu receptors (Gp1 mGluRs: mGluR1 and mGluR5) activity observed in preCGG neurons suggests that a defect in Glu transport and signaling in

astrocytes could also contribute to PM pathology (Cao et al. 2012), and is consistent with results from a more recent study indicating that astrocytic cultures from frontal cortex of preCGG mice have altered asynchronous Ca^{2+} oscillations and glutamatergic responses associated with modest reductions of both GLT1 and GLAST expression (Cao et al. 2013). Moreover, these changes have been also observed in aged mice, which show 25–30% reduction in the expression of GLT1 and GLAST in preCGG mice compared to aged matched wild type mice (Cao et al. 2013). Thus, neurons (Cao et al. 2012) and astrocytes (Cao et al. 2013) cultured from preCGG mice show significant sensitivity to Glu-triggered Ca^{2+} signals that is primarily attributed to mGluR5 hyperactivity.

In fragile X syndrome (FXS), a related *FMR1* associated disorder and the most common cause of inherited early onset intellectual impairment, the *FMR1* CGG repeat expansion exceeds ≥200 CGG repeats (termed a full mutation), which leads methylation of the *FMR1* promoter resulting in little to no expression of FMRP (Pieretti et al. 1991, Bagni et al. 2012, De Rubeis et al. 2012). During the past decade, it has become apparent that the dysregulated activation of the mGluR5, a translational target of FMRP, is a major contributing factor to FXS neuronal network dysfunction, and could explain both the psychiatric and neurological deficits observed in FXS patients (Bear et al. 2004, Auerbach and Bear 2010, Bear 2005, Osterweil et al. 2012). These findings prompted us to investigate whether similar abnormalities would be observed in the PM brain of patients affected by FXTAS as lower FMRP expression can also be observed in some PM. Here we report results from decreased cerebellar expression of astrocytic Glu transporters EAAT1 and of the mGluR5 in 16 human PM cases, all of whom developed FXTAS during life, compared to 8 age matched controls providing additional evidence of CGG-dependent glutamatergic dysregulation in PM and FXTAS.

3. Methods

3a. FXTAS stages

FXTAS stages are based on the degree of clinical involvement of tremor and/or gait ataxia, and are defined as follows: Stage 0 is considered asymptomatic, with no evidence of either tremor or ataxia; Stage 1 involves subtle or questionable tremor and/or balance problems; Stage 2 presents with clear intentional tremor and/or balance problems, with little or no compromise of activities of daily living (ADL); Stage 3 involves moderate tremor and/or balance problems with significant interference in ADLs; Stage 4 describes severe tremor and/or balance problems necessitating the use of a cane or walker; Stage 5 requires the use of a wheelchair on a daily basis. Symptoms progress in severity of tremor and ataxia such that, in stage 6, patients are bedridden (Bacalman et al. 2006). FXTAS progression also includes brain atrophy and white matter disease with gradual loss of cognitive function. Most individuals who die of FXTAS were at FXTAS stage 6.

3b. Brain tissue samples

Frozen post-mortem human cerebellum tissue from 16 PM cases (mean age 78, range= 64–108 CGG repeats; mean= 87) were obtained from the MIND Institute Brain Repository at the University of California at Davis in Sacramento, CA. All of the 16 PM cases were

diagnosed with FXTAS at the time they were seen clinically and were bedridden at the time they died (stage 6). Post-mortem tissue from 8 age matched typical developing (TD) controls (mean age 67, range: 21–42 CGG repeats; mean= 30) were obtained frozen from the Harvard Brain Tissue Resource Center at McLean Hospital in Belmont, MA. (n= 1); from the Brain and Tissue Bank for Developmental Disorders of the National Institute of Child Health and Human Development at the University of Maryland in Baltimore, MD (n= 2); and from the Miami Brain Endowment Bank at the University of Miami at Florida (n= 5).

3c. CGG repeat sizing

CGG repeat sizing was determined by Polymerase Chain Reaction (PCR) and Southern blot (SB) analysis as previously described (Tassone et al. 2008, Filipovic-Sadic et al. 2010, Fernandez-Carvajal et al. 2009). Genomic DNA was obtained from peripheral blood and brain regions using a Qiagen DNA isolation kit following standard procedures (Qiagen, Valencia, CA). When necessary, a dounce homogenizer was used to promote dissolution of lysates, which were then spun at 13,000 rpm for 10 minutes twice, and DNA was resuspended in TE buffer.

3d. Gene expression assay

Total RNA from cerebellum tissue was isolated using standard procedures (Trizol; Life Technologies, Carlsbad, CA). mRNA expression levels in the cerebellum was quantified by Real Time quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using Taqman gene expression assays (Assay on demand, Applied Biosystems, Foster City, CA). Probes included *EAAT2* (Hs01102415), *EAAT1* (HS00904818_m1), *GRM1 or mGLUR1* (HS00168250_m1), and *GRM5 or mGLUR5* (HS00168275_m1) probes; as well as a custom designed probes for *FMR1* (5' 6 FAM - TGATGAAGTTGAGGTGTATTCCAGAGCAAATGA - 3' TAMRA) and β-glucuronidase (*GUS*) (5' 6 FAM - TGAACAGTCACCGACGAGAGTGCTGG - 3' TAMRA) (Tassone et al. 2000). The β-*GUS* gene was used for normalization.

3e. Protein extraction and Western blot (WB) analysis

Frozen cerebellum tissue preserved at −80 °C from 16 PM carriers (CGG ranging from 64 to 108 repeats) and 8 age matched controls (CGG ranging from 21 to 42 repeats) was grounded in liquid nitrogen and resuspended in 1× RIPA buffer (Cell Signaling, Beverly, MS) complemented with complete ULTRA protease inhibitor tablets (Roche Applied Science, Indianapolis, IN) and AEBSF (Sigma-Aldrich, Saint Louis, MO). The lysates were kept on ice until homogenized by sonication using 15 seconds on/off intervals after which, followed by centrifugation at 18,000 rpm in a Sorvall centrifuge at 4°C. The supernatants containing the protein extraction were collected and protein concentrations were measured using a BCA Protein Assay (Thermo Scientific, Rockford, IL). Sample concentrations were adjusted for SDS-PAGE electrophoresis in Laemmli buffer containing 100mM DTT. The proteins were separated by SDS-PAGE electrophoresis using Criterion XT pre-cast gels in 1× MOPS buffer running at a constant current of 25 mA for 30 minutes followed by 40 mA for 4–6 hours and transferred to a PVDF membrane (Millipore, Billerica, MA) in 20% methanol

Tris/Glycine/SDS buffer at a constant current of 150 mA for 16 hours at 4 °C. Membranes were blocked in 1× TBST (USB Corporation, Cleveland, OH) with 5% milk and probed overnight at 4 °C with either mouse anti-FMRP (Chemicon, Temecula, CA, 1:1,000 dilution), mouse anti-GAPDH (Chemicon, Temecula, IL, 1:80,000 dilution), rabbit anti-EAAT2 (Aviva Systems Biology, San Diego, CA, 1:1,000 dilution), mouse anti-EAAT1 (Novacastra, Leica Microsystems, United Kingdom, 1:2,000 dilution), or mouse anti-mGluR1/5 (NeuroMab, Davis, CA, 1:500 dilution). The membranes were then washed 3 times, each for 15 minutes in 1× TBST and incubated in a1:5000 diluted goat anti-mouse (BioRAD Laboratories, Hercules, CA) or goat anti-rabbit (Cell Signaling Technologies, Inc.) horseradish peroxidase-coupled secondary antibodies for 2 hours at room temperature. The membranes were then washed and analyzed by chemiluminescence using SuperSignal West Dura extended duration substrate (Thermo Scientific, Rockford, IL). Band intensities were measured by densitometry using AlphaView SA software, version 3.2.2.0, from Cell Biosciences, Inc. WBs were also performed using LICOR (LICOR Biosciences, Lincoln, NE). Briefly, following transfer, the PVDF membranes were blocked in LICOR blocking buffer and hybridized overnight at 4 °C with either mouse anti-FMRP (Chemicon, Temecula, CA, 1:1,000 dilution), mouse anti-GAPDH (Chemicon, Temecula, IL, 1:200,000 dilution), rabbit anti-EAAT2 (Aviva Systems Biology, San Diego, CA, 1:1,000dilution), mouse anti-EAAT1 (Novacastra, Leica Microsystems, United Kingdom, 1:2,000 dilution), sheep anti-mGluR5 (R&D Systems, 1:500 dilution), or sheep anti-mGluR1 (R&D Systems, 1:500 dilution). The membranes were washed in 1× TBST followed by 2 hours incubation at RT with the corresponding IRDye 680RD goat anti-rabbit or IRDye 800CW goat anti mouse secondary antibodies (LICOR Biosciences, Lincoln, NE). The membranes were then washed in 1× TBST containing 0.02% SDS. Bands were imaged at 169 μm resolution using the Odyssey infrared scanner following manufacture recommendations. Mouse protein extracts were prepared from cerebellum tissue derived from preCGG and WT mice at different developmental stages following the protocol as described above. WB were performed using chemiluminescence and analyzed as described above.

3f. Immunohistochemistry

Sections of cerebellum from PM and control subjects were paraffin embedded, thin sectioned on a microtome and mounted on glass slides. Sections were deparafinized for 15 minutes in SafeClear (Fisher, City, state) and hydrated by sequential 15 minutes rinses in ethanol (100%, 95%, 70%, 50%) followed by distilled H_2O for 5 minutes and 0.1 M PBS (pH 7.4). Sections were treated for antigen retrieval for 15 min in boiling citrate buffer (10 mM) and cooled to room temperature. Endogenous peroxidase was quenched by treating sections with 3% H_2O_2 for 20 minutes after which sections were blocked for one hour in 10% donkey serum, 0.2% gelatin, and 1% Triton X-100 diluted in PBS. Sections were incubated overnight in either mouse anti-FMRP (Chemicon, Temecula, CA), mouse anti-EAAT1 (Novacastra, Leica Microsystems, United Kingdom, 1:2,000 dilution), rabbit anti-EAAT2 (Aviva Systems Biology, San Diego, CA; 1:1,000 dilution), or sheep anti-mGluR5 (R&D, Minneapolis, MN; 1:500 dilution), in incubation buffer (2% donkey serum, 0.04% gelatin, and 0.2% Triton X-100 diluted in PBS). Sections were rinsed in PBS and incubated for one hour at room temperature in donkey anti-sheep biotinylated antibody (Jackson, West Gove, PA; 1:200 dilution) diluted in incubation buffer. Biotinylated antibodies were

visualized with an ABC Kit (Vector) d in incubation buffer eveloped with DAB. Sections were then rinsed in distilled H_2O for 5 minutes followed by sequential 15 minute rinses in ethanol (50%, 70%, 95%, 100%), two 15 minute rinses in SafeClear, and coverslipped with DPX (Sigma, St. Louis, MO). Sections were imaged on an Olympus BX61 and figures arranged with Photoshop (Adobe Systems Incorporated, v. 7.0).

3g. Statistical analysis

Protein data were normalized for differences between gels as follows: A linear mixed model including fixed effects for gel and group (TD vs. PM) and a random effect for sample was fitted to log transformed data. The estimated gel effects from this model were exponentiated to obtain the geometric mean ratio between each gel and a reference gel, and data from each gel were divided by the corresponding geometric mean ratio. Protein data were normalized separately for each protein. Following normalization, data were averaged across replicates. Protein data were log transformed prior to analysis in order to symmetrize the data. mRNA data were not log transformed as no skewness was apparent in plots. Differences in protein expression or mRNA expression between groups were analyzed using two-sample t-tests. The relationship between protein/mRNA expression and CGG length was analyzed using linear regression, which examines the incremental change in expression with each additional CGG repeat. Analyses were conducted using R, version 2.15.2 (R Core Team, 2012).

4. Results

4a. Subjects description

Sixteen PM cases were included in the analysis. Among these individuals, all were confirmed cases with FXTAS with a FXTAS stage varying between 4 and 6 obtained at the time they were seen clinically and all were bedridden at the time of death (FXTAS stage 6). The description of demographic, molecular and clinical information is presented in Table 1.

4b. *FMR1* and FMRP expression is altered in the brain of PM carriers

SB analysis and PCR were used to determine the CGG size. Eight cases with no history of any neurodegenerative disorders were identified as unaffected TD controls and their genotype confirmed with detection of a CGG repeat allele ranging between 21 and 42 CGG repeats. Sixteen cases with CGG repeats, which ranged between 64 and 108 CGG repeats, were confirmed PM carriers (Fig. 1). SB analysis of blood did not detect the presence of methylated alleles in any of the PM samples analyzed.

We focused our investigation on the cerebellum as this area of the brain is highly affected in FXTAS and tissue was available for all PM cases and TD controls. *FMR1* mRNA expression in the cerebellum estimated by qRT-PCR showed an increased *FMR1* transcript levels, particularly in the higher CGG range as was reported previously (Garcia-Arocena et al. 2010, Tassone et al. 2004b) (Fig. 2a). Expression levels assessed by WB analysis in cerebellar protein lysate extracts obtained from the same tissue blocks used for DNA and RNA measures showed a significant reduction in FMRP level with increasing number of CGG repeats, with an estimate 1.7% decrease in FMRP expression for each additional CGG repeat (p= 0.004 from linear regression analysis) (Fig 2b, c). These results indicate that

FMRP is less abundant in the cerebellum of PM individuals and more so in those with larger expanded alleles. These results are consistent with previous observations of lower FMRP expression in human brain as well as in human peripheral blood leukocytes and lymphoblastoid cell lines (Tassone et al. 2000, Kenneson et al. 2001, Garcia-Arocena et al. 2010, Tassone et al. 2004b, Allen et al. 2005, Peprah et al. 2010). Importantly, this data also demonstrate that increased *FMR1* mRNA and decreased expression of FMRP levels in the brain of PM are correlated to the length of the CGG expansion although the correlation is not as strong as the one observed in peripheral blood (Tassone et al. 2000, Tassone et al. 2004a, Greco et al. 2002, Greco et al. 2006, Garcia-Arocena et al. 2010).

4c. EAAT1 and mGluR5 expression are downregulated in the cerebellum of PM carriers

To investigate whether expression of the main astrocytic Glu transporters EAAT2 and EAAT1 are affected by reduced FMRP expression, we measured their mRNA levels from cerebellum derived from the same subject. Our findings indicated that *EAAT2* mRNA expression in PM tended to decrease with increasing CGG repeat numbers, but the association did not reach statistical significance ($p = 0.662$ from linear regression analysis) (data not shown). By contrast, *EAAT1* mRNA expression decreased marginally significantly with increasing length of CGG repeats, with an estimated change of ~0.004 in EAAT1 expression for each additional CGG repeat ($p = 0.049$ from linear regression analysis) (Fig. 3a). We also measured the expression levels of EAAT2 and EAAT1 Glu transporter proteins using WB analysis. Although EAAT2 expression was not significantly different between PM and TD ($p = 0.138$ from two sample t-test) (data not shown), we observed a significant lower levels of EAAT1 protein expression that was correlated with the length of the CGG repeat, with an estimated 1.9% decrease in EAAT1 expression for each additional CGG repeat ($p = 0.002$ from linear regression analysis) (Fig. 3b, c). EAAT2 and EAAT1 mRNA and protein levels were also measured in frontal cortex derived from the majority of the same subjects (n=15) for which tissue was available, but no statistically significant difference was observed when compared to TD (n= 3).

Targets of FMRP regulation are mGluR1 and mGluR5, which have been both implicated in neuronal network impairments associated with FXS (Bear et al. 2004). To investigate whether altered expression of mGluR proteins might be affected with expanded CGG repeat in the PM range, the expression of *GRM1* and *GRM5* mRNA, and of their respective protein products mGluR1 and mGluR5, were measured in cerebellum. Although qRT-PCR of *GRM1* (data not shown) and *GRM5* (Fig. 4a) showed decreased expression levels in most of the PM cases with increased CGG repeat, differences were not statistically significant compared to TD ($p = 0.455$ and 0.115, respectively from two-sample t-tests). However, using specific antibodies, we detected a significant reduction of mGluR5 protein expression with increasing number of CGG repeats by WB in PM compared to TD, with an estimated 1.0% decrease in mGluR5 expression for each additional CGG repeat ($p = 0.021$ from linear regression analysis) (Fig 4b). No differences in mGluR1 expression were detected by WB analysis (data not shown). Gene expression of *GRM1* and *GRM5* mRNA was also measured in frontal cortex but no differences were detected and protein expression levels were not measured.

Using immunohistochemistry (IHC) we also determined the cell distribution of FMRP, EAAT1 and mGluR5 expression in cerebellar sections from subjects in our study. FMRP was expressed in the molecular and granule cell layers of the cerebellum, with marked expression in Purkinje cells. Our qualitative analysis indicates a similar cellular distribution between TD and PM cases with reduced overall immunoreactivity of the FMRP antibody in the PM cases as compared to TD (Fig.5a and 5b). IHC analysis of EAAT1 showed numerous EAAT1 positive cells with an overall reduction in immunoreactivity in the granule cell layer of PM and lack of immunoreactivity in Purkinje cells for both TD and PM (Fig.5c and 5d). IHC analysis also showed a similar overall distribution of mGluR5 immunoreactivity between TD and PM subjects (Fig.5e and 5f), suggesting that the observed detected decreased expression is likely not the results of altered cellular distributions.

4d. Protein expression levels in the mouse model of premutation (preCGG)

Protein expression levels of GLT1, GLAST and mGluR1/5 were measured in whole cerebellum extracts of preCGG mice (170 CGG repeats) at different developmental stages (P0, P14, P21, P35, and at 20, and 52 weeks) (Fig. 6). WB analysis of mGluR1/5 was performed only in the 52 week-old mice. Differences between WT and preCGG mice did not reach statistical significance at any of the age analyzed. However, we observed a trend for lower GLAST in preCGG cerebellum at P21and 20 weeks. The small number of mice (n=5) and large variability between samples may have precluded us from detecting any differences.

5. Discussion

In this study, we observed a CGG dependent reciprocal regulation of *FMR1* mRNA (increase) and FMRP (reduction) expression levels in postmortem cerebellum derived from male PM carriers who had received a clinical diagnosis of FXTAS. We identified a significant reduction of EAAT1 Glu transporter at both mRNA and protein levels, and of mGluR5 at protein level, in the cerebellum of FXTAS subjects with larger CGG repeats. In addition, IHC indicates that the distribution of EAAT1 and mGluR5 proteins in the cerebellar cortex is similar between PM and TD. Our study shows a reduction of EAAT1 expression in human cerebellar granule cell layer in PM carriers, which could be related to the downregulation of mGluR5 observed in the same individuals and could indicate a potential trigger for excessive glutamatergic signaling in the PM as detected in FXS (Bear et al. 2004). It is possible that downregulation of Glu uptake, retaining higher concentrations of Glu in the synaptic cleft, results in overstimulation of Glu activity leading to toxicity. However, it has been observed that mGluRs become rapidly downregulated in response to overstimulation (Javitt et al. 2011) which might explain the reduced mGluR5 expression levels we observe in the study. The recent observation of enhanced mGluR mediated long term depression in hippocampal slides from preCGG mice further support a role of mGluR mediated synaptic translation in FXTAS (Iliff et al. 2013).

Notably, it was recently reported that Glu uptake is reduced in cortical extracts of the *FMR1* KO mice, although this is thought to be mediated by GLT1; Downregulation of mGluR5 and

GLAST were also observed in these fractions at early but not at later developmental stages (Higashimori et al. 2013). The preCGG KI mice show rotarod deficits (Van Dam et al. 2005), and subtle but significant increased foot slips on the ladder rung test that could be analogous to a mild ataxia (Hunsaker et al. 2011), and poor skilled forelimb motor learning (Diep et al. 2012). However, the preCGG mice do not develop severe ataxia and premature mortality seen in some FXTAS patients. Because GLAST is the primary Glu transporter expressed in the cerebellum it is possible that the lack of significantly reduced GLAST expression in the cerebellum of preCGG KI mice may explain the much milder motor deficits in the mice compared to FXTAS patients, as well as the lack of tremor activity. In fact, evidence linking EAAT1 in humans with ataxia includes a cysteine to serine mutation (C186S) in a transmembrane domain of EAAT1 which has been shown to reduce Glu uptake and is explicitly linked to episodic ataxia in three human cases carriers of this mutation (de Vries et al. 2009). Similar studies also link EAAT1 to seizures and vertigo (Jen et al. 2005, Ueda et al. 2001).

Downregulation of the vesicular glutamate transporters VGLUT and VGAT in preCGG hippocampal neurons at 21 DIV is associated with abnormal clustered burst (CB) firing electrical activity as well as abnormal patterns of synchronized calcium oscillations in the cytosol (Cao et al. 2012). Addition of the type I mGluR activator dihydroxyphenylglycine (DHPG) or the GABAA antagonist picrotoxin to wild type neurons produces CB firing electrical activity that mimics those observed in preCGG cultures. Moreover, CB firing activity can be normalized by pharmacological suppression of type I mGluRs activity with addition of either 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP), an antagonist of mGluR5 receptors, or (−)-ethyl (7E)-7-hydroxyimino-1,7a-dihydrocyclopropa[b]chromene-1a-carboxylate (CPCCOEt), an mGluR1 antagonist. Moreover, enhancement of postsynaptic GABAA receptor activity with the positive allosteric modulator allopregnanolone also effectively normalizes CB firing in preCGG neuronal networks. Collectively these data strongly suggested an altered exitatory-inhibitory balance of metabotrophic glutamatergic and ionotrophic GABAergic signaling exists in neuronal networks formed by preCGG mice (Cao et al. 2012). These functional impairments are directly pertinent to the preCGG pathology, such as altered dendritic morphology in cultured hippocampal neurons (Chen et al. 2010) and mouse brain (Berman et al. 2012). More recently, we identified 10% reduction of GLT1 (EAAT2) and GLAST (EAAT1) in enriched cortical astrocytic cultures from preCGG mice, which is consistent with about 10% reduction on the Glu uptake in the preCGG cortical cultures. Consistent with astrocyte cultures *in vitro*, the cerebral cortex of aged preCGG KI mice also displayed reduced GLAST (72.3±3.1% of WT) and GLT-1 expression (77.2±17.8% of WT). The reduced Glu transporters expression / uptake were demonstrated to contribute to the Ca^{2+} oscillatory behaviors in the preCGG cortical astrocytes. Direct evidence for mGluR5 impairment has been reported in preCGG cortical astrocytes (Cao et al. 2013). PreCGG cortical astrocytes display altered Glu-stimulated Ca^{2+} response which can be completely blocked by mGluR5 antagonist (Cao et al. 2013). These findings indicated that functional defects in preCGG astrocytes, especially in Glu signaling, may contribute to the development of neuronal pathology in FXTAS.

Similarly, our findings support the hypothesis that Glu transporters and mGluR5 might be involved in the pathology of FXTAS. These observations may establish a correlate between mechanisms of dysregulation in neuronal activity between FXS and FXTAS that may be significant in establishing similar potential avenues for pharmacological treatments for PM and FXTAS. Current treatments for FXTAS are mostly limited to comorbid symptoms of the disorder such as depression, anxiety and GI problems as the incessant progression of the disabling disorder is thus far unavoidable (Leehey and Hagerman 2012). Importantly, a deficit in dendritic complexity and altered synaptic morphology, well documented in the *fmr1* KO mice has also been observed in preCGG mice (Berman et al. 2012) and in cultured neurons between 7 and 21 DIV (Chen et al. 2010), which can ultimately affect synaptic integration and potentially contribute to the neurodevelopmental and cognitive deficits seen in premutation carriers particular later in life. The use of mGluR5 antagonists currently under investigation in FXS human clinical trials might therefore be helpful in the treatment of other fragile X associated disorders such as FXTAS and autism.

In conclusion, our observations of decrease expression of EAAT1 transporter and mGluR5 receptor in human PM cases with FXTAS point toward a defect in Glu signaling in human PM carriers and suggests that this type of dysregulation may contribute to the pathology and possibly affect the onset of FXTAS.

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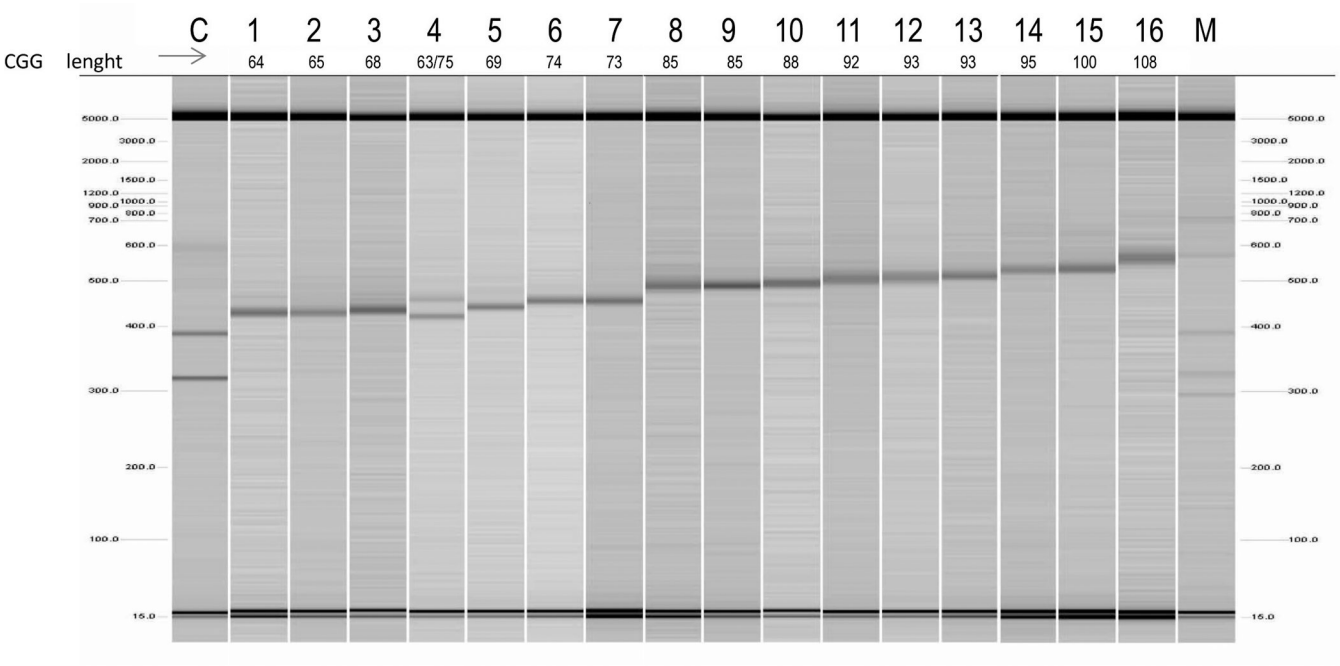

Figure 1.

CGG repeat sizing by gel electrophoresis *FMR1* allele CGG sizing of cerebellum samples from male PM carriers included in this study. C= control female with 30 and 55 CGG repeats. M= DNA size marker. CGG repeat number is shown for each sample on the top of the gel.

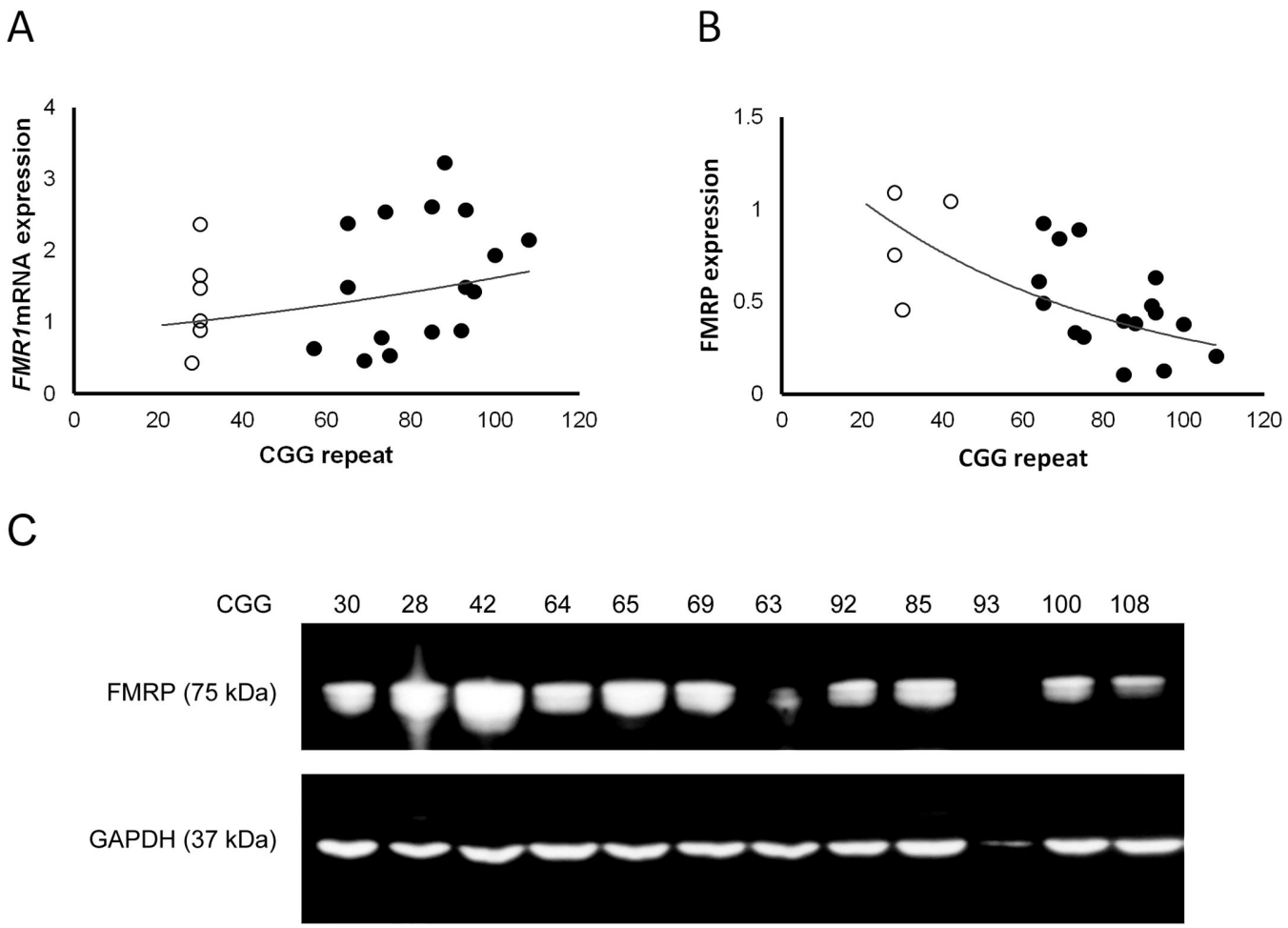

Figure 2.

FMR1 mRNA and FMRP expression levels. Scatter plots showing expression of *FMR1* mRNA and FMRP as a function of CGG length. Controls cases are depicted with open circles and PM cases with dark circles. A) Relative *FMR1* mRNA expression levels (relative to b-glucoronidase, y axis) plotted as a function of CGG length (X axis) show higher levels particularly in PM carriers with larger CGG repeat range. B) FMRP expression levels determined by WB analysis are plotted as a function of CGG repeat length and show that FMRP expression in PM carriers is reduced especially in the higher CGG repeat range. C) Representative Western blot image of FMRP expression in PM cerebellum samples. CGG repeat size is indicated on the top of the gel.

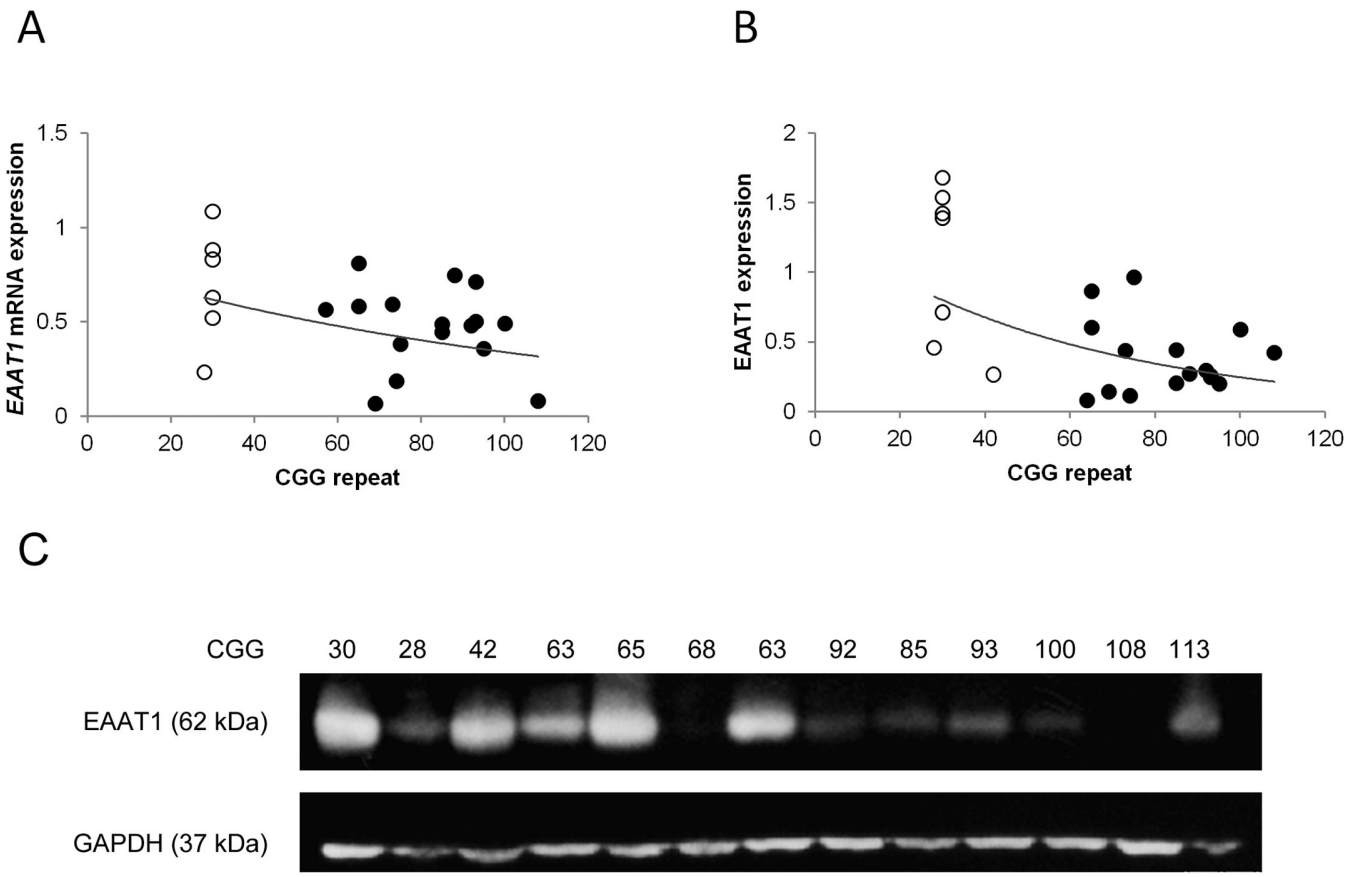

Figure 3.

EAAT1 mRNA and protein expression levels. Scatter plots showing *EAAT1* mRNA and protein expression as a function of CGG length. Controls cases are depicted with open circles and PM cases with dark circles. A) qRT-PCR derived CT values for *EAAT1* decrease with increasing CGG length. B) EAAT1 expression levels (detected by WB using a specific mouse anti-*SLC1A3* (EAAT1) antibody) are reduced in individuals with greater CGG repeat length. C) Representative Western blot image of EEAT1 expression in PM cerebellum samples. CGG repeat size is indicated on the top of the gel.

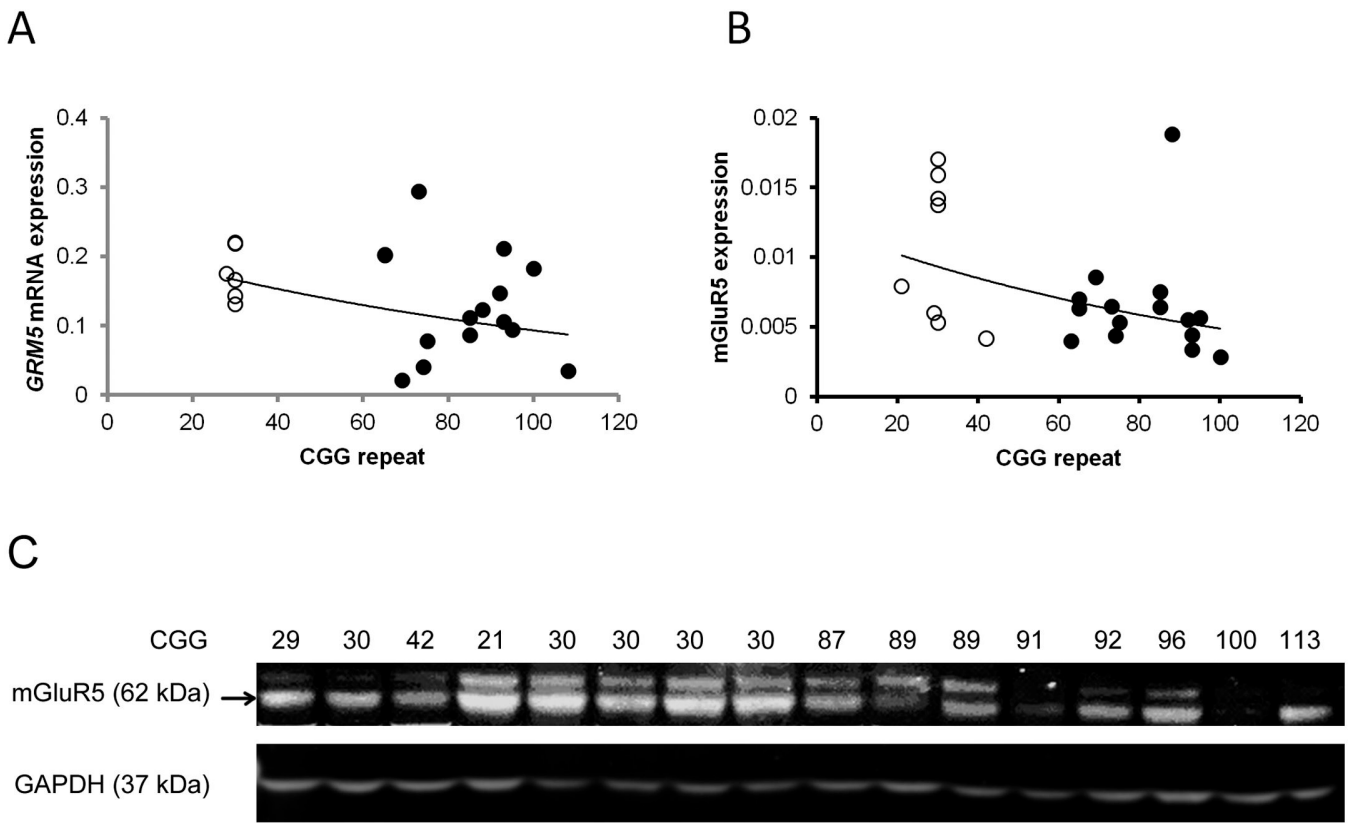

Figure 4.

GRM5 mRNA and mGluR5 protein expression levels. Scatter plots show mGluR5 expression as a function of CGG length. Controls cases are depicted with open circles and PM cases with dark circles. A) qRT-PCR derived CT values for *GRM5* decrease with increasing CGG length. B) Statistically significant decreased mGluR5 protein expression levels are observed by WB in PM cases, particularly in the higher CGG repeat range. C) Representative Western blot image of mGluR5 expression in PM cerebellum samples. CGG repeat size is indicated on the top of the gel.

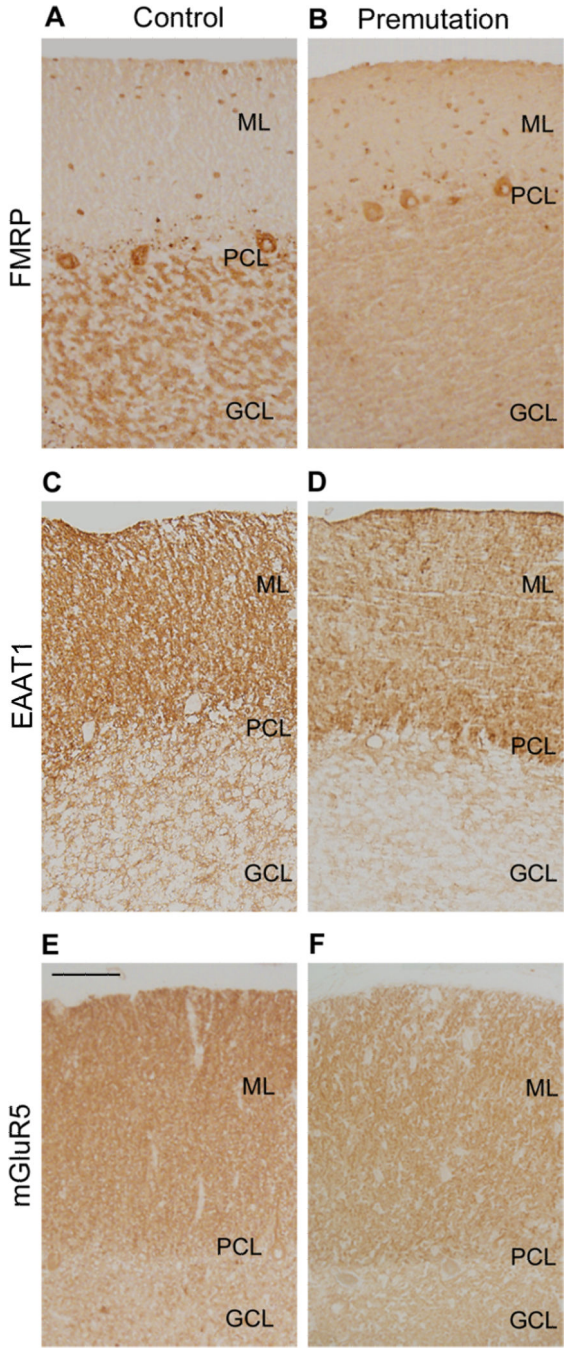

Figure 5.

Detection of FMRP, EAAT1 and mGluR5 in PM and control cerebellar tissue. Immunohistochemistry (IHC) shows depletion of FMRP in the PM (B) when compared to control (A); overall reduced staining in the granule cell layer for EAAT1 in the PM (D) when compared to control (C) and a similar distribution with reduced immunoreactivity for mGluR5 in PM (F) compared to control (E). IHC was carried out in 5 cases (2 TD and 3 PM). The IHC experiments showed in this figure are representative of the entire set analyzed.

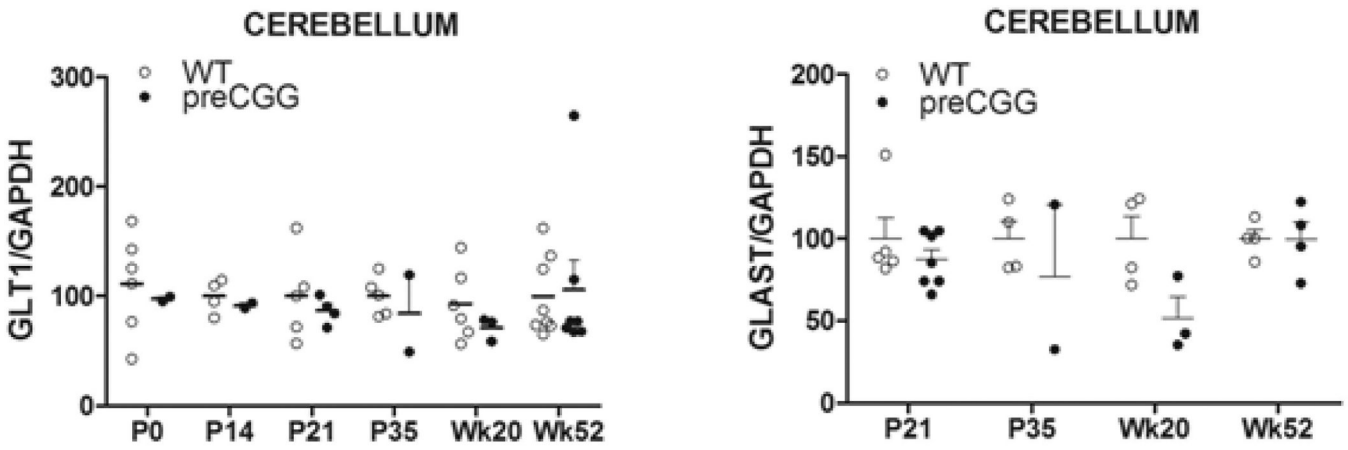

Figure 6.

GLT1 and GLAST expression in the cerebellum of preCGG KI mouse during post-natal developmental stages. GLT1 and GLAST expression was measured by WB in WT mice and in preCGG mice with ≥170 CGG repeats. Although a tendency for lower GLT1 and GLAST expression was observed in the preCGG mice, except at 52 weeks, these differences were statistically not significantly different than WT.

Table 1

Demographic, molecular and clinical description of PM cases in this study.

Case	Gender	Race	CGG length in cerebellum	Age at death	PMI (hrs)	Diagnosis
1	male	Caucasian	64	76	n/a	FXTAS
2	male	Caucasian	65	87	n/a	FXTAS
3	male	Caucasian	68	82	n/a	FXTAS
4	male	Caucasian	63/75	72	2.5	FXTAS
5	male	Caucasian	69	82	4.75	FXTAS
6	male	Caucasian	74	77	24	FXTAS
7	male	Caucasian	73	87	n/a	FXTAS
8	male	Caucasian	85	85	22.5	FXTAS
9	male	Caucasian	85	85	29.5	FXTAS
10	male	Caucasian	88	75	5	FXTAS
11	male	Caucasian	92	75	n/a	FXTAS
12	male	Caucasian	93	82	n/a	FXTAS, Lewy body dementia
13	male	Caucasian	93	81	n/a	FXTAS
14	male	Caucasian	95	68	7.16	FXTAS, AD
15	male	Caucasian	100	67	n/a	FXTAS
16	male	Caucasian	108	78	n/a	FXTAS

n/a = not available

FXTAS= Fragile X Tremor Ataxia Syndrome

AD=AlzheimersDisease