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Abstract

Many clinical and molecular features of the fragile X syndrome, a common form of intellectual disability and autism, can be modeled by deletion of the *Fmr1* protein (Fmrp) in mice. Previous studies showed a decreased expression of several components of the GABAergic system in *Fmr1* knockout mice. Here, we used this mouse model to investigate the functional consequences of Fmrp deletion on hippocampal GABAergic inhibition in the CA1-region of the hippocampus. Whole-cell patch-clamp recordings demonstrated a significantly reduced amplitude of evoked inhibitory postsynaptic currents (eIPSCs) and a decrease in the amplitude and frequency of spontaneous IPSCs. In addition, miniature IPSCs were reduced in amplitude and frequency and decayed significantly slower than mIPSCs in controls. Quantitative real-time PCR revealed a significantly lower expression of α2, β1 and δ $GABA_A$ receptor subunits in the hippocampus of the juvenile mice (P22) compared to wild-type littermates. Correspondingly, we found also at the protein level reduced amounts of α2, β1 and δ subunits in *Fmr1* knockout mice. Overall, these results demonstrate that the reduction in several components of the GABAergic system is already present at young age and that this reduction results in measurable abnormalities on $GABA_A$ receptor-mediated phasic inhibition. These abnormalities might contribute to the behavioral and cognitive deficits of this fragile X mouse model.

Keywords: fragile X syndrome, *Fmr1* knockout mice, $GABA_A$ receptor subunit, inhibitory postsynaptic current, hippocampus

Highlights:

- The expression of α2, β1 and δ $GABA_A$ receptor subunit mRNA is significantly decreased in young mice.
- The expression of $GABA_A$ α2, β1 and δ subunits is significantly reduced at the protein level.

- Evoked, spontaneous and miniature IPSCs in CA1 pyramidal neurons are reduced.
- GABAergic dysfunction contributes to behavioral and cognitive deficits of *Fmr1* mice.

Abbreviations:

AMPA: 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid

AP5: 2-amino-5-phosphonovaleric acid

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

eIPSC: evoked inhibitory postsynaptic current

FMR1: fragile X mental retardation 1

FMRP: fragile X mental retardation protein

GABA: gamma aminobutyric acid

Gp1 mGluR: group 1 metabotropic glutamate receptor

IEI: inter-event interval

KO: knockout

KS-test: Kolmogorov-Smirnov test

mIPSC: miniature inhibitory postsynaptic current

NMDA: *N*-methyl-D-aspartate

P22: postnatal day 22

PPR: paired-pulse ratio

RE: relative expression

RM-ANOVA: Two-way repeated measures ANOVA

sIPSC: spontaneous inhibitory postsynaptic current

TTX: tetrodotoxin

WT: wild-type

1. Introduction

Fragile X syndrome is the most common form of inherited intellectual disability with a prevalence of approximately 1 in 5000 (Coffee et al., 2009). In addition to cognitive impairment, patients are characterized by typical facial dysmorphic features, macroorchidism, and several behavioral problems including hyperactivity, enhanced fear and social anxiety, aggression and autistic-like behavior (Abbeduto et al., 2014; Hagerman, 2002; Loesch et al., 2007; Santos et al., 2014; Smith et al., 2012). Moreover, 20% of patients suffer from epileptic seizures (Musumeci et al., 1999). The disease is typically caused by a CGG repeat expansion in the 5' untranslated region of the fragile X mental retardation 1 (*FMR1*) gene (Verkerk et al., 1991). Expansion of this repeat above the threshold of 200 units induces hypermethylation of the repeat itself and a CpG island in the associated promoter region, resulting in transcriptional silencing and, consequently, absence of the encoded fragile X mental retardation protein (FMRP) (Pieretti et al., 1991). FMRP is an RNA binding protein that interacts with many neuronal mRNAs and is thought to be involved in the regulation of mRNA transport, translation and stability (Bassell and Warren, 2008; De Rubeis and Bagni, 2010).

Studies in animal models of fragile X syndrome have proven to be essential for unraveling the molecular mechanisms underlying the disease and led to the identification of potential therapeutic targets (Bagni et al., 2012; Braat and Kooy, 2014; Darnell and Klann, 2013; Heulens and Kooy, 2011; Wijetunge et al., 2012). Exaggerated group 1 metabotropic glutamate receptor (mGluR) signaling (Bear et al., 2004) in parallel with impaired GABAergic signaling (Braat and Kooy, 2015) are among the targets identified, suggesting the clinical consequences of the absence of FMRP are at least in part due to a disturbance of the inhibition/excitation balance (Contractor et al., 2015).

. Several studies have revealed brain region-specific deficits in the inhibitory GABAergic system of *Fmr1* knockout mice (Adusei et al., 2010; D'Antuono et al., 2003; D'Hulst et al., 2006; El Idrissi et al., 2005; Gantois et al., 2006; Kratovac and Corbin, 2013; Vislay et al., 2013). Underexpresssion was confirmed in patients (D'Hulst et al., 2015).

Gamma aminobutyric acid type A ($GABA_A$) receptors are the principle receptors mediating fast inhibition in the central nervous system (Olsen and Sieghart, 2008). These are heteropentameric ligand-gated chloride channels, assembled as a nonrandom combination of the 19 known subunits [α(1-6), β(1-3), γ(1-3), δ, ε, ρ(1-3), θ and π] with further increased variability by alternative splicing (Farrant and Nusser, 2005; Huntsman et al., 1998; Jin et al., 2004; Piton et al., 2013). The resulting distinct subtypes have an unique developmental, regional and (sub)cellular expression pattern, distinct physiological properties and sensitivities to GABA and allosteric modulators (Farrant & Nusser, 2005)(D'Hulst et al., 2009). Interestingly, $GABA_A$ receptors are involved in processes such as anxiety, epilepsy, insomnia, depression and learning and memory (Rudolph and Knoflach, 2011), all implicated in fragile X syndrome (D'Hulst and Kooy, 2007).

Of the many open questions around GABA-ergic dysfunction in fragile X syndrome we focused in this study on the functional consequences of the absence of Fmrp on $GABA_A$ receptor-mediated phasic inhibition.

2. Material and methods

2.1. Mouse breeding and genotyping

Male *Fmr1* knockout mice and wild-type littermates were generated by crossing females heterozygous for the *Fmr1* mutation (B6.129P2-Fmr1^{tm1Cgr}/Ant or *Fmr1* KO2 backcrossed for more than 20 generations to C57BL/6J) and C57BL/6J wild-type males (Charles River, Wilmington, MA, USA). Genotypes were determined by PCR on DNA isolated from tail biopsies (Bakker et al., 1994)(Mientjes et al., 2006). All animals were housed in mixed genotype groups of approximately 5 littermates in standard mouse cages under conventional laboratory conditions (food and water ad libitum, constant room temperature and humidity, 12:12 h light-dark cycle). Mice were between the age of 3 and 4 weeks at the time of the experiments. All experiments were carried out in compliance with the European Community Council directive (2010/63/EU) and approved by the Animal Ethics Committee of the University of Antwerp.

2.2. Whole-cell patch-clamp recordings

$GABA_A$ receptor-mediated inhibition was studied in transverse slices (400 µm thick), prepared from the medial hippocampus of *Fmr1* knockout and wild-type mice at an age of 4 weeks using a vibratome (Microm HM 650 V, Thermo Scientific, Waltham, MA, USA). Thereafter, slices were placed for about 90 min in an incubation chamber containing ACSF (in mM: NaCl, 124; KCl, 4.9; NaH_2PO_4, 1.2; $NaHCO_3$, 25.6; $CaCl_2$, 2; $MgSO_4$, 2; glucose, 10; saturated with 95% O_2 and 5% CO_2, pH 7.3–7.4) continuously perfused with 95%O_2/ 5%CO_2 at 32^{o}C. Whole-cell voltage clamp recordings were made from CA1 pyramidal neurons at 32^{o}C using a MultiClamp 700B patch-clamp amplifier and data were collected using pClamp software (Axon Instruments, Union City, CA, USA). The pipette solution contained (in mM): 140 CsCl, 10 Na-HEPES, 10 EGTA, 2 MgATP, 5 QX-314, pH 7.3 (pipette resistance 3-5 MΩ). Access resistance (RS) was 10-20 MΩ and was then compensated to 75%. Only recordings where RS remained below 20 MΩ and did not increase by more than 20% during the course of experiment were included in the analysis. 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 µM) and 2-amino-5-phospho-novaleric acid (AP5, 40 µM) were present in the bath medium to block 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA) receptor and *N*-methyl-D-aspartate (NMDA) receptor-mediated currents, respectively. $GABA_B$ receptor-mediated currents were blocked by CsCl and QX-314 in the pipette solution.

A sequence of stimulating protocols was applied to the recorded neuron: (a) an input/output protocol to evaluate stimulus-response function; (b) paired-pulse stimulation to detect tentative presynaptic effects; (c) trains of 200 stimuli at 10 Hz and then recovery at 0.1 Hz to measure synaptic fatigue and subsequent recovery. Electrical stimuli (10-100 µA) were applied through a PI-ir electrode placed in the CA1 stratum lacunosum-moleculare to generate eIPSCs (Banks et al., 2002). Cells were held at -60 mV. The eIPSC amplitude was measured with 5 IPSCs averaged for each neuron per stimulation strength. 29 wild-type and 28 *Fmr1* knockout neurons of each 10 animals were analyzed. For the paired-pulse stimulation protocol two identical stimuli were applied at an interval of 50 ms and 80 ms

(Centonze et al., 2008). The stimulus intensity was adjusted to evoke responses of 50-60% of the size obtained with 100 µA stimulus current. The paired-pulse ratio (PPR) was calculated as the ratio of the amplitude of the second response to that of the first. To examine presynaptic fatigue, IPSCs were recorded during a train of 400 stimuli applied at 10 Hz with the same stimulus strength as in paired-pulse protocol. Immediately thereafter, recovery of IPSCs was examined by recording at 0.1 Hz (Ferguson et al., 2007).

Spontaneous IPSCs (sIPSCs) and then, following application of 1 µM tetrodotoxin (TTX), miniature IPSCs (mIPSCs) were recorded at a holding potential of -60 mV. Data were low-pass filtered at 2 kHz and acquired at 10 kHz using Digidata 1440 and pClamp 10 software. Off-line analysis of sIPSCs and mIPSCs was performed using MiniAnalysis software (v.6.0.7, Synaptosoft, Decatur, GA, USA), MATLAB R2011b (MathWorks, Natick, MA, USA), and IBM SPSS Statistics 19 (IBM, Armonk, NY, USA). For amplitude analysis, 100 individual sIPSCs were randomly selected from the event list files thereby ensuring that each wild-type and *Fmr1* knockout neuron was equally represented in the allied data subsets. For frequency analysis (inter-event intervals), entire populations of the event list files were analyzed. Kinetic analysis was performed on ensemble mIPSCs that were created by aligning all mIPSCs from each event list file to 50% rise time. In order to allow a fit of all mIPSCs, two-phase exponential fits of the 90–10% decay phase with the least-squares simplex method were calculated (Picton and Fisher, 2007). In total, sIPSCs from 27 neurons of 10 wild-type mice and from 21 neurons of 10 *Fmr1* knockouts were analyzed. mIPSC data were collected from 27 neurons of 10 wild-type mice and 25 cells of 10 *Fmr1* knockout mice.

In a separate series of experiments, tonic inhibition which is mediated by extra-synaptic $GABA_A$ receptors (Haas and Macdonald, 1999; Nusser and Mody, 2002; Nusser et al., 1998), was measured by adding 50 µM bicuculline methbromide (BIC) to the ACSF superfusate during whole cell recording. The tonic current was calculated as the difference between the holding current before and after the addition of BIC. The mean current values for this calculation were obtained from Gaussian fits to all-point amplitude histograms (Bright

and Smart, 2013; Nusser and Mody, 2002). The difference between peak values of two simulated Gaussians was used as the measure of the tonic current.

Data are presented as mean ± SEM unless otherwise indicated. Statistical significance was determined using a two-way repeated measures ANOVA (RM-ANOVA) for the eIPSC data, Kolmogorov-Smirnov and Wilcoxon Rank-Sum Test for comparing cumulative probabilities and Welch's unpaired t-test for decay time values (IBM SPSS 19, Armonk, NY, USA and GraphPad Prism 5, La Jolla, CA, USA).

2.3. Real-time PCR

Fmr1 knockout and wild-type mice (10 per genotype) were euthanized by cervical dislocation at postnatal day 22 (P22). Immediately after isolation, hippocampi were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Brain samples were homogenized using the Dispomix v1.4 homogenizer (Miltenyi Biotec, Bergisch Gladbach, Germany). Total RNA was then isolated using Trizol (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After RNase-free DNase treatment (Ambion, Life Technologies, Carlsbad, CA, USA), the quality of the RNA samples was assessed with the automated gel electrophoresis Experion system (Biorad, Hercules, CA, USA). mRNA expression was analyzed using a two-step real-time PCR assay. First, cDNA was obtained by reverse transcription of mRNA with the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Penzberg, Germany). First strand cDNA was subsequently used as a template for real-time PCR with the qPCR MasterMix Plus for SYBR Green 1 No Rox (Eurogentec, Seraing, Belgium) and Lightcycler 480 thermocycler and detection platform (Roche Applied Science, Penzberg, Germany). Intron flanking or intron spanning primers (Integrated DNA technologies, Coralville, IA, USA) were used to prevent amplification of gDNA (for Primer sequences in Supplementary Table A.1). The specificity of the amplification was checked by performing meltcurve analysis after PCR amplification (cycling conditions: 2 min 50°C; 10 min 95°C; 45 cycli with 15 s 95°C and 1 min 60°C; 5 s

95°C;1 min 65°C; increase to 97°C at 11°C/s; 5 s 40°C). The obtained results were analyzed with qBase Plus software (Biogazelle, Ghent, Belgium). This analysis included quality control of the raw data, assessment of the reference gene stability and calculation of the normalization factors. More specifically, reference gene stability was assessed by the gene stability value (M<0.5) and coefficient of variation (CV<25%) (Supplementary Table A.2). mRNA expression levels were normalized by the geometric mean of the three most stably expressed reference genes (Gapdh, Htatsf1 and Zfp91) selected from the geNorm^{Plus} Kit with advanced reference genes (Primer Design, Southampton, UK). Then the relative expression (RE) was calculated, i.e. the ratio of the geometric mean of the normalized expression levels of the wild-type animals and that of the *Fmr1* knockout mice. The real-time PCR experiments were repeated on a second batch of cDNA obtained from the original mRNA samples and similar results were observed (data not shown). Statistical analysis of the quantitative real-time PCR data was done with the Mann-Whitney U non-parametrical test (IBM SPSS 19, Armonk, NY, USA).

2.4 Western blot analysis.

Subcellular fractionation and Western blot analysis were performed at postnatal age p21 as described in Gardoni *et al.* (2006; 2001). After the Triton-soluble fraction (TSF) and Triton-insoluble fraction (TIF) were prepared, the latter was thoroughly tested for the absence of the presynaptic marker synaptophysin and for the presence of post-synaptic marker PSD95. Membrane-associated Cyp46 (Pasciuto et al., 2015) and transferrin receptor, respectively, were used as normalisers. The following specific antibodies were used for Western blotting: synaptophysin (abcam ab8049), cyp46 (ab82814), Fmrp (Ram II), transferrin R (Abcam ab84036), GABA A α2 (Abcam ab72445), GABA A ß1 (Abcam ab16703), GABA A δ (Millipore Ab9752), PSD95 (Thermo Scientific 6G6-1C9).

3. Results

3.1. Stimulus-evoked $GABA_A$-ergic inhibition of Fmr1 knockout mice is markedly reduced

We examined functional properties of $GABA_A$-ergic inhibition in the hippocampus of *Fmr1* knockout mice using whole-cell voltage clamp recordings of CA1 pyramidal neurons. As shown in Fig. 1A, *Fmr1* knockout mice had significantly reduced eIPSC amplitudes in response to stimulation intensities varying from 10 to 100 µA ($p=0.013$, RM-ANOVA). An average reduction of about 30% indicated a deteriorated inhibition in *Fmr1* knockout mice.

To investigate whether the decreased inhibition was a consequence of changes in neurotransmitter release, we first analyzed paired-pulse responses of IPSCs evoked at interstimulus intervals of 50 ms and 80 ms (Centonze et al., 2008). We did not find significant differences between *Fmr1* knockout mice and their littermate controls (Fig. 1B). Likewise, there were no genotype effects in synaptic fatigue (Fig. 1.C) and recovery (Fig. 1D). Together, these results point to postsynaptic rather than presynaptic mechanisms as the main cause of the decreased inhibition.

3.2. Spontaneous and miniature IPSCs of Fmr1 knockout mice show a significant reduction in amplitude and frequency

We examined phasic $GABA_A$ receptor-mediated inhibition in *Fmr1* knockout mice in detail and measured spontaneous IPSCs (sIPSCs) and miniature IPSCs (mIPSCs) (Fig. 2A). As shown in Fig. 2B,C cumulative probabilities of sIPSCs and mIPSCs in *Fmr1* knockout mice revealed a significant reduction in the amplitudes and increase in inter-event intervals (IEI) as compared to wild-type mice (sIPSC median amplitude: KO 36.7 pA, WT 38.2 pA, $p=0.004$; sIPSC median IEI: KO 108 ms, WT 96 ms, $p<0.001$; mIPSC median amplitude: KO 26.2 pA, WT 30.3 pA, $p<0.001$; mIPSC median IEI: KO 143 ms, WT 127 ms, $p< 0.001$, KS-test). Additionally, we determined the decay time constants of mIPSCs by non-linear curve fits of a two-phase exponential decay time function. As depicted in Fig. 2D, *Fmr1* knockout mice had a significantly increased current decay time of the fast component (tau1) as compared with wild-type littermates (mean ± SEM, KO: 10.68 ± 0.55 ms, n=25; WT: 8.47 ± 0.38 ms, n=27; $p = 0.002$, $t=3.332$, $df=43$, Welch-test). This genotype difference in decay was primarily due to the faster decay of the first component because the decay of the second

component was considerable longer and did not differ between genotypes (KO: 20.68 ± 2.58 ms, n=25; WT: 24.91 ± 5.88 ms, n=27; p = 0.515, t=0.658, df=33, Welch-test).

3.3. Real-time PCR reveals lower expression level of $GABA_A$ receptor subunits

Prompted by the significant changes in electrophysiological readouts of $GABA_A$-ergic inhibition in *Fmr1* knockout mice, we analyzed the hippocampal expression of relevant $GABA_A$ receptor subunits at postnatal day 22 (P22). Previous expression studies have focused primarily on adolescent mice (2-3 months) (El Idrissi et al., 2005; Gantois et al., 2006; Hong et al., 2011). Based on our earlier findings in adult mice (D'Hulst et al., 2006; Gantois et al., 2006), we focused on the expression of $GABA_A$ receptor subunits α1, α2, α3, α5, β1, β2, δ, γ1 and γ2. The mRNA expression levels of these subunits were assessed using real-time PCR, with normalization using three reference genes (Gapdh, Htatsf1 and Zfp91). The reference gene stability was analyzed using qBase and met the predetermined criteria for stable expression (Supplementary table A.2.). Subsequently, we calculated the relative expression (RE) level, i.e. the ratio of the geometric mean of the normalized expression values of wild-types and those of Fmr1 knockout mice (Supplementary table A.3.). As depicted in Fig. 3A, *Fmr1* knockout mice showed a significantly reduced expression of α2 (72% of wild-type), β1 (84%) and δ (80%) subunits (p=0.007, p=0.022 and p=0.027, respectively; Mann-Whitney U test). The expression of major isoforms of the rate-limiting enzyme for GABA synthesis, GAD65/67 was unchanged in the hippocampus (Fig. 3B).

3.4. Western blotting demonstrates reduced expression of GABA A α2, ß$_1$ and δ protein in Fmr1 knockout mice

Given the consistent downregulation of mRNA expression levels of the α2, ß$_1$ and δ $GABA_A$ subunits in *Fmr1* knockout mice in this and a previous study (Gantois et al., 2006), we were interested to check whether this reduction can be also found at the protein level. When the expression of these subunits was examined by Western blotting, we found that the protein

level of all subunits was reduced in the Triton-insoluble fraction of *Fmr1* knockout mice resulting in levels between 60 and 70% as compared to WT controls (Fig. 4; α2: 60.4 %, p=0.006; ß1: 62.2 %, p=0.024; δ: 66.3%, p=0.023).

3.5. Tonic inhibition in Fmr1 knockout mice is reduced

Since we found a significant reduction in mRNA expression and protein level of the $GABA_A$ δ subunit which has been described to have a role in tonic inhibition mediated by extrasynaptic $GABA_A$ receptors (Nusser et al., 1998;Haas and Macdonald, 1999;Nusser and Mody, 2002), we examined whether we can detect a reduction in this type of inhibition in Fmr1 knockout mice. As an estimate of tonic inhibition we measured the shift of the baseline current following the application of the $GABA_A$ receptor blocker bicuculline methbromide (BIC). As shown in Fig. 5, this shift was significantly lower in Frm1 KO mice (14.2 ± 2.0 pA) as compared to WT controls (24.7 ± 3.9 pA; p = 0.025, t-test).

4. Discussion

Proper brain function depends on a correct balance between excitatory and inhibitory signaling (Gatto and Broadie, 2010; Yizhar et al., 2011). Recent studies support the hypothesis that this balance is disturbed in animal models of fragile X syndrome and underlies the cognitive impairment and behavioral abnormalities (Contractor et al., 2015; Gibson et al., 2008; Paluszkiewicz et al., 2011a)(Braat and Kooy, 2015). Deficits of hippocampus-dependent functions are of particular interest in fragile X syndrome because of the central role of this brain structure in cognitive functions (Battaglia et al., 2011; Eichenbaum, 2003; Shapiro and Eichenbaum, 1999; Squire, 1993; Vann and Albasser, 2011). Therefore, we examined for the first time characteristic features of inhibition in the hippocampus of *Fmr1* knockout mice. Previous studies have centered on other brain regions such as the subiculum (Curia et al., 2009), the amygdala (Olmos-Serrano et al., 2010; Vislay et al., 2013), the somatosensory cortex (Paluszkiewicz et al., 2011b), forebrain (Adusei et al., 2010) and striatum (Centonze et al., 2008) (See Supplementary Table A.4).

Here we demonstrate not only a significant reduction in the amplitude of eIPSCs**,** but also in the amplitude and frequency of both, sIPSCs and mIPSCs. The changed frequency of mIPSCs is indicative of changes in presynaptic functions which is in apparent contrast to unchanged paired-pulse ratio, synaptic fatigue and recovery in these mice. Thus, it seems that the changed presynaptic function of *Fmr1* knockout mice is only becoming overt in some electrophysiological measures but not in others. Together, our results point to a combination of pre- and postsynaptic changes underlying the deficit in $GABA_A$ receptor-mediated phasic inhibition in the hippocampus.

The abnormalities in phasic inhibition in the hippocampus differ from those in other brain regions of *Fmr1* knockout mice. In the subiculum, tonic inhibition was reported as impaired (-90%), possibly due to reduced expression of $GABA_A$ receptor subunits α_5 and δ, which constitute tonic $GABA_A$ receptors (Curia et al., 2009). Phasic inhibition, in contrast, was reported unaltered in the subiculum but in this study only sIPSCs were analyzed. The amygdala of *Fmr1* knockout mice is characterized by impairments in both tonic and phasic (sIPSCs and mIPSCs) currents (Olmos-Serrano et al., 2010). This reduced inhibition is thought to be associated with decreased GABA synthesis and release, reduced synaptic connectivity and interneuron activity. In the somatosensory cortex, no abnormalities were reported in phasic GABA receptor-mediated inhibition (sIPSCs and mIPSCs) (Paluszkiewicz et al., 2011b). In striatal neurons increased phasic inhibition was reported, as increased frequencies of sIPSCs and mIPSCs and decreased paired-pulse ratio were observed (Centonze et al., 2008). Thus, it appears that the abnormalities of the GABAergic system vary across different brain regions in *Fmr1* knockout mice.

Prompted by the significant changes in electrophysiological readouts of $GABA_A$-ergic inhibition in *Fmr1* knockout mice, we analyzed the hippocampal expression of relevant $GABA_A$ receptor subunits at this developmental stage (P22) by real-time PCR. This analysis revealed that *Fmr1* knockout mice have significantly decreased levels of α2, β1 and δ subunits in hippocampal tissue. The decrease in δ subunit at P22 is in accordance with our earlier whole-genome mRNA expression analysis in adult *Fmr1* knockout mice (D'Hulst et al.,

2006; Gantois et al., 2006) and findings in the subiculum (Curia et al., 2009). mRNA and protein levels of the β1 subunit were also shown to be reduced in adult cerebellum, of the ß2 subunit in cerebellum and hippocampus, and of the ß3 subunit in cortex (El Idrissi et al., 2005; Hong et al., 2012), although in a single study, mRNA expression of the ß3 subunit was reported as increased in the hippocampus of Fmr1 knockout mice (Hong et al., 2012). A decreased expression of the α2 subunit in *Fmr1* knockout mice has not been reported by others.

Of the many combinations of the 19 $GABA_A$ receptor subunits only a few dozen have been shown to exist and only a few combinations are abundant in certain brain regions and interneuron subtypes (Farrant and Nusser, 2005). The most abundantly expressed receptor subtype is formed from α1, β2 and γ2 subunits, but other combinations of α, β and γ2 subunits are common as well. Less common are subtypes in which the γ2 subunit is replaced by γ1, γ3, or δ (Farrant and Nusser, 2005). The α2 subunit, which showed a marked reduction by about 30% in our study, is highly abundant in the hippocampus with a relatively homogenous distribution across dendritic and cell body layers (Pirker et al., 2000; Prenosil et al., 2006). Together with the α1 subunit, it provides the bulk of eIPSCs in CA1 pyramidal neurons (Prenosil et al., 2006). Recordings indicated that the α2 subunit is primarily responsible for phasic synaptic inhibition on the soma (Prenosil et al., 2006) and immunogold stainings revealed the presence of this subunit on axon initial segments in about 80% of synapses (Nusser et al., 1996). α2-containing receptor subtypes deactivate more slowly than those containing the α1 subunit (Goldstein et al., 2002; Lavoie et al., 1997; McClellan and Twyman, 1999; Okada et al., 2000). A reduced expression of subunits with slower deactivation rates would be expected to lead to a diminished inhibitory tone (Picton and Fisher, 2007). Thus, a reduction of α2-containing subtypes at young age may contribute to the increased propensity to undergo epileptic seizures in childhood but not in adults as reported for fragile X patients (Musumeci et al., 1999).

The mechanism causing the impairments in $GABA_A$ receptor expression remains to be determined. Since FMRP is an RNA binding protein involved in the regulation of mRNA

transport and translation, absence of FMRP is expected to have a broad impact on its mRNA targets. Reports on the binding of FMRP to $GABA_A$ receptor subunit mRNAs are conflicting. Whereas $GABA_A$ receptors were not identified as FMRP targets in some studies (Brown et al., 2001; Darnell et al., 2001), others identified the $GABA_A$ receptor subunits ß1 and δ as FMRP mRNA targets (Summarized in Pasciuto and Bagni, 2014)]. Direct binding between FMRP and $GABA_A$ δ subunit mRNA was demonstrated using antibody positioned RNA amplification (APRA) (Miyashiro et al., 2003) and electrophoretic mobility shift assays (EMSAs) (Braat et al., 2015). Miyashiro et al. (2003) found the same subcellular localization of the δ subunit mRNA in the hippocampus and cerebellum of *Fmr1* knockout mice and WT animals but did not examine the mRNA abundance. Here we report for the first time that the down-regulation of the $GABA_A$ δ subunit at the mRNA level is also found at the protein level in the hippocampus of *Fmr1* knockout mice, in agreement with forebrain data from Adusei et al. (2010) who described reduced protein levels of the same subunit in 12-day- and 2-month-old *Fmr1* knockout mice. The absence of FMRP might thus have a direct effect on the quantity of certain δ subunit-containing $GABA_A$ receptors. Direct protein-protein interactions between FMRP and the $GABA_A$ δ subunit are also supported by their immunohistochemical colocalization, for example, in fiber cells of the mature rabbit eye lens which share several molecular, sub-cellular and morphological hallmarks with neurons (Frederikse et al., 2015). Further, we confirm that the reduction in mRNA expression of α2 and β1 subunits is paralleled by a similar decrease in their protein levels.

The values of the mIPSC decay time constant tau determined in our study for hippocampal pyramidal neurons are in the same range as reported for cortical neurons (Dunning et al., 1999). The decay of IPSCs reflects the ion channel deactivation following ligand removal and is determined by the microscopic transition kinetics of the receptors which is highly dependent on the subunit composition (Farrant and Nusser, 2005). The slower decay of IPSCs in *Fmr1* knockout mice, as described here, is expected to increase the inhibitory tone (Picton and Fisher, 2007). This would partially counteract the reduced inhibition by the diminished α2-expression and could reflect, therefore, a partial

developmental compensation. However, inhibitory net effects are also modulated by subunit-dependent single channel conductance and agonist affinity (Bohme et al., 2004) as well as by the strength of tonic inhibition (Bai et al., 2001; Semyanov et al., 2003). Of note, our experiments revealed a reduction in tonic inhibition of *Fmr1* knockout mice at this age. Since the δ subunit was shown to be present exclusively at extrasynaptic hippocampal sites (Farrant and Nusser, 2005; Nusser et al., 1998; Pirker et al., 2000), its downregulation in *Fmr1* knockout mice found in our study is most likely a involved in the diminished tonic inhibition. Persistently active δ–$GABA_A$ receptor openings provide a major contribution to the total charge that flows across the membrane (Brickley and Mody, 2012) thereby modulating neuronal activity and network behavior (Farrant and Nusser, 2005).

In conclusion, our results demonstrate a reduced expression of several subunits in the hippocampus of juvenile *Fmr1* knockout mice which is paralleled by a multi-faceted dysregulation of $GABA_A$-mediated inhibition. Together, these findings further advocate inhibitory GABAergic transmission as an attractive target for rational therapies for fragile X syndrome.

Captions to Figures

Fig. 1. Evoked IPSCs in CA1 pyramidal neurons of *Fmr1* knockout mice

(A) eIPSC amplitudes in *Fmr1* knockout (KO) mice (red) were significantly reduced as compared with wild-type (WT) littermates (black; p=0.013, RM-ANOVA). eIPSC amplitude was assessed by averaging 5 eIPSCs for each neuron per stimulation strength (29 WT and 28 *Fmr1* KO neurons). Data were averaged per animal (n=10), error bars represent SEM. Insets depict representative eIPSCs for WT (left traces) and *Fmr1* KO mice (right traces). (B) The paired pulse ratio (PPR; amplitude of the second eIPSC / amplitude of the first eIPSC) did not differ significantly between both genotypes, neither at 50 ms nor at 80 ms interstimulus interval (p>0.05, Welch-test). eIPSC amplitudes were calculated by averaging 5 IPSCs of each neuron per interstimulus interval (25 WT and 23 *Fmr1* KO neurons). Data were averaged per animal (n=10), error bars represent SEM. (C) During sustained 10 Hz stimulation, eIPSCs of *Fmr1* KO and WT declined due to presynaptic fatigue (24 WT and 23 *Fmr1* KO neurons of each 10 animals). (D) Recovery of eIPSCs during subsequent 0.1 Hz stimulation (24 WT and 22 *Fmr1* KO neurons of 10 and 9 animals, respectively).

Fig. 2. Characteristics of spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs) in CA1 pyramidal neurons of *Fmr1* knockout and wild-type mice.

(A) Representative traces of sIPSCs (upper sweeps) and mIPSCs (lower sweeps) recorded from CA1 pyramidal neurons in slices of WT (black) and *Fmr1* KO (red) mice. (B) Cumulative probability curves for the sIPSC amplitude (left) and inter-event intervals (IEIs) (right) in WT (black) and *Fmr1* KO (red) pyramidal cells. For the amplitude analysis, a total of 100 individual sIPSCs were randomly selected from the event list files. The median amplitudes of the arrays were 38.2 pA (WT) and 36.7 pA (*Fmr1* KO). Populations were significantly different (p=0.004, KS-test). For the frequency analysis (f = 1 / IEI), the total number of events analyzed was 16129 in WT and 15724 in *Fmr1* KO. The median values of IEI distributions were 96 ms (WT) and 108 ms (*Fmr1* KO), (p<0.001, KS-test); (C) Cumulative probabilities for the mIPSC amplitude (left) and IEIs (right) in WT (black) and *Fmr1* KO (red)

pyramidal cells. There was a significant difference in both parameters between genotypes ($p<0.001$ (amplitude); $p< 0.001$ (IEI), KS-test). For the amplitude analysis, a total of 100 individual mIPSCs were randomly selected from the event list files (2700 and 2500 IPSCs, respectively). Median amplitudes of the arrays were 30.3 pA (WT) and 26.2 pA (*Fmr1* KO). For the frequency analysis (IEIs) the total number of events analyzed was 16038 in WT and 15804 in *Fmr1* KO. The median values of IEI distributions were 127 ms (WT) and 143 ms (*Fmr1* KO). (D) Bar graph showing the averaged decay time constant τ_1 of the fast component obtained by a two-phase exponential decay fit by non-linear regression. (mean ± SEM: KO: 10.68 ± 0.55 ms, n=25; WT: 8.47 ± 0.38 ms, n=27; $p=0.002$, $t=3.332$ $df=43$, Welch-test). Analogue traces on the right display superimposed average representative mIPSC traces for WT (black) and *Fmr1* KO (red) neurons. Note the increased decay time of *Fmr1* KO mIPSCs.

Fig. 3. mRNA expression of $GABA_A$ receptor subunits and GABA metabolism enzymes.

(A) Expression difference (%) of $GABA_A$ receptor subunits in hippocampus on mRNA level. $GABA_A$ receptor subunits α2, β1 and δ were significantly reduced in *Fmr1* knockout (n=9) compared to wild-type mice (n=10). *p<0.05, **p<0.01, Mann-Whitney U-test; Error bars represent SEM.

(B) The expression difference of the GABA metabolic enzymes between *Fmr1* KO and WT. The expression of the GABA synthesizing enzymes glutamic acid decarboxylase 65 (Gad65) and 67 (Gad67) and the GABA degradation enzyme GABA-transaminase (GABA-T) did not differ between Fmr1 knockout mice and wild-type littermates (p>0.05, Mann-Whitney U-test). Error bars represent SEM.

Fig. 4. The expression of $GABA_A$ α2, β1 and δ subunit protein is significantly reduced in *Fmr1* knockout mice.

(A) Representative Western blot for the $GABA_A$ α2 subunit in the Triton-insoluble fraction (TIF) of WT and Fmr1 KO mouse cortices. The TIF preparation was tested for the absence of presynaptic marker synaptophysin, and transferrin receptor was used as normalizer (TOT: total, TSF: triton soluble fraction; TIF: triton insoluble fraction). The bar graphs on the right illustrate the reduction in α2 subunit protein levels to 60.4% (p=0.006, n=6, t-test). (B) Representative Western blot for the $GABA_A$ ß1 subunit in the Triton-insoluble fraction (TIF) of WT and Fmr1 KO mouse cortices. Transferrin receptor was used as normalizer. Protein levels were reduced to 62.2% (p=0.024, n=5). C) Representative Western blot for the $GABA_A$ δ subunit. Cyp46 (a non FMRP target) was used as normalizer. Quantification revealed a decline in the protein level to 66.3% (p=0.023, n=6). Error bars represent the standard error of the mean (SEM); * p < 0,05 t-test, ** p < 0.01 t-test.

Fig. 5. Reduced tonic inhibition in CA1 pyramidal neurons of *Fmr1* knockout mice. (A) Representative traces recorded from WT (black) and *Fmr1* KO (red) mice. The tonic current was calculated as the difference between the holding current in the absence and presence of 50 µM bicuculline (BIC). All-point amplitude histograms of control and bicuculline epoches from both genotypes are shown on the right. (B) Bar plot (mean ± SEM) illustrating the significant reduction of tonic inhibition in CA1 pyramidal neurons from *Fmr1* KO mice (14.2 ± 2.0 pA) as compared with WT controls (24.7 ± 3.9 pA; p=0.025, t-test; 20 cells from 6 animals in each group were analyzed)

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