Early Developmental Alterations in GABAergic Protein Expression in Fragile X Knockout Mice

by

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Abstract

The purpose of this study was to examine the expression of GABAergic proteins in Fmr1 knockout mice during brain maturation and to assess behavioural changes potentially linked to perturbations in the GABAergic system. Quantitative western blotting of the forebrain revealed that compared to wild-type mice, the GABA_A receptor $\alpha 1$, $\beta 2$, and δ subunits, and the GABA catabolic enzymes GABA transaminase and SSADH were down-regulated during postnatal development, while GAD65 was up-regulated in the adult knockout mouse forebrain. In tests of locomotor activity, the suppressive effect on motor activity of the GABA_A $\beta 2/3$ subunit-selective drug loreclezole was impaired in the mutant mice. In addition, sleep time induced by the GABA_A $\beta 2/3$ -selective anaesthetic drug etomidate was decreased in the knockout mice. Our results indicate that disruptions in the GABAergic system in the developing brain may result in behavioural consequences in adults with fragile X syndrome.

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Fmr1 knockout mice

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List of Abbreviations

ADHD	attention-deficit hyperactivity disorder
APRA	antibody-positioned RNA amplification
CNS	central nervous system
Co-IP	co-immunoprecipitation
DCN	deep cerebellar nuclei
dFMRP	<i>drosophila</i> FMRP
DTT	dithiothreitol
<i>Fmr1</i>	fragile X mental retardation-1
FMRP	fragile X mental retardation protein
FXTAS	fragile X tremor/ataxia syndrome
FXS	fragile X syndrome
GABA	γ -aminobutyric acid
GABA-T	GABA transaminase
GAD	glutamic acid decarboxylase
GAPDH	glyceraldehyde 3-phosphate-dehydrogenase
GAT	GABA transporter
HPLC	high-performance liquid chromatography
KO	knockout
LGIC	ligand-gated ion channel
LTD	long-term depression
LTP	long-term potentiation
mGluR	metabotropic glutamate receptor
mRNP	messenger ribonucleoprotein
nACh	nicotinic acetylcholine
qRT-PCR	quantitative real-time polymerase chain reaction
PND	postnatal day
POF	primary ovarian failure
SSADH	succinic semialdehyde dehydrogenase
WT	wild-type

Introduction

Fragile X syndrome: clinical features

Fragile X syndrome (FXS) is the most common inherited form of mental retardation, occurring in roughly 1 in 4000 males and 1 in 8000 females (O'donnell and Warren, 2002). FXS is caused by a mutation in the *Fmr1* gene on the X chromosome which leads to the loss of its encoded protein, Fragile X Mental Retardation Protein (FMRP). Reduction in intellectual ability ranges from mild to moderate and there is a significant positive correlation between the levels of FMRP and intellectual ability (Loesch et al., 2004). The behavioural phenotype of FXS patients also includes hyperactivity, anxiety, impaired visuo-spatial processing, and developmental delay (Hagerman *et al.*, 2009). Girls with FXS usually suffer from milder cognitive and behavioural deficits than boys primarily due to the fact females still express some FMRP, depending on the ratio of X chromosome inactivation (Freund et al., 1993; Lachiewicz et al., 2006). Most boys with FXS display some autistic behaviour and 30% meet formal criteria outlined by the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* for autistic spectrum disorder (Hagerman et al., 2009). FXS patients diagnosed with autism display a more severe social impairment, as well as having lower cognitive and language ability, academic achievement and adaptive behaviour, than seen in FXS individuals without autism (Bailey, Jr. et al., 2001; Hagerman, 2006).

Attention-deficit hyperactive disorder (ADHD) symptoms are more prevalent in children with FXS compared to the general population, or with other disorders with intellectual disabilities (Munir *et al.*, 2000; Backes *et al.*, 2000; Cornish *et al.*, 2005). FXS patients under the age of 5 years old are particularly difficult to treat, as stimulants that are routinely used to

treat older patients are not as effective (Hagerman and Hagerman, 2002). The use of clonidine and guanfacine, agonists of the α -adrenergic receptor, are useful in younger children and are without the side effects of stimulants (Hagerman et al., 2009). Clonidine has also been used to treat sleep disturbances, which in some cases have been reported in up to one-third of patients with FXS (Ingrassia and Turk, 2005; Kronk et al., 2010).

FXS patients also suffer from an increased susceptibility to epileptic seizures. The incidence rate is roughly between 13%-18% in males and 5% in females (Musumeci et al., 1999; Sabaratnam et al., 2001). The occurrence of seizures usually begins in early childhood (between 6 months and 4 years of age), but the majority of patients experience a resolution of seizures by adolescence (Berry-Kravis, 2002). Recurring seizures are associated with a high incidence of autism in FXS, since FXS patients with autism are three times more likely to experience seizures compared to FXS patients (Garcia-Nonell et al., 2008). The use of anticonvulsants must be carefully evaluated in these patients because many drugs can unintentionally exacerbate other symptoms of FXS. Common anticonvulsants such as phenobarbital and gabapentin are generally avoided, because they tend to increase hyperactivity in individuals with FXS (Hagerman et al., 2009).

The fragile X mental retardation gene and protein

FXS is caused by a CGG trinucleotide repeat expansion in the 5' untranslated region of the *Fmr1* gene located at Xq27.3 (Bakker et al., 1994). The human *Fmr1* gene spans approximately 38 kb in length and is composed of 17 exons that encode a 4.4 kb mRNA transcript. Alternative splicing in exons 12, 14, 15, 17, and in the 3' untranslated region result in several isoforms of the encoded protein, FMRP (D'Hulst and Kooy, 2009). *Fmr1* along with gene family members fragile X related gene 1 and 2 (*Fxr1* and *Fxr2*) are highly conserved

among vertebrates (Zhang et al., 1995). The *Fmr1* gene contains a TATA-less promoter region and three initiator-like sequences that correspond to three transcription start sequences (Weis and Reinberg, 1992; Chow et al., 1995). The CpG island of the promoter region of *Fmr1* is affected by the CGG trinucleotide expansion and through a poorly understood mechanism promotes the deacetylation of histones, possibly in an effort to halt the CGG expansion (Figure 1; Richards et al., 1993). As a result, the chromatin structure is altered from a relaxed to a condensed state by histone remodelling complexes that impede transcriptional machinery from binding the promoter region. This is followed by hypermethylation and transcriptional silencing of the *Fmr1* gene (Richards et al., 1993; Darlow and Leach, 1998; Kho et al., 1998). Studies have shown that the size of the CGG repeat alters the relative utilization and activity of the three transcriptional start sites present on the *Fmr1* gene, suggesting that the CGG expansion directly affects transcriptional initiation (Beilina et al., 2004).

Normal individuals carry anywhere from 5–55 CGG repeats, while people with 55 to 200 repeats are considered FXS premutation carriers. For CGG repeat expansions in the premutation range, *Fmr1* mRNA levels are elevated by as much as 5-fold (Tassone et al., 2000; Kenneson et al., 2001). The reason mRNA levels are elevated in premutation carriers is not known, as it does not appear to be attributable to increased mRNA stability. Furthermore, in spite of the increased levels of *Fmr1* mRNA, FMRP levels are in fact moderately reduced in premutation carriers (Tassone et al., 2000). However, the premutation is unstable and commonly expands to over 200 repeats during intergenerational transmission, leading to FXS (Nolin et al., 2003). The CGG repeat is more stable during male transmission; therefore the full mutation can only be inherited from maternal lineage (Bardoni et al., 2000).

Premutation carriers can develop a neurodegenerative syndrome later in life called fragile X tremor/ataxia syndrome (FXTAS). This disorder is characterized by symptoms such as progressive cerebellar gait ataxia, intention tremor, memory deficits and depression (Jacquemont et al., 2007). FXTAS is prevalent in male premutation carriers, with roughly 40% of aging males developing the disorder while only 4-8% of females develop FXTAS (Jacquemont et al., 2007).

Figure 1. A model of the trinucleotide repeat instability associated with hypermethylation on the *Fmr1* gene. The 5' UTR of the fragile X mental retardation-1 (*Fmr1*) gene contains a stably inherited CGG repeat that ranges in size from 5 to 50 repeats in normal individuals. The presence of 200 or more repeats causes fragile X syndrome. In affected individuals, the CCG expansion region of *Fmr1* becomes hypermethylated (black circles), which possibly triggers the recruitment of chromatin remodelling complexes, resulting in heterochromatin formation and transcriptional repression of the *Fmr1* gene.

Roughly 20% of female *Fmr1* premutation carriers develop a condition known as premature ovarian failure (POF; Sherman, 2000). The disorder is characterized by earlier menopause in premutation carriers compared to non-carriers (Murray et al., 2000; Hunscheid et al., 2001) and risk for POF significantly increases if the CGG repeat size exceeds 80 repeats (Sullivan et al., 2005). In contrast, the risk of developing POF in women without the premutation allele (non-carriers) is only 1% (Murray et al., 2000). Even female premutation carriers not diagnosed with POF have elevated follicle-stimulating hormone levels, an indicator of ovarian dysfunction (Hunscheid et al., 2001). It is hypothesized that POF is caused by toxicity from excess *Fmr1* premutation mRNAs in reproductive cells (e.g. oocytes, granulosa cells), resulting in apoptosis of follicles (Sullivan et al., 2005).

The *Fmr1* gene encodes the fragile X mental retardation protein (FMRP) that acts as an RNA-binding protein (Ashley et al., 1993). FMRP is maximally 631 amino acids in length, typically ranging from between 60-80 kDa depending on its 12 potential isoforms (D'Hulst and Kooy, 2009). However, the physiological role of these isoforms and how they differ are not known. FMRP is widely, but not ubiquitously, expressed in human tissues, with particularly high levels of expression in the brain and testes (Bakker et al., 2000). Patterns of expression for *Fmr1* transcripts and FMRP isoforms have been found to be similar in most tissues amongst different species (Khandjian et al., 1995; Verheij et al., 1995). FMRP and the two paralogous proteins FXR1P and FXR2P are highly conserved in mammals. In *Drosophila* there is only single orthologous protein, dFMR1 (Bassell and Warren, 2008). FMRP is localized predominately in the cytoplasm, although the protein contains highly conserved nuclear localization and nuclear export signals (Eberhart et al., 1996).

FMRP contains two coiled coil domains that are believed to play a part in protein-protein interactions (Bassell and Warren, 2008). FMRP associates directly with FXR1P/FXR2P, as well as nuclear FMRP interacting protein 1, 82 kDa FMRP interacting protein and microsphere protein 58 (Bardoni et al., 2006). The best characterized motifs in FMRP are the hnRNP-K-homology (KH) domains and an arginine-glycine-glycine (RGG box), both of which in part mediate its RNA-binding activity. Several transcripts have been validated to bind FMRP; many possess a G-quartet structure recognized by FMRP *in vitro*, including *Psd95*, *Map1b*, *CamKIIa*, *Sema3F*, and *Fmr1* itself (see Table 1; Brown et al., 2001; Darnell et al., 2001; Schaeffer et al., 2001). Another class of transcripts that contain U-rich sequences have also been shown to be a target of FMRP (Chen et al., 2003; Dolzhanskaya et al., 2003).

Although the function and roles of FMRP in the central nervous system are not fully understood, the protein is primarily thought to play a key role in synaptic plasticity through its ability to transport mRNAs and regulate local protein synthesis at synapses (Jin et al., 2004). The proposed mechanism of action of FMRP has been based on a combination of findings (Figure 2; Jin and Warren, 2003). Cytoplasmic FMRP is dimerized and transported into the nucleus via its nuclear localization signal. In the nucleus FMRP aggregates with specific mRNAs and proteins to form a messenger ribonucleic protein complex (mRNP), which is then transported out of the nucleus through its nuclear export signal.

Once back in the cytoplasm, the FMRP-mRNP complex interacts with members of the RNA induced silencing complex (RISC) before associating with actively translating ribosomes. The FMRP-mRNP complex regulates protein synthesis in the cell body of the neuron or the complex could be transported into the dendrites to regulate local protein synthesis of specific mRNAs in response to synaptic stimulation signals such as group I metabotropic glutamate

Table 1 - Validated Targets of FMRP				
Gene	Description	G-quartet-like Structure	Method	Reference
<i>CamKIIα</i>	Calcium/Calmodulin Kinase II alpha subunit	Yes	Co-IP	Bramham and Wells, 2007; Muddashetty et al., 2007; Hou et al., 2006; Zalfa et al., 2003
<i>App</i>	Amyloid Precursor Protein	No/Unknown	Co-IP	Westmark and Malter, 2007
<i>Arc</i>	Activity-regulated cytoskeleton-associated protein	No/Unknown	Co-IP	Steward and Worley, 2001; Waung et al., 2008; Park et al., 2008
<i>eEF1A</i>	Elongation factor-1A	No/Unknown	Co-IP, <i>In vitro</i>	Huang et al., 2005; Sung et al., 2003
<i>Fmr1</i>	Fragile X mental retardation 1	Yes	<i>In vitro</i>	Weiler et al., 1997; Antar et al., 2004; Schaeffer et al., 2001
<i>GluR1/2</i>	Glutamate receptor, ionotropic, AMPA 1	No/Unknown	Co-IP	Muddashetty et al., 2007
<i>Map1b</i>	Microtubule-associated protein 1B	Yes	Co-IP; in vitro; biophysical	Brown et al., 2001; Darnell et al., 2001; Antar et al., 2005; Davidkova and Carroll, 2007; Hou et al., 2006; Menon et al., 2008
<i>Psds95</i>	Post-synaptic density 95	Yes	Co-IP; in vitro; reversible crosslinking-IP	Todd et al., 2003; Zalfa et al., 2007; Muddashetty et al., 2007
<i>Sapap3/4</i>	SAP90/PSD95-associated protein 3	No/Unknown	Co-IP	Brown et al., 2001; Kindler et al., 2004; Narayanan et al., 2007; Dictenberg et al., 2008
<i>Sema3F</i>	Semaphorin-3F	Yes	Co-IP, biophysical	Darnell et al., 2001; Menon and Mihailescu, 2007
<i>Rgs5</i>	Regulator of G-protein signaling 5	No/Unknown	APRA, <i>in vitro</i>	Miyashiro et al., 2003; Dictenberg et al., 2008
<i>Gabrd</i>	GABA _A receptor delta subunit	No/Unknown	APRA, qRT-PCR, <i>in vitro</i> *	Dictenberg et al., 2008; Gantois et al., 2006; Miyshiro et al., 2003;

* *in vitro* includes filter binding, gel-shift, affinity capture, and UV crosslinking.

Figure 2. The role of FMRP in transcriptional regulation. At the subcellular level, FMRP is found in the cytoplasm, primarily associated with actively translating ribosomes. FMRP is believed to play a key role in synaptic plasticity through regulation of mRNA transport and translational regulation of local protein synthesis at synapses. FMRP is transported into the nucleus through its nuclear localization signal. In the nucleus, it assembles into a messenger ribonucleoprotein (mRNP) complex, allow it to associate with specific mRNAs and proteins. The FMRP–mRNP complex is transported out of the nucleus using a nuclear export signal; the complex then interacts with cytoplasm-specific proteins to facilitate movement along dendrites necessary for the regulation of synaptic protein synthesis (figure adapted from Bagni et al., 2005).

receptor (mGluR) activation (Huber et al., 2002; Bassell and Warren, 2008). Evidence suggests that FMRP can regulate translation through the RNA interference pathway, as the drosophila form of FMRP (dFMRP) was shown to interact with two RISC proteins, Argonaute 2 and Dicer (Ishizuka et al., 2002). Although FMRP's role in RNA interference is still unclear, it has been

postulated that FMRP may recruit the RISC complex and micro RNAs to facilitate recognition of micro RNAs with its specific target mRNAs (Bassell and Warren, 2008). Another mechanism by which FMRP can possibly regulate translation is through phosphorylation of FMRP itself. Both mammalian FMRP and drosophila dFMRP can be phosphorylated *in vivo* at a phosphorylation site (Ceman et al., 1999). The removal of the phosphate by activated phosphatase may signal FMRP to release the translational suppression and allow local protein synthesis of a target mRNA (Bassell and Warren, 2008).

The aforementioned mechanisms would explain the up-regulation of mRNAs in the absence of FMRP, but not the reduction of certain mRNAs that have been found in *Fmr1* knockout mice (Miyashiro et al., 2003; Gantois et al., 2006). Additionally, FMRP may have a role in stabilizing mRNA transcripts. In mice, FMRP binds the transcript encoding PSD-95, an adaptor protein that regulates synaptic signalling and has a role in learning and cortical plasticity (Zalfa et al., 2007). This interaction occurs through the 3' untranslated region of the PSD-95 mRNA, increasing its stability. Thus in the absence of FMRP, a disruption in the stability of target mRNAs leads to lower levels of mRNA expression.

The mouse model and molecular mechanisms of fragile X syndrome

Animal models which lack the expression of FMRP have greatly improved our understanding of the molecular mechanisms behind FXS. Mice share a high level of nucleotide and amino acid homology with humans (Verkerk et al., 1991; Ashley et al., 1993). In addition, the tissue specificity and temporal and spatial expression profile of FMRP are similar in both species, thus making the mouse a suitable model for the study of this disorder (Hinds et al., 1993; Bakker et al., 2000). *Fmr1* knockout mice do not possess a CGG repeat expansion in their gene

as in humans; rather exon 5 of the *Fmr1* gene is disrupted by the insertion of a neomycin cassette (Bakker et al., 1994). However, this mutation similarly leads to a transcribed mRNA transcript but a loss of the encoded protein.

The *Fmr1* knockout mice show both morphological abnormalities and changes in behaviour. As in humans, *Fmr1* knockout mice have macroorchidism, which is evident from PND 15 onwards and results in an increase of testicular weight by 30% after 6 months (Bakker et al., 1994; Kooy et al., 1996; Kooy et al., 1998). Dendritic spine abnormalities are a hallmark of FXS patients (Hinton et al., 1991) and this is recapitulated in *Fmr1* knockout mice. Long, thin immature spines are observed in these mice, with an overall increase in dendritic spine density indicative of defective pruning in development (Irwin et al., 2001; de Vrij et al., 2008).

Another clear neurological parallel between the mouse model and FXS patients is an increased susceptibility to seizures. However, as opposed to the spontaneous seizures observed in FXS patients, seizures in *Fmr1* knockout mice are specifically triggered by auditory stimuli (Musumeci et al., 2000; Chen and Toth, 2001). Seizure incidence in knockout mice have been shown peak in the third week of postnatal life (Yan et al., 2005). Finally, several independent studies have shown knockout mice to be hyperactive in the open field test (Bakker et al., 1994; Kooy et al., 1996; Mineur et al., 2002).

Due to the cognitive defects associated with FXS, a number of studies have focused on examining synaptic plasticity by measuring hippocampal long-term potentiation (LTP) in *Fmr1* knockout mice. Initial electrophysiological studies examining the NMDA receptor-dependent LTP in the hippocampus did not find a difference in early- or late-phase LTP between wild-type and *Fmr1* knockout mice (Godfraind et al., 1996; Paradee et al., 1999). Interestingly, it was

Figure 3. The GABAergic synapse. Schematic diagram of the synthesis and transport of GABA at synapses. GABA (red dots) is synthesized in inhibitory neurons from glutamate by the enzyme glutamic acid decarboxylase (GAD). Vesicular neurotransmitter transporters (VGAT) package GABA into vesicles for release. GABA can then bind to GABA_A and GABA_B receptors that are located at pre-, post-, and extra-synaptic sites. The GABA transporters GAT-1, GAT-2, and GAT-3 facilitate the recycling of GABA by reuptake to the pre-synaptic neuron. Alternatively, GABA can be transported into surrounding glial cells, where GABA is metabolized in the Krebs cycle or transported to the pre-synaptic neuron conversion to glutamine (green dots).

later discovered that a protein synthesis-dependent form of hippocampal long-term depression (LTD) induced by group I mGluRs was exaggerated in the *Fmr1* knockouts (Huber et al., 2002).

The activation of group I mGluRs triggers the rapid translation of certain mRNAs at synapses, including FMRP (Weiler and Greenough, 1993; Weiler et al., 1997). The translation of these mRNAs at post-synaptic dendrites stimulates the internalization of surface expressed AMPA and NMDA receptors. In the absence of FMRP, an increase in the translation of target mRNA occurs *in vivo* (Zalfa et al., 2003; Lu et al., 2004; Muddashetty et al., 2007). Thus, exaggerated LTD in *Fmr1* knockout mice was indicative of FMRP playing a role in the regulation of mRNA translation downstream of mGluR signalling.

The culmination of data that implicated exaggerated mGluR signalling in the phenotypical profile of *Fmr1* knockout mice became known as the 'mGluR theory of fragile X' (Huber et al., 2002). This theory posits that the loss of FMRP resulting in increased mGluR-mediated signalling is responsible for several aspects of the FXS phenotype, due to excess translation of target mRNAs at synapses. A dysfunction in local protein synthesis would affect synaptic plasticity by exaggerating LTD, during the critical period of synaptogenesis, therefore contributing to the developmental delay and cognitive impairment associated with FXS. In order to test the theory, double mutant mice were bred by crossing mGluR5 knockout mice (Lu et al., 1997) with *Fmr1* knockout mice in an attempt to correct established phenotypes of FXS by the genetic reduction of mGluR expression by 50% (Dolen et al., 2007). Remarkably, they found that audiogenic seizure susceptibility, increased basal protein synthesis, dendritic spine density and increased body weight were all rescued in *Fmr1*/mGluR5 double mutant mice. Subsequent studies have further validated the theory by pharmacologically rescuing audiogenic seizure, hyperactivity and dendritic spine morphology in *Fmr1* knockout mice using the mGluR5 antagonist MPEP (Yan et al., 2005; de Vrij et al., 2008). Overall, the use of the mouse model

and other models such as the drosophila and zebrafish models of FXS has led to increasing evidence of defective excitatory neurotransmission in FXS.

The GABAergic system

γ -Aminobutyric acid (GABA) is the major inhibitory neurotransmitter found in the mammalian peripheral and central nervous system (CNS). GABA, along with its metabolic precursor, glutamate, are important in maintaining the balance between inhibitory and excitatory neuronal activity, respectively (Chebib, 2004). The majority of the physiological actions produced by GABA are through interactions with ionotropic or metabotropic GABA receptors localized presynaptically, postsynaptically and extrasynaptically. The GABA_A receptors are members of the ligand-gated ion channel superfamily (LGIC), which include nicotinic acetylcholine (nACh), strychnine-sensitive glycine and 5-HT₃ receptors (Martin et al., 2009). GABA_A receptors are present along proximal dendrites of glutamatergic neurons, often situated at dendritic spines, allowing for the inhibitory regulation of glutamatergic signalling (Bassell and Warren, 2008). The metabotropic GABA_B receptors couple to G-proteins (e.g., G_{i/o}) which can act downstream to activate inwardly rectifying K⁺ channels or inhibit high voltage-activated Ca²⁺ channels and regulate of cyclic AMP or inositol phosphate signaling (Bowery et al., 2002; Bettler et al., 2004).

The GABA_A receptors are chloride ion (Cl⁻) channels formed from pentameric membrane-spanning proteins. These channels are assembled from a family of 19 homologous subunit gene products. These include 16 subunits (α 1–6, β 1–3, γ 1–3, δ , ϵ , θ , π) combined as GABA_A, and 3 rho (ρ) subunits, which were formally called the GABA_C receptors (Barnard et al., 1998; Olsen and Sieghart, 2009). When bound by its endogenous GABA ligand, or other exogenous agonists, the receptor changes its conformation to allow Cl⁻ to pass down an

electrochemical gradient. In mature neurons, activation of the GABA_A receptor leads to the reduction of neuronal excitability, as the influx of Cl⁻ into the cell leads to hyperpolarization and a subsequent decrease in the probability of an action potential. However, in immature neurons, the reversal of the electrochemical gradient results in activation of GABA_A receptors leading to membrane depolarization due to efflux of Cl⁻ from the neuron. This reversal of the electrochemical gradient is due to changes to the developmental expression of chloride transporters. In immature neurons, Na⁺-K⁺-2Cl⁻ co-transporter 1 (NKCC1) expression predominates and functions to increase [Cl]_i, while higher expression K⁺-Cl⁻ co-transporters (KCC2) in mature neurons lowers [Cl]_i below its equilibrium potential (Ben-Ari, 2002).

GABA is synthesized from glutamate in the presynaptic neuron by the enzyme glutamic acid decarboxylase (GAD). GAD exists in two major isoforms, GAD67 and GAD65, encoded by two distinct genes, *gad1* and *gad2* respectively. GABA is taken up into synaptic vesicles within the presynaptic terminal by vesicular neurotransmitter transporter (VGAT) and is expelled to the synaptic cleft through calcium-dependent exocytosis, where GABA can then bind GABA_A and GABA_B receptors (Chaudhry et al., 1998). Neurotransmission is terminated through the reuptake of GABA into presynaptic nerve terminals in a recycling process mediated by GABA transporters (GATs), which also co-transport for sodium and chloride ions (Takayama and Inoue, 2005). Four distinct GABA transporters, GAT-1, BGT-1, GAT-2, and GAT-3 have been identified in several mammalian species (Christiansen et al., 2007). GABA uptake can also occur to surrounding glia where GABA transaminase (GABA-T) converts GABA into succinic semialdehyde. GABA-T also synthesizes glutamate using α -ketoglutarate from the Krebs cycle (Figure 3; D'Hulst and Kooy, 2007). Succinic semialdehyde dehydrogenase (SSADH) converts succinic semialdehyde into succinate, which is used in the Krebs cycle. Glutamate produced by

GABA-T in glial cells can be converted into glutamine and transported into the presynaptic neuron, where it is used as a substrate for GABA.

GABA_A receptor activity can be modulated by a diverse range of pharmacological agents. GABA or GABA_A agonists are able to bind two molecules at the interface α and β site of the receptor (Martin et al., 2009). Other drugs such as benzodiazepines, barbiturates, and steroids bind at allosteric binding sites (Figure 4; Smith and Olsen, 1995; Sieghart et al., 1999). Additionally, GABA_A receptors have been shown to be involved in modulating epilepsy, anxiety, learning and memory (DeLorey et al., 1998; Crestani et al., 1999; Mihalek et al., 1999).

The GABAergic system and fragile X syndrome

Recently, the role of GABAergic dysfunction in FXS has gained attention in light of several new findings in fly and mouse models of the disorder (see Table 2). Studies performed in *dFmr1* null mutant flies revealed that the 3 genes that comprise the GABA_A receptor in flies (*Rdl*, *Grd*, *Lcch3*), were significantly under-expressed (D'Hulst et al., 2006). Another study found that *dFmr1* mutant flies died on a high glutamate diet, but survived when this diet was supplemented with GABA, or the GABA reuptake inhibitor, nipecotic acid (Chang et al., 2008). GABA treatment also rescued abnormal male courtship behaviour, mushroom bodies defects, and *futsch* over-expression (ortholog of *Map1b*), in *Fmr1* mutant flies.

One of the earliest findings of GABAergic dysfunction in *Fmr1* knockout mice was established by an electrophysiological study of the GABAergic and cholinergic systems (D'Antuono et al., 2003). The cholinergic system is linked with the GABAergic system in that cholinergic activation mediates the release of GABA from some interneuron terminals, resulting in a depression of excitatory responses (Pitler and Alger, 1992). D'Antuono et al. treated

subicular neurons with carbachol and witnessed a depression of excitatory responses in wild-type mice. However, LTP was observed in *Fmr1* knockout mice, a response which was similar to carbachol-treated wild type mice treated with the GABA_A receptor antagonist picrotoxin. The

Table 2 – Summary of Alterations to the GABAergic System in FXS Reported in the Literature

	Species	Up-regulation/ Down-regulation	Brain Region(s)	mRNA/Protein	Reference
GAD1 GAT1 GAT4 SSADH gephyrin	<i>Fmr1</i> KO Mouse (adult)	Down-regulation	Frontal cortex	mRNA (qRT-PCR)	D'Hulst <i>et al.</i> (2009)
GABA _A receptor $\alpha 5$ GABA _A receptor δ	<i>Fmr1</i> KO Mouse (adult)	Down-regulation	Subiculum	Protein (WB)	Curia <i>et al.</i> (2008)
Vesicular GABA Transporter (VGAT)	<i>Fmr1</i> KO Mouse (adult)	Down-regulation	Cultured Striatal Neurons	Protein (IF)	Centonze <i>et al.</i> (2008)
Ubiquilin (Plic-1)	Human FXS subjects	Down-regulation	Frontal cortex	mRNA (microarray; qRT-PCR)	Bittel <i>et al.</i> (2007)
GABA _A receptor $\alpha 1$ GABA _A receptor $\alpha 3$ GABA _A receptor $\alpha 4$ GABA _A receptor $\beta 1$ GABA _A receptor $\beta 2$ GABA _A receptor δ GABA _A receptor $\gamma 1$ GABA _A receptor $\gamma 2$	<i>Fmr1</i> KO Mouse (adult)	Down-regulation	Frontal cortex	mRNA (qRT-PCR)	D'Hulst <i>et al.</i> (2006)
GABA _A receptor δ	<i>Fmr1</i> KO Mouse (adult)	Down-regulation	Hippocampus Neocortex	mRNA (qRT-PCR)	Gantois <i>et al.</i> (2005)
GABA _A receptor β GAD65/67	<i>Fmr1</i> KO Mouse (adult)	Down-regulation Up-regulation	Cortex Diencephalon Hippocampus Brainstem	Protein (WB)	El Idrissi <i>et al.</i> (2005)
GABA	<i>Fmr1</i> KO Mouse (female; PND28-31)	Up-regulation	Striatum	Amino Acid (HPLC)	Gruss <i>et al.</i> (2003)
	<i>Fmr1</i> KO Mouse (male; PND28-31)	Up-regulation	Brainstem	Amino Acid (HPLC)	Gruss <i>et al.</i> (2001)

GABA_A Receptor

Figure 4. Structure and drug binding sites of GABA_A receptors. GABA_A receptors are pentameric chloride ion channels formed by the assembly of 5 copies of 16 possible subunits (α 1–6, β 1–4, γ 1–3, δ , ϵ , θ , π). The most common receptor subtype is composed of two α subunits, two β subunits, and one γ subunit. At various extra-synaptic sites, the γ subunit is often replaced by the δ subunit to promote receptors with exceptionally high sensitivity to GABA. GABA_A receptors are targets of various classes of clinically relevant drugs, including benzodiazepines, barbiturates, and general anaesthetics, which modulate receptor function through allosteric binding sites.

authors concluded that the *Fmr1* knockout mice have some impairment in GABA_A receptor-mediated function, supported by the increased excitability in knockout subicular slices.

Another electrophysiological study noted that although GABAergic synapses appeared to be reduced in the striatum of knockout mice, there was a significant increase in the frequency of spontaneous and miniature inhibitory postsynaptic currents, which was suggestive of abnormal GABA transmission (Centonze et al., 2008). Furthermore, HPLC analysis of amino acids in PND 28–31 female knockout mice brains in another study revealed a significant increase in

GABA levels in the striatum (Gruss and Braun, 2004). In contrast, reduced tonic GABA_A currents were observed in the subiculum of *Fmr1* knockout mice, coupled with the down-regulation of $\alpha 5$ and δ protein expression (Curia et al., 2008). Similarly, significant reductions in the frequency/amplitude of phasic IPSCs and a decrease in tonic inhibitory currents were observed in the basolateral nucleus of the amygdala of *Fmr1* knockout mice (Olmos-Serrano et al., 2010). Additionally, a down-regulation of GAD65/67 expression and reduced GABA concentration observed at the synaptic cleft was indicative of a presynaptic defect in GABAergic transmission in *Fmr1* knockout mice.

Using an antibody recognizing all three β subunit isoforms, El Idrissi et al. (2005) found a reduction in expression of β subunit, while GAD65/67 levels were increased in the hippocampus, cortex, diencephalon, and brain stem of *Fmr1* knockout mice. Additionally, immunohistochemical analysis revealed that expression of the β subunit was decreased in the pyramidal layer of the hippocampal CA1 region. A subsequent study in *Fmr1* knockout hippocampus and neocortex analyzed global gene expression using differential display and real-time PCR (Gantois et al., 2006). They found that three cDNAs were consistently under-expressed in *Fmr1* knockout mice, including the GABA_A receptor δ subunit, previously shown to be a target for direct FMRP binding (Miyashiro et al., 2003). Subsequent in-depth studies focusing primarily on GABAergic transcripts revealed that the mRNAs for 8 GABA_A receptor subunits (δ , $\alpha 1$, $\alpha 3$, $\alpha 4$ and $\beta 1$, $\beta 2$ and $\gamma 1$, $\gamma 2$), the 67 kDa isoform of GAD67, SSADH, gephyrin, and GABA transporters 1 and 4 were significantly under-expressed in the cortex of *Fmr1* knockout mice (D'Hulst et al., 2006; D'Hulst et al., 2009). Overall, several lines of evidence have been presented that implicate the direct or indirect involvement of FMRP in GABAergic synaptic function.

The cerebellum and deep cerebellar nuclei

In addition to its role in motor function, growing evidence of cerebellar abnormalities in neurodevelopmental disorders implicates the role of the cerebellum in higher cognitive functioning. Recently, our laboratory in collaboration with the Henkelman lab used high resolution magnetic resonance imaging (MRI) to compare the neuroanatomical structure of *Fmr1* knockout and wild-type mice. The volume of the arbor vitae in *Fmr1* knockout mice was reduced, with significant decreases in the volume of the deep cerebellar nuclei (DCN), particularly the interpositus and fastigial nuclei (Ellegood et al., 2010).

Excitatory and inhibitory output from the cerebellum occurs through the axons of the DCN, which receives GABAergic input from Purkinje cells and glutamatergic input from the mossy fiber and climbing fiber pathways (Bauman and Kemper, 2005). There are three major deep nuclei: the dentate, interpositus, and the fastigial nuclei. Output from the DCN travels from the superior cerebellar peduncle, through the red nucleus, then to the thalamus. The thalamus then relays the input to the upper neurons of the motor cortex (Bauman and Kemper, 1994). Although the borders between the nuclei are not well defined, each receives input from different regions of the cerebellar cortex. There has also been some evidence for a role of the dentate nucleus in spatial learning and memory, as lesions introduced into the dentate nucleus of rats impaired place learning in the Morris water maze, a task commonly associated with hippocampal function (Joyal et al., 2001; Noblett and Swain, 2003).

The cerebellar vermis is a structure that is anatomically connected to the interpositus nucleus and hippocampus (Hessl et al., 2004), believed to play a role in motor behaviour, auditory processing, and language (Steinlin, 2008). Decreases in the cerebellar posterior vermis size has been found in patients with FXS (Mostofsky et al., 1998). Children with cerebellar

hypoplasia present not only with congenital ataxia but also with mild to moderate speech and cognitive developmental problems (Shevell and Majnemer, 1996). Additionally, GABAergic dysregulation in the cerebellum has been associated with neuroanatomical abnormalities in the cerebellum. Purkinje cell size and GABAergic protein expression in the cerebellum is reduced in patients with autism (Fatemi et al., 2002; Fatemi et al., 2009). Finally, GABA_A receptor $\beta 3$ knockout mice display significant decreases in surface area of cerebellar vermal lobules II–VII compared to wild-type mice (DeLorey et al., 2008).

Objectives and Rationale

Several studies have shown that the expression of GABA_A receptor subunits and GABAergic enzymes are reduced in adult *Fmr1* knockout mice (D'Hulst et al., 2006; Gantois et al., 2006; D'Hulst et al., 2009). Previous reports indicating the GABA_A receptor δ subunit mRNA is a target for FMRP (Miyashiro et al., 2003) may suggest a direct role for FMRP in the transport or localization of GABA_A receptor subunits. However, a change in mRNA expression does not necessarily indicate changes in protein expression, and tests to verify changes in protein expression have been very limited (El Idrissi et al., 2005; Curia et al., 2008). Moreover, previous studies only focused on adult tissues and did not examine expression during the critical period of development in early postnatal life when synaptogenesis and neuronal maturation occurs.

I **hypothesize** that the absence of FMRP results in alterations of the dynamic interactions that occur between GABA_A receptor and GABA enzymes during brain development, resulting in long-lasting disruptions to synaptic transmission that contribute to the aberrant behavioural profile in *Fmr1* knockout mice. My first objective was to show using quantitative western blotting that protein expression of GABA_A receptors and GABA metabolizing enzymes are altered during the course of postnatal development. We chose three points that represent different stages in GABAergic development. At PND 5, GABA is excitatory; PND 12 represents a period around the transition from excitatory to inhibitory GABAergic transmission (Ben-Ari, 2002); finally, GABA is inhibitory in adult mice. Analysis of these three periods provided us with a picture of when defects in GABAergic transmission could affect development in *Fmr1* knockout mice. We anticipated that the pattern of expression of GABA_A receptors and metabolizing enzymes may differ in *Fmr1* knockout mice throughout development compared to wild-type mice. A second objective was to assess if alterations in GABAergic protein expression

would affect three behavioural phenotypes of *Fmr1* knockout mice; seizure susceptibility, locomotor activity, and sleep times. Specifically, we examined the efficacy of pharmacological agents that target the GABA_A receptor in *Fmr1* knockout and wild-type mice to determine if there is a difference between the genotypes as a result of altered GABAergic transmission.

As mentioned above, there is growing evidence for cerebellar abnormalities in FXS that suggest a developmental role for the structure in higher cognitive functioning and learning. Purkinje cells have been shown to possess abnormal dendritic morphology in *Fmr1* knockout mice (Koekkoek et al., 2005). It is plausible that GABAergic input from Purkinje cells to the DCN is perturbed, and that this might result in excitotoxic damage to DCN neurons. Thus, further analyses of the DCN was required at the cellular level to more clearly delineate how and why the morphometric changes observed in the MRI analysis (Ellegood et al., 2010) might be induced in *Fmr1* knockout mice. The dysfunction of GABAergic signalling in *Fmr1* knockout mice may be associated with these anatomical changes. Therefore, we hypothesized that GABAergic abnormalities throughout development, particularly in the DCN, contribute to anatomical changes of the cerebellum. To this end, I performed comparative morphometric analysis, as assessed via cresyl violet staining on thin section of the cerebellum, and also compared the protein expression of GABAergic and glutamatergic proteins and neuronal markers in the cerebellar cortex and DCN of wild-type and *Fmr1* knockout mice. We anticipated that volumetric decreases as assessed by cresyl violet staining in the DCN would coincide with the findings of the MRI study (Ellegood et al., 2010). In addition, we expected to see alterations in the expression of GABA_A receptors and GABA metabolizing enzymes in the DCN which may be related to reduced GABAergic input from afferent fibers.

Methods and Materials

Mouse model on FXS

All animal experiments were carried out in accordance with the guidelines set out by the Canadian Council on Animal Care and were approved by the University Animal Care Committee of the University of Toronto. All wild-type and knockout mice used were derived on the C57/Black 6 strain (kindly provided by Dr. William Greenough, University of Illinois). Mice were assessed at different developmental age points depending on the experiment.

Western blotting

For quantitative western blotting of the forebrain and whole cerebellum, mice were analyzed at postnatal days (PND) 5, 12 and adulthood (2 to 3 months old). Duke Chen (summer student, University of Toronto) and Clara Li (summer student, McGill University) assisted in the completion of this analysis. For western blotting of the DCN mice were tested at PND 30. Brain tissue from wild-type and *Fmr1* knockout mice were quickly dissected, weighed and transferred to a 10 or 2 ml glass tube. The tissue was homogenized on ice in cold lysis buffer containing 50 mM Tris-HCl, 1% SDS (pH 7.4) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Oakville, ON) to prevent degradation of proteins in the tissue. Protein samples were frozen at -70°C in 100 µl aliquots until they were needed for sample preparation. The volume of lysis buffer used for each sample that would yield a protein concentration of approximately 3 µg/ml was determined by this equation: $\text{Weight of wet tissue (mg)} / 30 * 100 = \text{Volume of lysis buffer (ml)}$.

Protein quantification was performed using the QuantiPro bicinchonic acid (BCA) assay kit (Sigma-Aldrich). The protein concentrations were measured using the DU 730 UV/Vis

scanning spectrophotometer (Beckman Coulter, Mississauga, ON) at 562 nm absorbance. A standard curve was generated based on the concentrations of the standards was used to determine protein concentrations of the tissue samples. Protein samples were prepared for SDS-PAGE in 4X sample buffer (8% SDS, 250 mM Tris, 40% glycerol, pH 6.8), 100 mM dithiothreitol (DTT) and 1 μ l of bromophenol blue. The samples were then sonicated for 10 seconds with a Microson™ ultrasonic cell disruptor (Heat Systems Ultrasonics, Farmingdale, NY) after which they were heated to 95 °C for 3 minutes.

Equal amounts of total protein from mouse brain tissue were loaded from the prepared samples into each lane of the stacking gel. Depending on the abundance of the target protein, between 3-20 μ g of protein was loaded in the lanes. The proteins were separated by SDS-PAGE on 10% polyacrylamide gels. Samples from wild-type and *Fmr1* mice were always analyzed simultaneously on the same gels and blots. The samples were run at 60V until the proteins had passed the stacking gel into the resolving gel, after which the voltage was increased to 100V until the samples had run the length of the gel. The gels and nitrocellulose membranes were then soaked in transfer buffer (48 mM Tris, 34 mM glycine, 1.5 mM SDS, pH 9.2) for 20 minutes. Afterwards, the gels were transferred for 1 hour at 20V onto nitrocellulose membranes using the Bio-Rad Trans-Blot semi-dry transfer system. The membranes were blocked with 5% non-fat milk in wash buffer containing 100 mM Tris 15 mM NaCl, 0.05% Tween-20 (pH 7.6) at 4°C overnight. The next day, membranes were washed 3 X 15 minutes at room temperature with wash buffer before incubation for 2 hours with the primary antibodies. The following antibodies were used: anti-FMRP antibody (clone 2F5-1; 1:2000; gift of Jennifer Darnell, Rockefeller University), anti-GABA-T (1:30; gift of Dr. Soo Young Choi, Hallym University), anti-GAD65 (1:400; Developmental Studies Hybridoma Bank, University of Iowa), anti-SSADH (1:2000; gift

of Dr. Michael K. Gibson, University of Pittsburgh), anti-GABA_A receptor α 1 (1:1000; Upstate), anti-GABA_A receptor β 1 (1:750; Abcam), anti-GABA_A receptor β 2 (1:400; Abcam), anti-GABA_A receptor β 3 (1:400; NeuroMab, UC Davis/NIH), anti-GABA_A receptor β 2/3 (BD17 clone, 1:1000; Chemicon), anti-GABA_B-R2 (1:400; NeuroMab, UC Davis/NIH), anti-GABA_A δ (1:10; NeuroMab, UC Davis/NIH), anti-NKCC1 (1:1000; Abcam), anti-KCC2 (1:1000; Upstate), anti-gephyrin (1:400; Abcam), anti-ubiquilin (1:1000; Abcam), anti-VGLUT1 (1:1000, Synaptic Systems), anti-VGLUT2 (1:1000, Synaptic Systems), anti-VGAT (1:1000, Synaptic Systems), anti-NeuN (1:1000, Millipore), anti-calbindin D-28k (1:1000, Swant) and anti-GAPDH (1:40000 or 1:80000; Sigma-Aldrich). After washing 3 X 15 minutes in wash buffer, the membranes were incubated for 2 hours at room temperature in donkey anti-mouse (1:5000; Jackson ImmunoResearch), goat anti-rabbit (1:30000; Jackson ImmunoResearch) or goat anti-chicken (1:10000; Jackson ImmunoResearch) horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected by incubating membranes with SuperSignal West Pico Chemiluminescence Substrate (Fisher Scientific, Pittsburgh, PA) for 5 minutes.

For quantification, the signal intensity was examined using the Alpha Innotech FluorChem[®] Chemiluminescent Imaging System. Nitrocellulose membranes were exposed for 10 minutes and immunolabelling density was analyzed using AlphaEaseFC image analysis software by measuring the intensity of the bands for each antibody and their corresponding loading control glyceraldehyde 3-phosphate-dehydrogenase (GAPDH). The intensity of the antibody bands were then calculated as a ratio to GAPDH intensity. The protein expression levels for *Fmr1* knockout mice are reported as a percentage of the wild-type expression $\pm$ S.E.M. The data from wild-type and *Fmr1* knockout mice were compared using the Student's t-test.

Quantitative morphometric analysis of wild-type and *Fmr1* knockout mice cerebellum

A quantitative morphometric analysis of the cerebellar lobules was performed in wild-type and *Fmr1* knockout mice. Animals were transcardially perfused with 4% PFA at room temperature, with their brains subsequently removed and post-fixed overnight at 4°C in the same solution. The following day, brains were placed in PBS containing 30% sucrose overnight at 4°C. Finally, the brains were flash frozen in -60°C isopentane then embedded sagittally in optimal cutting temperature (OCT) mounting medium.

Coronal serial sections (50 µm) were cut with a Leica CM 3050 S cryostat, thaw-mounted on gelatinized slides and allowed to dry for 3 hours. Every third section was collected for measurement (interval thickness of 150 µm). Sections used for morphometric analysis were stained at room temperature with a protocol using 0.1% cresyl violet (Sigma-Aldrich), dissolved in deionized water. Slides were rinsed in water for 10 minutes to remove OCT from the sections. The sections were then dehydrated in successive 70%/95%/100% ethanol baths for 3 minutes each. The sections were de-fatted in xylene for 3 minutes before rehydration in 100%/95%/70% ethanol and water for 3 minutes each. The slides were then placed in cresyl violet for 3-5 minutes until sections were a deep purple colour. Then, sections were quickly washed twice in water, agitated in 70% ethanol for 3 minutes until dye began to fade. Slides were then dehydrated in 95%/100% ethanol for 3 minutes each before they were cleared in xylene (2 changes) for 5 minutes. Slides were then coverslipped with Permount and allowed to dry overnight.

The morphometric analysis was performed on digital images of sections captured using a Hamamatsu ORCA 285 CCD camera mounted on a Nikon E1000 microscope at 4X magnification. Two cerebellar regions were considered: the medial part of the vermis (midline

to +0.5 mm laterally) and the intermediate cortex (+0.7 to +1.2). Several parameters were analyzed; granule layer thickness, molecular layer thickness, lobule/cerebellar circumference ratio, and cerebellar area. For parameters that analyzed individual lobules, four cerebellar lobules were analyzed; II, III, VI, and VIII. All images were analyzed using SimplePCI software (Hamamatsu). The average area of cerebellar cortex in mm^2 was obtained by outlining the contour of sections. Three sections per animal were measured in each cerebellar regions. Statistical comparisons between the genotypes were made using a student's t-test.

A quantitative morphometric analysis of the DCN in wild-type and *Fmr1* knockout mice was performed in coronal cerebellar sections. Mice were transcardially perfused with 4% PFA, then their brains were post-fixed overnight at 4°C in the same fixative. The following day, brains were cryoprotected with 30% sucrose overnight at 4°C. Coronal serial sections (50 μm) were collected on gelatinized slides and stained with cresyl violet with the procedure described above. Every other section was selected for measurement (interval thickness of 100 μm). The morphometric analysis was performed on digital images of sections captured using a Hamamatsu ORCA 285 CCD camera mounted on an Nikon E1000 microscope at 4X magnification. The area measurements for the dentate, interpositus, and fastigial nuclei from both hemispheres were analyzed with SimplePCI software (Hamamatsu). Measurements were made by identifying and tracing a boundary around each nuclei. The volume was calculated by adding the sum of the areas of each section multiplied by the interval thickness. Measurements of the average intensity of the right DCN were made using ImageJ software (NIH). The mean grey value of the DCN gives the sum of the intensity of each pixel divided by the number of pixels in the area selected. The grey value ranges between 0 (black) and 255 (white) while normalizing for area, which yields an estimate of neuron density in the DCN. A mean value was calculated by averaging the

values for each nuclei in every section for each animal. Statistical comparisons between the genotypes were made using a student's t-test.

Micro-dissection of the DCN

The brains of wild-type and *Fmr1* knockout mice were quickly excised after cervical dislocation and washed in ice cold 0.1 M PBS. The cerebellum was then dissected and mounted coronally onto a Vibratome stage with Krazy Glue® (Elmer; Columbus, OH). The tissue was then cut in ice-cold PBS with the Vibratome series 1000 tissue sectioning system. Initially, the cerebellum was gradually sectioned until the region that contains the DCN was reached. At this point, a 1.5 mm section was cut, collected onto a microscope slide and placed on dry ice. After the section had frozen, the regions surrounding the DCN, which includes the cerebellar cortex, were excised and only the region of arbor vitae encompassing the DCN was retained. The remaining tissue from the cerebellum was also collected separately and subsequently weighed and prepared for SDS-PAGE.

Audiogenic seizure testing

For audiogenic seizure testing, the apparatus consisted of a plexiglass mouse cage (28 × 17 × 14 cm) with a 135 dB alarm (Piezo siren, Electrosonic model XL-5530LW300-S-R P.V.I.) attached to the lid and extending 5 cm down into the cage. Female *Fmr1* knockout mice (PND 26-30) were intra-peritoneally injected with vehicle (0.3% Tween 80 in saline), diazepam (1.5 mg/kg), phenobarbital (15 mg/kg) or loreclezole (25 mg/kg) 45 minutes prior to seizure testing, at a volume of 0.1mL/10g body weight. The mice were then placed individually into the testing apparatus and were allowed to acclimate to the cage for 2 minutes, after which the alarm was rung for 2 minutes. Seizure activity was observed and scored using a seizure severity scale: wild

running = 1; clonic seizure = 2; tonic seizure = 3; status epilepticus/respiratory arrest/death = 4. Animals were considered to have had a seizure if the seizure severity score was greater than 1. Animals that did not suffer respiratory arrest were sacrificed after seizure testing by cervical dislocation. All seizure testing was performed between 1:00 p.m. and 5:00 p.m. To determine if the drug treatments had an effect on seizure incidence, statistical analysis was carried out using Fisher's exact probability test.

Open field activity testing

Open-field performance was assessed in male *Fmr1* knockout mice at 6 weeks of age (testing was performed by Dr. Laura K.K. Pacey). An automated movement detection system was used to assess locomotion of experimental animals in the open-field arena (AM1053 activity monitors; Linton Instrumentation, UK). This apparatus consisted of a plexiglass box (24 × 45 cm) surrounded by a housing frame (46 × 27 cm) from which 24 infrared beams form a grid across two levels. When the mouse moved, a beam was broken and an activity count, dependent on the type of movement, was registered by the monitoring system. Three behavioural parameters were analyzed: total activity, which counts the number of beam breaks in both horizontal and vertical planes; distance travelled, defined as the total distance travelled in metres in the horizontal plane; center field rearing, an exploratory behaviour which is measured by amounts of entries into the vertical plane. Wild-type and *Fmr1* knockout mice were dosed intraperitoneally with vehicle (0.3% Tween 80 in saline), diazepam (1 mg/kg) or loreclezole (30 mg/kg) and placed immediately into individual activity monitors for a total of 90 minutes with activity recorded in 5 minute epochs. All motor activity measurements were conducted between 9:00 a.m. and 2:00 p.m. to minimize circadian effects. Each subject was examined in the open

field only once. The data were analyzed using two-way ANOVA with Bonferroni's post hoc test.

Phenobarbital and etomidate sleep time test

The hypnotic effects of phenobarbital and etomidate were compared between wild-type and *Fmr1* knockout mice by determination of the duration of the loss of righting reflex. Mice used for phenobarbital testing were 2-3 months old, while mice used for etomidate testing were between 5-7 months old. Loss of righting reflex is defined as the inability of the mouse to right itself three times within a 1 minute span after being placed in the supine position. The mice were intraperitoneally injected with phenobarbital (100 mg/kg) or etomidate (15 mg/kg). Phenobarbital (120 mg/ml stock solution) was dissolved readily in 0.3% Tween 80/saline and injected at a volume of 10 μ l per gram mouse body weight. Etomidate (2 mg/ml stock solution) was supplied in an aqueous, nonpyrogenic solution containing 35% propylene glycol and injected based on body weight per gram of the mouse. The mice were placed in their home cage at room temperature until they lost the righting reflex then were placed in a V-shaped trough in the supine position until recovery. The time from when the mice lost its righting reflex to the point it regains it was measured as the sleep time. The mice were judged to have regained the righting reflex when they could right themselves three times within 30s after being placed in the supine position. One wild-type mouse was excluded from the analysis because it slept significantly longer than the other mice from both genotypes (> 6 hours). Sleep time data were analyzed using a student's t-test.

Results

Developmental expression of the GABA_A receptor subunit in *Fmr1* knockout mice

Previous studies have shown that the mRNA levels of the $\alpha 1$, $\alpha 3$, $\alpha 4$ subunits are down-regulated (D'Hulst et al., 2006), while $\alpha 5$ protein expression is reduced in the subiculum of adult *Fmr1* knockout mice (Curia et al., 2008). We examined the protein expression of two α subunits, $\alpha 1$ and $\alpha 4$. GABA_A receptors containing $\alpha 1$ subunits are most abundantly expressed in the CNS, comprising roughly half of all GABA_A receptors (Mohler, 2007; Olsen and Sieghart, 2009), while $\alpha 4$ -containing GABA_A receptors are expressed primarily at extrasynaptic sites (Liang et al., 2008). Quantitative western blot analysis of samples from mouse forebrain revealed that the $\alpha 1$ subunit was significantly down-regulated in PND 5 ($43 \pm 7.1\%$ of wild-type values, $P < 0.05$) and day 12 ($56 \pm 7.6\%$, $P < 0.05$) *Fmr1* knockout mice compared to wild-type mice (Figures 5 and 6). However, by adulthood, $\alpha 1$ expression was restored to near the normal level and was not significantly different from wild-type mice ($83 \pm 4.8\%$, $P > 0.05$). No significant changes in the expression of the $\alpha 4$ subunit were detected at any of the time points examined (Figure 6).

The assembly of functional GABA_A receptors *in vivo* requires the presence of β subunits. It has been previously reported that protein expression of the GABA_A receptor β subunit is down-regulated in various regions of the adult *Fmr1* knockout mouse forebrain (El Idrissi et al., 2005). To determine which specific β subunit isoform(s) were down-regulated in *Fmr1* knockout mice, we used antibodies specific for each of the three β subunits. Quantification revealed that expression of the $\beta 2$ subunit was significantly down-regulated in PND 12 ($67 \pm 8.2\%$, $P < 0.05$) and adult ($59 \pm 3.7\%$, $P < 0.05$) but not at PND 5 ($87\% \pm 7.9$, $P > 0.05$) in *Fmr1*

knockout versus wild-type mice (Figures 5 and 7). In contrast, there were no significant changes in the expression of the $\beta 1$ and $\beta 3$ subunits at any developmental time points (Figure 7). These data demonstrate that the GABA_A $\beta 2$ subunit is selectively down-regulated in *Fmr1* knockout mice, with no compensatory changes in the other β subunits.

Most GABA_A receptor pentameric complexes also contain either a $\gamma 2$ or a δ subunit. Although the $\gamma 2$ subunit was not detected at PND 5, no significant changes were seen at PND 12 or in adults. However, expression of the δ subunit was transiently and significantly decreased at

Figure 5. Quantitative western blot analysis of the expression of FMRP and GABA related proteins in wild-type and *Fmr1* mice forebrain. For each protein, sets of four wild-type and four *Fmr1* mouse whole brain samples were run side-by-side on the same gel and transferred to nitrocellulose. Western blots were quantitated with a scanning densitometer and normalized relative to the expression of GAPDH. Representative blots shown are from mice of different ages: FMRP, GAD65, and gephyrin are from adults, GABA-T, SSADH (higher band) and GABA_A receptor $\beta 2$ are from PND 12 mice, and GABA_A receptor $\alpha 1$ is from PND 5 mice.

Figure 6. Quantitative analysis of GABA_A receptor α1, α4, δ, and γ2 subunit expression in mice forebrain. Summary of western blot analyses of GABA_A receptor subunit expression relative to GAPDH expressed as a percentage of the wild-type level. The α1 subunit was significantly down-regulated (* P < 0.05) in PND 5 and 12 *Fmr1* knockout mouse forebrain samples compared to wild-type mice, while the expression of the δ subunit was significantly decreased (* P < 0.05) at PND 12 but not at PND 5 or in adults. No significant change in the expression of γ2 was observed at day 12 or in adult brain while γ2 expression was too low to quantitate at PND 5. For each analysis, 4 wild-type and 4 *Fmr1* mice were assessed at each time point. Error bars represent S.E.M.

PND 12 (60 ± 5.5% of wild-type), but not at PND 5 and in adult *Fmr1* knockout mice relative to wild-type mice (Figure 6).

GABA_B receptor expression in *Fmr1* knockout mice forebrain

The GABA_B receptor is a heterodimeric G-protein coupled receptor composed of two subunits, GABA_B-R1 and GABA_B-R2. Both subunits are required for functional assembly of the receptor. The GABA_B-R1 subunit contains the GABA binding site and undergoes a

conformational change when an agonist binds, allowing for the coupled GABA_B-R2 subunit to interact with the G-protein coupled signalling system (Bowery et al., 2002). GABA_B-R2 is responsible for the translocation of the receptor from the endoplasmic reticulum to the cell surface membrane and is required for G-protein coupling (Chung et al., 2008; Lagerstrom and Schioth, 2008; Wang et al., 2009). The expression of the both GABA_B subunits did not differ between wild-type and *Fmr1* knockout mice at any developmental time point (Figure 8).

GABA metabolic enzyme expression in the *Fmr1* knockout forebrain

We examined the expression levels of enzymes involved in GABA synthesis and catabolism. Western blots of forebrain samples of wild-type and *Fmr1* knockout mice were probed with antibodies specific for the GAD65, (GABA-T) and SSADH (Figure 5). GAD65 is responsible for the synthesis of GABA from glutamate, while GABA-T and SSADH catabolize GABA into succinate. Although no significant changes in GAD65 expression were observed in PND 5 and 12, expression was significantly increased in adult *Fmr1* knockout mice ($150 \pm 9\%$, $P < 0.05$) compared to wild-type adult mice (Figure 9). GAD65 was the only protein examined in this study that was significantly increased in the knockout mice relative to control mice. Interestingly, both SSADH and GABA-T were significantly decreased at PND 12 ($72 \pm 4.6\%$ and $55 \pm 2.1\%$ of wild-type values respectively; both $P < 0.05$) but not at PND 5 or in adult *Fmr1* knockout mice (Figure 9). Together, these results indicate that there is an increase in the GABA synthetic enzyme and a decrease in GABA catabolic enzymes in *Fmr1* knockout mice; these alterations in enzyme expression could conceivably cause an elevation in brain GABA levels at different stages of development.

Figure 7. Developmental expression of GABA_A receptor β subunits in *Fmr1* knockout mice forebrain. Summary of western blot analyses of GABA_A receptor β1, β2, and β3 expression. The β2 subunit was significantly down-regulated (* $P < 0.05$) in PND 12 and adult *Fmr1* knockout mice forebrain samples compared to wild-type mice (N = 4 at each time point).

NKCC1, KCC2, gephyrin and ubiquilin expression in the forebrain

The developmental switch from excitatory to inhibitory GABAergic transmission coincides with a switch in the expression of two ion transporters, Na⁺-K⁺-2Cl⁻ co-transporter 1 (NKCC1) and K⁺/Cl⁻ co-transporter 2 (KCC2). NKCC1 is responsible for high intracellular chloride levels in GABAergic neurons early in postnatal development, leading to GABA-mediated depolarization of immature neurons. However, as development progresses, KCC2 expression predominates in the cell thereby mediating a reversal in the intracellular chloride gradient and leading to the switch from excitatory to inhibitory GABAergic transmission

(Ganguly et al., 2001). We postulated that the developmental down-regulation of GABA_A receptor expression in *Fmr1* knockout mice might be accompanied by alterations in NKCC1 or KCC2 expression that may contribute to perturbations in GABAergic transmission. However, no significant differences were found in the expression of NKCC1 and KCC2 in *Fmr1* knockout mice at any developmental stage (Figure 10A), suggesting that the GABA_A receptor alterations observed in *Fmr1* knockout mice are not associated with changes in these transporters.

Figure 8. Developmental expression of the GABA_B receptor in *Fmr1* knockout mice forebrain. Western blot analysis of GABA_B-R1 and GABA_B-R2 subunit expression. No significant difference ($P > 0.05$) in either subunit was observed between *Fmr1* knockout mice and wild-type mice ($N = 4$ at each time point).

Ubiquilin (also known as Plic-1) is a protein that facilitates the membrane insertion of GABA_A receptors by increasing the stability of GABA_A subunits in the endoplasmic reticulum.

Ubiquilin may promote the accumulation of GABA_A receptors at synapses; peptides that disrupt the interaction between ubiquilin and GABA_A receptor subunits cause a decrease in cell-surface expression and subunit half-life (Bedford et al., 2001; Saliba et al., 2008). Moreover, a microarray study performed on frontal cortex samples of FXS patients revealed that the gene that encodes ubiquilin (UBQLN1) was significantly down-regulated (Bittel et al., 2007). However in the present analysis, no significant difference ($P > 0.05$) in the expression of ubiquilin was observed in *Fmr1* knockout mice compared to wild-type mice (Figure 10B). Gephyrin is an anchoring protein required for postsynaptic clustering of $\gamma 2$ -containing GABA_A receptors

Figure 9. Developmental expression of GABAergic enzymes in *Fmr1* knockout mice forebrain. Summary of western blot analyses of GAD65, GABA transaminase (GABA-T) and succinyl semialdehyde dehydrogenase (SSADH) expression. GAD65 expression was significantly increased in adults while GABA-T and SSADH were significantly decreased at PND 12 when compared to wild-type mice (* $P < 0.05$). For each analysis, 4 wild-type and 4 *Fmr1* mice were assessed at each time point.

Figure 10. D developmental expression of NKCC1, KCC2, gephyrin and ubiquilin in *Fmr1* knockout mice forebrain. Western blot analyses of A) $\text{Na}^+\text{--K}^+\text{--}2\text{Cl}^-$ co-transporter 1 (NKCC1), $\text{K}^+\text{--Cl}^-$ co-transporter 2 (KCC2), B) gephyrin and ubiquilin. Although there was no significant difference ($P > 0.05$) in the expression of these proteins, gephyrin was reduced to 72% of wild-type levels in adult *Fmr1* knockout mice. Expression of KCC2 at PND 5 was not detectable ($N = 4$ at each time point).

(Essrich et al., 1998; Tyagarajan and Fritschy, 2010). A reduction in GABA_A receptors may indicate a decrease in gephyrin-mediated clustering of these receptors. Gephyrin mRNA levels were previously reported to be reduced in the cortex of adult *Fmr1* knockout mice (D'Hulst et al.,

2009). Quantification of protein expression revealed that gephyrin expression was not significantly reduced in *Fmr1* knockout mice; however expression was reduced to 72% of wild-type levels in adult knockout mice.

Quantitative morphometric analysis of wild-type and *Fmr1* knockout mice cerebellum

A recent study conducted in conjunction with our laboratory examining anatomical changes in *Fmr1* knockout mice revealed significant volumetric changes in the arbor vitae of the cerebellum (Ellegood et al., 2010). Additionally, studies have shown defects in cerebellar vermis develop in both FXS patients and GABA_A receptor $\beta 3$ deficient mice (DeLorey et al., 1998; Mostofsky et al., 1998). We thus undertook a morphological analysis of the cerebellar lobules of adult wild-type and *Fmr1* knockout mice (Figure 11A). Measurements taken from cerebellar sagittal sections in revealed that the total circumference and area of the cerebellum were not significantly different between the two genotypes in the vermis or in the intermediate cortex (Table 3). The length of each of the cerebellar lobules in the vermis and intermediate cortex were was not statistically significant between wild-type and *Fmr1* knockout mice (Figure 11B). Additionally, when the lobules were normalized to the circumference of the whole cerebellum to account for variations in brain size, no significant difference in lobular length/circumference ratio was found between wild-type and knockout mice in both regions measured (Figure 11C). The cerebellar cortex exhibits normal layering in *Fmr1* knockout mice, as the thickness of both the granule and molecular layers measured were comparable to wild-type mice (Figure 11D and E). Overall, the analysis suggests that there are no gross morphological abnormalities to the cerebellar cortex in *Fmr1* knockout mice.

Table 3 – Cerebellar area and circumference in wild-type and <i>Fmr1</i> KO mice		
	Vermis	Intermediate Cortex
Section area (mm²)		
Wild-type	30.88 ± 1.25 (n = 4)	29.59 ± 1.80 (n = 4)
<i>Fmr1</i> KO	30.17 ± 0.80 (n = 4)	27.10 ± 1.12 (n = 4)
P value (t-test)	0.65, n.s.	0.33, n.s.
Circumference (mm)		
Wild-type	10.90 ± 0.17 (n = 4)	10.61 ± 0.40 (n = 4)
<i>Fmr1</i> KO	10.61 ± 0.13 (n = 4)	10.14 ± 0.17 (n = 4)
P value	0.23, n.s.	0.29, n.s.

Quantitative morphometric analyses of the DCN with serial cerebellar sections have been previously described in *pcd* and *Lurcher* mutant mice (Triarhou et al., 1987; Heckroth, 1994). In light of the finding that the interpositus and fastigial nuclei in the *Fmr1* knockout cerebellum are significantly reduced (Ellegood et al., 2010), we determined if we could detect volumetric changes in these nuclei using a histological approach (Figure 12A). Volumetric measurements of the individual subdivisions were performed separately in the left and right DCN. The average combined volume of the left and right nuclei in wild-type and knockout mice revealed that the interpositus nucleus (composed of both the anterior and posterior regions) is the largest component. The fastigial nucleus was smaller than the interpositus, while dentate nucleus was the smallest subdivision. There were no significant differences in the volume of any of the nuclei between the left and right hemispheres (data not shown). Volumetric comparisons between wild-type and *Fmr1* knockout DCN revealed no significant differences in the interpositus (WT: $0.24 \pm 0.03 \text{ mm}^3$ vs. KO: $0.26 \pm 0.01 \text{ mm}^3$), fastigial (WT: $0.15 \pm 0.006 \text{ mm}^3$ vs. KO: $0.17 \pm 0.014 \text{ mm}^3$) and dentate (WT: $0.09 \pm 0.03 \text{ mm}^3$ vs. KO: $0.10 \pm 0.005 \text{ mm}^3$) nuclei (Figure 12B). An estimate of neuronal density was calculated in the right hemisphere DCN by measuring the average grey intensity of each subdivision. This mean grey value represented an approximation of the number of neurons in each region of the DCN. Intensity comparisons

between wild-type and *Fmr1* knockout DCN revealed no significant differences of the the mean grey value in the interpositus (WT: $214.11 \pm 8.70 \text{ mm}^3$ vs. KO: $217.21 \pm 3.56 \text{ mm}^3$), fastigial (WT: $219.87 \pm 8.70 \text{ mm}^3$ vs. KO: 224.74 ± 0.94) and dentate (WT: $223.87 \pm 6.44 \text{ mm}^3$ vs. KO: $231.76 \pm 0.60 \text{ mm}^3$) nuclei (Figure 12C). Our results show that a difference cannot be detected between wild-type and *Fmr1* knockout mice in the volume or neuronal density of the DCN using this histological method.

GABAergic protein expression in the developing *Fmr1* knockout mouse cerebellum

We sought to determine if the alteration to GABAergic protein expression that we found in the forebrain were also reflected in the cerebellum. Some studies on *Fmr1* knockout mice did not reveal any changes in mRNA or protein expression in the cerebellum (El Idrissi et al., 2005; D'Hulst et al., 2006), while another study showed a reduction in mRNA expression of the 67 kDa isoform of glutamic acid (GAD67), SSADH, and GABA transporters 1 and 4 (GAT-1, GAT-4; D'Hulst et al., 2009). In my study, quantitative western blot analysis of samples from mouse cerebellum revealed that the $\alpha 1$ subunit, like the forebrain, was significantly down-regulated in PND 5 (Figure 13; $53.8 \pm 7.1\%$, $P < 0.05$), but not in PND 12 ($97.0 \pm 14.0\%$, $P > 0.05$) or adult *Fmr1* knockout mice ($77.0 \pm 3.8\%$, $P > 0.05$). Analysis of the GABA_A β 2/3 subunits, which

Figure 11. Quantitative morphometric analyses of the cerebellar cortex in wild-type and *Fmr1* knockout mice. A) Coronal serial sections (50 μm) in the vermis and intermediate cortex were collected on gelatinized slides and stained with cresyl violet. Every third section was selected for measurement (interval thickness of 150 μm). Lobular length (B), lobular length as a ratio of cerebellar circumference (C), molecular layer thickness (D), and granule layer thickness (E) were measured in lobules II, III, VI/VIII, and VIII in wild-type (N =4) and *Fmr1* knockout mice (N = 4) using SimplePCI software. There were no significant differences lobular length, cerebellar circumference or granule and molecular layer thickness. Statistical comparisons were made using a student's t-test.

Figure 11

Figure 12. Comparative morphometric analysis of the DCN in cresyl violet stained sections of wild-type and *Fmr1* knockout mice. A quantitative morphometric analysis of the DCN in wild-type (N = 4) and *Fmr1* knockout mice (N = 4) was performed in cerebellar. A) Coronal serial sections (50 μ m) were collected on gelatinized slides and stained with cresyl violet. Every other section was selected for measurement (interval thickness of 100 μ m). B) The area measurements for the dentate, interpositus, and fastigial nuclei from both hemispheres were analyzed with SimplePCI software (Hamamatsu). The volume was calculated by adding the sum of the area of each section multiplied by the interval thickness. There were no significant differences in volumes between wild-type and knockouts. C) Measurements of the average intensity of the right DCN as a measure of neuronal density was made using ImageJ software (NIH). The mean grey value of the DCN gives the sum of the intensity of each pixel divided by the number of pixels in the area selected. There were no significant differences in intensity between wild-type and knockouts.

Figure 13 . Developmental expression of GABAergic proteins in *Fmr1* knockout mice cerebellum. Summary of western blot analyses of GABAergic proteins expression in the whole cerebellum. The α 1 subunit was significantly down-regulated in postnatal day 5 knockouts relative to wild-type mice ($53.8 \pm 7.1\%$, $P < 0.05$), but not at day 12 or in adult. GABA-T and SSADH were not changed in the cerebellum at day 12 and GAD65 was not changed in adult knockout mice. For each analysis, 4 wild-type and 4 *Fmr1* cerebella were examined at each time point (* $P < 0.05$).

were decreased in adult and PND 12 forebrain, revealed no significant change in expression in the adult cerebellum ($101.0 \pm 12.2\%$, $P > 0.05$). GABA-T expression at PND 12, which was significantly increased in *Fmr1* knockout forebrain, was not significantly changed in the cerebellum GABA-T of knockout mice ($115.8 \pm 8.3\%$; $P > 0.05$). SSADH, which was also found at day 12 to be decreased in the *Fmr1* knockout forebrain, was near wild-type levels in the cerebellum ($84.3 \pm 2.3\%$, $P > 0.05$). Finally, the expression of GAD65, which was up-regulated in adult knockout forebrain, was not statistically different from wild-type adult cerebellum (83.7

$\pm 5.0 \%$, $P > 0.05$). Overall, the results show that the expression of GABAergic proteins we examined that were altered in the forebrain were not reflected similarly in the cerebellum.

Micro-dissection and quantitative western blotting of the DCN in *Fmr1* knockout mice

Morphological changes to the DCN in *Fmr1* knockout mice may be the result of altered GABAergic and/or glutamatergic protein expression and function. Thus, we compared the expression of several proteins in the DCN from micro-dissected wild-type and knockout cerebellar sections (Figure 14A). We performed an initial screen using quantitative western blotting on one set of mice ($N = 4$ WT + 4 KO) to detect any differentially expressed proteins. The expression of several proteins were found to have near wild-type level expression in *Fmr1* knockout mice (GABA_A receptor $\alpha 1$, GABA_{B-R2}, NMDA receptor 1, SSADH, VGLUT1) and were not examined in additional mice (Figure 14B, D). However, in our first set of wild-type and knockout mice, we found a significant increase in GABA-T expression (Figure 14B; $178.8 \pm 28.8\%$, $P < 0.05$). We examined the reproducibility of the expression changes in GABA-T, as well other proteins that showed potential differences in expression (GABA_A receptor $\beta 2/3$, VGAT, VGLUT2, GAD65, GluR2/3, calbindin and NeuN) in additional sets of mice. However, after examining three additional independent sets of wild-type and *Fmr1* knockout mice, we did not detect a significant difference in the expression of the proteins in wild-type and *Fmr1* knockout DCN (Figure 14B, C, D).

The effects of GABAergic potentiating drugs on seizures in *Fmr1* knockout mice

Fmr1 knockout mice are susceptible to audiogenic seizures (Musumeci et al., 2000; Dolen et al., 2007; Musumeci et al., 2007). We tested the anticonvulsant effects of drugs that potentiate GABA_A receptor activity in *Fmr1* knockout mice. Diazepam and phenobarbital both

Figure 14. Micro-dissection and quantitative western blotting of the DCN in *Fmr1* knockout mice. A) The cerebellum was dissected from 30 day old wild-type and *Fmr1* knockout mice and sectioned coronally (1.5 mm thickness) with a Vibratome series 1000 tissue sectioning system. The regions surrounding the DCN, which includes the cerebellar cortex, were excised and only the region encompassing the DCN was retained (shown under the red rectangle). Quantitative western blot analysis was performed micro-dissected wild-type and knockout DCN for protein expression of B) GABAergic and glutamatergic receptors, C) GABA enzymes and GABA/glutamate transporters, D) cell markers. There was a significant increase in the expression of GABA-T in the first batch of mice ($P < 0.05$), however this was not reproduced in subsequent sets of mice (batches 2-4). Batches 1, 3 and 4 had a sample size of $N = 4$ WT + 4 KO; $N = 3$ WT + 3 KO for batch 2.

Figure 15. The effects of diazepam, phenobarbital and loreclezole on seizures in *Fmr1* knockout mice. Mice were tested for audiogenic seizures after treatment with vehicle (N = 21), diazepam (N = 9; 1.5 mg/kg), phenobarbital (N = 7; 15mg/kg), or loreclezole (N = 8; 25 mg/kg). All three drugs significantly reduced audiogenic seizure incidence in *Fmr1* knockout mice as compared to vehicle controls * P < 0.05.

bind to the α subunits while loreclezole is a $\beta 2/3$ -specific GABA_A receptor agonist (Groves et al., 2006). Female *Fmr1* knockout mice were exposed to a 135 dB alarm for 2 minutes and seizure susceptibility was evaluated as previously described (Pacey et al., 2009). In total, 47% of *Fmr1* knockout mice treated with vehicle exhibited sound-induced seizures. No seizure activity was observed in knockout mice treated with diazepam (1.5 mg/kg, p < 0.05), phenobarbital (15 mg/kg, p < 0.05) or loreclezole (Figure 15; 25 mg/kg, p < 0.05). These results demonstrate that, at the doses tested, these three drugs that target the GABA_A receptor still possess potent anti-convulsant activity in *Fmr1* knockout mice.

The effects of diazepam and loreclezole on motor activity in *Fmr1* knockout mice

To determine if the down-regulation of $\alpha 1/\beta 2$ subunits altered the sedative effect of GABA_A agonists in *Fmr1* knockout mice, spontaneous locomotor activity was measured over 90 minutes following administration of diazepam (1 mg/kg) or loreclezole (30 mg/kg) in adult (6 week old) mice (Figure 16). The doses chosen for diazepam and loreclezole in this study were examined previously in $\alpha 1$ and $\beta 2$ knockout mice, respectively (Kralic et al., 2002; Groves et al., 2006). Three parameters were analyzed: total activity (number of beam breaks in both horizontal and vertical plane), distance travelled (in meters), and total rearing activity. Analysis by two-way ANOVA showed a significant main effect of drug treatment on all three parameters, with diazepam and loreclezole significantly reducing distance travelled, total activity, and total rearing as compared to vehicle controls ($P < 0.05$ to $P < 0.001$). In vehicle-treated mice the *Fmr1* knockouts were not significantly more active than wild-type mice (two-way ANOVA with Bonferonni post-hoc analysis, $P = 0.091$).

For diazepam, no significant genotype x treatment interaction was observed for any of the three activity parameters measured. Diazepam reduced total activity by 38% and 27% in wild-type and *Fmr1* knockout mice respectively (Figure 16A). Distance traveled was reduced by 24% in wild-types and 14% in knockouts, while total rearing decreased by 68% and 52% respectively (Figures 16B and C). For loreclezole, wild-type mice displayed a 71% reduction in total activity while activity in *Fmr1* knockout mice was reduced by only 42% (Figure 16A, genotype X treatment interaction, $P = 0.057$). In the distance travelled parameter, wild-type mice showed a 72% reduction in distance travelled while knockouts were reduced by 39% (Figure 16B, genotype X treatment interaction, $P = 0.078$). A significant genotype x treatment interaction was

Figure 16. The effects of diazepam and loreclezole on locomotor activity. Spontaneous locomotor activity was measured over 90 min. following i.p. administration of diazepam (1 mg/kg) or loreclezole (30 mg/kg). Drug-treated wild-type and *Fmr1* mice were assessed for total activity (A), distance travelled in meters (B), and total rearing (C). In the loreclezole treated mice there was a significant genotype x treatment interaction in total rearing (panel C; * $P < 0.05$) and a strong trend towards significance for both distance travelled (panel B, $P = 0.078$) and total activity (panel A, $P = 0.057$), indicating that *Fmr1* knockout mice were less susceptible to the sedative effects of loreclezole compared to wild-type controls (N = 9-11 for each group).

observed for total rearing in the loreclezole-treated animals. Loreclezole reduced total rearing activity by 85% in wild-type but only by 48% in *Fmr1* knockout mice (Figure 16C, $P < 0.05$). These findings demonstrate that *Fmr1* knockout mice are significantly less sensitive to the sedative effects of the $\beta 2/3$ agonist loreclezole, but show a similar response to diazepam when compared to wild-type controls.

The effect of phenobarbital and etomidate on wild-type and *Fmr1* knockout mice sleep times

The hypnotic effects of phenobarbital and the GABA_A receptor $\beta 2/3$ specific agonist etomidate (Reynolds et al., 2003) was assessed in wild-type and *Fmr1* knockout mice, in light of our findings of GABAergic protein dysregulation in the forebrain (Figures 5-8). Mice were examined for the duration of sleep after i.p. administration of phenobarbital (100 mg/kg) or etomidate (15 mg/kg). The duration of phenobarbital-induced loss of righting reflex was 232% longer in *Fmr1* knockout than in wild-type mice (Figure 17; KO: 32.5 ± 13.6 vs. WT: 14 ± 1.5 min), with this difference approaching significance ($P = 0.07$). In contrast, etomidate-induced loss of righting reflex was 35% shorter in knockout mice, but did not reach statistical significance (Figure 17; KO: 44.2 ± 8.8 vs WT: 70.0 ± 19.2 min, $P > 0.05$). One wild-type mouse injected with phenobarbital did not regain consciousness after 6 hours and one knockout mouse injected with etomidate did not lose its righting reflex; both were excluded from the analysis. The results suggest that *Fmr1* knockout mice were more sensitive to the hypnotic effects of phenobarbital, but less potentially sensitive to the GABA_A $\beta 2/3$ specific drug etomidate. Because of the shortage of mice of the correct age (3-6 months old) at the time of this analysis, only a small number of mice were studied in the sleep time test.

Figure 17. The effects of phenobarbital and etomidate on sleep times in wild-type and *Fmr1* knockout mice. The hypnotic effects of phenobarbital (100 mg/kg) in adult wild-type (N = 3) and *Fmr1* knockout mice (N = 4), and etomidate (15mg/kg) on a separate group of wild-type (N = 4) and *Fmr1* knockout mice (N = 3) were assessed by measuring the duration of sleep time as defined by the loss of the righting reflex. One WT mouse injected with phenobarbital failed to regain the righting reflex within 6 hours and one etomidate injected *Fmr1* mouse failed to lose the righting reflex; both mice were excluded from the analysis. *Fmr1* knockout mice injected with phenobarbital experienced longer sleep times compared with wild-type littermates that approached statistical significance (P = 0.073, Student's t-test), while etomidate injected *Fmr1* mice slept less than WT mice, although this was not statistically significant. Data represent the mean $\pm$ SEM.

Discussion

GABAergic protein expression and neuronal development in the forebrain

A key finding was that the level of GABA_A α 1 subunit expression in the forebrain was significantly reduced at PND 5 and 12 but then increased to near normal levels in adults in the *Fmr1* mouse (although not significantly different, the expression was approximately 80% of the level seen in adult wild-type animals), and that the GABA_A β 2 subunit was normal at day 5 but became significantly depressed at day 12 and in adults. In the mammalian brain, a developmental switch from GABAergic excitation to inhibition occurs early in the postnatal period. In rodents, GABA activation of GABA_A receptors causes excitation up to the first 6-10 days of postnatal life and then abruptly switches to inhibition due to changes in extracellular and intracellular chloride ion concentrations (Ben-Ari, 2002). This selective down-regulation of α 1 and β 2 subunits in *Fmr1* knockout mice may cause a reduction in excitatory activity over the first two postnatal weeks. GABAergic synaptic activity during this critical excitatory period is thought to play a vital role in the maturation of functional inhibitory synapses, cell proliferation and cell death (Gubellini et al., 2001; Cancedda et al., 2007).

As GABAergic signalling is vital for neuronal development, it is conceivable that postnatal alterations in GABA receptor expression and function could lead to structural alterations at synapses, for example, the characteristic presence of immature dendritic spines observed in both FXS patients and *Fmr1* knockout mice (Weiler and Greenough, 1999; Irwin et al., 2001; de Vrij et al., 2008). Thus, the potential disruption in synaptic transmission within the first two weeks after birth might induce long-lasting deleterious effects which could contribute to the aberrant cognitive profile in FXS. Of interest is the observation that *Fmr1* knockouts are

similar to mice lacking the GABA_A α 1 subunit in that the latter mice also display dendritic spines that fail to fully mature (Heinen et al., 2003). This prompts the intriguing question as to whether deficient GABA receptor expression during maturation of the CNS might be linked to the spine abnormalities seen in FXS. Normally in development, an increase in α 1 subunit expression is paired with a decrease in α 2 subunits after the first few weeks of postnatal development. This switch from α 2 to α 1 subunit expression coincides with the formation of synapses and is associated with the beginning of synaptic inhibition (Hornung and Fritschy, 1996). Therefore, the α 1 down-regulation we observed in *Fmr1* knockout mice (during the excitatory period) may lead to a disruption of the developmental switch in excitation/inhibition and synapse formation. Although the expression of the GABA_A α 1 subunit was not significantly decreased in the adult *Fmr1* mouse brain (approximately 80% of the level seen in adult wild-type animals), the potential disruption in synaptic transmission early in development might induce long-lasting deleterious effects which could contribute to the aberrant cognitive profile in FXS.

The reduction in β 2 subunit expression in knockout forebrain is possibly related to the early alterations in α 1 expression. The α 1 β 2 γ 2 subtype is the most abundant GABA_A receptor in the mature CNS, comprising roughly 50% of all GABA_A receptors (Mohler, 2007). Unlike the α 1 subunit, expression of β 2 is relatively high in the early postnatal period of wild-type mice (Hornung and Fritschy, 1996). Thus, we may normally expect to see an increase in the association of β 2 subunits with α 1 subunits as the expression of α 1 rapidly increases in the first few weeks of postnatal life. It is plausible that the observed down-regulation of the β 2 subunit in *Fmr1* knockout mice was a consequence of the initial decrease in α 1 subunit expression at PND 5, resulting in a lack of the ability to form α 1 β 2 γ 2 GABA_A receptor complexes.

The down-regulation of the two GABA metabolizing enzymes (GABA-T and SSADH) and the up-regulation of the GABA synthetic enzyme (GAD65) could induce a reduction in GABA breakdown and an increase in GABA synthesis respectively. This dual effect on GABA enzyme levels is consistent with a previous report indicating that the concentration of GABA is increased in *Fmr1* knockout mice (Gruss and Braun, 2004). The down-regulation of GABA-T and SSADH may serve to counterbalance the decreased levels of $\alpha 1$ and $\beta 2$ by increasing the amount of GABA available in early development in an attempt to increase the excitation of immature neurons. Similarly, GAD65 over-expression in adult *Fmr1* knockout mice may be the result of cellular compensation for decreased GABA receptor-mediated inhibition. The results from a study demonstrating an increase in the frequency of GABAergic spontaneous and miniature inhibitory postsynaptic currents in acute brain slices from the striatum of adult *Fmr1* knockout mice were interpreted as being indicative of increased GABA release at nerve terminals (Centonze et al., 2008). These effects might be a consequence of altered GABAergic enzyme expression and the accompanying down-regulation of the $\alpha 1$ and $\beta 2$ subunits.

There were discrepancies between the protein and mRNA expression of the $\alpha 4$, $\beta 1$ and $\gamma 2$ subunits in *Fmr1* knockout mice. D'Hulst et al. (2006) showed that mRNA levels of $\alpha 4$, $\beta 1$ and $\gamma 2$ were significantly reduced in the cortex of adult *Fmr1* knockout mice, while we did not find these changes at the protein level. However, we examined the whole forebrain which perhaps is better suited for detecting global changes as opposed to specific changes to subregions of the forebrain. Furthermore, Curia et al. (2008) found a significant decrease in the expression of the $\alpha 5$ subunit in *Fmr1* knockout mice subiculum that was not detected in mRNA analyses of the cortex. Analyzing the protein expression of individual structures within the forebrain can be useful in determining if mRNA expression studies coincide with protein expression findings for

the $\alpha 4$, $\beta 1$ and $\gamma 2$ subunits. Alternatively, it is possible that there was simply an excess of $\alpha 4$, $\beta 1$ and $\gamma 2$ mRNA transcripts that were not needed to maintain wild-type level protein expression in *Fmr1* knockout mice. The expression and distribution of mRNA for GABA_A receptor subunits have shown to not always correspond with the protein (Fritschy and Mohler, 1995).

We did not see changes in GABA_B-R1 nor GABA_B-R2 protein expression in the forebrain of *Fmr1* knockout mice. Only a few studies to date have investigated GABA_B signalling in *Fmr1* knockout mice (Zupan and Toth, 2008; Pacey et al., 2009). Zupan and Toth (2008) showed that *Fmr1* knockout mice are more sensitive to the sedative effects of GABA_B agonist baclofen, while our laboratory has previously showed that audiogenic seizures in *Fmr1* knockouts can be rescued with baclofen (Pacey et al., 2009). Our results suggest that increased sensitivity of GABA_B receptors in *Fmr1* knockout mice was likely not related to differences in GABA_B receptor protein expression in the forebrain.

We note that the transient down-regulation of GABA-T and SSADH coincided with a transient under-expression of the GABA_A δ subunit; all three proteins were expressed at wild-type levels at PND 5 and again in adults, but were significantly under-expressed at PND 12. These were the only proteins that displayed this particular aberrant pattern of expression. Pentameric receptor complexes incorporating the δ subunit are known to possess high affinity for GABA and to be localized primarily extrasynaptically where they contribute to tonic inhibition (You and Dunn, 2007; Kaur et al., 2009). Moreover, the GABA_A δ subunit displays a high degree of plasticity triggered by changes in inhibitory tone (Shen and Smith, 2009). It is possible that a transient increase in brain GABA levels caused by abnormally low GABA-T and SSADH expression may induce a compensatory decrement in the δ subunit, suggesting that the δ subunit may be responsive to changes in extracellular GABA concentrations. It was recently shown that

GABA concentrations are reduced in the amygdala *Fmr1* knockout mice and that treatment with gaboxadol, an agonist of the high-affinity extrasynaptic GABA_A receptors, was able to rescue cellular hyperexcitability. Thus, activity of high-affinity GABA_A receptors in knockout mice in response to ambient GABA concentration may be reduced.

GABAergic signalling plays a prominent role in neurite outgrowth and synapse formation in the developing CNS. Chronic GABA_A receptor activation in early development is necessary for the switch from GABA excitation to inhibition in the brain (Ganguly et al., 2001) and in the retina (Leitch et al., 2005). Furthermore, GABA itself promotes neurite length and branching in inhibitory and excitatory cortical neurons from neonatal rats and embryonic chicks (Baloyannis et al., 1983; Spoerri, 1988; Maric et al., 2001). In *Fmr1* knockout mice, the dysregulation of GABA_A receptor and GABAergic enzyme expression may affect GABA-mediated activity during the critical period. Our results and findings reported by others (Gruss and Braun, 2004) suggest that GABA levels are elevated in the brains of *Fmr1* knockout mice. This potential increase in GABA levels may be an attempt to promote neurite development in the brain by compensating for the lack of GABAergic activity as a result from GABA_A receptor down-regulation in knockout mice. In support of this theory, GABA_A receptor antagonists (e.g. bicuculline) or agents that block GABA synthesis inhibit neuritic arborization of cortical neurons (Ben-Ari et al., 1994; Maric et al., 2001). Thus, a deficiency in GABA_A receptors may perturb the maturation of the inhibitory neural network, in spite of elevated GABA levels that encourage axonal and dendritic development. In summary, our findings lead us to speculate that the GABAergic alterations we observed in *Fmr1* knockout mice forebrain might cause a disruption in early neurodevelopment that could have permanent long-term developmental consequences in the CNS.

The cerebellum of *Fmr1* knockout mice

Our study of cerebellar abnormalities in *Fmr1* knockout mice began with a morphological analysis on the size, lobulation, and layering of the cerebellum. Cerebellar hypoplasia has been previously identified in FXS patients (Mostofsky et al., 1998) and in other neurodevelopmental disorders such as autism and Down's Syndrome (Shevell and Majnemer, 1996; Aylward et al., 1997; Baxter et al., 2000; Necchi et al., 2008). Additionally, DeLorey et al. (1998) observed significant hypoplasia of the cerebellar vermal lobules in GABA_A receptor $\beta 3$ knockout mice. We sought to independently confirm the changes found in the cerebellum of *Fmr1* knockout mice from MRI analysis (Ellegood et al., 2010) using a histological analysis of thin sections from the cerebellum.

Analysis of cresyl violet stained wild-type and *Fmr1* knockout cerebellar sections did not identify any gross differences in the size of the lobules relative to the whole cerebellum nor in the changes to the molecular and granule cell layers, consistent with our previous finding in which no gross neuroanatomical abnormalities were found in the cerebellar cortex of *Fmr1* knockout mice (Ellegood et al., 2010). However, our morphometric analysis of the DCN reported in this thesis did not coincide with our previous findings of volumetric decreases in the dentate and fastigial nuclei using MRI (Ellegood et al., 2010). There are several possibilities for explaining the discrepancies between the results. In *Lurcher* and *staggerer* mutant mice, where similar histological methods of DCN analysis was utilized, significant cerebellar atrophy observed in these mutants allowed for easier detection of volumetric reductions (Triarhou et al., 1987; Heckroth, 1994). As the MRI scan revealed more subtle volumetric decreases of the DCN

in *Fmr1* knockout mice, our morphometric analysis may have been incapable of detecting any changes.

Alternative explanations address the manner in which our analysis was performed and interpreted. Firstly, the boundaries of the DCN are not well defined and the morphology of each nuclei changes rapidly within the section (see Figure 14A). As a result, the definition of the boundaries and estimations of DCN volumes are open to an amount of interpretation. Secondly, cells that were split during sectioning can lead to errors in the estimation of volume and cell number, as cells can be duplicated among sections (Roffler-Tarlov and Turey, 1982). Therefore, it is possible the interpretation of the DCN boundaries taken together with the limitations of our method did not permit our analysis to detect a relatively subtle reduction in the *Fmr1* knockout DCN.

The expression profile of GABAergic proteins in the cerebellum in *Fmr1* knockout mice were markedly different from what was observed in the forebrain. The decreases in $\beta 2$ expression and the increase in GAD65 expression that was observed in adult *Fmr1* knockout forebrain were not present in the cerebellum. Additionally, $\alpha 1$, SSADH and GABA-T expression at PND 12 was not decreased compared to wild-type mice in the cerebellum of *Fmr1* mice, as was seen in the forebrain. The only difference between wild-type and knockout mice in terms of cerebellar expression was a reduction in the $\alpha 1$ subunit at PND 5. The relatively normal levels of $\alpha 1$ subunit and GAD65 expression in adult *Fmr1* knockout cerebellum did however coincide with previous mRNA expression analyses (D'Hulst et al., 2006; D'Hulst et al., 2009). It is intriguing that the first alteration in GABAergic expression that is observed in knockout mice, the down-regulation of $\alpha 1$ subunit, is mirrored in both the forebrain and cerebellum. Because Purkinje cells almost exclusively express $\alpha 1$ -containing GABA_A receptors (Fritschy et al., 2006),

the reduction of $\alpha 1$ at PND 5 may disrupt the maintenance of GABA synapses formed by stellate cells on Purkinje cell dendrites. The early down-regulation of the $\alpha 1$ subunit in the cerebellum, along with the down-regulation of GABA_A receptors in the forebrain may contribute to the behavioural phenotype of *Fmr1* knockout mice. However, the results are preliminary and a complete investigation of GABA_A receptor subunits and GABA metabolic enzymes, particularly at PND 5, is needed to fully elucidate the extent of GABAergic dysfunction in the cerebellum. Analysis of GABA_A receptor subunits predominately expressed in the cerebellum (e.g. $\alpha 4$, $\alpha 6$, δ) will also be performed.

We attempted in our study to micro-dissect the DCN from the cerebellum in wild-type and *Fmr1* knockout mice, and quantify GABAergic protein expression using immunoblot analysis. Our results showed a high amount of variability in the expression levels of all the proteins examined within the sets of mice. For instance, we observed a significant increase in the GABA-T expression in DCN mice in the first set of knockouts mice, which was not replicated in subsequent sets of mice (Figure 14D). Consequently, it is difficult to draw conclusions on GABAergic expression in the DCN from the results. It is likely that the amount of variability observed was related to the preciseness of the micro-dissection. The DCN is a non-uniform structure situated deep within the cerebellum that changes in shape from the anterior to posterior end (Figure 14A). Thus, dissecting the DCN from the anterior end does not necessarily correspond to the same region of dissection further into the cerebellum. That is, surrounding regions may be dissected with the DCN which possibly confounded the results of the analysis. One particular cell population in close vicinity to the DCN is the granule cell layer from the cerebellar cortex. Cerebellar granule neurons are the most abundant class of neurons in the central nervous system (Heckroth, 1994), while only 8,500 principal neurons are estimated in the

entire DCN (Heckroth, 1994). Thus, various amounts of granule cell contamination in each DCN sample may account for a significant proportion of the variability seen in protein expression levels. This effect can either lead to an over-representation or under-representation of the protein analyzed in each DCN sample. Further characterization of GABAergic expression in the DCN may be possible after the completion of western blot analysis protein in the whole cerebellum, which may serve to identify proteins that are dysregulated in *Fmr1* knockout mice.

Behavioural phenotype of *Fmr1* knockout mice

In light of our finding of $\alpha 1$ down-regulation in young mice and $\beta 2$ subunit under-expression in older mice, we evaluated the protective effect of anticonvulsants on audiogenic seizure susceptibility in *Fmr1* knockout mice. We hypothesized that the down-regulation of GABA_A receptor subunits might be reflected in changes in seizure suppression for drugs that target the receptor. However, at the doses tested, both diazepam and phenobarbital blocked audiogenic seizures in *Fmr1* knockout mice. Similarly, diazepam was as effective in knockout mice as in wild-type mice in reducing locomotor activity, as measured by the open field. Benzodiazepines such as diazepam bind indiscriminately to $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ containing receptor complexes (Fradley et al., 2007). Additionally, $\alpha 1$ protein expression was not measured at the age of audiogenic seizure testing (PND 26-30) making it unclear how the level of $\alpha 1$ compared with wild-type mice at this age. Overall, the ability of diazepam to interact with the other benzodiazepine-sensitive α subunits combined with potentially higher $\alpha 1$ expression at PND 26-30 may potentially account for the effectiveness of diazepam in the seizure suppression of *Fmr1* knockout mice.

Based on the known subunit specificity of loreclezole which targets the GABA_A β 2/3 subunits, we postulated that the progressive down-regulation of the β 2 subunit into adulthood in *Fmr1* knockout mice would be reflected in reduced sensitivity to the anticonvulsant and/or sedative effects of this positive allosteric modulator. A previous study demonstrated that mutant knock-in mice containing a loreclezole-insensitive β 2 subunit were significantly less protected from pentylenetetrazole-induced seizures when treated with loreclezole (Groves et al., 2006). However in our study, loreclezole was still effective in blocking audiogenic seizures in *Fmr1* knockout mice. Given that *Fmr1* knockout mice maintain some β 2 subunit expression and normal β 3 expression, it is possible that the dose of loreclezole used in this study (25 mg/kg) was sufficient to saturate the remaining GABA_A β 2/3-containing receptors in *Fmr1* knockout mice to provide seizure suppression. Lower doses of the drug might be required in order to observe differential effects of loreclezole on seizure activity in *Fmr1* knockout mice. Alternatively, it is plausible that increased susceptibility to audiogenic seizures in *Fmr1* knockout mice is relatively more dependent on alterations in GABA_B receptor function vs. GABA_A receptor function. We have previously shown that GABA_B receptor activation can strongly attenuate audiogenic seizures in *Fmr1* knockout mice (Pacey et al., 2009). Increased susceptibility to audiogenic seizures in knockout mice may depend more on changes in group I mGluRs (Giuffrida et al., 2005) and GABA_B receptor-mediated signalling than on GABA_A receptor activity.

In contrast to the results from the seizure experiments, we found that *Fmr1* knockout mice were less sensitive than wild-type mice to loreclezole-induced suppression of motor activity. This finding is similar to that reported for knock-in mice possessing a loreclezole-insensitive GABA_A β 2 receptor (Groves et al., 2006). As observed here in *Fmr1* knockout mice, the loreclezole-insensitive β 2 receptor mice showed less loreclezole-induced sedation, as

measured by spontaneous motor activity, compared to wild-type mice. In our present study, locomotor activity between vehicle-treated *Fmr1* knockout and wild-type mice were not significantly different. However, in other behavioural experiments conducted in our laboratory, and in studies reported by others (Bakker et al., 1994; Ventura et al., 2004), *Fmr1* knockout mice have shown significantly higher motor activity compared to control mice. The hyperactivity observed in these knockout mice is reminiscent of the well-established hyperactivity seen in FXS in humans (Hagerman et al., 2009). Interestingly, GABA_A receptor β 2 but not GABA_A receptor α 1 knockout mice display increased locomotor activity (Sur et al., 2001). Based on the observations of Sur et al., 2001 using β 2 knockout mice, and those of (Groves et al., 2006) with the loreclezole-insensitive GABA_A β 2 receptor mice, together with the data presented here, we suggest that hyperactivity in *Fmr1* knockout mice could be a consequence of the deficiency in β 2-containing GABA_A receptors. Several forebrain subregions including the hippocampus, thalamus, and hypothalamic nuclei are known to participate in the sedative/ hypnotic actions of GABAergic drugs (Sukhotinsky et al., 2007). The reduction of β 2 expression in *Fmr1* knockout mice forebrain in turn may reduce the ability of β 2-containing GABA_A receptors to mediate sedation.

In the sleep time tests, we observed that adult *Fmr1* knockouts displayed an increase in phenobarbital-induced sleep but a decrease in etomidate-induced sleep times. An increase in sleep times was previously observed in α 1 knockout mice treated with diazepam, which was attributed to the increased contribution of other α subunits (Kralic et al., 2002). It is possible that increased sleep times in phenobarbital-treated *Fmr1* knockout mice suggests an enhanced activity of other α subunits. Thompson et al. (1996) showed that the affinity and efficacy of barbiturates was significantly dependent on the α subunit, with α 1 subunit showing the lowest

potentiation to GABA out of the 6 α subunits. Furthermore, β 1-containing GABA_A receptors have higher affinity and efficacy for barbiturates than β 2-containing GABA_A receptors (Thompson et al., 1996). Therefore, the significant decrease of β 2 subunit expression in the forebrain combined with contribution from other non- α 1 subunits in *Fmr1* knockout mice may have resulted in increased sensitivity to barbiturates, resulting in a longer duration of sleep. Future analysis of the protein expression of the remaining GABA_A receptor α subunits in the forebrain may also be useful in determining if there is a compensatory up-regulation which would explain an increase in sleep times.

One caveat is if there was indeed a compensatory increase in the expression of remaining α subunits, we might have expected that the sedative effect of diazepam in the open field would be enhanced in *Fmr1* knockout mice. On the contrary, diazepam was just as effective in sedating knockout mice compared to wild type mice (see Figure 16). Another possible explanation is that a reduction in α 1 β 2 γ 2 receptors results in an increased availability of δ - and α 4-containing extrasynaptic GABA_A receptors, which have been shown to be more sensitive to the effects of GABA and also barbiturates (Wohlfarth et al., 2002; Wallner et al., 2003; Stell et al., 2003; Hemmings, Jr. et al., 2005). Biochemical studies have demonstrated that the α 4 and δ subunits are often present in the same receptor complex (Sur et al., 1999). Furthermore, α 4 β δ receptor expression has been shown to be very plastic in the CNS, with α 4 protein levels subject to rapid regulation after administration of GABAergic drugs (Birzniece et al., 2006; Liang et al., 2007). Thus, phenobarbital may bind a higher proportion of δ and α 4 subunit-containing GABA_A receptors in *Fmr1* knockout mice, enhancing tonic inhibition and resulting in longer sleep times.

The selectivity of etomidate (as well as loreclezole) is dependent on an asparagine residue at position 265 of the GABA_A receptor β 2 subunit (Wingrove et al., 1994). The results

from our sleep time study, together with the results from the motor activity analysis, lend credence the notion that a decrease in $\beta 2$ protein expression translates to a functional loss of the GABA_A receptor activity. Blednov et al. (2003) showed a significant decrease in etomidate-induced sleep times in $\beta 2$ knockout mice when compared to wild-type mice, although another study did not observe this effect (O'Meara et al., 2004). The reasons for the discrepancy between the studies are unclear but might be related to differences in the doses and route of administration and dose (40 mg/kg intraperitoneal vs. 5-5 mg/kg intravenous). Overall, our preliminary results hinting at differences in sleep times in *Fmr1* knockout mice treated with etomidate or phenobarbital are suggestive of altered GABA_A receptor activity due to reduced $\beta 2$ subunit expression. Additional sleep test experiments with larger sample sizes are required to test the validity of this proposal.

Conclusions and Clinical Significance

Our study in *Fmr1* knockout mice has demonstrated the down-regulation of the GABA_A receptor $\alpha 1$, $\beta 2$, and γ subunits at specific points in brain development. In addition, changes in the expression of the GABAergic enzymes GAD65, GABA-T and SSADH are suggestive of elevated brain GABA levels. The role of FMRP in the change of GABAergic protein expression might be explained by either a direct or indirect interaction of FMRP with the GABAergic system. In a direct interaction scenario, FMRP would regulate the translation and/or stability of mRNA transcripts that code for GABA_A receptor subunits or GABA metabolic enzymes. In the absence of FMRP, the loss of stability/translation of GABAergic mRNA would result in reduced levels of protein expression. To date only the δ subunit has been shown to interact directly with FMRP; however the studies examined changes in global gene expression (Miyashiro et al., 2003; Gantois et al., 2006; Dichtenberg et al., 2008). Future studies that probe for specific FMRP-GABAergic mRNA complexes using RNA-protein co-immunoprecipitation techniques (Peritz et al., 2006) could be useful in testing the validity of this theory.

Alternatively, FMRP may have a neurodevelopmental role in the establishment of functional GABAergic synapses. Electrophysiology studies have previously showed that *Fmr1* knockout neurons are less successful than wild-type neurons in forming functional excitatory synapses (Hanson and Madison, 2007). It has recently been speculated that activity-dependent local protein synthesis necessary for synaptic plasticity may also occur proximal to GABAergic synapses (Mackie and Katona, 2009). Therefore, it will be of interest to determine if another role of FMRP involves the transport and regulation of mRNAs or proteins important for synaptic plasticity at GABAergic synapses.

Novel pharmacological interventions are emerging for the treatment of FXS. Currently, several mGluR5 antagonists such as fenobam and STX107 and others are being tested in clinical trials of FXS (Cornish et al., 2008; D'Hulst and Kooy, 2009). Drugs such as stimulants used to treat ADHD or phenobarbital used for treating seizures can often exacerbate behavioural problems and cause undesirable side effects for FXS patients (Hagerman et al., 2009), increasing the need for targeted therapeutics. Although several studies have identified the GABA_A receptor as a possible therapeutic target for FXS, there has been very little discussion of how precisely to approach treatment that would be beneficial to patients. One possibility for treating FXS is with the use of GABA agonists specific to the $\alpha 1$ and $\beta 2$ subunit, although it is unclear whether or not targeting the already down-regulated GABA_A receptor subunits would result in increased GABAergic function. Our study suggests that GABAergic drugs which target $\alpha 1$ and $\beta 2$ subunits may actually be less effective in patients with FXS compared to non-FXS patients, if our findings extend to humans. Alternatively, allosteric modulators that bind GABA_A receptors subunits shown to be unaltered in FXS (e.g. $\alpha 4$, $\beta 1$, $\beta 3$) may result in better treatment in this syndrome. Indeed, ganaxolone, a GABA_A receptor agonist that preferentially binds to $\alpha 2$ and $\alpha 3$ subunit-containing receptors (Carter et al., 1997) has been approved for clinical trials in FXS patients (Cornish et al., 2008; D'Hulst and Kooy, 2009). New insights into the role of GABA in early brain development may generate the design of specific GABAergic drugs for use in FXS and other neurodevelopmental disorders in which GABAergic dysfunction has been implicated, such as autism spectrum disorders, Rett and Angelman's syndrome.

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