SLOW GABAERGIC TRANSMISSION DEFICITS IN THE BASOLATERAL AMYGDALA IN A MOUSE MODEL OF FRAGILE-X SYNDROME

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ABSTRACT

Fragile-X Syndrome (FXS), caused by transcriptional silencing of the FMR1 gene, is the leading cause of inherited intellectual disability. Comorbidities of FXS such as autism are typically linked to excitatory/inhibitory (E/I) imbalance and amygdala dysfunction yet the role of the amygdala in FXS is poorly understood. Principal excitatory neurons (PNs) of the basolateral amygdala (BLA) in the Fmr1 knockout (KO) mouse model of FXS display a hyperexcitable phenotype (decreased action potential threshold) that may partially underlie amygdala dysfunction in FXS, but the etiology of this phenomenon remains unknown. Tonic GABA\(_A\) mediated transmission can powerfully affect cell and network excitability and regulate synaptic plasticity so could therefore underlie hyperexcitability in the Fmr1 KO BLA and/or provide a potentially relevant therapeutic target to rescue that hyperexcitability. Accordingly, we designed whole-cell patch clamp studies to test the hypothesis that tonic transmission is deficient in the Fmr1 KO BLA and as such, likely participates in the maintenance of PN excitability and synaptic E/I balance. We show that tonic transmission is comprised of both \(\delta\)- and \(\alpha_5\)-subunit containing GABA\(_A\) receptors and is reduced in Fmr1 KO PNs. Furthermore, the \(\delta\)-specific agonist THIP rescues the decreased firing threshold observed in Fmr1 KO PNs. Moreover in both genotypes, \(\alpha_5\)-subunit blockade enhances the amplitude and kinetics of relatively distal synaptic events indicating that these receptors preferentially modulate dendritic compartments of these cells. Examination of synaptic balance with evoked synaptic conductance experiments
reveal altered feedforward inhibition in *Fmr* KO PNs that reflects a change in E/I balance related to altered excitatory response kinetics and is consistent with sharpened neuronal tuning. This state is replicated in WT PNs by $\alpha_5$-subunit blockade. Therefore our data suggest that deficient tonic GABA$_A$ transmission in the FXS BLA contributes to cellular hyperexcitability and synaptic E/I imbalance in PNs, and may represent a therapeutic target for treatment of amygdala-based symptoms in FXS.
I dedicate this dissertation to my family and friends without whom this research would not have been possible. I’d like to especially thank my parents, Rick and Shirley Martin, for their unwavering emotional support and encouragement during my graduate training and throughout my life. To my mentor, Dr. Molly Huntsman, many thanks for the steadfast scientific enthusiasm, advice, and support. Special thanks go to my committee members for their guidance as well. Finally, I’d like to thank my fellow students and postdocs associated with the Interdisciplinary Program in Neuroscience (IPN) for their undying friendship and support.

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CHAPTER I: INTRODUCTION

A. FRAGILE-X SYNDROME

FXS is an X-linked, single gene disorder caused by dysfunction in the transcription of the $fmr1$ gene found on the Xq27.3 site on the human X chromosome that codes for fragile-X mental retardation protein (FMRP) (Fu et al., 1991; Verkerk et al., 1991). The syndrome results from an irregular expansion of CGG repeats in the 5’ untranslated region of the $FMR1$ gene which normally consists of ~55 repeats or less. Although not yet conclusively determined for the $FMR1$ gene, this repeat may expand because of DNA slippage during DNA replication associated with the instability of the CGG repeat in which the daughter DNA strand acquires extra repeat nucleotides. Additional trinucleotide repeats induce a nondeleterious frame shift that further destabilizes the region (Petruska et al., 1998). A moderate increase in CGG repeats at this locus (~45-200 base pairs) leads to a “premutation” phenotype resulting in distinctive clinical features such as primary ovarian insufficiency and fragile x associated tremor/ataxia syndrome or FXTAS (Hagerman et al. 2009; Kenneson et al. 2001). This condition occurs in a proportionally larger population of individuals than does the full syndrome (1 in 130-250 females and 1 in 250-800 males) (Hagerman et al. 2009). Greater than ~200 repeats of this trinucleotide sequence (~1 in 3600 individuals) promotes hypermethylation and chromatin condensation upstream of the coding region causing transcriptional silencing of $FMR1$ and a subsequent lack of expression of its protein product FMRP (Fu et al., 1991). In FXS patients, the loss of FMRP results in a variety of neurological symptoms widely associated with imbalances in excitation/inhibition and dysfunctional plasticity in critical brain regions such as the cortex, hippocampus, and amygdala. These symptoms include mild to severe intellectual disability, social anxiety and autistic behaviors, increased incidence of epilepsy, attention-deficit
hyperactivity disorder (ADHD), and sensory hypersensitivity (Miller et al., 1999; Musumeci et al., 1999; Berry-Kravis, 2002; Hagerman and Stafstrom, 2009; Hagerman et al., 2009; Berry-Kravis et al., 2010).

For clarity, excitation/inhibition (E/I) imbalances in neuronal networks can be defined in various ways including as gross imbalances in basal excitatory and inhibitory transmission as seen in the FXS amygdala for example (see below) (Olmos-Serrano et al., 2010; Martin et al., 2012), or as imbalances in specific circuits within a network as seen in the FXS somatosensory cortex in which there is decreased excitatory drive onto specific inhibitory neuron subtypes (Gibson et al., 2008). In the context of discussion within this dissertation, “E/I imbalance” refers broadly to all of these types of network or circuit changes as common hallmarks of neurodevelopmental and neurological disorders regardless of whether the E/I balance is tipped toward excitation or inhibition. We discuss specific examples of these types of imbalances in FXS and related disorders throughout and elaborate further on their affects on overall network function.

**B. FMRP FUNCTION**

FMRP is expressed in a variety of mammalian tissues but is highly concentrated in the brain and testes (Devys et al., 1993; Feng et al., 1997; Tamanini et al., 1997; Wang et al., 2004; Olmos-Serrano et al., 2010). In the brain FMRP is located both pre- and postsynaptically and functions mainly as a translational regulator, especially at the synapse (Feng et al., 1997; Laggerbauer et al., 2001; Li et al., 2001; Huber et al., 2002; Antar et al., 2006; Christie et al., 2009). It is known to associate with a myriad of neuronal mRNA molecules and at least 8% of synaptically targeted mRNA (Brown et al., 2001; Darnell et al., 2001; 2005; Bassell and Warren,
Studies also show that FMRP contains both nuclear and cytosolic localization sequences, nuclear export sequences (Eberhart et al., 1996; Sittler et al., 1996), and can function in the nucleus as an mRNA chaperone, binding specific mRNA as part of a ribonucleoprotein (RNP) complex to transport it from the nucleus to the appropriate cytosolic location for protein translation (Fridell et al., 1996; Bagni and Greenough, 2005; Bassell and Warren, 2008). Evidence supporting this role for FMRP comes from studies that show that the protein has at least two common mRNA binding motifs, a hnRNP-K-homology (KH) domain (two subtypes), and the arginine-glycine-glycine domain (RGG box) (Bassell and Warren, 2008). These motifs are crucial for ribosomal interactions and interactions with guanine (G)-rich mRNA regions like the stem-G-quartet loop found in many mRNA cargoes of FMRP, respectively. These cargoes include those important for neuronal development such as MAP1b, a microtubule-associated protein important for axonal development, and the intercellular signaling molecule semaphorin 3F, as well as those crucial for synaptic function such as the SNARE-associated protein, Munc 13, which is involved in neurotransmitter release (Brown et al., 2001; Darnell et al., 2001; Miyashiro et al., 2003). FMRP dysfunction can result in up- or down-regulation of various mRNAs (Brown et al., 2001; Miyashiro et al., 2003) or proteins (Liao et al., 2008; Schütt et al., 2009) consistent with a role as a repressor or promotor of protein translation. Furthermore, evidence reveals activity-dependent shuttling of mRNAs by FMRP to dendritic compartments (Antar et al., 2004; Dictenberg et al., 2008).

C. THE FMR1 KO MOUSE MODEL OF FXS

Shortly after the fmr1 gene and its protein product were identified as the source of the disorder (Fu et al., 1991; Verkerk et al., 1991), an Fmr1 KO mouse model of the disease was
generated to study the consequences of the loss of FMRP in FXS neuronal networks (The Dutch-Belgian Fragile X Consortium, 1994). These mice express no FMRP and display phenotypes consistent with the symptoms of FXS in humans including abnormalities with learning and memory, social interaction, hyperactivity, hypersensitivity, and susceptibility to seizures (The Dutch-Belgian Fragile X Consortium, 1994; Kooy et al., 1996; Paradee et al., 1999; Chen and Toth, 2001; Zhao et al., 2005; Dölen et al., 2007; Hayashi et al., 2007; Musumeci et al., 2007; Bassell and Warren, 2008; McNaughton et al., 2008; Paylor et al., 2008; Moy et al., 2009). Importantly, and of particular relevance to autism-like abnormalities such as social avoidance and repetitive behaviors, Fmr1 KO mice phenotypes show particular dependence on background strain consistent with the existence of modifiers genes that can enhance or diminish the effects of FMRP loss (Moy et al., 2009).

D. FMRP, THE SYNAPSE, AND THE MGLUR THEORY OF FXS

Early studies of FMRP indicated that the protein was highly expressed in the dendritic shafts and spines of neurons (Weiler et al., 1997; Antar et al., 2005; 2006; Schütt et al., 2009). Interestingly Fmr1 KO mice have a higher density and higher proportion of elongated dendritic spines in hippocampus and cortex similar to humans with FXS (Hinton et al., 1991; Comery et al., 1997; Irwin et al., 2000; Mckinney et al., 2005). These collective observations led researchers to hypothesize that FXS might primarily be a synaptic plasticity disorder (Huber et al., 2002; Martin and Huntsman, 2012). Synaptic plasticity is commonly associated with functional changes of pre- and postsynaptic neuronal elements such as synaptic vesicle release probability and postsynaptic receptor kinetics following patterned activity that discretely strengthen (potentiation) or weaken (depression) synapses. FMRP was first connected to
synaptic plasticity when researchers identified the protein as upregulated in response to the Group I metabotropic glutamate receptor (subtypes 1 and 5) (GpI mGluR) agonist 3,5-dihydroxyphenylglycine (DHPG) (Weiler et al., 1997). This compound induces GpI mGluR-dependent and protein translation-dependent long-term depression (LTD) in the CA1 region of the hippocampus. During GpI mGluR dependent LTD, ionotropic glutamate receptors, α-amino-3-hydroxy- 5-methyl-4-isoxazolepropionic acid (AMPARs), undergo internalization decreasing synaptic strength in response to low frequency stimulation or DHPG (Huber, 2000). This process is restrained by GpI mGluR-induced, FMRP-dependent shuttling of mRNAs to dendritic spines in response to activity (Dictenberg et al., 2008). Soon after the discovery, Huber and colleagues showed the first evidence of pathological plasticity in FXS in the form of enhanced GpI mGluR-dependent LTD in CA1 slices from Fmr1 KO mice (Huber et al., 2002) that involves the failure of FMRP-dependent restraint of protein synthesis (Antar et al., 2004; Muddashetty et al., 2007). Because FMRP can function as a negative regulator of translation (Laggerbauer et al., 2001; Li et al., 2001) and is upregulated in response to mGluR activation (Weiler et al., 1997; Bear et al., 2004) “the mGluR theory of FXS” was proposed. According to the theory, AMPAR receptor internalization and synaptic destabilizing protein-dependent processes go unchecked in mice lacking functional FMRP. Unexpectedly though, the exaggerated LTD observed in hippocampal area CA1 in Fmr1 KO mice is not protein translation dependent (Nosyreva and Huber, 2006) consistent with basal increases in protein synthesis in the Fmr1 KO mouse. Therefore, mGluR dependent protein synthesis is dysregulated and often reduced in Fmr1 KO mice (Huber et al., 2002; Todd et al., 2003; Bear et al., 2004; Weiler et al., 2004; Muddashetty et al., 2007; Westmark and Malter, 2007) while basal levels of protein synthesis stay increased consistent with normally FMRP-regulated mRNAs free to chronically
associate with actively translating polyribosomes (Zalfa et al., 2003; Qin et al., 2005; Dölen et al., 2007).

The theory has been strengthened in recent years by evidence that mGluR5 antagonists or genetic reduction of mGluR5 expression can at least partially rescue both synaptic and behavioral phenotypes in Fmr1 KO mice (Gasparini et al., 1999; Aschrafi et al., 2005; McBride et al., 2005; Yan et al., 2005; Dölen et al., 2007; Pan and Broadie, 2007; de Vrij et al., 2008; Pan et al., 2008; Repicky and Broadie, 2009; Michalon et al., 2012; Silverman et al., 2012). However, more detailed examinations into the role of FMRP in controlling activity dependent protein translation reveal a more complex role of the protein in the regulation of various types of plasticity including activity-dependent, cell intrinsic, and homeostatic plasticity.

Since the initial proposal of the mGluR theory, mGluR dependent and independent synaptic plasticity mechanisms have been thoroughly evaluated in the Fmr1 KO mouse. The results of these studies reveal highly region and modality-specific dysfunction in postsynaptic plasticity mechanisms as a result of the loss of FMRP that may or may not respond to a reduction in Gp I mGluR activity. In the cerebellum of Fmr1 KO mice for instance, mGluR1-dependent LTD is enhanced similar to hippocampal area CA1 (Koekkoek et al., 2005). However, N-Methyl-D-aspartic acid (NMDA) receptor mediated non mGluR-dependent long-term potentiation (LTP) is not affected in hippocampal circuits in these mice (Godfraind et al., 1996; Paradee et al., 1999; Huber et al., 2002; Li et al., 2002) revealing the specificity of FMRP for regulating mGluR-dependent plasticity. In other regions such as deep somatosensory cortical layers in which non mGluR-dependent and mGluR-dependent LTP mechanisms coexist, mGluR-dependent LTP is not enhanced but absent (Wilson and Cox, 2007). Furthermore the mGluR5 selective antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) cannot rescue this phenotype.
in Fmr1 KO mice (Wilson and Cox, 2007). Similar deficits in mGluR-dependent LTP were revealed in the basolateral amygdala of these mice (Suvarathan et al., 2010). Although seemingly contradictory to the “overactivation of mGluR mediated protein synthesis” hypothesis put forth by the mGluR theory, these results might be explained by an upregulation of mGluR dependent processes during development (basal protein synthesis as mentioned above) that may have washed out and/or eliminated this type of plasticity from the particular synapse. In the basolateral amygdala deficits in mGluR-dependent LTP were accompanied by decreases in basal synaptic transmission (Suvarathan et al., 2010) so plasticity deficits may not be wholly activity-dependent but arise subsequently to developmentally altered circuits. In accordance with these possible network alterations, similar attenuations in non-mGluR dependent LTP exist in areas like the anterior cingulate cortex and lateral amygdala (Zhao et al., 2005).

Recent studies also characterize deficits in presynaptic plasticity in FXS related to the loss of FMRP in presynaptic terminals. Using isolated sensory-to-motor neuron co-cultures derived from Aplysia, Till and colleagues (2011) knocked down the Aplysia homolog of FMRP (ApFMRP) in either the presynaptic or postsynaptic neuron and evoked LTD with pulses of the inducing agent FRMF-amide. They identified enhanced LTD consistent with mGluR-dependent hippocampal LTD if the FMRP knockdown was applied to the postsynaptic cell or the presynaptic cell indicating an additional crucial role of presynaptic protein regulation to modulate LTD (Till et al., 2011). Another study examined presynaptically regulated short-term depression (STD) in Fmr1 KO hippocampal excitatory synapses. Neurons from Fmr1 KO mice exhibited enlarged vesicle pools and increased vesicle turnover that correlated with reduced STD when compared to wild-type mice. Consequently, these synapses showed increased responses to replicated high-frequency place field stimuli. These data indicate a strong presynaptic
requirement for regulation by FMRP in this type of processing (Deng et al., 2011). Additionally, it is unknown whether mGluR activity reduction can improve these deficits.

**E. THE GABAERIC SYSTEM AND FXS**

While there is strong evidence in support of excessive glutamatergic-mediated signaling via Gp I mGluRs, a number of recent studies identify deficient GABAergic inhibitory transmission as a contributor to FXS phenotypes. Complementary deficits in these two receptor systems support a prevalent causal theory for many of the symptoms of FXS including anxiety, autistic behaviors, epilepsy, and cognitive impairment, as well as related developmental disorders and ASD - hyperexcitability and excitatory/inhibitory (E/I) imbalance in key brain regions (Rubenstein and Merzenich, 2003; Zoghbi, 2003; Belmonte and Bourgeron, 2006; Gibson et al., 2008; McNaughton et al., 2008; Moy and Nadler, 2008; Berry-Kravis et al., 2010; Chao et al., 2010; Wetmore and Garner, 2010; Cordeiro et al., 2011; Hays et al., 2011; LeBlanc and Fagiolini, 2011). In fact, many of the FXS comorbidities such as anxiety and epilepsy can be corrected with GABAergic agonists aimed to reduce hyperexcitability and maintain E/I balance (D'Hulst and Kooy, 2007; Olmos-Serrano et al., 2011; Heulens et al., 2012). Therefore, the GABAergic system remains a potentially viable, underexplored complement to currently emerging FXS treatments aimed at excessive mGluR signaling. The next sections (E to G) describe GABAergic inhibitory transmission and summarize evidence for pre- and postsynaptic deficits in FXS. This introduction pays particular attention to specialized, extrasynaptic tonic GABAergic inhibition that can have profound effects on cellular and network excitatory/inhibitory (E/I) balance and excitability (Farrant and Nusser, 2005).
GABAergic transmission is comprised of two main types of GABA receptors, GABA_A receptors and GABA_B receptors. Briefly, GABA_B receptors are G-protein coupled receptors (GPCRs) arranged as heterodimers of GABA_B1 and GABA_B2 subunits, each with seven transmembrane domains similar to metabotropic glutamate receptor subunits (Bettler, 2004). Via linkage to Gαi/o and through the βγ subunits of these GPCRs, activation of GABA_B receptors inhibits Ca2+ receptors and thereby neurotransmitter release. This activation occurs primarily at presynaptic receptors, but also likely at postsynaptic receptors especially in the dendrites, via paracrine or autocrine release of GABA (Chalifoux and Carter, 2011). Postsynaptically these receptors also activate G-protein coupled inwardly-rectifying (GIRK) K+ channels to induce a hyperpolarizing K+ efflux in adult neurons (Bowery et al., 2002; Calver et al., 2002; Bettler and Tiao, 2006). In contrast GABA_A receptors are ionotropic and can be divided into 2 groups, GABA_A and GABA_A-ρ (formerly GABA_C) receptors (Olsen and Sieghart, 2008). GABA_A receptors function as heteropentamers made up of a combination of receptor subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3) (Whiting, 2003; Olsen and Sieghart, 2008; 2009). GABA_A receptors (including the GABA-ρ subclass) primarily function postsynaptically in adult neurons with low internal Cl- concentrations to enable a hyperpolarizing influx of Cl- ions when activated. However GABA_A receptors can also function presynaptically to control neurotransmitter release (Trigo et al., 2008). The combination of GABA_A receptor subunits that form a functional receptor (usually 2 α subunits, 2 β subunits, and either γ or δ) determines the regional and developmental expression, physiological and pharmacological properties, and importantly the subcellular location of the receptor (Hevers and Lüddens, 1998; Pirker et al., 2000; Mody and Pearce, 2004; Brickley and Mody, 2012). Synaptically located receptors perform fast, phasic inhibition by responding to large concentrations of GABA in the synapse (mM) with a low
affinity, high efficacy, and high desensitization rates. Contrastingly, peri- and extrasynaptic receptors perform slow, tonic inhibition by responding to relatively low concentrations of GABA (nM to µM) with very high affinity, low efficacy, and lower desensitization rates than their synaptic counterparts (Semyanov et al., 2004; Farrant and Nusser, 2005). These properties in particular enable these particular GABAA receptors to modulate a potent, dynamic tonic conductance many times larger than that of the collective fast, phasic inhibition (Brickley et al., 1996; Rossi et al., 2003). We describe tonic inhibitory conductance and its importance to network excitability in more detail below (Section G).

In general optimal GABAergic inhibitory transmission employs a diverse population of GABAergic interneurons distinct in their morphological, biochemical, and physiological properties (Kawaguchi and Kubota, 1997; Markram et al., 2004; Somogyi and Klausberger, 2005; Petilla Interneuron Nomenclature Group et al., 2008). These populations tend to form exclusive subtype-specific electrical connections via gap junctions (Galarreta and Hestrin, 1999; Gibson et al., 1999; Beierlein et al., 2000; 2003) and preferentially synapse on distinct subcellular locations of their principal neuron targets consistently across brain regions (i.e. soma or dendrites) (Kawaguchi and Kubota, 1997; Somogyi et al., 1998; Somogyi and Klausberger, 2005; Muller et al., 2006; 2007). Additionally, these subpopulations typically form inhibitory synapses with specific postsynaptic GABAA receptor subtypes that define their kinetics and pharmacological properties (Nusser et al., 1996; Fritschy et al., 1998; Nyíri et al., 2001; Klausberger et al., 2002; Fritschy and Brünig, 2003). Therefore, each interneuron subtype controls a defined circuit that can be specifically manipulated via targeted pharmacology (Olsen and Sieghart, 2009). These circuits perform various roles crucial to information processing and cognition in the brain including regulation of excitability, controlling spread of activity, and
modulating inter-region connectivity and network processing via oscillations (Porter et al., 2001; Cardin et al., 2009; Sohal et al., 2009; Wang, 2010). Naturally then, disruption in inhibitory circuitry via loss of synapses or presynaptic interneuron subtypes is a hallmark of many neurological diseases including FXS, Rett Syndrome, Down’s Syndrome, schizophrenia, and epilepsy (Buckmaster and Jongen-Rêlo, 1999; Cossart et al., 2001; Lewis et al., 2005; Selby et al., 2007; Gogolla et al., 2009; Chao et al., 2010; Lewis et al., 2011; Rissman and Mobley, 2011).

F. GABAERGIC ABNORMALITIES IN FXS

Over the past ten years numerous studies have reported extensive alterations in critical components of the GABAergic system in behaviorally relevant forebrain regions such as the amygdala, cortex, and hippocampus in FXS (summarized in Figure 1, Table 1) (Idrissi et al., 2005; D’Hulst et al., 2006; Centonze et al., 2008; Chang et al., 2008; D’Hulst et al., 2009b; Olmos-Serrano et al., 2010). FMRP is broadly expressed in GABAergic inhibitory interneuron populations throughout development, indicating that it is involved in normal interneuron maturation and function (Feng et al., 1997; Schütt et al., 2009; Olmos-Serrano et al., 2010). Our laboratory and others show that the loss of FMRP function presents many problems for presynaptic GABA release including the altered expression of scaffolding proteins, changes in glutamic acid decarboxylase levels (GAD65/67) required to synthesize GABA, decreases in GABA transporter expression (GAT1), and decreases in enzymes for GABA catabolism (GABA-T and SSADH) (Liao et al., 2008; Curia et al., 2009; Schütt et al., 2009; D’Hulst et al., 2009b; Adusei et al., 2010; Olmos-Serrano et al., 2010). On the other side of the synapse postsynthetically, Fmr1 KO mice display broad decreases in GABA_A receptor subunits and key
GABAergic synaptic components such as gephyrin (Idrissi et al., 2005; Curia et al., 2009; D'Hulst et al., 2009b; Adusei et al., 2010). Studies of mRNA expression in the brains of \textit{Fmr1} KO mice revealed prominent reductions in the expression of \(\delta\), as well as \(\alpha\), \(\beta\), and \(\gamma\) subunits in behaviorally relevant brain regions such as the cortex and hippocampus (D'Hulst et al., 2006; Gantois et al., 2006). Importantly the severities of these various pre- and postsynaptic deficits in FXS are region-specific similarly to abnormal mGluR transmission detailed above, and therefore may have diverse effects on FXS phenotypes (Figure 2) (Centonze et al., 2008; Gibson et al., 2008; Olmos-Serrano et al., 2010; Zhang and Alger, 2010; Paluszkiewicz et al., 2011b). For instance, while we show that inhibition in the basolateral amygdala is globally decreased in \textit{Fmr1} KO mice including decreases in synaptic number (Olmos-Serrano et al., 2010; Martin et al., 2012), basal inhibitory transmission in the striatum is \textit{increased} but with a decrease in synaptic density (Centonze et al., 2008). These changes may play a role in FXS phenotypes associated with frontostriatal dysfunction such as ADHD and repetitive behaviors (Reiss et al., 1995; Menon et al., 2004; Hoeft et al., 2007). However contrary to a synaptic reduction, studies of hippocampal CA1 inhibitory synapses actually show increases in synaptic number (Dahlhaus and El-Husseini, 2010) indicating highly divergent effects of the absence of FMRP on region-specific network development. Furthermore, as described in this dissertation, changes in some forms of GABAergic transmission such as slow, tonic transmission can occur with (i.e. basolateral amygdala) or without (i.e. subiculum) complementary changes in phasic GABAergic transmission depending on the brain region examined.

These abnormalities in molecular expression and basal transmission expand to functional deficits in \textit{Fmr1} KO mice that again, are region specific. For example, presynaptic excitatory transmission onto parvalbumin-positive (PV+) interneurons in Layer IV somatosensory cortex is
weakened in *Fmr1* KO mice contributing to decreased feedback inhibition and increased excitability in this critical input circuit (Gibson et al., 2008). This deficit in excitatory drive onto interneurons correlates with an increase in the duration of UP states that represent coordinate excitatory/inhibitory network activity as well as a decrease in gamma frequency synchronization between excitatory cells receiving thalamic input. These network changes downstream of inhibitory dysfunction illustrate the importance of coordinated activity-dependent inhibition to cortical function that is disrupted in FXS. These coordinated changes in excitation/inhibition may contribute to symptoms of FXS such as epilepsy, abnormal EEG, cognitive deficits, and sensory hypersensitivity (Miller et al., 1999; Musumeci et al., 1999; Berry-Kravis, 2002; Hagerman and Staffstrom, 2009; Berry-Kravis et al., 2010).

Exaggerated signaling through mGluR5 receptors integrates with the GABAergic system as well and can functionally alter the strength and duration of inhibitory neurotransmission in a region-specific way. GABA release is modulated by both membrane depolarization and through presynaptic receptors that act to reduce the amount of neurotransmitter in the synapse (Misgeld et al., 1995; Wilson, 2002; Bacci et al., 2004). One mechanism to control presynaptic release in the CA1 region of the hippocampus involves the synthesis and release (or mobilization) of endocannabinoids - endogenous neuromodulatory lipids that target type 1 cannabinoid receptors (CB1Rs) on the presynaptic terminals of inhibitory interneurons (Wilson, 2002). Activation of Gp1 mGluRs enable the mobilization of endocannabinoids in the postsynaptic neuron and retrogradely modulate GABA release through a mechanism known as depolarization induced suppression of inhibition (DSI) (Varma et al., 2001). The binding to CB1Rs on the presynaptic terminal of the inhibitory interneuron leads to a transient suppression of voltage-gated calcium channel activity thus inhibiting GABA release. These mechanisms require heightened neuronal
activity - an environment that exists in brain circuitry of Fmr1 KO mice (Gibson et al., 2008; Olmos-Serrano et al., 2010). In the CA1 region of the hippocampus in Fmr1 KO mice, enhanced mGluR signaling leads to excessive endocannabinoid mobilization and enhanced suppression of inhibitory transmission (Zhang and Alger, 2010). This increase in the suppression of inhibition could potentially contribute to hyperexcitability in the Fmr1 KO hippocampus and enhanced mGluR-dependent LTD (Huber et al., 2002). In hippocampal circuitry, endocannabinoid modulation of DSI likely involves specific inhibitory circuits relegated to perisomatic targeting interneurons (Lee et al., 2010b). Therefore, with respect to endocannabinoid mobilization in the FXS brain, the loss of FMRP may selectively affect specific inhibitory circuits and leave other circuits intact. Group I mGluR-eCB-dependent suppression of inhibition also exists in the striatum and is enhanced in Fmr1 KO mice (Maccarrone et al., 2010), therefore this particular disruption of inhibitory transmission may represent a more common mechanism of inhibitory dysfunction in FXS.

In the cerebral cortex endocannabinoid mobilization can modulate the retrograde release of presynaptic GABA (Trettel and Levine, 2002) or act to hyperpolarize a specialized type of inhibitory interneuron known as the low threshold spiking (LTS) cell through endogenous autocrine release (Bacci et al., 2004). In this mechanism, sustained action potential activity activates voltage-gated calcium channels for the influx of calcium in LTS interneurons that triggers the synthesis of endocannabinoids. The binding of endocannabinoids to CB1Rs expressed within the same neuron function to activate G-protein-coupled inward-rectifying potassium (GIRK) current, resulting in a prominent hyperpolarization that can last for several minutes (Bacci et al., 2004). This mechanism is known as slow self-inhibition (SSI) and is specific to cortical LTS interneurons (Bacci et al., 2005). While there is no known abnormality
in FXS for this type of interneuron it is likely affected in FXS. Both Group I and Group II mGluRs selectively activate cortical LTS interneurons causing sustained action potential firing during agonist application (Beierlein et al., 2000; Fanselow et al., 2008). Our studies show that DHPG-induced mGluR activation of LTS interneurons is abnormal in Fmr1 KO mice leading to abnormalities in pyramidal neuron synchrony (Paluszkiewicz et al., 2011b). mGluR activation of LTS interneurons in the developing and mature brain is critical for the proper synchronization of cortical excitatory neurons at behaviorally relevant frequencies (Beierlein et al., 2000; Long et al., 2005; Fanselow et al., 2008; Fanselow and Connors, 2010). Therefore GABAergic transmission alterations in association with the loss of FMRP and abnormal mGluR signaling in this specific type of interneuron likely has wide-reaching ramifications in developing and mature cortical networks.

Finally, although not extensively studied yet in Fmr1 KO mice, GABA_B transmission may also be altered in FXS. Functional studies investigating this aspect of GABAergic transmission are lacking, but at least one behaviorally study indicates that increasing GABA_B transmission with the agonist R-baclofen can improve seizure susceptibility in Fmr1 KO mice (Pacey et al., 2009). Since GABA_B receptors can affect presynaptic release of glutamate and GABA as well as postsynaptic excitability, the mechanism of R-baclofen rescue is unclear but likely involves mostly increases in postsynaptic inhibitory currents. Regardless clinical trials with R-baclofen are currently underway and show promise for supplementing mGluR antagonists as FXS treatments (Pacey et al., 2011)(clinicaltrials.gov).

G. TONIC GABA_A TRANSMISSION IN FXS
The region-specific molecular and functional abnormalities described here show the pervasiveness and diversity of abnormalities that result from the loss of FMRP. These abnormalities involve disruptions on both the excitatory and inhibitory sides of neurotransmission that integrate to produce remarkable imbalances in excitation/inhibition in multiple circuits that likely underlie FXS symptoms. One other form of GABAergic transmission plays a vital role in the maintenance of E/I balance: tonic GABA_A transmission. Tonic GABA_A receptors lie peri- (just outside) or extrasynaptically on neuronal membranes and can sense very low concentrations of GABA (nM to µM) compared to their synaptic counterparts (Figure 1) (Farrant and Nusser, 2005). These receptors have a high affinity but low efficacy for GABA with low desensitization rates so that they can maintain a persistent background inhibitory conductance that affects not only intrinsic neuronal excitability (Bonin et al., 2007), but also the integration of synaptic inputs (Mitchell and Silver, 2003; Semyanov et al., 2004), and synaptic plasticity (Martin et al., 2010). Over two decades of research have shown that deficits in this paracrine form of GABAergic transmission can contribute to network dysfunction in a range of neurological diseases including schizophrenia, epilepsy, sleep disorders, Parkinson’s Disease, and Down Syndrome by pathologically altering the E/I balance (Maguire et al., 2005; Ade et al., 2008; Wafford and Ebert, 2008; Maldonado-Avilés et al., 2009; Macdonald et al., 2010; Braudeau et al., 2011).

Tonic currents through these specialized receptors are typically observed in voltage clamp recordings by applying a GABA_A antagonist (gabazine or bicuculine) and observing a shift in the holding current required to maintain the cell at voltage (Farrant and Nusser, 2005; Glykys and Mody, 2007a). This decrease in input conductance is often accompanied by a decrease in the recording trace variance consistent with a decrease in the number of open
GABA$_A$ receptor channels (Brickley et al., 1996). Tonic GABA$_A$ receptors consist of a variety of combinations that confer subcellular locations, kinetics, and pharmacological properties crucial to their activity (Farrant and Nusser, 2005). The stoichiometry of the GABA$_A$ receptor pentamer predicts 2 subunits from the $\alpha$ subunit subclass, 2 subunits from the $\beta$ subclass, and 1 subunit of either the $\gamma$ class or $\delta$ subclass. Typically, receptors containing $\alpha$4-, $\alpha$5-, or $\alpha$6-subunits tend to express extrasynaptically (Farrant and Nusser, 2005). Similarly, receptors containing the $\delta$-subunit in place of a $\gamma$-subunit in the typical $\alpha$-$\beta$-$\alpha$-$\beta$-x pentamer also express extrasynaptically (Nusser et al., 1998; Farrant and Nusser, 2005). Furthermore $\alpha$4- and $\alpha$6 subunits tend to express with $\delta$ subunits while $\alpha$5 subunits likely express mostly with $\gamma$2. The relative expression of these tonic receptor subtypes is brain region specific. For example, $\delta$-subunit containing receptors express with $\alpha$4 rather ubiquitously in the brain while $\alpha$5-subunit containing receptors are highly expressed in certain regions such as the hippocampus (Caraiscos et al., 2004) and Layer V of the cerebral cortex (Ali and Thomson, 2008).

The relative strength of a tonically active conductance as well as its sign (hyperpolarizing or depolarizing) critically depends on several dynamic factors. One of the most crucial factors is the source and amount of GABA that acts on these tonic receptors (Farrant and Nusser, 2005). The most obvious and common source of GABA is via synaptic spillover (Glykys and Mody, 2007b) from phasic synaptic release. Early studies on tonic currents in the hippocampus showed that tonic currents increased proportionally with synaptic activity (Frerking et al., 1999) emphasizing an intimate link between tonic and phasic GABAergic transmission. However extrasynaptic GABA does not require synaptic activity. For example in cerebellar granule cells a developmental shift occurs so that extrasynaptic GABA$_A$ receptors initially supplied solely by synaptically released GABA from an abundance of Golgi cell axon terminals transition to a
primarily nonsynaptic source as the axon terminals are pruned (Wall and Usowicz, 1997; Rossi et al., 2003). After the initial discovery of this phenomenon, subsequent studies revealed mechanisms by which glial cell and neuronal GABA transporters can reverse polarity from GABA uptake to GABA extrusion in a voltage and concentration dependent manner (Wu et al., 2006; 2007) and therefore supply this nonsynaptically released GABA. These studies emphasize the role of GABA transporters (GAT-1, -2, -3, BGT-1) in modulating the extrasynaptic GABA concentration by dynamically regulating the rate and sign of their activity to regulate GABA in the synaptic/extrasynaptic space (Brickley and Mody, 2012). More recently, another mechanism, the astrocytic anion channel Bestrophin 1 (Best1), has been identified that likely plays a dominant role as a source for GABA in the cerebellum (Lee et al., 2010a).

Another pair of factors that crucially determines the magnitude and sign of the tonic GABAergic conductance are the reversal potential for GABA ($E_{\text{GABA}}$) at the site of the tonic receptors and the membrane potential at the point in time of receptor channel opening. The $E_{\text{GABA}}$ is generally maintained by the relative expression of two opposing chloride transporters, KCC2 and NKCC1 (Payne et al., 2003; Rivera et al., 2005). KCC2 extrudes Cl- ions in exchange for K+ and NKCC1 accumulates Cl- in exchange for K+ and Na+ ions (Rivera et al., 2005). Immature neurons typically express at higher ratio of NKCC1 to KCC2 and therefore have an $E_{\text{GABA}}$ that remains above the resting membrane potential (RMP) of the cell. Therefore, GABA channel opening results in depolarization. This type of transmission likely allows for calcium influx and is important for neuron development and differentiation (Ben-Ari, 2002; Owens and Kriegstein, 2002; Nguyen et al., 2003). In contrast mature neurons typically express a lower proportion of NKCC1 to KCC2 and therefore have $E_{\text{GABA}}$ less than the RMP so that tonic GABAergic transmission is typically hyperpolarizing (inhibitory) (Farrant and Nusser,
However, in some disease states, NKCC1/KCC2 ratios can become pathologically high in mature neurons resulting in hyperexcitability of the affected cell/network. In pediatric focal cortical dysplasia for example, an abnormal mixture of cells with depolarizing and hyperpolarizing tonic GABAergic currents may contribute to network excitability and the generation of epileptiform activity (Figure 3).

Given the close relationship between phasic and tonic GABAergic transmission described above, deficits in one likely affects the other. A small body of evidence shows that this condition is true for FXS. In fact strikingly, one of the first mRNA substrates of FMRP discovered was that of the δ-subunit of the GABA_A receptor (Miyashiro et al., 2003), the most common extrasynaptic subtype (Brickley and Mody, 2012). Additionally, the global decrease of GABA_A receptors in the Fmr1 KO brain include those necessary for tonic transmission (α5 and δ) (Idrissi et al., 2005; Curia et al., 2009; Adusei et al., 2010). The subiculum of the hippocampus shows reductions in mRNA and protein α5- and δ-subunit-containing receptor (Curia et al., 2009). Electrophysiological recordings also reveal reduced total tonic currents in pyramidal cells in this area. Interestingly, there was no change in basal inhibitory synaptic transmission indicating that the dominant reduction was likely at the GABA_A receptor expression level and not dependant on the availability of GABA. Regardless, given the broad, dramatic reductions in GABA levels and receptor expression, tonic GABAergic transmission is likely heavily compromised generally in the Fmr1 KO brain contributing to the overall reduction in GABAergic tone. Despite this early evidence that the GABAergic system and tonic GABAergic conductance in particular may be affected in FXS, detailed electrophysiological studies examining the state of excitatory/inhibitory balance and the involvement of the GABAergic
system in maintaining that balance have not been extensively conducted in key brain regions such as the cortex or amygdala.

**H. THE BASOLATERAL AMYGDALA**

One particular region of interest crucial to the FXS phenotype is the amygdala. This subcortical structure consists of 13 diverse nuclei essential for regulating emotional saliency, processing social and non-social emotional responses, and neuronal coding underlying innate and acquired fear (LeDoux, 2003; Ehrlich et al., 2009). Despite implications of the amygdala in many of the symptoms of FXS including epilepsy, hyperactivity, ADHD, autism, and social anxiety (Sanders and Shekhar, 1995a; Truitt et al., 2007; McNaughton et al., 2008; Hagerman et al., 2009; Truitt et al., 2009; Posner et al., 2011), physiological studies on this highly integrated subcortical structure had not been performed in a model of FXS until recently.

Information flows into the amygdala from thalamic, cortical, and subcortical structures primarily into the basolateral nucleus (BLA) where it is processed and returned through reciprocal connections to areas like the prefrontal cortex and hippocampus. The basolateral nucleus also projects to the main output of the amygdala, the central nucleus (CeN), which affects autonomic responses to fearful stimuli via projections to the brainstem and hypothalamus (Sah et al., 2003; Ehrlich et al., 2009). The BLA resembles other parts of the telencephalon such as the cortex and hippocampus, made up of a majority of excitatory principal neurons (PNs, ~80%) and diverse populations of GABAergic interneurons (INTs, ~20%) distinct in their physiology, biochemistry, and connectivity (McDonald and Mascagni, 2002; Sah et al., 2003; Muller et al., 2006; Rainnie et al., 2006; Muller et al., 2007; Woodruff and Sah, 2007; Jasnow et al., 2009; Sosulina et al., 2010). Although glutamatergic transmission is the main contributor to
the BLA, GABAergic transmission via the diverse pool in INTs crucially regulates all aspects of
BLA and therefore amygdala processing (Ehrlich et al., 2009). Consequently, early studies
showed that modulation of GABA in the BLA affected fear and anxiety in rodents (Harris and
Westbrook, 1995; Pesold and Treit, 1995; Sanders and Shekhar, 1995b; 1995a) and many
anxiolytic compounds likely act on GABAergic transmission in the region (Mohler et al., 2004).
GABAergic transmission in the BLA not only fundamentally regulates generalized anxiety, but
also controls activity-dependent synaptic plasticity necessary for accurate fear acquisition,
expression, and extinction (Ehrlich et al., 2009).

Given the implications of the amygdala in FXS symptoms and the global compromise of
the GABAergic system in FXS, our laboratory conducted studies to investigate the state of
GABAergic transmission in the BLA (Olmos-Serrano et al., 2010). Initial findings show that in
the BLA of the Fmr1 KO mouse there are reductions in GABAergic inhibitory efficacy that
include reduced GABA production and release, decreased frequency and amplitude of action-
potential (AP) dependent and independent inhibitory post-synaptic potentials (IPSCs), and
decreased GABAergic synapses. These deficits occur in association with increased excitability
of excitatory PNs as indicated by a reduced threshold to fire action potentials in Fmr1 KO PNs
compared to wild-type. Given the compromised GABA availability and obvious excitatory
imbalance, tonic GABAergic transmission possibly represents an intriguing cause and/or cure for
abnormal inhibition and excitability in the BLA relevant to FXS treatment for amygdala based
symptoms.

I. SIGNIFICANCE AND AIMS
The GABAergic system is increasingly viewed as a potential therapeutic option for FXS, however few studies have revealed specific physiological mechanisms by which GABAergic transmission can affect relevant phenotypes such as BLA PN hyperexcitability. Tonic GABAergic transmission in the BLA represents one possible mechanism that remains unexplored. In addition, although the GABAergic system is reduced in the Fmr1 KO BLA, overall synaptic E/I balance that would affect network coding and excitability in the region is unknown. Therefore we designed studies to characterize tonic GABAergic transmission in the BLA and assess its role in the maintenance of excitability and E/I balance. Specifically, experiments aim 1) to identify the components of tonic GABAergic transmission in the BLA including relevant receptors and the source of GABA; 2) identify and characterize deficits in the Fmr1 KO mouse; 3) investigate tonic GABAergic transmission as a pharmacological treatment of PN hyperexcitability; and 4) determine how tonic GABAergic transmission regulates synaptic balance in the normal and Fmr1 KO BLA. Most significantly, the studies were intended to establish tonic GABAergic transmission as an important regulator of synaptic integration and neuronal excitability in the BLA and establish tonic GABA_A receptors as viable pharmacological targets to improve amygdala function in FXS.

J. GENERAL METHODS

Animal use. Control and Fmr1 knock-out (KO) mice on the congenic FVB background were obtained from Jackson Laboratory. For the purpose of delineation, we refer to control animals as wild-type (WT) throughout the text and figures in this dissertation. Animals were housed and utilized in accordance with protocols approved by Children’s National Medical Center, Institutional Animal Care and Use Committee.
Slice Preparation for Electrophysiology. Acute slices were prepared from male WT or \textit{Fmr1} KO mice (\textit{Fmr1} \textsuperscript{−/−}), age postnatal day 21 to day 30 (P21-P30). Animals were briefly anesthetized with CO\textsubscript{2} and decapitated. Brains were removed quickly and placed in cold (4°C) sucrose-based oxygenated (95% O\textsubscript{2}/5% CO\textsubscript{2}) cutting solution composed of (in mM): Sucrose (234), Glucose (11), NaHCO\textsubscript{3} (26), KCl (2.5), NaH\textsubscript{2}PO\textsubscript{4}*H\textsubscript{2}O (1.25), MgSO\textsubscript{4}*7H\textsubscript{2}O (10), and CaCl\textsubscript{2}*H\textsubscript{2}O (0.5). Coronal slices containing the basolateral amygdala (BLA) were obtained using a slicing vibratome (Leica 1200) by removing the cerebellum with a perpendicular cut to the rostral-caudal plane and gluing the caudal side down on the vibratome stage submerged in cold cutting solution. Slice thickness was 300 µM for all experiments with the exception of conductance experiments in which case slices were cut at 400 µM. The slices were immersed in oxygenated (95% O\textsubscript{2}/5% CO\textsubscript{2}) artificial cerebral spinal fluid (ACSF) at 34°C for 30-45 minutes. ACSF was composed of (in mM): NaCl (126), NaHCO\textsubscript{3} (26), Glucose (10), KCl (2.5), NaH\textsubscript{2}PO\textsubscript{4}*H\textsubscript{2}O (1.25), MgCl\textsubscript{2}*7H\textsubscript{2}O (2), and CaCl\textsubscript{2}*2H\textsubscript{2}O (2); pH 7.4; osmolarity maintained at 290-300 mOsm.

Electrophysiology. For all experiments slices were placed in a submerged slice chamber and continuously perfused with ACSF at 2-4 ml/min maintained at 25-27°C with an inline heater system (Warner Instruments). Slices were visualized on a fixed stage upright microscope (Nikon E600 FM) equipped with 10x and 60x objectives using differential interference contrast (DIC) optics, infrared illumination, and an infrared-sensitive camera (COHU). Whole-cell patch clamp recordings were performed with glass pipettes with an access resistance of 2.0-4.0 MΩ when filled with intracellular solution. Data were acquired with a Multiclamp 700A amplifier and digitized with a Digidata 1322A using pClamp 9.2 acquisition software (Molecular Devices).
Neuronal Morphology. For post hoc morphological analyses, biocytin (1%, Sigma) was included in the intracellular recording solution. After recording the firing properties of a given cell, biocytin was injected by delivering a series of short depolarizing current pulses (1 nA). After removal from the recording chamber, slices were fixed overnight in 4% paraformaldehyde in 0.1 M PBS. Slices were then incubated in 0.6% H2O2 for 30 min, and then twice in 50% ethanol for 10 min. After washing twice in PBS, slices were incubated for 1 h in PBS containing fluorescein-tagged Avidin D (10 µl/ml, Vector Laboratories), 10% horse serum, 5% Triton X-100, and 0.02 g/ml bovine serum albumin (Sigma). Following three more washes in PBS, slices were mounted and covered with mounting medium (Vectashield). Filled cells were visualized using an Olympus Fluoview laser-scanning confocal microscope.

Statistical Analysis. All recordings were analyzed off-line (Clampfit v 9.2, Molecular Devices, Minianalysis v 6.0.7, Synaptosoft, Microsoft Excel, and Matlab). Expressed values are the means ± standard error (SE). Statistical analyses utilized the two-tailed Student’s t test or the nonparametric Mann-Whitney U test where appropriate for measures of tonic currents, sIPSC amplitudes, and properties of synaptic conductance (Origin v 7.0552 OriginLab). The Kolmogorov–Smirnov test (K-S test) was used to compare probability distributions of frequency, amplitude, and decay of sIPSCs and mIPSCs (Matlab) (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Gramacidin Perforated-Patch Recordings of Tonic Current. Perforated-patch experiments were conducted on 300 micron slices of dysplastic neocortex acutely resected from pediatric patients undergoing tissue resection for intractable seizures associated with focal cortical dysplasia. Recordings were made with an intracellular solution containing (in mM): K-Gluconate (70), KCl (70), HEPES (10), EGTA (1), MgCl2 (2), Mg-ATP (4), Na-GTP (0.3), 100 µg/ml gramicidin (dissolved in DMSO), E_cl = -16 mV. GABAergic currents were isolated by...
blocking ionotropic glutameric transmission with 6,7-dinitroquinoxaline-2,3-dione (DNQX) (20 µM final concentration, AMPA/kainate antagonist, Tocris Bioscience) and DL-2-amino-5-phosphonopentanoic acid (APV) (50 µM final concentration, NMDA receptor antagonist, Tocris Bioscience) added in the ACSF. To establish perforated-patch configuration, a GΩ seal was formed with the cell membrane and the cell was monitored until the seal resistance stabilized between 25-50 MΩ (10-30 minutes). Next the resting membrane potential (RMP) of the cell was established in current clamp. Then 5 µM exogenous GABA was added to the bath and allowed to wash in for at least 10 minutes to allow synaptic GABA_A receptors to desensitize thereby minimizing their contribution to the measured tonic current. In voltage clamp a step protocol was applied stepping from -100 mV to +20 mV in 15 mV intervals for 3 seconds every 5 seconds. Next we bath applied the GABA_A receptor antagonist bicuculline methiodide (BMI, 100 µM) to block all GABAergic transmission and repeated the step protocol. An average of 3 step protocols from each condition was used for analysis. The corresponding voltage steps of the BMI protocol were subtracted from the GABA steps to reveal the tonic current at each holding potential. An I-V curve was then generated for each cell to assess the level of tonic current and establish the relationship between E_{GABA} and their RMP.
Figure 1. Several GABAergic synapse components exhibit altered expression in the Fmr1 KO mouse model of FXS. Numbers identify key synaptic proteins disrupted in Fmr1 KOs, including GABA<sub>A</sub> receptors, enzymes involved in GABA production and catabolism (GAD, SSADH, GABA-T) and GAT (inset legend; Table 1). (Taken from (Paluszkiewicz et al., 2011a)).
Table 1. GABAergic synapse components with altered expression in *Fmr1* KO mice.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Function</th>
<th>Brain region</th>
<th>Expression</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  GABA&lt;sub&gt;α&lt;/sub&gt; receptor subunits (α, β, γ, δ)</td>
<td>Ionotropic GABA receptor function, localization</td>
<td>Cortex, Subiculum, Hippocampus</td>
<td>↓ mRNA, protein</td>
<td>El Idrissi et al., 2005; D’Hulst et al., 2006; Gantois et al., 2006; Curia et al., 2008; Adusei et al., 2010</td>
</tr>
<tr>
<td>2  GAD</td>
<td>GABA synthesis</td>
<td>Cortex, Cerebellum</td>
<td>↓ mRNA</td>
<td>D’Hulst et al., 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amygdala</td>
<td>↓ protein</td>
<td>Olmos-Serrano et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whole forebrain, Cortex</td>
<td>↑ protein</td>
<td>El Idrissi et al., 2005; Adusei et al. 2010</td>
</tr>
<tr>
<td>3  GABA</td>
<td>Ligand</td>
<td>Amygdala</td>
<td>↓ release</td>
<td>Olmos-Serrano et al., 2010</td>
</tr>
<tr>
<td>4  Gephyrin</td>
<td>GABA receptor clustering</td>
<td>Cortex</td>
<td>↓ mRNA</td>
<td>D’Hulst et al., 2009</td>
</tr>
<tr>
<td>5  GAT1,4</td>
<td>GABA reuptake</td>
<td>Whole forebrain, Cortex, Cerebellum</td>
<td>↓ mRNA, protein</td>
<td>Liao et al., 2008; D’Hulst et al., 2009; Adusei et al., 2010</td>
</tr>
<tr>
<td>6  GABA-T</td>
<td>GABA catabolism</td>
<td>Cortex</td>
<td>↓ mRNA, protein</td>
<td>D’Hulst et al., 2009; Adusei et al. 2010</td>
</tr>
<tr>
<td>7  SSADH</td>
<td>GABA catabolism</td>
<td>Cortex, Cerebellum</td>
<td>↓ mRNA, protein</td>
<td>D’Hulst et al., 2009; Adusei et al. 2010</td>
</tr>
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(Taken from (Paluszkiewicz et al., 2011a)).
Figure 2. Alterations in inhibitory neurotransmission in the Fmr1 KO mouse brain are pervasive, but region-specific. Regional inhibitory deficits are associated with a number of common FXS phenotypes (inset table). Affected regions include the basolateral amygdala, cerebral cortex, striatum and hippocampus/subiculum. Color-coding in the illustration corresponds to the adjacent inset table. (Taken from (Paluszkiewicz et al., 2011a)).
Figure 3. Hyperexcitable cortex of human cortical dysplasia displays a mixture of hyperpolarizing and depolarizing tonic currents. (A) A representative example of a malformed pyramidal cell from the upper layers of dysplastic cortex acquired from a 8 month old male undergoing resection for hemi-megalencephaly. Notice the atypically bifurcated apical dendrite that does not point toward the pial surface (dotted line). (B,C) In another sample taken from a focal cortical dysplasia in the orbitofrontal cortex of a 4 year old male, gramicidin perforated patch recordings were used to record current responses during a step protocol from $-100$ mV to $+20$ mV ascending in 15 mV increments. Currents were measured first in the presence of 5 $\mu$M GABA and then after application of 100 $\mu$M bicuculline methiodide (BMI).
Tonic GABA currents at different voltages were calculated by subtracting the BMI currents from corresponding GABA currents. (B) Recordings from a pyramidal cell in Layer II/III of the cortex reveal a non-rectifying, hyperpolarizing tonic GABAergic current indicated by the linear I-V curve (red dotted line) and more negative $E_{\text{GABA}}$ compared to the resting membrane potential (RMP). Inset calibration: calibration 500 ms, 100 pA. (C) Nearby, a presumed interneuron displays an outwardly rectifying, depolarizing tonic GABAergic current characterized by a higher conductance at depolarized membrane potentials (red dotted line, Boltzmann function) and an $E_{\text{GABA}}$ more positive than the RMP of the cell.
A. BRIEF OVERVIEW

The amygdala is essential for regulating emotional saliency, processing social and non-social emotional responses, and neuronal coding underlying innate and acquired fear (LeDoux, 2003; Ehrlich et al., 2009). The BLA in particular is crucial for these functions and integrates multiple afferent inputs from primary sensory and associational cortex, hippocampus, and other amygdala nuclei to control amygdala output via inputs to the central nucleus (reviewed in (Pape and Pare, 2010)). Numerous studies show that modulation of GABAergic inhibitory tone is required for proper BLA function (Ehrlich et al., 2009; Pape and Pare, 2010). Patients with FXS display symptoms associated with amygdala defects including social anxiety, autistic behaviors such as social avoidance and repetitive behaviors, increased incidence of epilepsy, and attention-deficit hyperactivity disorder (ADHD) (Musumeci et al., 1999; Rogers et al., 2001; Berry-Kravis, 2002; Clifford et al., 2007; Posner et al., 2011) as well as structural and functional amygdala abnormalities (Gothelf et al., 2008; Watson et al., 2008). A common hallmark of these symptoms is a general imbalance of excitation and/or inhibition in relevant neuronal networks. In accordance with this feature, data from our laboratory indicates that GABAergic inhibition in the BLA of the Fmr1 KO mouse model of FXS is drastically reduced with lower GABA production and release and fewer, poorly functioning inhibitory synapses (Olmos-Serrano et al., 2010). These deficits occur in correlation with a hyperexcitability phenotype of principal excitatory neurons (PNs) that control BLA output.
Tonic GABAergic conductance, which relies on GABA persistently acting on extrasynaptic GABA\textsubscript{A} receptors, can powerfully control neuronal excitability (Semyanov et al., 2004) and tonic GABAergic defects at least partially underlie disruptions in excitatory/inhibitory (E/I) balance involved in many CNS disorders including epilepsy (Maguire et al., 2005), learning and memory problems (Martin et al., 2010; Braudeau et al., 2011), anxiety (Tasan et al., 2011), schizophrenia (Hashimoto et al., 2008), recovery from neural injury (Clarkson et al., 2010), and autism (Fatemi et al., 2009; 2010). Similarly studies in the Fmr1 KO mice illustrate disruptions in tonic conductance in the subiculum (Curia et al., 2009) and a global decrease in expression of the tonically active δ-GABA\textsubscript{A} receptor subunit (D’Hulst et al., 2006). In addition, FMRP binds δ-subunit mRNA (Brown et al., 2001) and therefore may directly affect δ-subunit expression.

Based on the importance of the BLA to dysfunction in FXS and the potential involvement of a deficient tonic conductance we conducted experiments designed to investigate functional tonic currents in both the WT and Fmr1 KO BLA. Using whole-cell patch clamp electrophysiological techniques, we demonstrate a marked reduction in tonic GABAergic currents in Fmr1 KO BLA PNs that involve reduced GABA availability and reduced tonic GABA\textsubscript{A} receptor expression. Furthermore these currents depend on action-potential (AP) mediated release of GABA and consist of both δ- and α5-GABA\textsubscript{A} receptor mediated components. In addition, we find that pharmacological augmentation of tonic inhibition rescues cellular hyperexcitability in the Fmr1 KO BLA. These findings reveal a strong association of phasic and tonic inhibitory deficits in the Fmr1 KO amygdala, indicate the importance of tonic current to regulate BLA excitability, and provide strong evidence that pharmacological targeting of the GABAergic system may correct amygdala-based phenotypes in FXS.
B. MATERIALS AND METHODS

Slice Preparation for Electrophysiology. Acute slices were prepared from male WT or \textit{Fmr1} KO mice (\textit{Fmr1} \textsuperscript{-/-}), age postnatal day 21 to day 30 (P21-P30). Animals were briefly anesthetized with CO\textsubscript{2} and decapitated. Brains were removed quickly and placed in cold (4\textdegree C) sucrose-based oxygenated (95\% O\textsubscript{2}/5\% CO\textsubscript{2}) cutting solution composed of (in mM): Sucrose (234), Glucose (11), NaHCO\textsubscript{3} (26), KCl (2.5), NaH\textsubscript{2}PO\textsubscript{4}*H\textsubscript{2}O (1.25), MgSO\textsubscript{4}*7H\textsubscript{2}O (10), and CaCl\textsubscript{2}*H\textsubscript{2}O (0.5). Coronal slices containing the basolateral amygdala (BLA) were obtained using a slicing vibratome (Leica 1200) by removing the cerebellum with a perpendicular cut to the rostral-caudal plane and gluing the caudal side down on the vibratome stage submerged in cold cutting solution. Slice thickness was 300 \textmu M for all experiments. The slices were immersed in oxygenated (95\% O\textsubscript{2}/5\% CO\textsubscript{2}) artificial cerebral spinal fluid (ACSF) at 34\degree C for 30-45 minutes. ACSF was composed of (in mM): NaCl (126), NaHCO\textsubscript{3} (26), Glucose (10), KCl (2.5), NaH\textsubscript{2}PO\textsubscript{4}*H\textsubscript{2}O (1.25), MgCl\textsubscript{2}*7H\textsubscript{2}O (2), and CaCl\textsubscript{2}*2H\textsubscript{2}O (2); pH 7.4; osmolarity maintained at 290-300 mOsm.

Electrophysiology. For all experiments slices were placed in a submerged slice chamber and continuously perfused with ACSF at 2-4 ml/min maintained at 25-27\degree C with an inline heater system (Warner Instruments). Slices were visualized on a fixed stage upright microscope (Nikon FN6?) equipped with 10x and 60x objectives using differential interference contrast (DIC) optics, infrared illumination, and an infrared-sensitive camera (COHU). Whole-cell patch clamp recordings were performed with glass pipettes with an access resistance of 2.0-4.0 M\Omega when filled with intracellular solution. Data were acquired with a Multiclamp 700A amplifier and digitized with a Digidata 1322A using pClamp 9.2 acquisition software (Molecular Devices). Recordings were made from principal excitatory neurons (PNs) identified first visually as having
a large, pyramidal-like soma with 2-7 primary dentrites, and then physiologically using prolonged depolarizing and hyperpolarizing current injections (600 ms). PNs typically display broad (~1.2 ms), accommodating action potentials (APs) in combination with long afterhyperpolarizing potentials (AHPs) (Sah et al., 2003). In experiments utilizing tetrodotoxin (TTX) to record AP-independent synaptic events or that used Cesium-based intracellular solution, visual identification combined with physiological responses to hyperpolarizing current injections recorded within 30 seconds of membrane rupture were used exclusively to identify PNs. GABAergic tonic currents and spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in voltage clamp and isolated by blocking ionotropic glutameric transmission with 6,7-dinitroquinoxaline-2,3-dione (DNQX) (20 µM final concentration, AMPA/kainate antagonist, Tocris Bioscience) and DL-2-amino-5-phosphonopentanoic acid (APV) (50 µM final concentration, NMDA receptor antagonist, Tocris Bioscience) in the ACSF. To determine δ-subunit mediated GABAergic tonic currents, a K-Gluconate based intracellular solution was used (in mM): K-Gluconate (70), KCl (70), HEPES (10), EGTA (1), MgCl2 (2), Mg-ATP (4), Na-GTP (0.3), ECl = -16 mV, Vhold = -60 mV. For tonic current capacity experiments, a Cesium-chloride based intracellular solution was used (in mM): CsCl (135), HEPES (10), EGTA (10), QX-314 (5), MgCl2 (2), Mg-ATP (4), and Na-GTP (0.3), ECl = 0 mV, Vhold = -70 mV. This solution reduces potassium channel currents allowing better visualization of distal events recorded at the soma. Both intracellular solutions allow visualization of GABAergic currents as downward when the holding potential is near rest (-60 mV to -70 mV, i.e.). For F–I plots (AP frequency for given depolarizing current pulses), the following intracellular solution was used (in mM): K-Gluconate (130), KCl (10), HEPES (10), EGTA (10), and MgCl2 (2); ECl = -60 mV, pH 7.3. Membrane potential was adjusted to -60 mV, and brief (600 ms) depolarizing current pulses
of increasing amplitude (10 pA interval) were used. AP threshold was defined as the lowest current step for which an action potential was generated. Threshold was measured under control conditions and in the presence of gaboxadol (THIP, 4,5,6,7-tetrahydroisoaxazolo[5,4-c]pyridin-3-ol, 10 µM). To record total tonic and α5-subunit mediated tonic current capacity, 5 µM GABA (Glykys and Mody, 2007b; Glykys et al., 2008) was included in the bath solution to normalize extracellular GABA between the WT and the GABA-deficient Fmr1 KO acute slice (Olmos-Serrano et al., 2010). Slices were allowed to equilibrate in 5 µM GABA for at least 10 minutes prior to recording.

Tonic currents were acquired and analyzed as reported in our previous work (Krook-Magnuson et al., 2008; Olmos-Serrano et al., 2010). Briefly, ten-second samples were taken from voltage clamp recordings ($V_h = -60$ mV, K-Gluconate based solution, $V_h = -70$ mV, CsCl-based solution) at each experimental condition (baseline ($I_{BSLN}$), α5ia ($I_{5ia}$, α5-subunit specific GABAR inverse agonist, 1.5 µM), gabazine ($I_{GBZ}$, GABAR antagonist SR-95531, 50 µM), or THIP ($I_{THIP}$, 10 µM). To minimize bias from phasic events, a Gaussian distribution was fit to the right side of an all-points histogram from each sample from a point 1–3 pA left of the peak (Glykys and Mody, 2007b). The Gaussian peak determined the mean current for the sample. Total tonic current capacity was calculated from the difference in mean baseline and gabazine currents ($I_{GBZ} - I_{BSLN}$) and α5-subunit mediated tonic current capacity was calculated from the difference in mean baseline and α5ia currents ($I_{5ia} - I_{BSLN}$). In a separate set of experiments, δ-subunit specific tonic currents were calculated (in the absence of extracellular GABA supplementation) from the difference in mean baseline and THIP currents ($I_{THIP} - I_{BSLN}$). To control for differences in cell size/capacitance, calculated currents were converted to tonic current densities for each cell based on cell capacitance (current density = current
(pA)/capacitance (pF)). Capacitance was determined in voltage clamp with brief 10 mV biphasic voltage steps delivered immediately after establishing whole-cell configuration. For tonic current measurements drugs were applied locally via gravity fed Y-tube application and for F-I plot measurements, drugs were bath applied.

**Statistical Analysis.** All recordings were analyzed off-line (Clampfit v 9.2, Molecular Devices, Minianalysis v 6.0.7, Synaptosoft, Microsoft Excel, and Matlab). Expressed values are the means ± standard error (SE). Statistical analyses utilized the two-tailed Student’s t test or the nonparametric Mann-Whitney U test where appropriate for measures of tonic currents, sIPSC amplitudes, and properties of synaptic conductance (Origin v 7.0552 OriginLab). The Kolmogorov–Smirnov test (K-S test) was used to compare probability distributions of frequency, amplitude, and decay of sIPSCs and mIPSCs (Matlab) (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

C. RESULTS

**Tonic current is reduced in principal neurons of the Fmr1 KO BLA**

Tonic inhibition is a major source of inhibitory tone (Semyanov et al., 2004) that complements AP-dependent and -independent phasic inhibitory transmission. Given our previously reported phasic and GABAergic system deficits in Fmr1 KO PNs (Olmos-Serrano et al., 2010), we investigated possible corresponding tonic deficits by measuring tonic inhibitory currents in principal neurons using local application of the GABA_A receptor antagonist gabazine. Tonic current was found to be significantly reduced in Fmr1 KO neurons (WT: 20.0 ± 5.2 pA, n = 10; Fmr1 KO: 4.9 ± 1.4 pA, n = 8; p = 0.021) as well as tonic current density which controls for differences in cell size/capacitance (WT: 0.080 ± 0.022 pA/pF; Fmr1 KO: 0.013 ± 0.006; p = 0.016; Figure 4A-C). Previous studies implicate AP-dependent release as a major source of
ambient GABA required to act on perisynaptic and extrasynaptic GABA\(_A\) receptors mediating tonic currents (Brickley et al., 1996; Bright et al., 2007; Glykys and Mody, 2007b). We therefore tested whether tonic inhibition in BLA PNs similarly depends on AP-dependent GABA release. Bath application of TTX (1µM) eliminated nearly all of the tonic inhibitory current in principal neurons in both WT and \(Fmr1\) KO mice (WT: 102.95 ± 17.97\%, \(n = 6\); \(Fmr1\) KO: 96.13 ± 9.25\%, \(n = 3\); \(p = 0.81\); Figure 4D-E), such that subsequent addition of gabazine had nearly no effect on holding current. Together, these findings indicate a global reduction in inhibitory neurotransmission in the BLA of \(Fmr1\) KO mice as compared to WTs and identifies a critical link between levels of tonic current and deficits in GABA production and release (Olmos-Serrano et al., 2010).

**GABA\(_A\) receptor subtype components essential to tonic GABAergic transmission are reduced in \(Fmr1\) KO principal neurons**

To determine whether deficits in tonic GABA\(_A\) receptor expression in addition to poor GABA availability contribute to the decreased tonic currents in \(Fmr1\) KO PNs, we performed whole-cell voltage clamp experiments to measure tonic current capacity in both WT and \(Fmr1\) KO PNs. We specifically targeted those currents mediated by the most common known tonically active GABA\(_A\) receptor subunits, the \(\delta\)- and \(\alpha5\)-subunit containing receptors [reviewed in (Brickley and Mody, 2012)], because immunohistochemical data shows significant BLA expression (Fritschy and Mohler, 1995; Pirker et al., 2000) and therefore these subunits may contribute to the overall tonic current. First we tested BLA PNs in WT and \(Fmr1\) KO mice for the presence of \(\delta\)-subunit mediated tonic currents using the \(\delta\)-subunit preferring GABA\(_A\) receptor superagonist THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol, 10 \(\mu\)M). As suggested by
immunocytochemical localization data, BLA principal neurons have the capacity for δ-subunit mediated tonic currents measured via changes in mean holding potential in response to THIP (WT: 16.44 ± 3.5 pA, n = 8; Figure 5). In addition THIP-induced tonic currents in Fmr1 KO principal neurons are considerably reduced (Fmr1 KO: 6.38 ± 1.13 pA, n = 8, p = 0.016). Overall current density in BLA neurons from Fmr1 KOs is also reduced three-fold (WT: 0.068 ± 0.014 pA/pF; Fmr1 KO: 0.024 ± 0.008 pA/pF, p = 0.018) (Martin et al., 2012).

In parallel experiments we assessed α5-subunit mediated current capacity and total tonic current capacity (Figure 6) using an α5-subunit specific inverse agonist, α5ia, and the nonspecific GABA_A receptor antagonist gabazine, respectively, to quantify the removal of baseline tonic current. Since extrasynaptic GABA levels differ between the two genotypes (Olmos-Serrano et al., 2010), we controlled for decreased GABA availability in the Fmr1 KO BLA by equalizing the extrasynaptic GABA between the two genotypes with 5 µM bath-applied exogenous GABA which more closely matches in vivo extracellular GABA concentration (Glykys and Mody, 2007b). Then application of α5ia (1.5 µM) revealed the α5-subunit specific tonic current capacity and subsequent application of gabazine (50 µM) revealed the total tonic current capacity (Figure 6A,B). Exogenous GABA was applied for at least 10 minutes prior to other drugs to allow synaptic receptors to desensitize to minimize their contribution to measured tonic currents. Additionally, we used a cesium-based intracellular solution (CsCl) in our recording pipettes. Replacing potassium with cesium increases voltage control of the membrane to enhance the strength of more distal synaptic transmission by blocking potassium channels whose conductance otherwise filters events occurring far from the somatic recording site (Nicoll et al., 1993; Stuart and Spruston, 1998; Day et al., 2006). Under these conditions application of α5ia showed that BLA principal neurons indeed have the capacity for α5-subunit specific tonic
current and that Fmr1 KO principal cells have reduced $\alpha_5$-subunit mediated tonic currents (WT: 13.0 ± 2.3 pA, n = 12 cells; Fmr1 KO: 5.28 ± 0.88 pA, n = 12, p = 0.007; Figure 6D) and current density (WT: 0.04 ± 0.020 pA/pF; Fmr1 KO: 0.02 ± 0.011 pA/pF, p = 0.012) compared to WT. Subsequent application of gabazine showed that total tonic current capacity is also reduced as indicated by reduced gabazine-dependent changes in holding current (WT: 74.61 ± 9.81 pA, n = 12; Fmr1 KO: 44.97 ± 5.56 pA, n = 12, p = 0.015; Figure 6C) and current density (WT: 0.24 ± 0.026 pA/pF; Fmr1 KO: 0.17 ± 0.015 pA/pF, p = 0.028) (Martin et al., 2012).

**THIP rescues neuronal hyperexcitability in BLA PNs in Fmr1 KOs**

In addition to a dampening of inhibitory tone in Fmr1 KO mice, we have previously observed increased principal neuron excitability. BLA excitatory neurons from Fmr1 KOs consistently fired a greater number of APs in response to a series of depolarizing current steps (WT: n = 29; Fmr1 KO: n = 44; Figure 7A,C (Olmos-Serrano et al., 2010)). The threshold for AP generation was also significantly lower in Fmr1 KOs (WT Control ACSF: 39.0 pA ± 3.5, n = 10; Fmr1 KO Control ACSF: 23.3 pA ± 4.2, n = 15; p = 0.009; Figure 7D) with no significant change in $R_{in}$ (WT: 301.5 ± 24.6 MΩ; Fmr1 KO: 392.0 ± 55.1 MΩ; p = 0.15). Closer examination of the input/output (I/O) curves up to a 200 pA current step injection (Figure 7E,F) show that throughout the I/O function, only the offset (threshold) of the two genotypes remain different while the slope of the functions remain basically unchanged. Given the decreased GABA availability and reduced tonic currents in Fmr1 KOs, we hypothesized that augmentation of tonic inhibitory tone might alleviate the observed hyperexcitability. Activation of tonic inhibitory currents is highly dependent upon the availability of ambient GABA, which is deficient in Fmr1 KO mice (Olmos-Serrano et al., 2010), and represents a means to decrease
membrane excitability by decreasing input resistance and increasing conductance (Semyanov et al., 2004). In an attempt to rescue cellular hyperexcitability in Fmr1 KO principal neurons, we injected depolarizing currents steps first in control ACSF, and then during bath application of THIP, a superagonist at δ subunit-containing perisynaptic and extrasynaptic GABA_A receptors known to mediate a strong tonic inhibitory conductance (Brown et al., 2002; Glykys et al., 2007). As expected, significant increases in the AP threshold were observed in both WT and Fmr1 KO mice with THIP application accompanied by changes in resting R_in (WT THIP: 219.2 ± 15.7 MΩ; WT decrease: 82.3 ± 8.9 MΩ; p = 0.0002; Fmr1 KO THIP: 258.0 ± 31.4; Fmr1 KO decrease: 134 ± 23.7 MΩ; p = 0.0014). These AP threshold increases were reflected in a change in I/O function offset with little to no change in slope (Figure 7E,F, dotted lines). Strikingly, the AP threshold in Fmr1 KOs was restored to WT levels by THIP application (Fmr1 KO THIP: 40.7 pA ± 4.6, n = 15; p = 0.78; Figure 7B,D). Thus, the neuronal hyperexcitability in the Fmr1 KO BLA can be dramatically rescued by augmenting tonic inhibitory tone.

D. DISCUSSION

The BLA of Fmr1 KO mice display major deficits in the GABAergic system that significantly affect inhibitory synaptic transmission in a critical brain region involved in the symptoms of FXS (Figure 1, Chapter I)) (Olmos-Serrano et al., 2010; Martin et al., 2012). In combination with studies of other neurodevelopmental disorders including ASD and their animal models, the prevailing hypothesis is that the primary dysfunction in these disorders rests at the level of the synapse (Rubenstein and Merzenich, 2003; Zoghbi, 2003; Belmonte and Bourgeron, 2006; Moy and Nadler, 2008). In this study however we provide evidence that deficits in synaptic inhibition expand beyond the synapse in FXS to affect a major contributor to
GABAergic inhibitory tone, tonic conductance. We report that tonic currents in PNs of the Fmr1 KO BLA are reduced and dependent on decreased functional expression of extrasynaptic GABA \( \alpha \) receptors as well as decreased GABA availability. Additionally both \( \delta \)- and \( \alpha 5 \)-subunit containing GABA \( \alpha \) receptors participate in tonic currents in BLA PNs. Finally, we determined that enhancement of tonic inhibitory transmission at \( \delta \)-GABA \( \alpha \) receptors can rescue cellular hyperexcitability in the BLA. Our study therefore provides basic evidence of the importance of tonic GABAergic transmission in the BLA to regulate neuronal excitability and highlights a novel pathogenic mechanism and potential pharmacological treatment for amygdala based symptoms of FXS and related autism spectrum disorders.

**Basolateral amygdala tonic currents in FXS**

The source of GABA for extrasynaptic receptors, and therefore tonic conductance, can originate from multiple and diverse vesicular and nonvesicular means including synaptic spillover (Glykys and Mody, 2007b), membrane potential dependent reversal of GABA transporters in neurons or astrocytes (Wu et al., 2006; 2007), or astrocytic release via Bestrophin (Best1) channels (Lee et al., 2010a). Studies show that these sources are at least partially region specific. For instance AP-independent release from astrocytes predominates as a source for GABA in cerebellar granule cells (Rossi et al., 2003), whereas in the hippocampus AP-dependent synaptic spillover plays a larger role in maintainance of extrasynaptic GABA concentration and tonic current (Glykys and Mody, 2007b) although some recent evidence also implicates astrocytic Best1 channels in hippocampal area CA1 as well (Yoon et al., 2011). In the BLA, we demonstrate that in both WT and Fmr1 KO PNs tonic currents rely heavily on AP-dependent release of GABA, likely from synaptic vesicles, and therefore probably do not rely
significantly on astrocytic release or reversal of GABA transporters. Thus, tonic current in the BLA is heavily associated with the level of phasic transmission (synaptic GABA release) as has been demonstrated in the hippocampus (Glykys and Mody, 2007b). Additionally our previous investigation of AP-dependent phasic spontaneous inhibitory postsynaptic currents (sIPSCs) in Fmr1 KO PNs showed significant reduction compared to WT (Olmos-Serrano et al., 2010). Therefore reduced interneuron activity as well as reduced GABA availability in the Fmr1 KO BLA likely contributes to deficient PN tonic currents. This close association between phasic inhibitory transmission and tonic transmission is unique to the BLA in Fmr1 KO mice at least compared to known tonic transmission deficits in the subiculum (Curia et al., 2009). In this brain region, tonic current deficits exist in the absence of phasic GABA_\text{A} inhibitory transmission changes demonstrating a region-specific effect of the loss of FMRP on neuronal network dysfunction.

Our complementary investigation of tonic current capacity in Fmr1 KO PNs confirms that in addition to decreased AP-dependent GABA availability, tonically active receptor expression is also decreased in these cells and contributes to their decreased tonic currents (Martin et al., 2012). For these experiments we equilibrated the amount of extrasynaptic GABA between slices and between cells within each slice by adding exogenous GABA (5 \mu M) to the bath solution. Based on previous studies this concentration should prevent major changes in extrasynaptic GABA at various levels within the slice (Glykys and Mody, 2006) and overcome the strong activity of GABA uptake transporters such as GAT-1 that significantly affect extrasynaptic GABA concentrations (Glykys and Mody, 2007a). These assumptions depend on a relatively equal activity level of these uptake transporters between WT and Fmr1 KO slices. However, recent preliminary data from our lab (data not shown) supports a higher level of GAT-
expression and activity in the Fmr1 KO BLA compared to WT. This increase could potentially decrease the effective concentration of GABA reaching extrasynaptic receptors below 5 µM in at least some subcellular locations in Fmr1 KO PNs that in turn would decrease our recorded tonic currents from these cells. However, in context with lower induced currents observed in Fmr1 KO cells with exposure to a direct GABA agonist (THIP) that supports a decrease in receptor expression and the ample time allowed for equilibration in exogenous GABA before recordings took place (at least 10 min), any changes induced by increased GAT-1 activity are likely minimal. Regardless, an increase in this activity would also represent yet another altered aspect of the GABAergic system that would affect inhibitory tone in the BLA network.

Importantly, our GABA_A receptor subunit-specific investigations show that tonic currents in these cells are comprised of at least 2 distinct components, δ- and α5-subunit containing GABA_A receptors, similar to analogous brain regions such as the hippocampus and cortex (Martin et al., 2012). It appears that most tonic transmission in BLA PNs relies on other tonic components rather than α5-GABA_A receptors since α5ia-sensitive tonic current is only ~5-10% of the total tonic capacity in both WT and Fmr1 KO PNs. However increased desensitization time compared to other tonic GABA_A receptors (Caraiscos et al., 2004) might underlie this low percentage. At any rate given the ubiquitous expression of α4 and δ subunits in the BLA (Fritschy and Mohler, 1995; Marowsky et al., 2012) and the presence of THIP-sensitive currents, α4/δ-subunit containing receptors likely make up a significant fraction, although δ containing receptors may contain other α subunits such as α1 (Oláh et al., 2009). However, a substantial portion of the tonic current capacity likely also includes another, albeit atypical component to tonic GABAergic transmission in BLA PNs, the α3-subunit containing receptor. According to a recent study GABA_A receptors containing the α3-subunit express on BLA PNs and mediate a
PN-specific tonic current that affects excitability (Marowsky et al., 2012). We did not directly assay α3-GABA	extsubscript{A} receptors here but given the global reductions in GABA	extsubscript{A} receptor subunit expression including α3-GABA	extsubscript{A} receptors in the Fmr1 KO mouse (Idrissi et al., 2005; D'Hulst et al., 2006; 2009a), the α3-GABA	extsubscript{A} receptor component of tonic transmission is also likely deficient in Fmr1 KO PNs. Interestingly, mass spectrometry studies demonstrate the promiscuity of the α5-subunit to form both α5-exclusive homomeric receptors as well as heteromeric receptors that incorporate other α subunits including α3 (Ju et al., 2009). Therefore although unlikely, tonic GABA	extsubscript{A} receptors of BLA PNs may contain a heterogeneous mix of these α subunits. Based on the major contribution of non-α5-GABA	extsubscript{A} receptors (δ- and α3-), total tonic transmission in BLA PNs most closely mirrors that of excitatory neurons in the upper cortical layers (i.e. Layer II/III, Layer IV) (Yamada et al., 2007; Krook-Magnuson et al., 2008; Sebe et al., 2010) in which α5-subunit expression is relatively low or absent compared to other tonic subunits. In contrast in hippocampal area CA1 α5-GABA	extsubscript{A} receptors mediate a substantially large component of the total tonic current (Caraiscos et al., 2004).

**Tonic inhibition as a therapeutic target in FXS**

Complementing our findings that tonic inhibitory transmission is altered in the BLA of Fmr1 KOs, we show that principal neuron excitability is restored to WT levels by pharmacological enhancement of tonic inhibitory transmission. Close investigation of the baseline input/output functions (F-I plots) show that there is no difference in gain (I/O slope and shape) between genotypes, but instead only a difference in the firing threshold, indicating that the main difference underlying the increased excitability in WT and Fmr1 KO PNs, at least in our *in vitro* assay, is likely the membrane offset with no significant contribution of any
differences in the level of spontaneous subthreshold synaptic input (Pavlov et al., 2009). Furthermore increasing the tonic conductance with THIP shifts the firing threshold in both genotypes by primarily affecting I/O offset and not gain consistent with THIP providing a shunting inhibition that minimally affects the cell’s sensitivity to subthreshold synaptic inputs (Ulrich, 2003; Pavlov et al., 2009). In our experimental paradigm we expect this effect because studies show that tonic inhibition primarily provides shunting inhibition during tonic excitation via constant current injection (Mitchell and Silver, 2003). Therefore the effect of THIP on PN gain requires further study but we assume given the decrease in \( R_{\text{in}} \) we observe at -60 mV with THIP application that gain of subthreshold synaptic events, and therefore synaptic integration are likely also affected (Mehaffey et al., 2005). Effect of THIP treatment on gain has important implications for treatment of excitability because unintended large gain shifts could possibly disrupt neuronal input coding in the BLA that relies on activity dependent plasticity at distinct synapses (Ehrlich et al., 2009).

Additionally, although we observe clear deficiencies in tonic currents consistent with a role of decreased inhibitory tone in the hyperexcitability of \( Fmr1 \) KO PNs, no significant differences in input resistance exist between genotypes and THIP affects both WT and \( Fmr1 \) KO PN excitability equally. Recent evidence indicates that outwardly rectifying tonic inhibitory currents can preferentially affect neuronal offset over gain and therefore control threshold without significantly altering subthreshold \( R_{\text{in}} \) (Pavlov et al., 2009). So tonic current deficiency could remain a primary mechanism underlying the hyperexcitability if analogous currents exist in the BLA. However other potential mechanisms that affect AP threshold independent of baseline \( R_{\text{in}} \) may also play a role such as pathological expression of voltage-gated sodium channels (Oliva et al., 2012) or T-type calcium channels (Zamponi et al., 2010). Thus, THIP
application would counter their effects. Regardless, our data shows that therapeutic intervention with tonic GABAergic enhancement can robustly correct a physiological phenotype in FXS. At least one study corroborates our physiological data in vivo by demonstrating that acute THIP treatment improves hyperactivity in Fmr1 KO mice (Olmos-Serrano et al., 2011), known to involve the BLA (Posner et al., 2011; Zhou et al., 2011). Therefore despite knowledge of the exact mechanism, our findings support the notion that inhibitory GABAergic transmission is an intriguing target for the treatment of at least some major symptoms of FXS (El Idrissi et al., 2005; D'Hulst et al., 2006; D'Hulst and Kooy, 2007).
Figure 4. Tonic inhibition is deficient in Fmr1 KO PNs and depends AP-mediated release of GABA. (A) Current-clamp trace from a WT principal neuron illustrating the typical regular spiking phenotype (+100 pA current injection) and membrane potential sag during hyperpolarization (-100 pA current injection). (B) Current clamp traces from representative WT (upper) and Fmr1 KO (lower) principal neurons, indicating the difference between the average baseline holding current and average holding current in the presence of gabazine (100 µM), as determined by fitted Gaussian curve (see Materials and Methods). (C) Total inhibitory tonic current (left) and current density (right) is significantly reduced in Fmr1 KO principal neurons as compared to WT. (D) Current clamp trace from a WT principal neuron illustrating its AP-dependent tonic GABAergic current and total tonic current. (E) The primary source of tonic inhibitory current in both WT and Fmr1 KO principal neurons is AP-dependent GABA release. *p < 0.05. (Modified from (Olmos-Serrano et al., 2010)).
Figure 5. *Fmr1* KO principal neurons of the BLA have reduced δ-subunit mediated tonic GABAergic currents. (A,B) Representative whole-cell voltage-clamp traces recorded from WT (A) and *Fmr1* KO (B) principal neurons showing 10 second samples before (baseline) and after (THIP) bath application of THIP, 10 μM. Gaussian distributions (right) for each sample indicate the differences in mean holding current at each condition. (C) Averaged group data reveal significantly reduced δ-subunit mediated current (left) and current density (right) in *Fmr1* KO cells versus WT. *p < 0.05. (Taken from (Martin et al., 2012)).
Figure 6. *Fmr1* KO principal neurons in the BLA have diminished α5-subunit specific and total tonic current capacity compared to WT cells. (A,B) Representative whole-cell voltage-clamp traces recorded from principal neurons of WT (A) and *Fmr1* KO (B) showing 10 second samples recorded at baseline (black), after application of α5ia (1.5 µM, red), and after application of gabazine (50 µM, gray). Gaussian distributions (right) for the samples indicate the differences in mean holding current at each condition. (C) Averaged group data reveal significantly reduced total tonic current capacity (left) and current density (right) in *Fmr1* KO cells versus WT. Similarly in (D) averaged group data reveal significantly reduced α5-subunit mediated current capacity (left) and current density (right) in *Fmr1* KO cells versus WT. *p < 0.05. (Taken from (Martin et al., 2012)).
Figure 7. THIP rescues neuronal excitability in Fmr1 KOs. (A, B) Concatenated traces from single cells in response to depolarizing current steps of increasing amplitude (10 pA steps). In control ACSF (A, B1), cells from Fmr1 KO mice exhibit higher action potential firing rates for a
given depolarizing current step, as well as a lower threshold for action potential generation than cells from WT mice. (C) Group $F-I$ plot illustrating hyperexcitability of cells in $Fmr1$ KOs. No differences in passive membrane properties were observed between cells in the two groups ($V_m$: WT, $-61.5 \pm 0.5$ mV; $Fmr1$ KO, $-61.2 \pm 0.7$ mV; $p = 0.80$; $R_m$: WT, $231 \pm 21$ M$\Omega$; $Fmr1$ KO, $242 \pm 12$ M$\Omega$; $p = 0.19$). (B2,D) Bath application of THIP increases action potential threshold in $Fmr1$ KOs to WT levels. (E) Group $F-I$ plot of the cohort from panel (D) shows that the input/output curves of each genotype and condition maintain the same overall shape. (F) Best fit lines to the initial 10 current steps demonstrate that initial slope (gain) is similar among all cell groups. Addition of THIP mostly affects offset inducing little change in gain of the input/output function. *$p < 0.05$; **$p < 0.01$; n.s. not statistically significant. (Modified from (Olmos-Serrano et al., 2010)).
A. BRIEF OVERVIEW

Imbalances in excitation and inhibition (E/I) of neuronal networks are hallmarks of many of the disorders associated with FXS including anxiety, epilepsy, attention-deficit and hyperactivity disorder (ADHD), and autism (Musumeci et al., 1999; Rogers et al., 2001; Berry-Kravis, 2002; Rubenstein and Merzenich, 2003; Clifford et al., 2007; Posner et al., 2011). E/I balance not only determines cell and network output but also is fundamental for regulating activity-dependent plasticity, which is disrupted in FXS in various brain regions including the amygdala (Suvrathan et al., 2010). In the BLA PNs receive various connections from multiple afferents including the thalamus, cortex, hippocampus, and intra-amygdalar inputs (Krettek and Price, 1978; Ottersen, 1982; McDonald and Mascagni, 1996; McDonald et al., 1996) that converge on the same dendrites in close proximity (Ehrlich et al., 2009). Proper neuronal coding and association of input activity essential to fear processing requires dynamic and distinct regulation of plasticity at these individual synapses. For instance fear conditioning requires coordinate plasticity at cortical and thalamic synaptic inputs to lateral amygdala principal neurons (Gewirtz and Davis, 1997; Weisskopf et al., 1999; Collins and Paré, 2000; Paré and Collins, 2000; Maren and Quirk, 2004). Likewise for fear extinction BLA PNs require a shift in synaptic weights at synapses that code for the conditioned stimulus (CS) in exchange for strengthening appropriate intraamygdalar connections that favor proper drive of output neurons (Herry et al., 2008). GABAergic inhibition in particular critically regulates these activity-dependent plasticity events (Mahanty and Sah, 1999; Bissière et al., 2003; Bauer and LeDoux, 2004; Shaban et al., 2006; Shin et al., 2006; Szinyei et al., 2007; Tully et al., 2007).
Tonic GABAergic conductance can regulate neuronal output (see Chapter II, Figure 7) (Olmos-Serrano et al., 2010), but it can also affect synaptic integration and efficacy essential to regulating plasticity (Martin et al., 2012). Therefore, defective tonic conductance in the FXS BLA may hinder synaptic E/I balance and adversely affect coding via activity-dependent plasticity that contributes to FXS phenotypes. Despite studies that investigate changes in excitation or inhibition in the amygdala in FXS (Olmos-Serrano et al., 2010; Suvrathan et al., 2010) no studies consider E/I inputs in concert. In this study we seek to determine the state of synaptic E/I balance in Fmr1 KO PNs compared to their WT counterparts and determine if tonic conductance affects this balance. Close examination of our previous experimental data (Chapter II, Figure 6) indicated that blockade of α5-GABA_A receptors with α5ia altered holding current required to hold the cell at -70 mV and appeared to increase sIPSC amplitude consistent with a change in synaptic efficacy (Martin et al., 2012). Therefore we first characterized the affect of α5-GABA_A receptors on synaptic efficacy and determined that these receptors modulate postsynaptic currents and functionally express away from the cell soma making them a potential tonic component that regulates synaptic balance. Subsequent investigation of evoked excitatory and inhibitory conductance revealed that E/I balance is altered in Fmr1 KO PNs associated with changes in response kinetics that depend on α5-GABA_A receptor activity. Thus deficient α5-GABA_A receptor mediated tonic conductance is likely a key mediator of abnormal synaptic integration in FXS that may affect accurate coding within the FXS amygdala network.

B. MATERIALS AND METHODS

Animal use. Control and Fmr1 knock-out (KO) mice on the congenic FVB background were obtained from Jackson Laboratory. For the purpose of delineation, we refer to control animals as
wild-type (WT) throughout the text and figures. Animals were housed and utilized in accordance with protocols approved by Children’s National Medical Center, Institutional Animal Care and Use Committee.

**Slice Preparation for Electrophysiology.** Acute slices were prepared from male WT or Fmr1 KO mice, age postnatal day 21 to day 30 (P21-P30). Animals were briefly anesthetized with CO₂ and decapitated. Brains were removed quickly and placed in cold (4°C) sucrose-based oxygenated (95% O₂/5% CO₂) cutting solution composed of (in mM): Sucrose (234), Glucose (11), NaHCO₃ (26), KCl (2.5), NaH₂PO₄·H₂O (1.25), MgSO₄·7H₂O (10), and CaCl₂·H₂O (0.5). Coronal slices containing the basolateral amygdala (BLA) were obtained using a slicing vibratome (Leica 1200) by removing the cerebellum with a perpendicular cut to the rostral-caudal plane and gluing the caudal side down on the vibratome stage submerged in cold cutting solution. Slice thickness was 300 µM for all experiments with the exception of conductance experiments in which case slices were cut at 400 µM. The slices were immersed in oxygenated (95% O₂/5% CO₂) artificial cerebral spinal fluid (ACSF) at 34°C for 30-45 minutes. ACSF was composed of (in mM): NaCl (126), NaHCO₃ (26), Glucose (10), KCl (2.5), NaH₂PO₄·H₂O (1.25), MgCl₂·7H₂O (2), and CaCl₂·2H₂O (2); pH 7.4; osmolarity maintained at 290-300 mOsm.

**Electrophysiology.** For all experiments slices were placed in a submerged slice chamber and continuously perfused with ACSF at 2-4 ml/min maintained at 26-28°C with an inline heater system (Warner Instruments). Slices were visualized on a fixed stage upright microscope (Nikon) equipped with 10x and 60x objectives using differential interference contrast (DIC) optics, infrared illumination, and an infrared-sensitive camera (COHU). Whole-cell patch clamp recordings were performed with glass pipettes with an access resistance of 2.0-4.0 MΩ when
filled with intracellular solution. Data were acquired with a Multiclamp 700A amplifier and digitized with a Digidata 1322A using pClamp 9.2 acquisition software (Molecular Devices). All recordings were made from principal excitatory neurons (PNs) identified first visually as having a large, pyramidal-like soma with 2-7 primary dentrites, and then physiologically using prolonged depolarizing and hyperpolarizing current injections (600 ms). PNs typically display broad (~1.2 ms), accommodating action potentials (APs) in combination with long afterhyperpolarizing potentials (AHPs) (Sah et al., 2003). In experiments utilizing tetrodotoxin (TTX) to record AP-independent synaptic events or that used Cesium-based intracellular solution, visual identification combined with physiological responses to hyperpolarizing current injections recorded within 30 seconds of membrane rupture were used exclusively to identify PNs. GABAergic tonic currents and spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in voltage clamp and isolated by blocking ionotropic glutameric transmission with 6,7-dinitroquinoxaline-2,3-dione (DNQX) (20 µM final concentration, AMPA/kainate antagonist, Tocris Bioscience) and DL-2-amino-5-phosphonopentanoic acid (APV) (50 µM final concentration, NMDA receptor antagonist, Tocris Bioscience) in the ACSF. To determine the effect of α5-GABA<sub>A</sub> receptor blockade on sIPSCs (Figure 9), a K-Gluconate based intracellular solution was used (in mM): K-Gluconate (70), KCl (70), HEPES (10), EGTA (1), MgCl<sub>2</sub> (2), Mg-ATP (4), Na-GTP (0.3), E<sub>Cl</sub>= -16 mV. To investigate the role of α5- GABA<sub>A</sub> receptors on sIPSCs recorded at the soma (Figure 10), a Cesium-chloride based intracellular solution was used (in mM): CsCl (135), HEPES (10), EGTA (10), QX-314 (5), MgCl<sub>2</sub> (2), Mg-ATP (4), and Na-GTP (0.3), E<sub>Cl</sub>= 0 mV. This solution reduces potassium channel currents allowing better visualization of distal events recorded at the soma. Both intracellular solutions allow
visualization of GABAergic currents as downward when the holding potential is near rest (-70 mV to -60 mV, i.e.).

To evaluate the role of α5-GABA<sub>A</sub> receptor-mediated tonic conductance in control of IPSC parameters recorded at the soma, AP-dependent IPSCs were recorded with K-Gluconate based internal solution alone (sIPSCs) and then in the presence of 1 µM tetrodotoxin (TTX) in order to block sodium channels (AP-independent miniature IPSCs (mIPSCs)). Subsequent experiments then utilized the CsCl based internal solution to better visualize distal-originating events at the soma by decreasing potassium conductance (Nicoll et al., 1993). IPSCs were analyzed for changes in frequency, amplitude, and kinetics before and after the application of α5ia (1.5 µM). For IPSC measurements drugs were applied locally via gravity fed Y-tube application and for evoked conductance experiments, drugs were bath applied.

Evoked conductance was derived from the slopes of synaptic current/voltage plots utilizing methods similar to those described in (Wehr and Zador, 2003; Cruikshank et al., 2007; Jiao et al., 2011). Excitatory and inhibitory components of total evoked synaptic conductance were then determined based on assumed reversal potentials for excitation and inhibition (Figure 1). First, synaptic currents were evoked in voltage clamp mode with at least 3 different holding potentials (typically -20 mV, -45 mV, -70 mV) utilizing a Cs-Gluconate based internal solution composed of (in mM): 130 Cs-gluconate, 4 KCl, 2 NaCl, 10 HEPES, 0.2 EGTA, 0.2 QX-314 (Br-), 4 ATP-Mg, 0.3 GTP-Tris and 14 phosphocreatine-Tris (pH 7.25, 290 mOsm, E<sub>Cl</sub> = -69 mV); and with 50 µM APV in the bath to remove any nonlinearities in the I/V relationship introduced by activation of synaptic NMDA receptors. Evoked currents were initiated by a 25-µM diameter concentric bipolar Pt-Ir external stimulating electrode (FHC) placed in the external capsule (mostly cortical inputs) at the level of the central nucleus of the amygdala. Threshold
stimulus intensity was determined using single 200 µS pulses at a holding potential of -70 mV (reversal potential of IPSCs) and was considered to be the stimulus amplitude at which there occurred a ~50% failure rate of monosynaptic evoked excitatory postsynaptic currents (eEPSCs), typically 15-25 µA. Stimulus intensity was then adjusted to 4 times the measured threshold amplitude for the duration of the experiment.

Stimuli were delivered at 3 to 4 different holding potentials using a step protocol beginning with the highest holding potential and descending to the lowest in 25 mV increments (i.e. 5 mV, -20 mV, -45 mV, -70 mV). Steps lasted at least 10 seconds at each holding potential before the stimulus was delivered and each stimulus was preceded 500 ms by a 5 mV biphasic voltage step to assess input resistance (R_in). The protocol was repeated 6-15 times (typically 10 times) and the responses at each step were averaged (Figure 8A). Series resistance was also determined before each group of descending voltage steps with the membrane test feature of Pclamp 9 using a 20 mV biphasic pulse at 20 Hz. Series resistance was compensated for offline to wholly account for voltage errors due to series resistance versus on-line methods that can leave up to 50% of the voltage error uncompensated. Holding potential was compensated for using the following equation:

\[ V_{corr}(t) = V_{rec}(t) - I_{rec}(t) \times R_s, \]

where \( V_{corr}(t) \) is the corrected voltage at time \( t \), \( V_{rec}(t) \) is the recorded voltage at time \( t \), \( I_{rec}(t) \) is the recorded current at time \( t \), and \( R_s \) is the series resistance measured before each set of voltage steps using the membrane test. Next, recorded current was compensated for input resistance and thus any nonsynaptic current affecting somatic voltage change (McNaughton et al., 1981) using the following equation to calculate the evoked synaptic current at each holding potential:

\[ I_{syn}(t) = \Delta I_{rec}(t) - \Delta V_{corr}(t) / R_{in}, \]
where \( I_{\text{syn}}(t) \) is the derived synaptic current at time \( t \), \( R_{\text{in}} \) is the input resistance derived using Ohm’s Law from the 5 mV voltage step preceding the stimulus,

\[
\Delta I_{\text{rec}}(t) = I_{\text{rec}}(t) - I_{\text{rec}}(\text{baseline, 10 ms before stimulus}),
\]

and \( \Delta V_{\text{corr}}(t) = V_{\text{corr}}(t) - V_{\text{corr}}(\text{baseline, 10 ms before stimulus}) \).

After deriving \( I_{\text{syn}}(t) \), an I-V curve for each point in the average response was generated using \( I_{\text{syn}}(t) \) values and corresponding \( V_{\text{corr}}(t) \) values (\( I_{\text{syn}}(t) \) vs \( V_{\text{corr}}(t) \)). The most depolarizing step (5 mV) was removed from analysis because as has been reported previously, the most depolarizing step introduced nonlinearities into the I-V relationship related to high voltage escape during synaptic responses (Cruikshank et al., 2007). The slope of the I/V relationship at each time point was the total synaptic conductance at each time point, \( G_{\text{syn}}(t) \) (Figure 8C). The X-intercept of the I-V plot was the synaptic reversal potential at each time point, \( E_{\text{syn}}(t) \). The excitatory synaptic reversal potential, \( E_{\text{e}} \), was assumed to be 0 mV and the inhibitory synaptic reversal potential, \( E_{\text{i}} \), was assumed to be -69 mV based on the calculated reversal potential of Cl- ions for the Cs-Gluconate based intracellular solution. Using these measures and the following equations (Cruikshank et al., 2007), total conductance (\( G_{\text{syn}}(t) \)) and the excitatory (\( G_{\text{e}}(t) \)) and inhibitory (\( G_{\text{i}}(t) \)) components of that conductance were calculated and plotted:

\[
G_{\text{syn}}(t) = G_{\text{e}}(t) + G_{\text{i}}(t)
\]

\[
E_{\text{syn}}(t) = \frac{G_{\text{e}}(t) \cdot E_{\text{e}} + G_{\text{i}}(t) \cdot E_{\text{i}}}{G_{\text{e}}(t) + G_{\text{i}}(t)}
\]

therefore,

\[
G_{\text{e}}(t) = G_{\text{syn}}(t) \cdot \left( \frac{E_{\text{syn}}(t) - E_{\text{i}}}{E_{\text{e}} - E_{\text{i}}} \right)
\]
\[ G_i(t) = G_{\text{syn}}(t) \ast \left( \frac{E_{\text{syn}}(t) - E_e}{E_i - E_e} \right) \]

**Statistical Analysis.** All recordings were analyzed off-line (Clampfit v 9.2, Molecular Devices, Minianalysis v 6.0.7, Synaptosoft, Microsoft Excel, and Matlab). Expressed values are the means ± standard error (SE). Statistical analyses utilized the two-tailed Student’s t test or the nonparametric Mann-Whitney U test where appropriate for measures of tonic currents, sIPSC amplitudes, and properties of synaptic conductance (Origin v 7.0552 OriginLab). The Kolmogorov–Smirnov test (K-S test) was used to compare probability distributions of frequency, amplitude, and decay of sIPSCs and mIPSCs (Matlab) (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

C. RESULTS

**α5-GABA\(_A\) receptors regulate synaptic efficacy in BLA principal neurons**

In addition to the change in holding current observed in the previous experiment by reducing α5-GABA\(_A\) receptor activity (Chapter II, Figure 6), we surprisingly observed an apparent increase in amplitude of GABA\(_A\) receptor-mediated spontaneous inhibitory post-synaptic currents (sIPSCs) as well in both WT and *Fmr1* KO PNs (Figure 6A,B (α5ia)) (Martin et al., 2012). We explored this observation in more detail by first recording sIPSCs with our standard potassium-gluconate (K-gluc) intracellular solution in our recording pipettes in the absence of exogenous GABA (Figure 9). Under these conditions we did not observe any significant changes in holding current after application of the α5-subunit specific inverse agonist α5ia (Figure 9A). However in both WT and *Fmr1* KO principal neurons, reduction of α5-subunit containing receptor activity caused an overall *increase* in inhibitory synaptic efficacy as recorded from the soma. Specifically, we observed a decrease in the inter-event interval of...
sIPSCs indicating an increase in sIPSC frequency in both WT and *Fmr1* KO cells. This increase was more significant in the WT than *Fmr1* KO principal cells (Figure 9A,B; WT: baseline, 203.25 ± 3.65 ms, n = 4498 events (from 13 cells); α5ia, 153.54 ± 2.63 ms, n = 5203 events (from 13 cells), K-S test, p <<< 0.0001; *Fmr1* KO: baseline, 244.57 ± 6.86 ms, n = 1499 events (from 7 cells); α5ia, 225.7 ± 6.26 ms, n = 1601 events (from 7 cells), K-S test, p = 0.02) (Martin et al., 2012). Additionally, the amplitude of these sIPSCs also increased in WT and *Fmr1* KO cells on α5ia application, however only the WT group showed a statistically significant increase (Figure 9A,C; WT: baseline, 19.36 ± 1.91 pA; α5ia, 21.52 ± 1.73 pA, n = 13 cells, paired t-test, p = 0.021; *Fmr1* KO: baseline, 15.36 ± 1.95 pA; α5ia, 16.44 ± 2.23 pA, n = 7 cells, paired t-test p = 0.182) (Martin et al., 2012). Furthermore the weighted decay time constant (tauD) of sIPSCs increased slightly but significantly after α5ia application while the mean 10-90% rise time showed no increase in either WT or *Fmr1* KO cells (Figure 9D, Table 2) (Martin et al., 2012). In general our results are consistent with our previous published data indicating that *Fmr1* KO BLA principal neurons display decreased baseline inhibitory synaptic efficacy compared to their WT counterparts (Olmos-Serrano et al., 2010) (inter-event interval, Figure 9B; WT baseline vs *Fmr1* KO baseline, K-S test, p <<< 0.0001; amplitude, Figure 9C; WT baseline vs *Fmr1* KO baseline, t-test, p = 0.10) with α5ia having the greatest effect on WT cells consistent with the conclusion that *Fmr1* KO principal neurons express less α5-GABA_A receptors.

Both pre- and/or postsynaptic actions of α5-GABA_A receptors could underlie the rapid increase in inhibitory synaptic efficacy (frequency and amplitude) following reduction of α5-containing GABA_A receptor activity. Since tonic conductance can modulate neuronal gain (Mitchell and Silver, 2003), we reasoned that the increase in inhibitory efficacy may have resulted from the removal of an α5-GABA_A receptor mediated conductance that unmasked more
distal synaptic events increasing detectability of our recordings. To test the possibility that blockade of α5-containing receptors uncovers more distally originating synaptic events, we recorded sIPSCs again in the absence of exogenous GABA using a cesium chloride (CsCl) based internal recording solution in our recording pipettes instead of potassium based (K- Gluc) as utilized above (Figure 9) to reduce potassium currents that filter current changes originating far from the recording site (Nicoll et al., 1993; Stuart and Spruston, 1998; Day et al., 2006). If a more complete sample of synaptic events including those that originate farther from the recording site are observed under baseline conditions with CsCl recording solution, any effect by α5-GABA_A receptors revealed by application of α5ia should be reduced. As we expected under these conditions that allow maximum detection of IPSCs, the increase in efficacy previously observed with K-Gluc intracellular solution after α5-GABA_A receptor blockade was partially occluded (Figure 10). Rather than an increase in frequency, we observed a slight decrease in frequency (Figure 10A, solid lines; inter-event interval: baseline, 55.27 ± 0.40 ms, n = 14293 events (from 8 cells); α5ia, 61.19 ± 0.46 ms, n = 14150 events (from 8 cells), K-S test, p << 0.0001) and no significant change in sIPSC amplitude (Figure 10B, left; amplitude: baseline, 34.13 ± 2.99 pA; α5ia, 33.41 ± 2.56 pA, n = 8 cells, paired t-test, p = 0.29). However there was still a slight increase in sIPSC tauD with α5- GABA_A receptor blockade (Figure 10C, left, Table 1) with no significant change in event 10-90% rise time (Martin et al., 2012).

Based on the evidence that simply replacing pipette potassium with cesium can prevent frequency increases in response to α5-GABA_A receptor blockade, it is unlikely that presynaptic mechanisms are involved in the observed increase in inhibitory efficacy (Figure 9). However, the possibility remains that an increase in the excitability of presynaptic interneurons may be induced by α5-GABA_A receptor blockade if these cells are heavily modulated by an α5-specific
tonic conductance. Reduction of that conductance might thereby increase the sIPSC frequency on principal neurons via an increase in presynaptic APs and GABA release. To control for this possibility we recorded miniature inhibitory postsynaptic currents (mIPSCs) with the CsCl-based recording solution in the presence of tetrodotoxin (TTX, 1 µM) to block APs. AP-independent mIPSCs showed a slight decrease in frequency rather than an increase similar to sIPSCs recorded with CsCl (Figure 10A, dotted lines; interevent interval: baseline, 96.71 ± 1.31 ms, 6304 events (from 9 cells); α5ia, 108.80 ± 1.61 ms, 6219 events (9 cells), K-S test, p = 0.0006). Under these conditions we also observed a slight increase in amplitude (Figure 10B, amplitude baseline, 31.05 ± 2.51 pA; α5ia, 33.22 ± 3.06 pA, n = 9, paired t-test, p = 0.027), an increase in tauD (Figure 10C, Table 2), and no change in event 10-90% rise time (Martin et al., 2012). We subsequently recorded mIPSCs using the K-Gluc based solution to record primarily more proximal somatic synapses. In these recording conditions we observed a trend towards an increase in frequency that was not significant (inter-event interval: baseline, 155.36 ± 2.47 ms, n = 4668 events (from 6 cells); α5ia 151.68 ± 2.31 ms, n = 4809 events (from 6 cells), K-S test, p = 0.159), no significant change in amplitude (amplitude: baseline, 21.69 ± 1.22 pA; α5ia, 21.10 ± 1.05 pA, n = 6 cells, paired t-test, p = 0.94), tauD, or event 10-90% rise time (Table 2) after the application of α5ia to block α5-GABA_A receptors (Martin et al., 2012).

α5-GABA_A receptors affect evoked response kinetics and synaptic balance in BLA PNs

BLA PNs receive a variety of direct afferent excitatory input from hippocampus and associational cortex traveling within the external capsule (Ottersen, 1982; McDonald and Mascagni, 1996; McDonald et al., 1996) as well as projections from output neurons in the lateral nucleus of the amygdala that relay sensory cortical and thalamic input to the BLA (Krettek and
Price, 1978). BLA PNs also receive a variety of heterogeneous feedforward and feedback inhibitory inputs from a diverse pool of interneurons that preferentially synapse in the perisomatic, proximal dendritic, or distal dendritic compartments of these cells (McDonald et al., 2005; Muller et al., 2007; Manko et al., 2012). The distinct ability of α5-GABA_A receptors to regulate synaptic efficacy in BLA PNs places the receptor subtype in a position to modulate integration of excitatory and inhibitory inputs to these cells locally on dendrites and at the soma and axon initial segment. Therefore, we examined if α5-GABA_A receptor mediated conductance affects the integration of excitatory and inhibitory synaptic transmission in BLA PNs by measuring evoked synaptic conductance in the presence or absence of α5-GABA_A receptor activity in both WT and Fmr1 KO amygdala slices (Figure 11) (Martin et al., 2012). Synaptic currents were evoked in BLA PNs at 3 different holding potentials in voltage clamp (-20 mV, -45 mV, -70 mV) by stimulating the external capsule at 4 times the threshold to evoke an inward excitatory current recorded at -70 mV (the reversal potential for Cl-) (Figure 11A,B inset). This stimulus strength was used because it reliably evoked strong outward inhibitory currents when the cell was held at -20 mV or above. Current thresholds for WT and Fmr1 KO slices were not significantly different from each other (WT: 19.1 ± 1.00 µV, n = 16; Fmr1 KO: 21.7 ± 1.76 µV, n = 18, p = 0.22). Total synaptic (Gtot), excitatory (Ge), and inhibitory (Gi) conductance was derived from I-V curves taken from average traces at each holding potential (7-15 sweeps/potential) using established methods (see Methods Section) (Figure 11A,B, inset). The total conductance, Gtot, was large with a fast onset (~3 ms, Figure 4F) and slow latency to peak (~11-14 ms) in both WT and Fmr1 KO slices. Ge and Gi onset occurred within 1-2 ms of each other (Figure 11A,B) indicating that stimulated external capsule afferents monosynaptically
innervate PNs and GABAergic interneurons of the BLA (Gabernet et al., 2005; Cruikshank et al., 2007).

Since overall inhibitory efficacy is decreased in Fmr1 KO PNs (Olmos-Serrano et al., 2010), we expected that total E/I synaptic balance in Fmr1 KO slices might be reduced owing to a severely reduced basal inhibitory component. To our surprise though, no significant differences in peak levels of total, excitatory, or inhibitory conductance existed between genotypes (Figure 11C, Table 3, reported as conductance density to control for differences in cell size/capacitance, nS/pF; WT: n = 9; Fmr1 KO: n = 11). Overall E/I balance as measured by the ratio of Ge to Gi over time (Figure 11A,B,C) was also unchanged between WT and Fmr1 KO genotypes (Area E/I (integral) = (Ge(ns) / time(ms))/(Gi(ns) / time(ms)) (Martin et al., 2012).

Instead of total conductance and overall E/I balance differences we did observe a striking difference between genotypes in the kinetics of the evoked responses. Evoked conductance in Fmr1 KO PNs showed a significant decrease in the time between peak Ge and peak Gi compared to WT PNs (Figure 11Aii,Bii,D; Gi peak time – Ge peak time, WT baseline: 2.41 ± 0.54 ms; Fmr1 KO baseline: 1.19 ± 0.28 ms; p = 0.023). The decreased Ge peak to Gi peak time was associated with significantly decreased E/I ratio at the Ge peak compared to WT (Figure 11E; WT baseline: 0.48 ± 0.05; Fmr1 KO baseline: 0.32 ± 0.02; p = 0.005). We investigated the underlying cause of this alteration in balance by examination of the conductance onset and peak latencies of each component conductance (Ge and Gi). We determined that the difference in Ge and Gi response kinetics correlated with a significant decrease in the Ge peak latency in WT PNs compared to Fmr1 KO PNs (Figure 11F; WT baseline: 11.72 ± 0.73 ms; Fmr1 KO baseline: 14.09 ± 1.06 ms; p = 0.048) but without a difference in the latency of Gi (Figure 11F; Gi latency WT baseline: 14.22 ± 0.58 ms; Fmr1 KO baseline: 15.22 ± 0.99 ms; p = 0.38). Therefore, these
data suggest that differences in kinetics in WT and Fmr1KO slices depend solely on the faster latency of Ge (faster rise time) in WT PNs and not on Gi latency (Martin et al., 2012). The increased Ge kinetics result in an increased window for cell responsiveness in WT PNs in which there is less overlap of excitation and inhibition compared to Fmr1KO PNs resulting in an increase in the E/I ratio at the Ge peak for WT neurons (Figure 11E). We reasoned that if α5-GABA_A receptors heavily regulate synaptic efficacy and integration, these receptors might underlie these kinetics, providing a tonic conductance to maintain local membrane resistance and ensure faster synaptic kinetics thereby controlling the spread of synaptic activity.

To determine whether this difference in Ge kinetics and altered E/I balance is related to the reduction/loss of α5-GABA_A receptors in Fmr1KO PNs we next examined evoked synaptic conductance in a separate set of cells in the absence of α5-GABA_A receptor activity. In recordings from both WT and Fmr1KO slices we bath-applied α5ia (1.5 µM) to the bath solution after threshold stimulation levels were established, then assessed evoked synaptic conductance as described above. The threshold stimulus did not significantly change for either group after application of α5ia (WT before: 21.1 ± 1.76 µV; WT after α5ia: 22.2 ± 1.31, n = 7; p = 0.66; Fmr1 KO before: 18.1 ± 2.85 µV; Fmr1 KO after α5ia: 18.7 ± 2.97 µV, n = 7; p = 0.89; WT vs Fmr1 KO, p = 0.39). Measurements of peak conductance density (Gtot, Ge, and Gi) did not differ significantly from WT baseline or Fmr1KO baseline groups for either genotype in the presence of α5ia (Figure 11C, Table 3) so α5-GABA_A receptor blockade does not affect overall conductance or components of that conductance (Ge and Gi) (Martin et al., 2012).

In WT slices, blockade of α5-GABA_A receptors significantly reduced the Ge peak to Gi peak time compared to the WT baseline group (Figure 11Aii,Aiii,D; WT α5ia: 0.67 ± 0.33 ms, n
Interestingly, peak-to-peak times mimicked those of both the *Fmr1* KO baseline and *Fmr1* KO α5ia conditions (WT α5ia vs *Fmr1* KO baseline, p = 0.26; vs *Fmr1* KO α5ia: 0.86 ± 0.18 ms, n = 7 cells, p = 0.63). Similarly blockade of α5-GABA<sub>A</sub> receptors in WT cells also resulted in significant reduction of the E/I ratio at the Ge peak compared to the WT baseline group and *Fmr1* KO slices in either the baseline or α5ia condition (Figure 11E; WT α5ia: 0.31 ± 0.03, vs WT baseline, p = 0.016, vs *Fmr1* KO baseline, p = 0.79; *Fmr1* KO α5ia: 0.37 ± 0.03, p = 0.19). Once again this reduction correlated with an increased latency to the Ge peak similar to both *Fmr1* KO groups (Figure 11F; WT α5ia: 13.8 ± 0.68 ms, vs WT baseline, p = 0.03, vs *Fmr1* KO baseline, p = 0.84; *Fmr1* KO α5ia: 13.2 ± 0.82 ms, p = 0.60) but without a change in the latency to the Gi peak (WT α5ia: 14.5 ± 0.54 ms, vs WT baseline, p = 0.67, vs *Fmr1* KO baseline, p = 0.58; *Fmr1* KO α5ia: 14.2 ± 0.79 ms, p = 0.69).

In *Fmr1* KO slices, blockade of α5-GABA<sub>A</sub> receptors did not result in any significant changes in any measure compared to the *Fmr1* KO baseline groups (Figure 11D,E,F; Ge to Gi peak time, p = 0.19; E/I ratio at Ge peak, p = 0.08; latency to Ge peak, p = 0.29; latency to Gi peak, p = 0.43) indicating a lack of effect of α5-GABA<sub>A</sub> receptor blockade on their responses consistent with a decreased functional expression of these receptors (Martin et al., 2012).

These data show that α5-containing GABA<sub>A</sub> receptors are involved in controlling evoked synaptic conductance balance in BLA PNs by primarily modulating Ge latencies. Summary data from all cells reveals that Ge latency negatively correlates with E/I balance at the Ge peak (Figure 11G) such that longer Ge latencies in the absence of α5-GABA<sub>A</sub> receptor activity generally coincide with lower E/I ratios (Martin et al., 2012).

**D. DISCUSSION**
This study reports three basic findings. First, for the first time we show that $\alpha_5$-GABA$_A$ receptors preferentially mediate tonic conductance in more distal cellular compartments in BLA PNs as opposed to somatic regions. Second, we provided evidence that this $\alpha_5$-GABA$_A$ receptor mediated tonic conductance controls synaptic balance by maintaining a strong temporal separation between excitatory and inhibitory synaptic inputs that correlates with increased E/I conductance balance at the peak of excitation. This separation is reduced in Fmr1 KO PNs in correlation with reduced E/I balance and deficient functional $\alpha_5$-GABA$_A$ receptor expression. Therefore tonic $\alpha_5$-GABA$_A$ receptors constitute a critical regulator of synaptic integration in BLA PNs, the loss of which may at least partially underlie problems with amygdala function in FXS.

**Modulation of synaptic efficacy in the BLA by $\alpha_5$-GABA$_A$ receptors**

Although $\alpha_5$-GABA$_A$ receptor-mediated tonic currents make up a very small proportion of the total tonic current in BLA PNs, we reveal a unique role of these receptors in these cells to regulate postsynaptic efficacy (Figure 9,10) (Martin et al., 2012). Blockade of $\alpha_5$-GABA$_A$ receptors in BLA PNs produced an increase in sIPSC efficacy consisting of increases in the frequency, amplitude, and tau$_D$ of events when recorded using K-Gluc based recording solution (Figure 9). Although pre- and postsynaptic mechanisms could mediate these changes, our data is consistent with a postsynaptic mechanism involving a reduction in $\alpha_5$-GABA$_A$ receptor-mediated conductance that increases membrane resistance (De Schutter, 2002; Wisden et al., 2002), thereby improving voltage control of our recordings and enabling synaptic events previously undetectable at the soma to suddenly become measurable. Data recorded using CsCl recording solution and therefore with stronger baseline voltage control (Nicoll et al., 1993)
(Figure 10) supports this conclusion because these sIPSCs did not show an increase in frequency that would indicate a possible presynaptic effect of α5ia on local BLA interneuron excitability. Yet we consistently observed increases in τD (Table 2) consistent with an increase in membrane resistance relating to postsynaptic α5-GABA_A receptor blockade. Recordings of mIPSCs with CsCl corroborated this finding showing increases in amplitude and τD after α5ia application. Interestingly, in both sIPSC and mIPSC recordings using CsCl (Figure 10) we observed a decrease in event frequency with α5ia application instead of a reduced increase or no change as we had expected. This relatively small frequency reduction may indicate the presence of a subpopulation of inhibitory synapses highly enriched with α5-GABA_A receptors that are blocked by α5ia. Given that the decrease was only observed in the presence of CsCl, this subpopulation is likely more distally located. Evidence shows that α5-GABA_A receptors express specifically in dendritic synapses in both cortical (Ali and Thomson, 2008) and hippocampal (Brünig et al., 2002; Christie and De Blas, 2002; Serwanski et al., 2006) pyramidal cells in synapses opposed to specific dendritically targeting interneuron subpopulations. Similar interneuron populations (i.e. somatostatin positive) have also been identified in the BLA (Muller et al., 2007; Spampanato et al., 2011) however no studies to date have explored the possible inclusion of α5-GABA_A receptors in their synapses. Further evidence supports a preferential localization of α5-GABA_A receptors to dendrites in BLA PNs. Our results show that reducing a tonic α5-mediated membrane conductance with α5ia increases visibility (frequency and amplitude) of sIPSCs recorded at the soma only when recording primarily proximal synaptic events with relatively weak membrane voltage control (K-Gluc recording solution) (Figure 9). Reducing membrane conductance by blocking α5-GABA_A Rs tightens the voltage control in more distal regions thereby increasing event frequency. This occurrence is consistent with a
preferential modulation of events originating farther away from the somatic recording site (i.e. proximal and distal dendrites) by $\alpha_5$-GABA$_A$ receptors since strong voltage control recordings with CsCl confine the effect of $\alpha_5$ia to amplitude and/or $\tau_{D}$ increases only. By detecting the maximum number of events under baseline conditions including more distal dendritic events, the observable change induced by a decrease in tonic membrane conductance by $\alpha_5$ia is reduced.

In general the effects of $\alpha_5$ia were muted in recordings of mIPSCs in the presence of TTX with essentially no effect of the compound observed in K-Gluc recordings. We have previously shown that essentially all of the tonic current in BLA PNs relies on action potential-dependent release of GABA (Chapter II) (Olmos-Serrano et al., 2010) so tonic $\alpha_5$-GABA$_A$ receptors were probably not sufficiently activated in our mIPSC recordings to the level necessary to observe an extensive effect of their blockade on synaptic efficacy. The small effects we did observe in our CsCl recordings with stronger voltage control (increased amplitude and $\tau_{D}$ of events) therefore further underscore the preferential regulation of more distal non-somatic membrane by tonic $\alpha_5$-GABA$_A$ receptors.

$\alpha_5$-GABA$_A$ receptor transmission maintains synaptic balance in BLA PNs

Numerous studies in brain areas analogous to the basolateral amygdala illustrate that E/I input timing can control neuronal tuning. For example in both the hippocampus (Pouille and Scanziani, 2001) and cortex (Gabernet et al., 2005) the kinetics of strong feedforward inhibition and excitation determines the window of responsiveness for PNs, permitting responses to a preferred set of inputs that arrive within the Ge to Gi integration window and suppressing others (Moore et al., 1999). Many pre- and postsynaptic factors such as the strength and kinetics of excitatory and inhibitory synaptic conductance (Cruikshank et al., 2007) as well as their relative
subcellular location (Pouille and Scanziani, 2001) may affect this separation. Here we show for the first time a distinct postsynaptic α5-GABA_\text{A} receptor-dependent mechanism to affect synaptic conductance balance in BLA PNs. Evoked stimulation of the external capsule afferents to the BLA produced a mixed excitatory and inhibitory conductance whose kinetics and E/I balance were influenced by the relative activity of α5-GABA_\text{A} receptors. Importantly, conductance kinetics in Fmr1 KO PNs that lack sufficient α5-GABA_\text{A} receptor activity show reduced Ge to Gi separation consistent with a restricted window of responsivity to coincident synaptic input (Pouille and Scanziani, 2001).

The excitatory conductance evoked by our stimulation of external capsule afferents to BLA PNs was tightly controlled by a large, fast inhibitory conductance (Figure 11) – this is expected given the considerable amount of feedforward inhibition from intercalated and local interneurons that tightly controls PN activity in the BLA (Li et al., 1996; Lang and Paré, 1997; Royer et al., 1999; Marowsky et al., 2005). External capsule stimulation produced a similar conductance profile to that generated in comparable, well-characterized feedforward circuits in Layer IV somatosensory cortex and hippocampal area CA1 (Pouille and Scanziani, 2001; Gabernet et al., 2005; Cruikshank et al., 2007). Our responses differed from those areas however with a longer overall latency to peak conductance (11-14 ms vs 5-7 ms) and longer decay times (100-200 ms vs 40-60 ms) even though stimulus strength (~40-120 μA, 100-200 μS) was comparable. This longer time course likely occurred either because of the preservation or presence of many more excitatory afferents and synapses onto PNs in our amygdala slices, or alternatively, as a consequence of unique BLA network architecture. PNs project a substantial number of synapses to dendrites of neighboring PNs (Smith and Paré, 1994) as well as local, primarily parvalbumin-positive inhibitory neurons that contribute feedback inhibition (Samson et
al., 2003). Therefore reverberant connections may have potentially reinforced our strong stimulus and recruited a larger summation of feedforward and feedback excitatory/inhibitory transmission. In general our responses are physiologically similar to in vivo synaptic responses of BLA PNs to robust sensory stimulation that induces mixed coincident excitatory/inhibitory synaptic conductance led by excitation and lasting ~500-1000 ms (Windels et al., 2010).

Despite known deficits in overall inhibitory efficacy in Fmr1 KO PNs (Olmos-Serrano et al., 2010) we unexpectedly observed no significant differences in overall conductance or Ge/Gi balance over time (Figure 11C). The presence of robust feedback inhibitory connections (Smith et al., 2000; Samson et al., 2003) and heavy PN to PN innervation (Smith and Paré, 1994) might underlie this discrepancy by overpowering individual synaptic deficiencies in Fmr1 KO slices with substantial excitatory drive. Lower stimulus levels or repetitive stimulation might be required then to observe underlying inhibitory deficits in Fmr1 KO PNs that would produce overall E/I imbalances in favor of excitation or reveal significantly reduced levels of synaptic conductance compared to WT.

Most importantly, despite an unchanged overall E/I balance, Fmr1 KO PNs display a significantly restricted integration window (Figure 11D,E) (Pouille and Scanziani, 2001; Gabernet et al., 2005) that results in more efficient containment of excitation reflected by a decreased E/I balance at the Ge peak. Reduction of α5-GABA_A receptor activity with α5ia in WT PNs reproduced this restricted Ge-Gi separation and E/I balance consistent with at least a partial role of α5-GABA_A receptors in maintenance of the response window. Importantly α5-GABA_A receptor blockade only significantly affected the excitatory conductance kinetics (Ge) in our evoked synaptic responses (Figure 11F) consistent with previous studies in Layer IV somatosensory cortex that highlight a similarly distinctive role of Ge latency to regulate the Ge-
Gi separation (Cruikshank et al., 2007). In the BLA a substantial portion of excitatory synapses exist on PN dendrites and dendritic spines versus the soma (Smith and Paré, 1994) and a significant number of feedforward and feedback inhibitory connections synapse on or near the soma (Smith et al., 2000). Therefore this preferential modulation of Ge versus Gi again supports a more dendritic concentration of α5-GABA_A receptors to primarily affect Ge latencies versus Gi latencies that in contrast remain unchanged.

Many different synaptic and cell intrinsic mechanisms could modulate Ge latency. In the BLA, we show that α5-GABA_A receptor activity represents one postsynaptic mechanism of Ge control. Tonic conductance modulates gain by altering membrane resistance thereby affecting synaptic efficacy and integration (De Schutter, 2002; Hamann et al., 2002; Wisden et al., 2002; Mitchell and Silver, 2003). The loss of α5-GABA_A receptor activity likely increases membrane resistance locally thereby increasing the membrane time constant, τ_m, and slowing the response rise time. This change supports the increased summation of synaptic events and longer Ge latency. Changes in τ_\text{D} in the presence of α5ia in our IPSC recordings further support this phenomenon. Although we observe no significant changes in Ge amplitude with α5ia application as expected with an increase in τ_m, any amplitude changes may be lessened by nearby non-α5 mediated inhibitory synaptic or extrasynaptic conductance. Notably although our data shows a clear role of α5-GABA_A receptors to modulate Ge kinetics, we cannot distinguish the participation of extrasynaptic versus synaptic receptors in this phenomenon. However given the power of tonic conductance over phasic conductance (Farrant and Nusser, 2005) and our failure to observe significant changes in synaptic efficacy when tonic conductance is significantly decreased in the presence of TTX, tonic α5-GABA_A receptor activity remains the more likely candidate.
Since α5ia had no further effect on the already altered kinetics and balance in Fmr1 KO PNs the lack of α5-GABA_A receptor activity in these cells may play a role in their significantly reduced Ge-Gi separation. However other synaptic or intrinsic changes in Fmr1 KO PNs and the BLA network could affect Ge latency and Ge-Gi separation independently of decreased α5-GABA_A receptor-mediated conductance. For instance, Fmr1 KO PNs in the lateral nucleus of the BLA have decreased expression of the AMPA receptor subunit, GluR1, and a lower presynaptic release probability of glutamate (Suvrathan et al., 2010) that results in a lower excitatory synaptic efficacy and could slow Ge latencies. In addition to these issues, a decrease in synapse number, a preferential location of synapses more distally from the soma, or a decreased synaptic density of excitatory synapses could also slow Ge latency, however these possibilities have yet to be explored in the Fmr1 KO BLA. A baseline decrease in membrane input resistance in Fmr1 KO PNs versus WT would also support slower Ge kinetics however no significant differences exist (Chapter II, (Olmos-Serrano et al., 2010)). Furthermore, possible increased excitability of PNs in response to α5-GABA_A receptor blockade does not likely affect Ge because in our recordings, we observe no corresponding increase in Ge or Gi amplitude compared to baseline or changes in Gi kinetics that would be expected with a corresponding increase in excitatory drive. Also, α5ia application did not affect threshold stimulation levels indicating little to no change in PN excitability.

**Physiological relevance of altered evoked conductance kinetics in the FXS BLA**

We have identified increased excitatory synaptic summation and an E/I imbalance in BLA feedforward circuitry in the Fmr1 KO BLA that results in a tighter integration time window in PNs, both of which could affect many aspects of BLA processing related to FXS phenotypes.
Numerous studies reveal the importance of GABAergic inhibition to the regulation of synaptic plasticity in the BLA [reviewed in (Ehrlich et al., 2009)]. In the lateral amygdala for instance, PNs receive convergent cortical and thalamic sensory afferents in close proximity to each other (Humeau et al., 2005) and GABAergic feedforward inhibition isolates activity-dependent plastic changes to these afferents (Shaban et al., 2006; Shin et al., 2006). Changes in inhibition via synaptic plasticity or neuromodulators such as dopamine thereby gate cortical and thalamic synaptic integration (Bissière et al., 2003; Tully et al., 2007). BLA PNs similarly receive convergent inputs from cortical, hippocampal, and lateral amygdala, for example. The balance of these inputs on two distinct subclasses of BLA PNs modulates fear recall and fear extinction, respectively [reviewed in (Paré and Duvarci, 2012)]. Therefore a disrupted integration window established by feedforward transmission could adversely affect coordination of these inputs during fear processing preventing accurate synaptic integration or supporting generalization of plastic changes required for accurate fear expression and/or extinction, which might relate to heightened anxiety in FXS patients (Hagerman et al., 2009). We have demonstrated that α5-GABA\(_A\) receptor activity at least partially controls this temporal window. Additionally, since PN tonic GABAergic transmission is modulated by synaptic activity (Chapter II, Figure 4) (Olmos-Serrano et al., 2010) and Fmr1 KO PNs lack sufficient tonic α5-GABA\(_A\) receptor mediated transmission, these PNs may not only have more narrow integration windows, but also reduced ability to properly modulate that window in an activity dependent manner as demonstrated in the related layer IV somatosensory cortex feedforward circuit (Gabernet et al., 2005).

FXS is a pervasive neurodevelopmental disorder marked with increased anxiety and social avoidance – symptoms that suggest amygdala involvement. Decades of investigation
reveal how deficits in tonic GABAergic conductance affect E/I balance and excitability in multiple brain regions and disease states. We have previously illustrated that tonic GABA conductance augmentation can rescue BLA PN hyperexcitability in Fmr1 KO mice (Chapter II, Figure 7) (Olmos-Serrano et al., 2010). In the present study these data expand the role of defective tonic conductance in FXS for the first time to the dysregulation of E/I balance during feedforward synaptic transmission as well as identify feedforward E/I integration windows generally as a possible essential component of network function in the BLA. Therefore in terms of E/I imbalance, deficient tonic conductance not only influences increased cell excitability but may also adversely affect synaptic integration and neuronal coding central to amygdala based symptoms of FXS.
Figure 8. Derivation of evoked synaptic conductance. Evoked synaptic conductance was derived from synaptic currents recorded at 3 different holding potentials (A) in response to external capsule stimulation at 4x the threshold for EPSCs as demonstrated in this example (A-D). (B) I/V curves for each time point were calculated from averages of 6-15 responses at each holding potential. This example represents the time point at 50% of the maximum conductance (A, gray line). (C) Using Ohm’s law total (Gtotal, black), inhibitory (Gi, red), and excitatory (Ge, purple) synaptic conductance was calculated at each time point from the I/V curve using equations detailed in Materials and Methods. (D) The synaptic reversal potential (Esyn) over time used in the calculation shows that early in the response when Ge is highest, Esyn is closest to the reversal potential for EPSCs (~0 mV). Then as Gi dominates, Esyn moves closer to the reversal potential for IPSCs (~70 mV). Then finally as the response terminates, Esyn moves back to the reversal potential for our intracellular solution (~50 mV).
Figure 9. Blockade of α5-GABA<sub>A</sub> receptors increases GABAergic inhibitory efficacy as recorded at the soma. (A,B) Application of α5ia (1.5 µM) increases the frequency of sIPSCs in both WT and Fmr1 KO principal neurons of the BLA shown here as a decrease in the distribution of the cumulative probability of the Inter-Event Interval (B) before and after application of α5ia. (C) In the presence of α5ia amplitude also increases in both WT and Fmr1 KO cells. However, both frequency (B) and amplitude (C) changes are reduced in the Fmr1 KO cells versus WT. (D) Average event fits from WT and Fmr1 KO sIPSCs (WT: left, baseline (black solid), α5ia (red solid); Fmr1 KO: right, baseline (gray dotted), α5ia (red dotted)) show
slight but significant increases in decay constant tau (Table 2). *p < 0.05. (Taken from (Martin et al., 2012)).
<table>
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<th>α5ia</th>
<th>p value</th>
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**Table 2. Summary of α5ia induced changes in rise time and tauD.**

(Taken from (Martin et al., 2012)).
Figure 10. Recording IPSCs with CsCl-based pipette solution occludes increases in inhibitory efficacy in response to α5-\textit{GABA}_A receptor blockade. (A) Application of α5ia (1.5 μM) decreases rather than increases sIPSC (solid lines) and mIPSC (dotted lines) as observed with K-Gluc recordings. (B) Amplitude increases only occur in the presence of TTX in CsCl recordings (mIPSCs, right) whereas no significant changes in amplitude occur in sIPSC recordings after application of α5ia (left). (C) In both sIPSC (left) and mIPSC (right) recordings the decay constant τ_d increases slightly and significantly in response to α5ia application (Table 2). *p < 0.05. (Taken from (Martin et al., 2012)).
Figure 11. The presence of α5-GABAAR activity affects evoked response kinetics and synaptic balance. (A,B) Representative conductance measurements (Gtot, black; Ge, blue; Gi, red) derived from I/V curves taken from evoked responses recorded in voltage clamp at 3 different holding potentials (inset: -20 mV, black; -45 mV, red; -70 mV, green) from WT (A) and KO (B) PNs. (Aii,Bii) Representative examples of baseline conductance kinetics for WT
(Aii) and Fmr1 KO (Bii). (Aiii,Biii) Representative examples of conductance kinetics in the presence of α5-GABA\(_A\) receptor blockade (α5ia, 1.5 μM) in WT (Aiii) and Fmr1 KO (Biii) cells. (C) Conductance measurements of WT and Fmr1 KO cells reveal no significant differences in G\(_{\text{tot}}\), Ge, or Gi between genotypes or conditions (baseline or α5ia) (left, conductance density (nS/pF)). In addition overall E/I balance is similar among genotypes and conditions (right, Conductance Area: Ge (nS/ms)/Gi (nS/ms)). (D) Conductance kinetics demonstrate a significantly longer duration between Ge and Gi peaks in WT baseline cells compared to WT cells in the presence of α5ia or Fmr1 KO cells (*p < 0.05). (E) The E/I conductance ratio at the Ge peak is increased in WT baseline compared to WT cells in the presence of α5ia and Fmr1 KO baseline cells (WT baseline vs WT α5ia and Fmr1 KO baseline; *p < 0.05; vs Fmr1 KO α5ia; p = 0.06). (F) Increased Ge to Gi peak times in WT baseline cells associate with changes solely in the Ge latency (middle) and not with the Ge onset (left) or Gi latency (right) compared to other conditions (WT baseline vs WT α5ia and Fmr1 KO baseline; *p < 0.05; vs Fmr1 KO α5ia; p = 0.09). (G) Summary data from all cells indicate that Ge latency (F) and E/I ratio at peak Ge (E) are negatively correlated (linear regression, r = -0.587, p < 0.0001). (Taken from (Martin et al., 2012)).
<table>
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</table>

Table 3. Total conductance and excitatory/inhibitory components are not significantly different between WT and Fmr1 KO slices and $\alpha$5ia does not affect conductance in either genotype.

(Taken from (Martin et al., 2012)).
CHAPTER IV: SUMMARY AND DISCUSSION

A. SUMMARY

The results presented in this dissertation provide significant, novel insights into tonic GABAergic modulation of neuronal transmission in the BLA, a key brain region involved in FXS phenotypes. We report increased excitability in Fmr1 KO PNs associated with decreased tonic currents that is dramatically rescued with supplementation of δ-subunit mediated tonic current in these cells (Olmos-Serrano et al., 2010). Furthermore investigations of synaptic conductance in these PNs reveal that despite basal inhibitory deficits, E/I balance of evoked synaptic transmission remains broadly unchanged (Martin et al., 2012). However, enhanced excitatory synaptic kinetics linked with a deficit in α5-subunit mediated tonic GABAergic transmission in Fmr1 PNs emphasize a functional role of tonic transmission to affect synaptic integration and highlight a fundamental alteration in the Fmr1 KO amygdala that has important implications for amygdala function in FXS (Martin et al., 2012). Taken together our findings establish tonic GABAergic transmission in the BLA as an important mediator of neuronal excitability and synaptic integration as well as a promising therapeutic target for amygdala-based FXS symptoms.

B. THE STATE OF TONIC TRANSMISSION IN THE MOUSE BLA

In combination with a small amount of recent data regarding tonic GABAergic transmission in the amygdala (Marowsky et al., 2012), for the first time our data illustrate the state of this powerful inhibitory conductance natively in mouse BLA PNs (Figure 12) (Martin et al., 2012). Tonic GABAergic currents are comprised of at least 3 distinct GABA_A receptor subtypes in the BLA of WT animals. Firstly, the δ-subunit containing GABA_A receptor plays a
substantial role in PN tonic currents given that the δ-preferring GABA_A receptor agonist shifts the I/O function of these cells in response to constant current injection (Olmos-Serrano et al., 2010). Secondly, the α3 subunit-containing GABA_A receptor has recently been identified to participate in the tonic current in BLA PNs (Marowsky et al., 2012). Until recently α3 subunits were not typically known for extrasynaptic expression (Fritschy and Mohler, 1995). However, in PNs a substantial portion of α3 subunit-containing GABA_A receptors do not co-express with the inhibitory postsynaptic marker, gephyrin, suggesting they reside outside the synapse. Furthermore, application of the α3-specific agonist TP003 induces an increase in tonic current in these cells (Marowsky et al., 2012). Finally, BLA PNs express a third tonic GABAergic component common to the cortex (Sur et al., 1999; Chen et al., 2010) and the hippocampus (Caraiscos et al., 2004), the α5 subunit-containing receptor. This GABA_A receptor subunit shows more diffuse expression in the BLA than δ and α3 subunit-containing receptors (Fritschy and Mohler, 1995). Nevertheless we show that BLA PNs do have small α5 subunit-mediated tonic currents revealed by blockade of α5 subunit-containing receptors with the α5-specific inverse agonist, α5ia (Martin et al., 2012). Interestingly, based on immunohistochemical and electrophysiological data (Fritschy and Mohler, 1995; Marowsky et al., 2012), recent studies including our data included in this dissertation indicate that unlike δ and α3-subunit containing receptors that likely express ubiquitously in extrasynaptic membranes of PNs, α5 subunit containing receptors are restricted to more dendritic synaptic and extrasynaptic sites (Serwanski et al., 2006; Martin et al., 2012). In order to observe α5 subunit-dependent tonic currents, cesium must be included in the recording pipette to block outward potassium currents that normally reduce the ability of the patch-clamp electrode to maintain voltage control far from the somatic recording site (Isaacson et al., 1993). Recordings of GABAergic currents without
cesium in the pipette and therefore of mostly proximal inhibitory currents show no evidence of α5 subunit-mediated tonic current (holding current shift) with addition of the α5-specific inverse agonist (Martin et al., 2012).

The likely distinct subcellular location and physiological roles of α5 subunit- vs α3- and δ subunit-containing receptors places each receptor subtype in the position to modulate distinct aspects of PN excitability and neuronal coding. Since α5-subunit mediated tonic inhibition remains concentrated more in dendritic synaptic compartments, this mode of tonic conductance is well positioned to modulate synaptic plasticity (i.e. LTP and LTD) that underlies amygdala coding by restricting excitation and phasic inhibition received from specific excitatory afferents and GABAergic circuits, respectively, to distinct dendritic compartments (Shin et al., 2006; Jang and Kwag, 2012). On the other hand overall PN excitability control is complementary to maintenance of proper dendritic coding (Mitchell and Silver, 2003; Cope et al., 2005). To this end, δ and α3 subunit-containing receptors that are conspicuously positioned closer to the soma and axon initial segment compared to α5 subunit-containing receptors probably play an important role (Olmos-Serrano et al., 2010; Marowsky et al., 2012; Martin et al., 2012) since they reside close to the site of postsynaptic potential summation for action potential generation (Kole and Stuart, 2012). Of course for tonic neurotransmission, receptor activation requires a sufficiently available pool of extrasynaptic GABA neurotransmitter. In the BLA, we show that this pool critically depends on action-potential mediated release of GABA. With the exception of the α3 component of tonic GABAergic transmission that is unexplored in our model, our data shows that Fmr1 KO mice have deficient tonic currents based on decreased functional receptor expression (at least α5- and δ-subunit mediated) and decreased activity dependent release of GABA from an inefficient presynaptic interneuron population (Olmos-Serrano et al., 2010;
Martin et al., 2012). Below we discuss the possible effects of these tonic transmission deficiencies in the BLA on cell and network excitability, synaptic plasticity and integration, and broadly in neuronal network development in FXS with an emphasis on implications for pharmacological treatment.

C. TONIC GABAERGIC MODULATION OF EXCITABILITY

The effects of tonic GABAergic conductance on neuronal excitability are well-established (Mitchell and Silver, 2003; Vida et al., 2006). Moreover, deficits in tonic transmission have been implicated in numerous disorders of E/I balance including anxiety (Maguire et al., 2005; Tasan et al., 2011; Marowsky et al., 2012) and epilepsy (Peng et al., 2004; Zhang et al., 2007; Macdonald et al., 2010) that are comorbid with FXS (Hagerman et al., 2009). For instance, deficits in α5-subunit expression in the central nucleus of the amygdala associate with high anxiety behavior in mice (Tasan et al., 2011). In women with premenstrual dysphoric disorder, a state of heightened anxiety, dysfunction in the proper cycling of δ-GABA_A receptors to the cell surface underlies an increase in excitability during late diestrus (Maguire et al., 2005). Similarly, δ-GABA_A receptor expression and tonic GABAergic currents are decreased in rodent models of temporal lobe epilepsy (Zhang et al., 2007). Our results indicating decreased tonic conductance (Olmos-Serrano et al., 2010; Martin et al., 2012) and increased excitability link amygdala dysfunction in FXS to these related disorders. Likewise, tonic transmission deficits contributing to pathological excitability confirm the similarity of FXS functional deficits to those of related ASDs. For example decreases in tonic inhibitory GABA_A receptor subunits exist in the brains of humans with autism (Fatemi et al., 2009) and a decrease in amygdala GABAergic inhibition associates with an increase in social anxiety in rats (Truitt et al., 2009).
The increased excitability we observe in Fmr1 KO PNs is reflected as a change in neuronal offset that affects AP firing threshold independently of gain. As discussed in Chapter II, the rightward shift of the I/O function (increases in AP threshold) in both WT and Fmr1 KO PNs in response to the δ-subunit preferring agonist THIP independent of a change in slope is expected given the known preferential affects of tonic conductance on responses to constant current injection versus stochastic synaptic input (Mitchell and Silver, 2003). Therefore we cannot determine the effects of THIP, and therefore δ-subunit specific augmentation, on the whole of the I/O function (offset and gain) with our experimental protocol. Increasing tonic conductance in BLA PNs with THIP does not likely only alter neuronal offset however, since we observe significant decreases in R\textsubscript{in} after drug application that would increase synaptic attenuation by reducing gain (Häusser and Clark, 1997). Regardless we have preliminary data from WT PNs that indicates that tonic currents in BLA PNs may outwardly rectify (Figure 13) consistent with a preferential effect of tonic conductance on neuronal offset over gain even in the presence of stochastic synaptic activity that normally exists in the network (Pavlov et al., 2009).

*This interpretation supports the conclusion that increasing tonic transmission will support a decrease in AP firing with minimal affect on subthreshold synaptic integration.* In the context of treatment for hyperexcitability in FXS, this preference permits an effect on a distinct aspect of excitability and consequently reduces potential of unintended side effects subsequent to a concurrent decrease in synaptic gain.

However importantly, the outward rectification includes all tonic current. Thus, we cannot distinguish the contribution of the various tonic GABAergic receptor components (δ, α5, and α3) to the overall outward rectification. We know from our measurements of synaptic currents that α5-GABA\textsubscript{A} receptors can significantly affect gain at near resting membrane
potentials (-60 mV) (Chapter III, Figure 9) (Martin et al., 2012), so this component may not preferentially modulate offset over gain. Thus, more investigation is needed to determine both the contributions of each receptor subtype to the overall current and also its mode of modulation (degree of rectification) to design targeted compounds that optimally affect tonic conductance to reduce excitability. Despite the unanswered questions, our investigations identify PN hyperexcitability as a functional abnormality in the BLA of Fmr1 KO mice that is positively affected by supplementation of tonic GABAergic conductance.

D. TONIC GABAERCIC MODULATION OF BLA SYNAPTIC PLASTICITY AND NEURONAL TUNING

Modulation of gain by tonic conductance can also critically affect synaptic integration and plasticity in multiple brain areas (Häusser and Clark, 1997; van den Burg et al., 2007; Andrásfalvy et al., 2008; Martin et al., 2010). For instance, in hippocampal CA1 pyramidal neurons, the loss of the dendritic potassium channel, Kv4.2, induces compensatory increases in dendritic tonic conductance that reduce synaptic strength postsynaptically and quench the progression of back-propagating action potentials from the soma to the apical dendrites (Andrásfalvy et al., 2008). Similarly in area CA1 of the hippocampus, δ-GABA_A receptors can restrict NMDA-dependent LTP by reducing EPSP efficacy (Shen et al., 2010). Tonic transmission in the BLA could conceivably control plasticity induction in a similar manner along with phasic inhibitory transmission, especially in the dendrites where plasticity at convergent cortical and thalamic inputs is known to be restricted by GABAergic inhibition (Mahanty and Sah, 1998; Bissière et al., 2003; Bauer and LeDoux, 2004; Shaban et al., 2006; Shin et al., 2006; Szinyei et al., 2007; Tully et al., 2007). In Fmr1 KO PNs that have decreased tonic receptor...
expression and decreased tonic currents, this diminished inhibition would result in enhanced
associations between nearby glutamatergic synapses affecting the specificity of dendritic coding
via activity-dependent plasticity (i.e. LTP and LTD) and therefore BLA output underlying
amygdala based behaviors.

Our investigations into feedforward synaptic integration and timing revealed the novel
finding that like other feedforward circuits in the brain, external capsule stimulation of BLA PNs
produces a mixed excitatory/inhibitory conductance in which inhibition proportionately balances
synaptic excitation (Pouille and Scanziani, 2001; Higley and Contreras, 2003; 2006; Isaacson
and Scanziani, 2011). This balance produces an integration window in the PN that is defined by
the timing of the opposing excitatory and inhibitory conductance (Chapter III, Figure 11) (Martin
et al., 2012) providing a discrete time window (~2 ms) to support precise spike timing (Higley
and Contreras, 2003; 2006; Cruikshank et al., 2007; Isaacson and Scanziani, 2011). We report
that in WT PNs, blockade of α5-GABA_A receptors sharpens PN tuning by increasing the latency
of the peak excitatory conductance (Ge) (Wilent and Contreras, 2005; Cruikshank et al., 2007;
Martin et al., 2012), thereby significantly reducing the window of opportunity for coincident
afferent synaptic activity to induce an AP in the cell (Wehr and Zador, 2003; Isaacson and
Scanziani, 2011). Furthermore Fmr1 KO PNs show significantly reduced time windows that are
unaffected by α5-GABA_A receptor blockade consistent with a possible role of the receptor in
modulating the integration window (Martin et al., 2012).

The presence of increased Ge latency in the Fmr1 KO PNs associated with α5-GABA_A
receptor deficiency predicts at least two conditions that would adversely affect synaptic
integration in the BLA: 1) increased synaptic summation in the dendritic tree that may
generalize otherwise distinct spike-timing dependent plasticity from diverse BLA afferents
(Pouille and Scanziani, 2001; Andrásfalvy et al., 2008; Martin et al., 2010), and/or 2) decreased coincidence detection since the dynamic range of input timing that the PN can respond remains narrow (Wehr and Zador, 2003; Isaacson and Scanziani, 2011).

In the case of event summation, one example from area CA1 of the hippocampus shows that α5-GABA_A receptors crucially determine the threshold for LTP induced by stimulation within a specific frequency range (10-20Hz). Importantly, absence of α5-GABA_A receptor activity increases the strength of LTP without affecting total cell excitability or input resistance so tonic transmission likely only affects local dendritic membrane properties rather than somatic (Martin et al., 2010). In the absence of α5-GABA_A receptors afferent input occurring within the specific frequency range sums easily postsynaptically to induce NMDA dependent LTP, therefore not only can tonic GABAergic transmission control plasticity (Shen et al., 2010), but it can control plasticity in stimulus pattern-specific and strength-specific ways. In the BLA, changes in afferent activity patterns critically determine fear acquisition and especially fear extinction that is known to rely heavily on substantial inhibitory tone (Likhtik et al., 2008; Ehrlich et al., 2009; Heldt and Ressler, 2010; Pape and Pare, 2010; Palomares-Castillo et al., 2012). Given that at least mGluR-dependent plasticity is already deficient at Fmr1 KO mice including in the amygdala (Huber et al., 2002; Suvrathan et al., 2010), the additional reduction of an important regulator in tonic transmission likely compounds the deficit.

In sensory cortex, the E/I balance of feedforward circuits determines the tuning of neuron receptive fields, and the integration window and dynamic range of coincidence detection (Isaacson and Scanziani, 2011). Across multiple modalities (i.e. somatosensory, auditory, visual, olfactory) broader windows allow the cell to respond to a broader range of stimuli while more narrow windows sharpen tuning to specific stimuli (Kyriazi et al., 1996; Isaacson and Scanziani,
Furthermore, the dynamic range of this window and the corresponding E/I balance can adjust in an activity-dependent manner (Gabernet et al., 2005) and can depend solely on conductance timing regardless of absolute amplitude of its excitatory and inhibitory components (Wilent and Contreras, 2005). Interestingly, PNs in the lateral nucleus of the amygdala also have receptive fields sharply tuned to stimulus features such as the frequency of auditory stimuli (Bordi and LeDoux, 1992). Neuronal receptive fields then defined by feedforward E/I balance, may represent a common neuronal substrate by which to code activity. Thus in the BLA these integration windows likely define PN responses to conditioned stimuli required for fear conditioning and extinction and determine the degree of discrimination and generalization of stimuli based on the range of coincidence detection allowed by E/I integration. In effect, the broader a neuron’s tuning, the more plastic its responses. This condition implies that Fmr1 KO PNs, which have sharpened neuronal tuning, are not as adaptable as their WT counterparts. One predicts then that in conjunction with a general hyperexcitability phenotype (Olmos-Serrano et al., 2010), Fmr1 KO BLA PNs do not fire action potentials accurately during coincident inputs representing conditioned and unconditioned stimuli, for example, required to support their appropriate association or dissociation. This characteristic defined by dysfunctional feedforward E/I balance may underline amygdala-based issues in FXS such as generalized anxiety (Cordeiro et al., 2011; Qin et al., 2011), but may also contribute to sensory processing issues, for example, if the comparable deficiency exists in analogous circuits such as the canonical Layer IV feedforward circuit of the somatosensory cortex (Kyriazi et al., 1996; Chen and Toth, 2001).

Furthermore, evidence from cortical studies shows that increased GABAergic inhibition broadens neuronal tuning and reduces selectivity of neurons (Pouille and Scanziani, 2001;
Isaacson and Scanziani, 2011), consistent with deficient inhibition in the \( Fmr1 \) KO BLA triggering the opposite and sharpening neuronal tuning. Also strikingly, in primary sensory cortex the narrowing of the integration window is also associated with an increase in the Ge latency (Wilent and Contreras, 2005) as described in this thesis, further supporting the similarity in BLA circuitry and analogous brain regions. Therefore we show that sharpened PN response tuning represents another phenotypic hallmark complementary to hyperexcitability in the FXS BLA that may respond to tonic GABAergic transmission augmentation.

If sharpened tuning results from a decrease in tonic inhibitory tone (Isaacson and Scanziani, 2011), then increasing tonic conductance potentially improves two compromised aspects of BLA processing in \( Fmr1 \) KO mice. Firstly, it improves hyperexcitability (Chapter II), and secondly, it broadens tuning for more dynamic, adaptable coding of inputs by PNs (Wilent and Contreras, 2005; Cruikshank et al., 2007; Pouille et al., 2009). These effects would at least partially normalize PN function by allowing accurate responses to activity dependent changes in activity and support the maintenance of those responses. For acquisition of fear extinction especially, dynamic changes in response to the conditioned stimulus must occur in two distinct populations of PN neurons to change the balance of BLA output (Herry et al., 2008; 2010). Sharpened PN tuning represents an intriguing mechanism that could hinder that transition in \( Fmr1 \) KO mice in combination with or directly caused by the known inhibitory deficits in these mice (Likhtik et al., 2008; Heldt and Ressler, 2010; Martin et al., 2012). Correlative evidence comes from studies that show that GABAergic components including the \( \alpha5 \)-subunit of GABA\(_A\) receptors tend to downregulate in the amygdala during fear conditioning. Conversely many GABAergic components such as gephyrin and \( \alpha2 \)-GABA\(_A\) receptors tend to upregulate during extinction (Heldt and Ressler, 2010). This contraction and expansion of inhibition in the BLA
would support a sharpening of neuronal tuning after conditioning (contraction) that would maintain tight spike-timing dependence in support of fear conditioning maintenance, while an expansion of inhibition in extinction supports a broadening of PN tuning increasing the dynamic range of the PN.

Finally, many mechanisms have been suggested for the way GABA transmission can modulate the integration window. These possibilities include differences in synaptic drive of interneurons, postsynaptic receptor composition (Cruikshank et al., 2007), synchrony of inhibitory transmission, and amount of GABA release onto PNs (Gabernet et al., 2005). Here we report the novel finding that participation of tonically active GABA$_A$ receptors is one of those mechanisms. Given the global GABAergic deficits in the Fmr1 KO BLA including GABA synthesis and release, we can conclude that the sharpened tuning in PNs likely results from both decreased tonic receptor expression ($\alpha_5$- and possibly $\delta$- or $\alpha_3$-GABA$_A$ receptors), decreased GABA availability, and may also involve phasic inhibitory transmission deficits as well.

E. TONIC GABAERGIC TRANSMISSION IN DEVELOPMENT

Tonic GABAergic inhibition not only plays a role in established, adult neuronal networks, but can affect developing networks as well. For instance, $\delta$-GABA$_A$ receptors express in star amacrine cells of the developing retina. Activation of these receptors by endogenous GABA decreases correlated firing of retinal ganglion cells (RGCs) during retinal waves that progress throughout the developing visual system (Wang et al., 2007). FMRP expression begins early in embryonic development and persists throughout development and into adulthood (Hinds et al., 1993; Wang et al., 2004). This pattern of expression also exists in humans. FMRP and $fmr1$ can be detected in embryonic, fetal, and adult brain (Abitbol et al., 1993; Devys et al.,
1993; Tamanini et al., 1997; Agulhon et al., 1999). If similar tonic GABAergic transmission
deficits related to the loss of FMRP exist in this developmental time period, increased correlative
firing in retinal ganglion cells as a result of a loss of tonic control could induce long lasting
changes in connectivity of RGCs that might affect visual acuity and visual processing in the
cortex (Firth et al., 2005). It is intriguing that this type of phenomenon early in development in
FXS could partially underlie sensory processing problems later in development and affect
network development via similar mechanisms as we have identified in the mature BLA network
(Chapter III) (Martin et al., 2012).

Multiple lines of evidence show that tonic transmission is an important trophic factor
early in brain development (Owens and Kriegstein, 2002). In the neocortical proliferative zone,
neuronal precursors (actively mitotic radial glia cells) express function GABA_A receptors
(LoTurco et al., 1995; Noctor et al., 2002). Although the exact subunit composition of these
subunits is unknown, α4-, β1-, and γ1-subunits appear to be the most highly expressed (Araki et
al., 1992; Laurie et al., 1992; Poulter et al., 1993). Like tonically active GABA_A receptors in the
adult brain, receptors on these precursor cells have high affinity for GABA and low
desensitization rates which enables them to respond with high efficacy to low GABA
concentrations in the extracellular space (Owens et al., 1999). Consequently, in whole-cell patch
clamp recordings from these radial glia, application of the GABA_A receptor antagonist
bicuculline revealed a tonic current (LoTurco et al., 1995). Since no evidence of synaptic
contacts exist in the ventricular zone, these cells probably receive GABA via a paracrine
mechanism from surrounding cells (Van Eden et al., 1989; Cobas et al., 1991; Taylor and
Gordon-Weeks, 1991; Behar et al., 1996). Accordingly GABAergic cells have been identified in
and just above the ventricular zone that likely supply GABA from their growth cones to
stimulate the tonic GABA\textsubscript{A} receptors (Van Eden et al., 1989; Taylor and Gordon-Weeks, 1991; Behar et al., 1996). These tonic currents are depolarizing owing to a high expression of the Cl-uptake transporter NKCC1 and a low expression of the Cl- extruder KCC2 in immature neurons (Rivera et al., 1999; Wang et al., 2002). The physiological relevance of GABAergic activation in the proliferative zone is currently controversial (Owens and Kriegstein, 2002). However, activation does appear to depolarize radial glia and induce Ca\textsuperscript{2+} entry through voltage-gated calcium channels that supports activation of 2\textsuperscript{nd} messenger systems (Yuste and Katz, 1991; Lin et al., 1994; Leinekugel et al., 1995; LoTurco et al., 1995; Owens et al., 1996; Ben-Ari, 2002) and a downregulation of DNA synthesis consistent with a reduction in cell proliferation and an exit from the mitotic state (LoTurco et al., 1995; Haydar et al., 2000). The role of FMRP in support of this tonic GABAergic transmission-dependent process is unknown. However intriguingly, recent data acquired using neural progenitor cultures from \textit{Fmr1} KO mouse and post mortem human fetuses with FXS report \textit{increases} in neuron proliferation and decreases in glial proliferation in the absence of FMRP in both samples (Castrén et al., 2005). Additional evidence from this study showed an increase in BrdU positive cells in the subventricular zone of the \textit{Fmr1} KO mouse compared to WT and complementary studies report increases in the Tbr glutamatergic cell progenitor population in the ventricular and subventricular zone at embryonic day 17 in \textit{Fmr1} KO mice (Tervonen et al., 2009; Saffary and Xie, 2011). Whether deficient tonically active GABA\textsubscript{A} receptor transmission in the \textit{Fmr1} KO mouse affects this increased proliferation is unknown but it is certainly a possibility given the known requirement of FMRP for effective tonic currents (Chapter II) (Curia et al., 2009; Olmos-Serrano et al., 2010; Martin et al., 2012).
In the developing hippocampus from P0 to P4, rather than reduce cell proliferation, activation of extrasynaptic GABA\textsubscript{A} receptors containing \(\beta 2/\beta 3\)-, \(\gamma 2\)-, and \(\alpha 5\)-subunits function to help initiate giant depolarizing potentials that are crucial for establishing and maintaining synaptic connections in the developing network (Sipilä et al., 2005; Marchionni et al., 2007). Defects in tonic GABA\textsubscript{A} transmission related to the loss of FMRP here would vastly affect synaptic development by reducing spontaneous coordinate network activity. Similarly, one study shows that newborn dentate granule cells in the adult hippocampus are affected by depolarizing tonic GABAergic currents from extracellular GABA in the surrounding network first before innervation with GABAergic and glutamatergic synapses. If this depolarizing tonic current is blocked, synaptic integration and dendritic arborization are greatly diminished (Ge et al., 2006). Therefore deficits in tonic GABAergic conductance related to the loss of FMRP in FXS have considerable implications on the maintenance of the adult hippocampus and consequently learning and memory as well.

F. TONIC GABAERGIC TRANSMISSION IN BLA INTERNEURONS

Until now the focus of this dissertation has rested on tonic GABAergic transmission in principal excitatory neurons. However, tonic GABA\textsubscript{A} receptors also express on a diverse population of interneurons (INTs) in the BLA that form unique inhibitory circuits and affect distinct aspects of inhibitory transmission onto PNs (Spampanato et al., 2011). These presynaptic cell populations are deficient in the \textit{Fmr1} KO BLA at synthesizing and releasing GABA (Olmos-Serrano et al., 2010) and their activity regulates tonic inhibitory conductance in PNs (Chapter II), therefore their regulation by tonic GABA\textsubscript{A} receptor agents has important implications for therapeutic rescue of FXS. BLA INTs arguably comprise a more diverse pool of
INT subtypes than in analogous brain regions like the cortex and hippocampus. In general, the latter brain regions have INT subtypes that can be relatively well separated by their calcium binding or neuropeptide expression and physiological properties (Petilla Interneuron Nomenclature Group et al., 2008). In the BLA, these INTs exhibit more heterogeneity making specific characterizations of synapse- and circuit-specific properties difficult (Sosulina et al., 2010). Consequently, detailed physiological studies on the roles of INT subtype-specific circuits are generally lacking in the BLA. For instance, in the cortex and hippocampus, INT that express the protein parvalbumin (PV+) typically exclusively fire fast APs (FS) in excess of 100 Hz with little accommodation when injected with constant current and tend to form synapses at the proximal dendrites, axon initial segment, and the soma (Kawaguchi, 1993; Kawaguchi and Kubota, 1997; Petilla Interneuron Nomenclature Group et al., 2008). In the BLA however, PV+ cells, which make up about 50% of the INT population, express at least four different distinct physiological firing patterns including FS, regular spiking, studdering, and accommodating subtypes (Rainnie et al., 2006; Woodruff and Sah, 2007; Spamanato et al., 2011). The heterogeneity of the PV+ group implies that different PV+ cell populations may play unique roles in BLA processing but this possibility has not been explored. In addition, except for the FS phenotype, these firing patterns greatly resemble the firing patterns of PV-negative INTs with similar passive membrane properties (like $R_{in}$ and $\tau_m$) that synapse in different locations on PNs such as the distal dendrites and therefore constitute distinct inhibitory circuits. These interneuron subtypes include those positive for cholecystokinin (CCK), somatostatin (SST) and calretinin (CR) (Jasnow et al., 2009; Sosulina et al., 2010).

We recorded $\delta$-subunit mediated tonic currents using the $\delta$-preferring GABA$_A$ receptor agonist THIP (see Chapter II) from several different interneuron subtypes in the BLA of both
WT and Fmr1 KO mice (Figure 14). These cells showed firing patterns consistent with PV+ (FS), low-threshold spiking (LTS), late-spiking non accommodating (LS), and highly accommodating (ACC) subtypes observed in the cortex and hippocampus. Using analogous data from the cortex, FS and ACC subtypes typically synapse somatically or perisomatically (Petilla Interneuron Nomenclature Group et al., 2008; Jasnow et al., 2009), the LTS subtype typically synapses in the distal dendrites (Gibson et al., 1999; Beierlein et al., 2003; Paluszkiewicz et al., 2011b), and the LS has been identified in both populations (Petilla Interneuron Nomenclature Group et al., 2008; Sosulina et al., 2010). Similar to Fmr1 KO PNs, INTs express significantly lower δ-GABA_A receptor mediated currents than their WT counterparts. This data is consistent with a global reduction of tonic inhibitory currents in the BLA that may represent a homeostatic reduction of currents to increase excitability of the INT pool to accommodate the decrease in overall inhibitory efficacy of these cells (Olmos-Serrano et al., 2010). In general, INTs of both groups expressed larger tonic currents than their PN counterparts (WT PNs = 0.08 vs WT INTs = 0.42 pA/pF; KO PNs = 0.013 vs KO INTs = 0.21 pA/pF), typical of INTs in analogous circuits (Krook-Magnuson et al., 2008). This condition may serve to normalize tonic tone throughout the network by adjusting tonic current capacity to relative extrasynaptic GABA concentrations surrounding PNs and INTs. Evidence shows that GABA concentrations surrounding INT can be significantly less than PNs (Pan et al., 2009). By normalizing tonic capacity with available GABA ligand, the network can adjust to changes in activity in unison and maintain its dynamic range of responses to incoming inputs in times of both low and high network activity. Any differences in tonic tone in a particular inhibitory circuit as a result of the loss of FMRP in FXS could result in network disorganization during these changes in activity. Therefore, subtype
specific changes in tonic GABAergic currents should be assessed in the future to determine if
one particular inhibitory circuit is more affected.

G. FXS TREATMENT BY GABAERGIC AGENTS

To date, a primary therapeutic target in FXS has been the metabotropic glutamate
receptor 5 (mGluR5), based on evidence of unregulated signaling downstream of this receptor in
Fmr1 KO mice (Chuang et al., 2005; Yan et al., 2005; Dölen et al., 2007; Jacquemont et al.,
2011). Given the increasingly understood prevalence of inhibitory dysfunction in FXS, however,
especially the powerful role of tonic conductance described in this dissertation, the GABAergic
system also presents a number of relevant and intriguing targets for treatment that are distinct
from therapeutics aimed at reducing mGluR signaling. As we described above, current evidence
generally reveals an extensive dampening of GABAergic function throughout the adult FXS
brain including decreases in tonic conductance, and GABAergic function in general has been
implicated in many of the hallmark symptoms of FXS, including anxiety, autistic behaviors,
epilepsy, and cognitive impairment. GABAergic compounds, in turn, have demonstrated
therapeutic efficacy in many of these disorders. Therefore, compounds targeting the GABAergic
system may provide novel, effective, and in some cases, currently available treatment options for
the symptoms of FXS (Figure 16, Table 4). Here we detail some of those possible treatments
with particular focus on those that work on tonic conductance, highlight positive and negative
aspects of their modes of action, and emphasize key factors that should be considered when
applying GABAergic treatments to FXS.

The first study that systematically identified GABAergic components as possible targets
for FXS treatment used the Fmr1 KO Drosophila melanogaster model of FXS to screen 2,000
compounds for their ability to rescue glutamate-induced toxicity in developing fly larvae related to hypertrophic mGluR signaling (Chang et al., 2008). Of the 9 compounds identified in this screen, 3 acted on the GABAergic system: nipecotic acid (a GAT blocker), creatinine (a GABA\textsubscript{A} receptor activator), and GABA itself. Not only did these compounds rescue glutamate-induced lethality, but they also rescued characteristic cellular, morphological, and behavioral phenotypes related to FXS in the adult \textit{Fmr1} KO flies. Therefore, compounds that increase the function of the GABAergic system, either by increasing the availability of GABA (nipecotic acid) or acting directly at GABA receptors (creatinine and GABA), can rescue FXS phenotypes.

GABAergic function can be enhanced not only by affecting tonic GABA\textsubscript{A} transmission but also via the activation of postsynaptic GABA\textsubscript{B} receptors, and stimulation of these receptors has shown promise as a treatment in animal models of FXS - administration of the GABA\textsubscript{B} receptor agonist baclofen successfully reduces audiogenic seizure susceptibility in Fmr1 KOs (Pacey et al., 2009). Accordingly, Phase II clinical trials for safety and efficacy of the most active isomer of baclofen, Arbaclofen, are currently being conducted in FXS patients (clinicaltrials.gov; NCT01282268). In support of this approach, we have preliminary evidence of disrupted postsynaptic GABA\textsubscript{B} receptor-mediated transmission in PNs of the \textit{Fmr1} KO mice (Figure 15). Slow voltage ramps in baseline conditions and in the presence of arbaclofen (R-baclofen, 10 \textmu M) were used to reveal voltage-dependent postsynaptic GABA\textsubscript{B} induced K\textsuperscript{+} currents (GIRK currents) in BLA PNs. \textit{Fmr1} KO PNs showed significantly reduced GABA\textsubscript{B} current capacity at more depolarized thresholds (-40 mV) around AP threshold. Deficits in these currents then may play a role in hindering repolarization of the cell during periods of high, coordinated network activity such as upstates (Sanchez-Vives et al., 2010) in which the membrane potential is chronically near AP threshold, resulting in extended upstate durations in
Fmr1 KO PNs (Wang et al., 2010). In addition decreased availability of GABA in Fmr1 KOs would function to compound these deficits (Olmos-Serrano et al., 2010). Importantly, although GABA\(_B\) receptors express presynaptically on excitatory and inhibitory terminals to reduce neurotransmitter release (Bettler and Tiao, 2006), any therapeutic actions of the GABA\(_B\) receptor agonist Arbaclofen likely center on postsynaptic receptors acting through GIRK channels (Bowery et al., 2002). However, if presynaptic actions play a role, they likely exclusively modulate glutamatergic transmission because GABA\(_B\) receptors do not express presynaptically in BLA interneuron terminals (Pan et al., 2009; Olmos-Serrano et al., 2010) and so would not act to reduce inhibitory transmission.

Two clinically available, FDA-approved anticonvulsants function like nipecotic acid in the Drosophila Fmr1 KO model to increase GABA availability and might therefore prove efficacious in FXS. The first, tiagabine, specifically blocks the presynaptic GABA Transporter 1 (GAT1), thus increasing synaptic GABA levels and enhancing phasic and tonic GABAergic inhibition (Nielsen et al., 1991). The second, vigabatrin, blocks the catabolism of GABA by inhibiting the function of GABA transaminase (GABA-T), which is required for the breakdown of GABA to glutamate. This blockade increases GABA availability, both intracellularly, for packaging into presynaptic vesicles, and extracellularly (French et al., 1996; Chiron et al., 1997). Although these compounds exhibit efficacy at improving GABAergic function, they do not work in all patients and have a high incidence of side effects, including, in the case of vigabatrin, retinal neuropathy (Frisén and Malmgren, 2003). Their GABAergic actions, however, may warrant investigation in FXS.

Many compounds act directly on GABA\(_A\) receptors, and therefore may also improve symptoms of FXS. For instance, the free amino acid taurine acts as an agonist at GABA\(_A\)
receptors, increases GAD expression, increases GABA levels, induces changes in GABA_{A} receptor subunit composition (L’Amoreaux et al., 2010), and decreases seizure susceptibility (Idrissi, 2008). This endogenous amino acid is developmentally dysregulated in Fmr1 KO mice (Gruss and Braun, 2004), and chronic taurine feeding improves cognition (Idrissi et al., 2010a) and neuroendocrine symptoms in these animals (Idrissi et al., 2010b).

Several other compounds that potentiate GABA_{A} receptor function have been identified and/or examined in preclinical and clinical trials for FXS or its related symptoms. One compound, riluzole, has multiple modes of action to decrease excitability, including GABA_{A} receptor potentiation (Jahn et al., 2008) and GAT blockade (Mantz et al., 1994). Riluzole is an FDA-approved anticonvulsant often utilized for amyotrophic lateral sclerosis treatment in adults and in one preclinical trial shows mild efficacy in improving attention deficit disorder with hyperactivity in adult FXS patients (Erickson et al., 2011). Benzodiazepines as well are proven, effective GABA_{A} receptor agonists, but often present unwanted side effects, including sedation and rebound symptoms such as anxiety when treatment is discontinued (Nemeroff, 2003), and therefore might not be the optimal agents to treat symptoms of FXS.

Interestingly, another class of molecules, the non-benzodiazepine neuroactive steroids (neurosteroids), increase GABAergic receptor function via positive allosteric modulation (PAM) (Belelli and Lambert, 2005). Natural neurosteroids such as allopregnanolone are not orally active, but one new synthetic neurosteroid ganaxolone, the 3β-methyl analogue of allopregnanolone, is orally active, lacks hormonal side effects, and has entered phase II clinical trials for infantile spasms, partial seizures, and catamenial epilepsy (Reddy, 2010). A neuroactive steroid like ganaxolone may be useful in the treatment of anxiety and seizures associated with FXS, and has advantages over benzodiazepines, given the low occurrence of side
effects and evidence of preferential effects at extrasynaptic, tonically active δ-subunit-containing receptors which are deficient in the Fmr1 KO amygdala and whose activation can potently rescue PN neuron hyperexcitability in the region (Chapter II) (Mihalek et al., 1999; Biagini et al., 2010; Reddy, 2010).

When considering the best possible scenario for treatment of FXS with GABAergic modulators, particularly tonic current modulators, several factors need to be considered. First one must consider the mode of action of the pharmacological agent. There are several ways to increase GABAergic tone: directly activate GABA\(_A\) receptors, increase GABA availability, and increase receptor efficacy. Secondly, one must consider the specificity of the GABAergic agent to particular GABA\(_A\) (or GABA\(_B\)) receptors that affect various aspects of cellular and network function including cell-specific aspects. For instance, benzodiazepines can greatly enhance affinity of GABA\(_A\) receptors for GABA and often have receptor subtype specificity but may not work well to specifically target tonic conductance (Nemeroff, 2003). Zolpidem for instance, which enhances GABA\(_A\) receptors at the benzodiazepine site, can readily affect mostly \(\alpha_1\)-, and also \(\alpha_2\)- and \(\alpha_3\)-GABA\(_A\) receptors, but has basically no efficacy at \(\alpha_5\)-containing receptors (Pritchett and Seeburg, 1990; Sanger, 2004). If the goal is to modulate tonic conductance then, zolpidem is not an ideal choice. However, PV\(^+\) interneuron synapses that express high levels of \(\alpha_1\)-GABA\(_A\) receptors would be greatly enhanced by zolpidem (Sanger, 2004).

In the case of tonic GABAergic modulation specifically in the BLA, current evidence shows (Chapter II, III, (Olmos-Serrano et al., 2010; Martin et al., 2012)) that the best therapeutic value may be achieved by increasing activity at tonically active GABA\(_A\) receptors specifically on PNs, as opposed to INTs. To this end THIP, or gaboxadol, a δ-preferring superagonist at δ-GABA\(_A\) receptors may not be the best choice because as a direct agonist, this agent likely has
more substantial effects on the excitability of INTs (Figure 14) than PNs in the BLA and other analogous brain regions. This effect predicts an overall increase in GABAergic tone by THIP that significantly reduces the ability of INTs to function as dynamic mediators of the network. Regardless, recent preclinical evidence shows that THIP can improve hyperexcitability in *Fmr1* KO mice *in vivo* (Olmos-Serrano et al., 2011) so further investigation is warranted. Alternatively agents such as tiagabine and vigabatrin that increase GABA availability globally in the extrasynaptic space would likely affect the BLA in a more advantageous way by increasing overall GABAergic tone. However side effects such as retinal neuropathy (Frisén and Malmgren, 2003) can arise because this therapeutic method does not have sufficient GABA<sub>A</sub> receptor specificity. A global increase in GABA increases activity at GABA<sub>B</sub> as well as GABA<sub>A</sub>-ρ receptors that function in the retina and may underlie this deleterious effect (Jones and Palmer, 2009).

To reach the best combination of enhancement and specificity of tonic GABAergic currents, perhaps neurosteroid compounds represent the best currently available agents (Belelli and Lambert, 2005). Firstly as positive allosteric modulators, neuroactive steroid compounds work with the current GABA availability to increase GABA efficacy at the receptor without adding exogenous agonist. So the effects at GABA<sub>A</sub> receptors on INTs in the BLA that normally experience much smaller extrasynaptic GABA concentrations (Pan et al., 2009) should be minimal, allowing them to retain their current level of excitability during treatment and continue to release the GABA that is crucial for tonic inhibitory tone on PNs (Figure 4). Furthermore, neuroactive steroids can possess a significant amount of specificity for GABA<sub>A</sub> receptor subtypes, whether synaptic or extrasynaptic (Belelli and Lambert, 2005). Ganaxolone for instance, can preferentially affect δ-GABA<sub>A</sub> receptors and therefore could function to reliably
and specifically enhance δ-mediated tonic current on Fmr1 KO PNs to reduce excitability in the BLA network and normalize BLA processing.

One final aspect of GABAergic treatment, the developmental time point, warrants consideration. Since GABAergic transmission early in neuronal development (before approximately postnatal day 14) is generally excitatory (Owens et al., 1999), increasing tonic GABAergic tone during this developmental window with GABAergic agents may be detrimental and result in excitotoxicity. However, as the summary above detailing the role of excitatory tonic currents in synaptic development and neuronal proliferation indicates, no matter the sign (depolarizing or hyperpolarizing), tonic GABAergic current is a crucial neuronal element throughout development. Therefore, tonic GABAergic augmentation may not only powerfully affect mature networks, but also support the proper development and maintenance of synaptic and network balance throughout life in FXS.
Figure 12. Distribution of extrasynaptic GABA\(_A\)Rs in proximal and distal regions of principal projection neurons in the WT and Fmr1 KO basolateral amygdala. In the WT situation, δ (green) and α3 subunit-containing receptors (light blue) are likely located ubiquitously in extrasynaptic membranes whereas α5 subunit-containing receptors (red) concentrate in the more distal (dendritic) extrasynaptic compartments. Extrasynaptic GABA\(_A\) receptors receive GABA from robust release from synapses that spills into the extrasynaptic space. In Fmr1 KO PNs extrasynaptic GABA\(_A\)R expression/function is globally reduced (the fate of α3 subunit-containing receptors is unknown). These receptor reductions are compounded by the weak production and release of GABA, thereby reducing the availability of GABA on an already deficient extrasynaptic receptor population.
Figure 13. Tonic currents in WT BLA PNs outwardly rectify. A step protocol was performed before and after the application of 100 µM gabazine similar to that described in Chapter I (Figure 3). Rather than gramicidin-perforated patch recordings, we performed whole-cell patch clamp recordings with a cesium-based intracellular solution with an $E_{Cl}$ of approximately 0 mV (X-intercept). Tonic currents were calculated by subtracting the steady-state currents recorded at each voltage in the presence of gabazine from the baseline currents at that voltage (recorded in the presence of endogenous GABA). The data is fitted with a Boltzmann function (red dotted line) and indicates a more positive slope (higher tonic GABAergic conductance) at depolarized membrane potentials compared to membrane potentials near the typical resting membrane potential of BLA PNs (approx. -70 to -60 mV). The rectification index (RI) of the sample was $3.64 \pm 1.84$ ($n = 3$) indicating a strong outward rectification (if RI = 1, no rectification; RI > 1, outward rectification; RI < 1, inward rectification). RI was calculated using the equation below.

$$RI = \frac{I_{40mV}}{I_{-60mV}} - \frac{I_{-60mV} - E_{Cl}}{40mV - E_{Cl}}$$

where $I = current, E_{Cl} = 0mV$
**Figure 14.** *Fmr1* KO interneurons also have decreased THIP-induced currents. (A-D) In both WT and *Fmr1* KO BLA slices we recorded from a diverse population of inhibitory neurons. These neurons displayed physiological firing patterns when injected with constant current (600 ms) consistent with those identified previously in the BLA and with related interneuron populations in the cortex and hippocampus (Spampanato et al., 2011). (A) This fast-spiking interneuron (FS) is likely PV+, has a relatively low input resistance, and fires in excess of 100 Hz at its maximal firing frequency (109 Hz) (Woodruff and Sah, 2007). Current injection: -100 pA (blue), 25 pA (threshold, red), 200 pA (black). (B) Low-threshold spiking (LTS) cells typically express SST in the cortex (Beierlein et al., 2003; Paluszkiewicz et al., 2011b) but may express both CCK or PV in the BLA (Sosulina et al., 2010), and likely synapse on distal dendrite of BLA PNs (Jasnow et al., 2009; Sosulina et al., 2010). Current Injection: -100 pA (blue), 10
pA (threshold, red), 100 pA (black). (C) This late-spiking, non-accommodating cell has a regular spiking firing pattern, may express PV or CCK/CB, and could synapse on the somatic or dendritic region (Jasnow et al., 2009; Spampanato et al., 2011). Current Injection: -100 pA (blue), 50 pA (threshold, red), 100 pA (black). (D) The accommodating (ACC) firing pattern is the most heterogenous among the identified population of neurons and therefore could express a diverse combination of markers (Jasnow et al., 2009; Sosulina et al., 2010; Spampanato et al., 2011). Current Injection: -100 pA (blue), 25 pA (threshold, red), 100 pA (black). (E) Bath application of THIP (10 µM) revealed a δ-subunit mediated tonic GABAergic current in both WT and Fmr1 KO PNs. The current density of the Fmr1 KO population was significantly smaller than WT (WT: 0.42 ± 0.11 pA/pF, n = 9; Fmr1 KO: 0.21 ± 0.03 pA/pF, n = 17; *p = 0.03).
Figure 15. R-baclofen induced currents are also deficient in Fmr1 KO PNs. (A) Slow voltage ramps from -150 mV to +10 mV over 1000 ms (Inset, calibration 50 mV, 500 ms) were performed using Cs-based solution ($E_{Cl} = 0$ mV) in baseline conditions first (black trace) and in the presence of 10 μM R-baclofen (blue trace). (B) Subtraction of baseline traces from R-baclofen traces revealed the inwardly rectifying GABAB specific current (expressed as current density) in WT and Fmr1 KO PNs consistent with activation of postsynaptic GIRK channels with a reversal potential near the $E_{K^+}$ of the intracellular solution (-85 mV). (C) Quantification of current densities revealed no significant differences in induced current at -70 mV (near RMP) (WT: $0.13 \pm 0.02$ pA/pF, n = 9; Fmr1 KO: $0.17 \pm 0.03$ pA/pF, n = 11, p = 0.13), but significant
differences in current at depolarized membrane potentials (+40 mV) (WT: 0.40 ± 0.08; Fmr1 KO: 0.26 ± 0.03, *p = 0.04). (D) The rectification index, RI, was not significantly different between groups (WT: 0.65 ± 0.06; Fmr1 KO: 0.58 ± 0.05, p = 0.36)

\[
RI = \frac{I_{-40mV}}{-40mV - E_{K+}} \quad \frac{I_{-130mV}}{-130mV - E_{K+}}
\]

where \( I = \text{current} \), \( E_{K+} = -85mV \)
Figure 16. Potential pharmacological targets for the treatment of FXS. Numbers identify loci of action of pharmacological compounds targeting components of the GABAergic system (inset legend; Table 4). Note: The presumed actions of arbaclofen are illustrated as predominately postsynaptic, since presynaptic modulation is expected to reduce GABA release via a reduction in voltage-dependent calcium influx. However, since glutamatergic terminals also express presynaptic GABA_{B} receptors, additional beneficial actions of arbaclofen could also lie in the reduction of glutamate release from excitatory synapses. (Taken from (Paluszkiewicz et al., 2011a)).
### Table 2. Potential FXS treatments targeting deficits in the GABAergic system

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Effect</th>
<th>FDA Approval</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ganaxolone, GABA receptors, synaptic and extrasynaptic; synaptoneurotic</td>
<td>Potentiates/activates GABA&lt;sub&gt;A&lt;/sub&gt; transmission</td>
<td>No Phase II clinical trials: infantile spasms, complex-partial and catamenial epilepsy</td>
<td>Reddy 2010; Biagini et al. 2010</td>
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<tr>
<td>2</td>
<td>Riluzole, GABA receptors, synaptic and extrasynaptic; GABA Transporters (GATs)</td>
<td>Potentiates GABA transmission; blocks GABA&lt;sub&gt;A&lt;/sub&gt; uptake</td>
<td>Yes Anticonvulsant, amyotrophic lateral sclerosis (ALS) treatment</td>
<td>Erickson et al., 2010; Jahn et al., 2008; Mantz et al., 2004</td>
</tr>
<tr>
<td>3</td>
<td>Gaboxadol, GABA&lt;sub&gt;B&lt;/sub&gt; receptors, preferential extrasynaptic (delta-containing)</td>
<td>Potentiates/activates GABA&lt;sub&gt;A&lt;/sub&gt; transmission</td>
<td>No Phase III clinical trials: insomnia (completed)</td>
<td>Lundahl et al., 2007; Deacon et al., 2007; Olmos-Serrano et al., 2010</td>
</tr>
<tr>
<td>4</td>
<td>Arbaclofen, GABA&lt;sub&gt;B&lt;/sub&gt; receptors</td>
<td>Activates GABA&lt;sub&gt;B&lt;/sub&gt; transmission</td>
<td>No Phase II clinical trials: FXS, Autism Spectrum Disorders;</td>
<td>Pacey et al., 2009</td>
</tr>
<tr>
<td>5</td>
<td>Tiagabine, GABA transporters (GAT1 selective)</td>
<td>Blocks GABA uptake</td>
<td>Yes Anticonvulsant, complex partial seizures</td>
<td>Nielsen et al., 1991</td>
</tr>
<tr>
<td>6</td>
<td>Vigabatrin, GABA-T</td>
<td>Inhibits GABA catabolism</td>
<td>Yes Anticonvulsant, complex partial seizures, infantile spasms</td>
<td>French et al., 1996; Chiron et al., 1997</td>
</tr>
</tbody>
</table>

**Table 4. Potential FXS treatments targeting deficits in the GABAergic system.** (Taken from (Paluszkiewicz et al., 2011a)).
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