Deficient tonic GABAergic conductance and synaptic balance in the Fragile-X Syndrome Amygdala

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Running title: Altered tonic inhibition in fragile X syndrome

Pages: 42, Tables: 2, Figures: 5

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ABSTRACT

Fragile X Syndrome (FXS) is the leading cause of inherited intellectual disability. Comorbidities of FXS such as autism are increasingly linked to imbalances in excitation and inhibition (E/I) as well as dysfunction in GABAergic transmission in a number of brain regions including the amygdala. However, the link between E/I imbalance and GABAergic transmission deficits in the FXS amygdala is poorly understood. Here, we reveal that normal tonic GABA<sub>A</sub> receptor-mediated neurotransmission in principal neurons (PNs) of the basolateral amygdala (BLA) is comprised of both δ- and α5-subunit containing GABA<sub>A</sub> receptors. Furthermore, tonic GABAergic capacity is reduced in these neurons in the Fmr1 knock-out (KO) mouse model of FXS (1.5-fold total, 3-fold δ-, and 2-fold α5-subunit mediated) as indicated by application of gabazine (50 μM), THIP (1 μM), and α5ia (1.5 μM) in whole-cell patch clamp recordings. Moreover, α5-containing tonic GABA<sub>A</sub> receptors appear to preferentially modulate nonsomatic compartments of BLA PNs. Examination of evoked feedforward synaptic transmission in these cells surprisingly revealed no differences in overall synaptic conductance or E/I balance between WT and Fmr1 KO mice. Instead, we observed altered feedforward kinetics in Fmr1 KO PNs that supports a subtle, yet significant decrease in E/I balance at the peak of excitatory conductance. Blockade of α5-subunit containing GABA<sub>A</sub> receptors replicated this condition in WT PNs. Therefore, our data suggest that tonic GABA<sub>A</sub> receptor mediated neurotransmission can modulate synaptic E/I balance and timing established by feedforward inhibition and thus may represent a therapeutic target to enhance amygdala function in FXS.

KEY WORDS: tonic inhibition, gaba, amygdala, fragile-x syndrome
INTRODUCTION

Patients with FXS display a variety of symptoms associated with autism spectrum disorders (ASDs) including mild to severe intellectual disability, social anxiety/withdrawal, increased incidence of epilepsy, attention-deficit hyperactivity disorder (ADHD), and sensory hypersensitivity (Fu et al., 1991; Verkerk et al., 1991; Hagerman et al., 2009). Current evidence implicates a number of developmental defects that likely underlie these symptoms including dysregulated synaptic plasticity (He and Portera-Cailliau, 2013), brain region connectivity (Geschwind and Levitt, 2007), and E/I imbalance in critical neuronal circuits such as the cerebral cortex, hippocampus, and amygdala (Gibson et al., 2008; Olmos-Serrano et al., 2010; Zhang and Alger, 2010; Hays et al., 2011; Paluszkiewicz et al., 2011b). From the standpoint of neuronal excitation, many of these defects in synaptic plasticity and network imbalance can be attributed to elevated basal and activity-dependent protein translation downstream of enhanced Group I metabotropic glutamate receptor signaling caused by the loss of a critical translational regulator, FMRP (Fragile-X Mental Retardation Protein) (Bear et al., 2004).

However, a growing literature also implicates widespread, brain-region specific disruption in GABAergic inhibitory transmission in FXS, particularly in brain regions such as the sensory cortices and amygdala that are crucially implicated in the most prominent features of the disorder (e.g. sensory hypersensitivity and social withdrawal) (Paluszkiewicz et al., 2011a). For instance, numerous studies identify decreased GABA receptor subunit expression (D'Hulst et al., 2006; Gantois et al., 2006), altered
expression of GABAergic system components such as GAD and GAT-1 (Idrissi et al., 2005; Adusei et al., 2010), and functional and anatomical disruption of inhibitory synapses (Olmos-Serrano et al., 2010; Vislay et al., 2013).

Accordingly in the basolateral amygdala (BLA), a region essential for sensory integration, assignment of emotional saliency, and regulation of acquired fear (Ehrlich et al., 2009), pervasive GABAergic deficits exist. These deficits include reductions in the number of GABAergic synapses, GABA production and release, and phasic and tonic inhibitory currents in principal excitatory neurons (PNs). Furthermore, this GABAergic dysfunction correlates with a neuronal hyperexcitable phenotype that is pharmacologically rescued by specifically enhancing tonic GABAergic transmission (Olmos-Serrano et al., 2010). Indeed, tonic GABAergic conductance, maintained by low levels of ambient GABA in the extrasynaptic space (Farrant and Nusser, 2005), provides a persistent background inhibitory conductance that regulates E/I balance to affect 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). Yet despite the clear potential of GABAergic inhibitory tone to regulate these common cellular and network dysfunctions in neurodevelopmental disorders including FXS and in particular, to regulate synaptic plasticity underlying fear processing (acquisition, expression, extinction) [reviewed in (Ehrlich et al., 2009)], this form of GABAergic transmission remains understudied in the amygdala and FXS.

Here we investigated the state of tonic GABAergic conductance in wild-type (WT) and Fmr1 KO mouse BLA to identify specific alterations in FXS that might inform the
role of tonic conductance in regulating cellular and synaptic balance in the region. We found that tonic conductance in BLA PNs is comprised of at least $\delta$- and $\alpha_5$-subunit containing receptors. In addition, total, $\delta$-, and $\alpha_5$-mediated tonic capacity are deficient in Fmr1 KO PNs. We also identify a functional preference of $\alpha_5$-containing tonic GABA$_A$ receptors for modulation of synaptic events distal from our somatic whole-cell patch clamp recording site. Furthermore we determined the dynamics of the feedforward circuit comprised of excitatory afferents within the external capsule and local BLA interneurons. Our results reveal that surprisingly, Fmr1 KO PNs exhibit overall balanced evoked excitatory ($G_e$) and feedforward inhibitory conductance ($G_i$) compared to WT. However, slower excitatory response kinetics in Fmr1 KO slices results in a more narrow time window between $G_e$ and $G_i$ peaks in the feedforward response. Furthermore blockade of $\alpha_5$-containing GABA$_A$ receptors in WT slices mimics this condition. Thus we reveal the importance of this selectively activated tonic GABAergic conductance to regulate E/I dynamics in BLA PNs. Therefore, augmenting tonic transmission in Fmr1 KO mice may enhance synaptic integration and network function in this important feedforward inhibitory circuit and thereby improve amygdala-based symptoms of this disorder.

**METHODS**

**Animal use.** Control (FVB.129P2, stock #4828) and Fmr1 knock-out (KO) mice (strain name: FVB.129P2-Fmr1$^{tm1Cgr}$/J; stock #4624) on the congenic FVB strain background were obtained from The Jackson Laboratory (Genetics Research, Bar Harbor, Maine, USA) and maintained as separate congenic stocks in our own facility. Here, we refer to
control animals as wild-type (WT) throughout the text and figures. Only male animals were included in the study. 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 CO$_2$ and decapitated. Brains were removed quickly and placed in cold (4°C) sucrose-based oxygenated (95% O$_2$/5% CO$_2$) cutting solution composed of (in mM): Sucrose (234), Glucose (11), NaHCO$_3$ (26), KCl (2.5), NaH$_2$PO$_4$·H$_2$O (1.25), MgSO$_4$·7H$_2$O (10), and CaCl$_2$·H$_2$O (0.5). Coronal slices containing the basolateral amygdala (BLA) were obtained using a slicing vibratome (Leica VT1200s) 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 to preserve afferent connections to PNs in the external capsule. The slices were immersed in oxygenated (95% O$_2$/5% CO$_2$) artificial cerebral spinal fluid (ACSF) at 34°C for 30-45 minutes. ACSF was composed of (in mM): NaCl (126), NaHCO$_3$ (26), Glucose (10), KCl (2.5), NaH$_2$PO$_4$·H$_2$O (1.25), MgCl$_2$·7H$_2$O (2), and CaCl$_2$·2H$_2$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 26-28°C with an inline heater system (Warner Instruments). We used temperatures below
physiological (~ 37°C) consistent with our previous investigations of the BLA and GABAergic transmission (Olmos-Serrano et al., 2010) to ensure cell and slice health over long recordings. These temperatures were essential in conductance experiments (Figure 5) to ensure robust and consistent evoked responses from the external capsule afferents over the length of the experiment. 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 resistance of 2.0-4.0 MΩ when filled with intracellular solution. Access resistance of recordings was < 25 MΩ and monitored throughout the experiment with brief 5 mV steps every 20 seconds. Data was discarded if the access resistance changed by > 25%. Membrane potentials were adjusted for junction potential (12 mV). 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 dendrites, and then physiologically using prolonged depolarizing and hyperpolarizing current injections (600 ms) (Figure 1A,B). 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
phasic spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in voltage clamp and isolated by blocking ionotropic glutamatergic 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 and the effect of α5-GABA_A receptor blockade on sIPSCs (Figures 1 and 3), a K-Gluconate based intracellular solution was used (in mM): K-Gluconate (70), KCl (70), HEPES (10), EGTA (1), MgCl_2 (2), Mg-ATP (4), Na-GTP (0.3), E_{Cl} = -16 mV. For tonic current capacity experiments and to investigate the role of α5- GABA_A receptors on sIPSCs recorded at the soma (Figures 2 and 4), a Cesium-chloride based intracellular solution was used (in mM): CsCl (135), HEPES (10), EGTA (10), QX-314 (5), MgCl_2 (2), Mg-ATP (4), and Na-GTP (0.3), E_{Cl} = 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).

Tonic currents were acquired and analyzed as reported in our previous work (Krook-Magnuson et al., 2008). 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_{α5ia}, α5-subunit specific GABAR inverse agonist, 1.5 µM), gabazine (I_{GBZ}, GABAR antagonist SR-95531, 50 µM), or THIP (I_{THIP}, 1 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, 2007). 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 $\alpha_5$-subunit mediated tonic current capacity was calculated from the difference in mean baseline and $\alpha_5\text{ia}$ currents ($I_{\alpha_5\text{ia}} - I_{BSLN}$). In a separate set of experiments, $\delta$-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.

To evaluate the role of $\alpha_5$-GABA$_A$ 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). 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) and were recorded without and in the presence of 1 $\mu$M tetrodotoxin (TTX) in order to block sodium channels (AP-independent miniature IPSCs (mIPSCs)). IPSCs were analyzed for changes in frequency, amplitude, and kinetics before and after the application of $\alpha_5\text{ia}$ (1.5 $\mu$M). 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
Excitatory and inhibitory components of total evoked synaptic conductance were then determined based on assumed reversal potentials for excitation and inhibition. 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, $E_{Cl} = -69$ 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 5A). 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:

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

where \( V_{\text{corr}}(t) \) is the corrected voltage at time \( t \), \( V_{\text{rec}}(t) \) is the recorded voltage at time \( t \),
\( I_{\text{rec}}(t) \) 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:

\[ I_{\text{syn}}(t) = \Delta I_{\text{rec}}(t) - \Delta V_{\text{corr}}(t)/R_{\text{in}}, \]

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, $G_{\text{syn}}(t)$. 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, $E_e$, was assumed to be 0 mV and the inhibitory synaptic reversal potential, $E_i$, 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 ($G_e(t)$) and inhibitory ($G_i(t)$) components of that conductance were calculated and plotted:

\[
G_{\text{syn}}(t) = G_e(t) + G_i(t)
\]

\[
E_{\text{syn}}(t) = \frac{(G_e(t) \cdot E_e + G_i(t) \cdot E_i)}{(G_e(t) + G_i(t))}\]

therefore,

\[
G_e(t) = G_{\text{syn}}(t) \cdot \frac{(E_{\text{syn}}(t) - E_i)}{(E_e - E_i)}
\]

\[
G_i(t) = G_{\text{syn}}(t) \cdot \frac{(E_{\text{syn}}(t) - E_e)}{(E_i - E_e)}
\]

**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). We used the paired Student’s $t$ test to determine significance for within group comparisons between conditions (i.e. baseline and drug) and ANOVA to compare baseline and drug conditions across the two genotypes (WT and $Fmr1$ KO). The Kolmogorov–Smirnov test (K-S test) was used to compare probability distributions of inter-event interval (frequency, sIPSCs and mIPSCs) for within group comparisons between conditions (i.e. baseline and drug) and across.
baseline conditions for the two genotypes (WT and Fmr1 KO) (Matlab) (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

RESULTS

Tonic GABA$_A$ mediated capacity in BLA principal neurons is reduced in Fmr1 KO mice

To determine whether deficits in tonic GABA$_A$ receptor expression contribute to the decreased tonic currents previously observed in Fmr1 KO PNs (Olmos-Serrano et al., 2010), 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 δ- and α5-subunit containing receptors [reviewed in (Brickley and Mody, 2012)]. These receptor subunits have been previously shown to be expressed in the BLA via immunohistochemistry (Fritschy and Mohler, 1995; Pirker et al., 2000), and therefore likely contribute to the overall tonic conductance. First we tested BLA PNs in WT and Fmr1 KO mice for the presence of δ-subunit mediated tonic currents using the δ-subunit preferring GABA$_A$ receptor super-agonist THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol, 1 μM). Recent studies conducted in δ-subunit knock-out mice (δ$^{-/-}$) combined with efficacy studies of THIP on various GABA$_A$ receptor subtype combinations have shown that concentrations of THIP specific for efficacy at δ-subunit containing GABA$_A$ receptors likely reside in the low micromolar to sub-micromolar range (Brown et al., 2002; Stórustovu and Ebert, 2006). Therefore we used THIP at 1 μM in our experiments. THIP application at this concentration generated δ-subunit mediated
currents in WT BLA PNs verifying the presence of δ-subunit mediated tonic currents in these cells (Figure 1Aii). These currents were significantly reduced in Fmr1 KO PNs by about 3-fold (Figure 1Bii) (WT: 4.47 ± 1.01 pA, n = 11; Fmr1 KO: 1.57 ± 0.42 pA, n = 10, t-test, p = 0.02; Figure 1C, left). Moreover 1 μM THIP-induced current densities were also significantly reduced in Fmr1 KO PNs (WT: 0.019 ± 0.005 pA/pF, n = 11; Fmr1 KO: 0.007 ± 0.002 pA/pF, n = 10, t-test, p = 0.04; Figure 1C, right).

In parallel experiments we assessed α5-subunit mediated current capacity and total tonic current capacity (Figure 2) using an α5-subunit specific inverse agonist, α5ia, and the GABA<sub>A</sub> receptor antagonist gabazine, respectively, to quantify the removal of baseline tonic current. Since our previous studies revealed that extrasynaptic GABA levels differ between the WT and Fmr1 KOs (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, 2007) and produces larger currents generally than our previous investigations with the lower concentrations of native extrasynaptic GABA in our submerged slices (Olmos-Serrano et al., 2010). 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 2A,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 (Stuart and Spruston, 1998). Under these conditions application of α5ia revealed that BLA PNs indeed have the capacity for α5-subunit specific tonic current and that Fmr1 KO PNs have reduced α5-subunit mediated tonic currents (WT: 13.0 ± 2.3 pA, n = 12 cells; Fmr1 KO: 5.28 ± 0.88 pA, n = 12, t-test, p = 0.007; Figure 2C) 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, t-test, p = 0.015; Figure 2D) and current density (WT: 0.24 ± 0.026 pA/pF; Fmr1 KO: 0.17 ± 0.015 pA/pF, t-test, p = 0.028). These data confer a reduced capacity for tonic currents (δ-subunit mediated, Figure 1; and α5-subunit mediated/total capacity, Figure 2) in Fmr1 KO PNs independent of reduced GABA availability (Olmos-Serrano et al., 2010) since direct activation and saturating GABA still produces smaller currents in these cells compared to WT.

**α5-GABA<sub>A</sub> receptors regulate synaptic efficacy in BLA principal neurons**

In addition to the change in holding current observed in our measurements of tonic capacity by reducing α5-GABA<sub>A</sub> receptor activity (Figure 2), we surprisingly observed an apparent increase in the amplitude of GABA<sub>A</sub> receptor-mediated spontaneous inhibitory post-synaptic currents (sIPSCs) in both WT and Fmr1 KO PNs (Figure 2Aii,Bii (α5ia)). To further explore this observation, we first recorded sIPSCs with our standard potassium-gluconate (K-gluc) intracellular solution in our recording
pipettes in the absence of exogenous GABA (Figure 3). Under these conditions we did not observe any significant changes in holding current after application of the \( \alpha \)-5-subunit specific inverse agonist \( \alpha \)-5ia (Figure 3A). However, in both WT and \( Fmr1 \) KO principal neurons, reduction of \( \alpha \)-5-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 PNs. This increase occurred in both WT and \( Fmr1 \) KO PNs (Figure 3A,B; WT: baseline, 203.25 ± 3.65 ms, \( n = 4498 \) events (from 13 cells); \( \alpha \)-5ia, 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); \( \alpha \)-5ia, 225.7 ± 6.26 ms, \( n = 1601 \) events (from 7 cells), K-S test, \( p = 0.02 \)). Additionally, the amplitude of these sIPSCs also increased in WT on \( \alpha \)-5ia application, but not \( Fmr1 \) KO cells (Figure 3A,C; WT: baseline, 19.36 ± 1.91 pA; \( \alpha \)-5ia, 21.52 ± 1.73 pA, \( n = 13 \) cells, paired t-test, \( p = 0.021 \); \( Fmr1 \) KO: baseline, 15.36 ± 1.95 pA; \( \alpha \)-5ia, 16.44 ± 2.23 pA, \( n = 7 \) cells, paired t-test \( p = 0.182 \)). Furthermore the weighted decay time constant (\( \tau_{aD} \)) of sIPSCs increased slightly but significantly after \( \alpha \)-5ia application while the mean 10-90% rise time showed no increase in either WT or \( Fmr1 \) KO cells (Figure 3D, Table 1). In general our results are consistent with our previous published data (Olmos-Serrano et al., 2010; Vislay et al., 2013) indicating that \( Fmr1 \) KO BLA principal neurons display decreased baseline inhibitory synaptic efficacy compared to their WT counterparts (inter-event interval, Figure 3B; WT baseline vs \( Fmr1 \) KO baseline, K-S test, \( p < 0.0001 \); amplitude, Figure 3C; WT vs \( Fmr1 \) KO, ANOVA with Bonferroni correction, \( p = 0.035 \)). Interestingly, \( \alpha \)-5ia has the most potent effect on WT cells (frequency and amplitude of
sIPSCs) versus Fmr1 KO cells (frequency only) consistent with the conclusion that Fmr1 KO principal neurons express less α5-GABA<sub>A</sub> receptors. We corroborated the specificity of α5ia effects on sIPSC properties by repeating the experiments in WT slices with another commonly utilized α5-subunit preferring inverse agonist of the imidazobenzodiazepine class, L655-708 (100 nM) (Quirk et al., 1996; Atack et al., 2006). This inverse agonist has less specificity and potency at α5- over α1-, α2-, and α3-GABA<sub>A</sub> receptors compared to α5ia (Atack, 2010). Application of this compound in recordings using K-Gluc based intracellular solution also produced no significant changes in holding current, however we did observe similar decreases in sIPSC inter-event interval determined as a significant leftward shift in the cumulative probability function (similar to Figure 3B, α5ia) (WT: baseline, 193.66 ± 3.89 ms, n = 3116 events (from 6 cells); L655-708, 178.49 ± 3.49 ms, n = 3457 events (from 6 cells), K-S test, p < 0.01) as well as a frequency increase by pair-wise comparison of mean frequency before and after L655-708 application (WT: baseline 5.16 ± 1.08 Hz; L655-708, 5.66 ± 1.15 Hz, n = 6 cells, paired t-test, p = 0.03). In addition, amplitude of sIPSCs increased in 4 out of 6 cells with L655-708 application (data not shown). These results support a consistent effect of α5-subunit inverse agonists on sIPSC properties in BLA PNs and additionally highlight a more potent action of α5ia versus L655-708. Therefore, we solely utilized α5ia for the rest of the study.

Both pre- and/or postsynaptic actions of α5-GABA<sub>A</sub> receptors could underlie the rapid increase in inhibitory synaptic efficacy (frequency and amplitude) following reduction of α5-containing GABA<sub>A</sub> 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 by increasing detectability of our recordings. To test the possibility that blockade of α5-containing receptors uncovers more distally originating synaptic events, we repeated sIPSC recordings in the absence of exogenous GABA using a cesium chloride (CsCl) based internal recording solution in our recording pipettes (Figure 4) instead of potassium based (K-Gluc) solution as utilized above (Figure 3) to reduce potassium currents that filter current changes originating far from the recording site (Stuart and Spruston, 1998). By observing a more complete sample of synaptic events including those that originate farther from the recording site under baseline conditions with CsCl recording solution, any effect by α5-GABA_A receptors revealed by application of α5ia should be reduced.

As expected, after α5-GABA_A receptor blockade, the increase in efficacy previously observed with K-Gluc intracellular solution was partially occluded (Figure 4). Rather than an increase in frequency, we observed a slight decrease in frequency (Figure 4A, 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 4B, 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 tau_D with α5-GABA_A receptor blockade (Figure 4C, left, Table 1) with no significant change in event 10-90% rise time. Furthermore, unlike cells recorded with K-Gluc intracellular (Figure 3), α5-GABA_A receptor blockade was also accompanied by decreases in tonic current in 6 out of 8 cells. As expected these currents were smaller than those observed with CsCl and
exogenous saturating GABA (Figure 2) (CsCl + 5 μM GABA, 0.04 ± 0.020 pA/pF, n = 12; CsCl + native GABA, 0.02 ± 0.005 pA/pF, n = 6, t-test, p = 0.03). The presence of 

α5-subunit mediated tonic currents in CsCl recordings with only native GABA levels suggests that lack of exogenous GABA in our K-Gluc recordings (Figure 3) did not contribute to our observation of no α5-GABA<sub>A</sub> receptor mediated tonic currents in those recordings. Instead, the results are consistent with an α5-subunit mediated tonic conductance that regulates neuronal membrane normally electrically out of reach of the K-Gluc intracellular recordings.

It is unlikely that presynaptic mechanisms are involved in the observed increase in inhibitory efficacy following α5-GABA<sub>A</sub> blockade (Figure 3) as simply replacing pipette potassium with cesium can prevent frequency increases in response to α5-GABA<sub>A</sub> receptor blockade (Figure 4). However, the possibility remains that an increase in the excitability of presynaptic interneurons may be induced by α5-GABA<sub>A</sub> 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 test for this possibility we recorded miniature IPSCs (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 4A, dotted lines; inter-event 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 4B, amplitude baseline, 31.05 ± 2.51 pA; α5ia, 33.22 ± 3.06 pA, n = 9, paired t-
test, p = 0.027), an increase in τD (Figure 4C, Table 2), and no change in event 10-90% rise time. We subsequently recorded mIPSCs using the K-Gluc based solution to record primarily more proximal somatic synapses. In these recording conditions we observed no significant change in frequency (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), τD, or event 10-90% rise time (Table 2) after the application of α5ia to block α5-GABA_A receptors. Collectively, these results suggest that α5-GABA_A receptors in BLA PNs have a functional preference to modulate postsynaptic events more distal from the soma than those typically recorded with K-Gluc intracellular solution.

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 frontal cortex traveling within the external capsule (Ottersen, 1982; McDonald and Mascagni, 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 more distal synaptic efficacy in BLA PNs places the
receptor subtype in a position to perhaps modulate integration of excitatory and inhibitory inputs locally on dendrites and subsequently affect summation at the soma and axon initial segment. Therefore, we examined if $\alpha_5$-GABA$_A$ receptor-mediated conductance affects the integration of excitatory and inhibitory synaptic transmission in BLA PNs. We investigated this possibility by measuring evoked synaptic conductance in the presence or absence of $\alpha_5$-GABA$_A$ receptor activity in both WT and $Fmr1$ KO amygdala slices (Figure 5). 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 5A,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: $19.1 \pm 1.00 \mu V$, $n = 16$; $Fmr1$ KO: $21.7 \pm 1.76 \mu V$, $n = 18$, $p = 0.22$). Total synaptic (G$_{tot}$), excitatory (G$_e$), and inhibitory (G$_i$) conductance was derived from I-V curves taken from average traces at each holding potential (7-15 sweeps/potential) using established methods (see Methods) (Figure 5A,B, inset). The total conductance, G$_{tot}$, was large with a fast onset (~3 ms, Figure 5G) and slow latency to peak (~11-14 ms) in both WT and $Fmr1$ KO slices. G$_e$ and G$_i$ onset occurred within 1-2 ms of each other (Figure 5A,B) indicating that stimulated external capsule afferents monosynaptically innervate PNs and GABAergic interneurons of the BLA. The conductance profile corresponds well with analogous feedforward circuits such as those in Layer IV somatosensory cortex (thalamocortical) and hippocampal area CA1 (Pouille and Scanziani, 2001; Gabernet et al., 2005; Cruikshank et al., 2007) with the exception
of 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) despite similar stimulus strength (~40-120 μA, 100-200 μS). Extensive BLA-specific reverberant synaptic connections in PNs (Smith and Paré, 1994) reinforced by our strong stimulus together with lower experimental temperatures (26-28° C vs 32° C) may support this uniquely longer time course by recruiting a relatively larger summation of feedforward excitatory/inhibitory transmission.

Since overall inhibitory synaptic efficacy is decreased in Fmr1 KO PNs (Olmos-Serrano et al., 2010), we expected that total evoked synaptic E/I balance in Fmr1 KO slices might be increased. However surprisingly, we instead detected no significant differences in peak levels of total, excitatory, or inhibitory conductance between WT and Fmr1 KOs (Figure 5C, Table 2, reported as conductance density to control for differences in cell size/capacitance, nS/pF; WT: n = 9; Fmr1 KO: n = 11). In addition, overall E/I balance as measured by the ratio of Ge to Gi over time (Figure 5A,B,D) was also unchanged between WT and Fmr1 KOs (Area E / Area I (integral) = (Ge(ns) / time(ms))/(Gi(ns) / time(ms))).

Interestingly, instead of total evoked conductance and overall synaptic E/I balance differences we observed 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 5Aii,Bii,E; Gi peak time – Ge peak time, WT baseline: 2.41 ± 0.54 ms; Fmr1 KO baseline: 1.19 ± 0.28 ms; ANOVA, p = 0.023). The decreased Ge peak to Gi peak time was associated with significantly decreased E/I conductance ratio at the Ge peak in Fmr1 KO PNs compared to WT (Figure 5F; WT baseline: 0.48 ± 0.05; Fmr1 KO
baseline: 0.32 ± 0.02; ANOVA, p = 0.005) consistent with a decreased window for synaptic integration in these Fmr1 KO cells (Pouille and Scanziani, 2001; Isaacson and Scanziani, 2011). We then investigated the underlying cause of this alteration in synaptic timing by determining 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 significantly decreased Ge peak latency in WT PNs compared to Fmr1 KO PNs (Figure 5G; WT baseline: 11.72 ± 0.73 ms; Fmr1 KO baseline: 14.09 ± 1.06 ms; ANOVA, p = 0.048) but without a difference in the latency of Gi (Figure 5G; Gi latency WT baseline: 14.22 ± 0.58 ms; Fmr1 KO baseline: 15.22 ± 0.99 ms; ANOVA, p = 0.38). Therefore, these data suggest that differences in kinetics in WT and Fmr1 KO slices depend solely on the faster latency of Ge (faster rise time) in WT PNs and not on Gi latency. 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 Fmr1 KO PNs resulting in an increase in the E/I ratio at the Ge peak for WT neurons (Figure 5F).

To determine whether this difference in Ge kinetics and altered E/I timing could be related to the reduction and/or loss of α5-GABA_{A} receptors, we next examined evoked synaptic conductance in a separate set of cells in the absence of α5-GABA_{A} receptor activity. If α5-GABA_{A} receptors heavily regulate synaptic efficacy and integration (Figures 3 and 4), these receptors might underlie the kinetic differences, providing a tonic conductance to maintain local membrane resistance and ensure faster evoked synaptic kinetics thereby controlling the spread of synaptic activity. In recordings from both WT and Fmr1 KO 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 $\alpha$5ia (WT before: $21.1 \pm 1.76 \mu V$; WT after
$\alpha$5ia: $22.2 \pm 1.31$, n = 7; paired t-test, p = 0.66; Fmr1 KO before: $18.1 \pm 2.85 \mu V$; Fmr1
KO after $\alpha$5ia: $18.7 \pm 2.97 \mu V$, n = 7; paired t-test, p = 0.89; WT vs Fmr1 KO, ANOVA,
p = 0.39). Measurements of peak conductance density (Gtot, Ge, and Gi) did not differ
significantly from WT baseline or Fmr1 KO baseline groups for either genotype in the
presence of $\alpha$5ia (Figure 5C, Table 2) so $\alpha$5-GABA_A receptor blockade does not affect
overall conductance or components of that conductance as recorded at the soma (Ge
and Gi).

In WT slices, blockade of $\alpha$5-GABA_A receptors significantly reduced the Ge peak
to Gi peak time compared to the WT baseline group (Figure 5Aii,Aiii,E; WT $\alpha$5ia: $0.67 \pm
0.33$ ms, n = 7 cells, vs WT baseline, t-test, p = 0.012). Notably peak-to-peak times
mimicked those of both the Fmr1 KO baseline and Fmr1 KO $\alpha$5ia conditions (WT $\alpha$5ia
vs Fmr1 KO baseline, t-test, p = 0.26; vs Fmr1 KO $\alpha$5ia: $0.86 \pm 0.18$ ms, n = 7 cells, t-
test, p = 0.63). Concurrently, blockade of $\alpha$5-GABA_A receptors in WT cells also resulted
in significant reduction of the E/I conductance ratio at the Ge peak compared to the WT
baseline group. Again these values were similar to those recorded from Fmr1 KO slices
in either the baseline or $\alpha$5ia condition (Figure 5F; WT $\alpha$5ia: $0.31 \pm 0.03$, vs WT
baseline, t-test, p = 0.016, vs Fmr1 KO baseline, t-test, p = 0.79; Fmr1 KO $\alpha$5ia: $0.37 \pm
0.03$, t-test, p = 0.19). In addition this reduction correlated with an increased latency to
the Ge peak similar to both Fmr1 KO groups (Figure 5G; WT $\alpha$5ia: $13.8 \pm 0.68$ ms, vs
WT baseline, t-test, p = 0.03, vs Fmr1 KO baseline, t-test, p = 0.84; Fmr1 KO $\alpha$5ia:
13.2 \pm 0.82 \text{ ms}, t\text{-test}, p = 0.60) but without a change in the latency to the Gi peak (WT \alpha 5ia: 14.5 \pm 0.54 \text{ ms}, vs WT baseline, t\text{-test}, p = 0.67, vs Fmr1 KO baseline, t\text{-test}, p = 0.58; Fmr1 KO \alpha 5ia: 14.2 \pm 0.79 \text{ ms}, t\text{-test}, p = 0.69). Consistent with a lack of \alpha 5-GABA_A receptor function in Fmr1 KO slices, blockade with \alpha 5ia did not result in any significant measureable changes compared to the Fmr1 KO baseline groups in control ACSF (Figure 5E,F,G; Ge to Gi peak time, t\text{-test}, p = 0.19; E/I conductance ratio at Ge peak, t\text{-test}, p = 0.08; latency to Ge peak, t\text{-test}, p = 0.29; latency to Gi peak, t\text{-test}, p = 0.43).

These data show that in WT slices, \alpha 5-containing GABA_A receptors are involved in controlling evoked synaptic conductance balance in BLA PNs by primarily modulating Ge latencies. Summary data from all PNs reveals that Ge latency negatively correlates with E/I balance at the Ge peak (Figure 5H) such that longer Ge latencies in the absence of \alpha 5-GABA_A receptor activity generally coincide with lower E/I ratios. In general, the Fmr1 KO PN population displays a tendency toward narrower Ge to Gi temporal windows associated with decreased \alpha 5-GABA_A receptor expression that may imply decreased capacity for accurate input integration and plasticity in a circuit that is crucial for regulating fear and anxiety.

**DISCUSSION**

Tonic GABAergic inhibition is a critical regulator of cellular and network E/I balance (Farrant and Nusser, 2005), however in neurodevelopmental disorders such as FXS that are characterized by a network imbalance, the role of this powerful conductance is poorly understood, particularly in key brain regions such as the
amygdala. Here we demonstrated that tonic GABAergic current capacity in BLA PNs of Fmr1 KO mice is reduced independently of decreased GABA availability, a prominent characteristic in the Fmr1 KO amygdala (Olmos-Serrano et al., 2010). Furthermore this tonic conductance consists of at least δ- and α5-GABA_A receptor components, both of which are reduced in Fmr1 KO cells. Interestingly, we show that α5-GABA_A receptors functionally modulate cellular compartments distal from the soma. In addition although overall E/I synaptic conductance balance appears to be maintained in Fmr1 KO slices in response to external capsule stimulation, the window of peak excitation to peak inhibition generated by feedforward inhibition is significantly narrowed compared to WT. Finally, deactivation of an α5-GABA_A receptor mediated conductance in WT slices tightens E/I timing to Fmr1 KO levels indicating that this tonic conductance can at least partially regulate synaptic E/I dynamics in BLA PNs.

**Tonic GABAergic neurotransmission in BLA Principal Neurons**

Tonic GABAergic conductance has been observed in a number of brain regions including the cerebral cortex, hippocampus, striatum, thalamus, and cerebellum, and is commonly mediated to varying degrees by both δ- and α5-GABA_A receptors in a region-specific manner (Farrant and Nusser, 2005). Our results show that BLA PNs utilize at least these two receptor subtypes to maintain tonic GABAergic conductance. Furthermore, the magnitude of both of these component currents is relatively small, consistent with the diffuse levels of protein expression of these subunits in the region (Fritschy and Mohler, 1995). The data here, combined with recent data implicating strong tonic modulation mediated by the atypical α3-subunit containing GABA_A receptor
in BLA PNs (Marowsky et al., 2012), underscores the diversity of GABA<sub>A</sub> receptor subtypes than can tonically control membrane excitability in these cells.

Recordings performed with two intracellular solutions that enable relatively strong (CsCl) and relatively weak (K-Gluc) voltage control demonstrate a functional preference of α5-GABA<sub>A</sub> receptors to affect synaptic conductance at membrane locations distal from our somatic recording site in BLA PNs. This preference implies that these receptors likely lie in proximal dendrites rather than the soma. However, this functional data only indirectly identifies this subcellular location preference for α5-GABA<sub>A</sub> receptors. Ultrastructural studies comparing α5-subunit expression surrounding somatic and dendritic synapses will be required to confirm these observations.

Nevertheless, the data is consistent with an established role of these receptors in analogous principal cells that express α5-GABA<sub>A</sub> receptors in other brain regions. For instance functional and ultrastructural studies of neocortical Layer 5 and hippocampal pyramidal neurons reveal a preference of these receptors to specifically modulate dendritic subcellular compartments (Christie and De Blas, 2002; Ali and Thomson, 2008).

**α5-GABA<sub>A</sub> receptor transmission affects synaptic balance in BLA PNs**

Integration windows (Lloyd, 1946), the time frame within which excitatory potentials can effectively summate to generate an action potential, are influenced by the relative timing and strength of synaptic excitatory and inhibitory input and have been well established to exist in feedforward circuits especially in sensory cortices (Isaacson and Scanziani, 2011). Most importantly, they determine the window of responsiveness to coincident activity in the neuron and determine the tuning of the neuron to incoming
stimuli (the population of inputs to which the neuron can respond (König et al., 1996)). The more narrow the response window (Ge to Gi), the more synchronized afferent inputs must be to generate an action-potential response in the neuron (Pouille and Scanziani, 2001; Gabernet et al., 2005). In contrast, the broader the integration window, the less sensitive the neuron is to input timing and therefore the broader the range of inputs to which the neuron can respond and the higher the coding capacity (Poo and Isaacson, 2009). In the present study we revealed that in WT BLA PNs, reduction of a distally located $\alpha$5-GABA$_A$ receptor mediated tonic conductance restricted the feedforward temporal window established by a mixed evoked excitatory/inhibitory conductance. Therefore, tonic $\alpha$5-GABA$_A$ receptor mediated conductance could at least partially modulate integration of postsynaptic excitatory and inhibitory potentials at the soma in these cells. In this manner, tonic conductance might support the proper feedforward integration of various concurrent afferent inputs to BLA PN dendrites from thalamus, lateral amygdala, prefrontal cortex, and hippocampus for example, for proper processing of conditioned fear and extinction via regulation of activity dependent synaptic plasticity and coincidence detection (Ehrlich et al., 2009). However, future direct investigations of the integration window are necessary to determine whether changes in this $\alpha$5-GABA$_A$ receptor mediated conductance indeed affect the spike probability of BLA PNs in response to coincident inputs.

Furthermore evidence shows that even small changes in inhibition can significantly alter E/I balance and network function (Isaacson and Scanziani, 2011). Although it is well established that GABAergic inhibition generally supports a more narrow integration window and increased neuron selectivity (Wehr and Zador, 2003),
studies to elucidate the role of GABAergic inhibition in these processes generally block all GABA$_A$ receptors (phasic and tonic) with an antagonist such as bicuculline, which limits the interpretation of experimental results. In contrast, using blockade of specifically $\alpha$5-GABA$_A$ receptors, we show that a tonic, perhaps dendritically based conductance, can function to support a broader E/I temporal window independent of the feedforward inhibitory phasic synaptic conductance (Gi), by primarily affecting Ge kinetics. Since tonic conductance modulates gain by altering membrane resistance (Mitchell and Silver, 2003), the loss of $\alpha$5-GABA$_A$ receptor activity could increase membrane resistance locally thereby increasing the membrane time constant and slowing the response rise time and decay in these cells. This situation could support the increased summation of synaptic events from multiple recurrent excitatory synaptic connections (Smith and Paré, 1994) to produce longer Ge latencies. Increases in $\tau_{D}$ in the presence of $\alpha$5ia in our IPSC recordings further support this phenomenon.

**Implications of reduced tonic GABAergic inhibition for FXS**

Our data showing compromised tonic GABA conductance in Fmr1 KO slices adds to the growing evidence of excitation/inhibition imbalances in FXS (Paluszkiewicz et al., 2011b) and to related autism spectrum disorders in general (Zhang et al., 2008; Markram and Markram, 2010). As a powerful regulator of cellular excitability, the significant reduction of this conductance likely directly underlies the hyperexcitability of Fmr1 KO BLA PNs (Olmos-Serrano et al., 2010). And although it has only specifically been examined in a limited number of circuits in FXS (Curia et al., 2009), decreased tonic GABAergic conductance may in fact represent a common feature of the disorder.
across the FXS brain given the broad reduction of GABAergic components, including the tonically active δ-subunit [reviewed in (Paluszkiewicz et al., 2011a)].

Our observations of significantly tightened E/I temporal windows in Fmr1 KO BLA PNs in response to afferent input represents a novel physiological phenotype that could also result from impaired tonic GABAergic inhibition, given that reduction of α5-subunit mediated tonic conductance in WT slices reproduces the Fmr1 KO phenotype. However we cannot discount other cellular and network changes that may occur in the Fmr1 KO BLA that could also account for the narrow temporal window including maturity, number, and location of excitatory and inhibitory synapses on Fmr1 KO PNs. For instance hyperconnectivity of neocortical Layer 5 pyramidal cells as has been observed in these mice (Testa-Silva et al., 2012). A similar condition in the BLA would support increased summation of recurrent excitatory synaptic conductance in our experiments independent of decreased α5-GABA_A receptor mediated conductance. Alternatively, intrinsic alterations in membrane properties related to ion channelopathies could alter summation of inputs at the soma and/or integration of orthodromic and anterograde dendritic E/I currents (Brager and Johnston, 2014) and modify the observed temporal window. These factors combined with our strong evoked stimulus could have similarly influenced the surprisingly unchanged total synaptic conductance and overall E/I balance we observed in Fmr1 KO slices compared to WT by reinforcing and/or amplifying recurrent connections. Also of note, although our recordings were performed at temperatures near those used in analogous examinations of feedforward circuits in acute brain slices (Gabernet et al., 2005; Cruikshank et al., 2007), they were below physiological temperature (~37° C). Given the variable temperature sensitivity of
excitatory and inhibitory synapses (Huntsman and Huguenard, 2000; Postlethwaite et al., 2007), the observed differences in integration windows between genotypes could be more or less pronounced \textit{in vivo} or at a higher temperature. Despite this potential caveat, our results show a clear difference in regulation of E/I inputs in \textit{Fmr1} KO PNs and a role of $\alpha$5-GABA$_A$ receptors in that regulation.

Even if decreased $\alpha$5-subunit expression is not the principal cause of the restricted E/I windows in \textit{Fmr1} KO PNs, augmentation of deficient tonic GABAergic conductance could still support more efficient BLA network function by increasing coding capacity (Isaacson and Scanziani, 2011) and improving cellular hyperexcitability (Olmos-Serrano et al., 2010). In fact, these cellular and network phenotypes may go hand in hand in FXS since narrow integration windows could be partially overcome with increased neuron excitability to support coincidence detection within a restricted time window. Consequently hyperexcitability could develop as a homoestatic response to narrow integration windows in FXS subsequent to abnormal synaptic development (He and Portera-Cailliau, 2013) or vice versa. Given the pervasiveness of cellular hyperexcitability and network hypersynchrony in FXS networks, particularly sensory cortices (Gibson et al., 2008; Gonçalves et al., 2013), and its association with narrowed E/I temporal windows here, altered E/I synaptic integration windows may represent a hallmark of FXS networks generally. If so, sensory integration dysfunction could be explained by cellular/network hyperexcitability during developmental critical periods in FXS (Gonçalves et al., 2013) combined with decreased coding capacity to affect experience dependent plasticity within sensory cortices as well as the proper assimilation of those networks with subcortical structures like the BLA. Enhancement of
tonic GABAergic inhibition in FXS then, may support both a normalization of coding capacity and network excitability to enhance brain function in patients with this disorder.

Author Contributions:

BSM and MMH designed the study. BSM collected and analyzed all data. BSM, JGC, and MMH wrote the manuscript.

Acknowledgements:

This work was supported by grants from NIH/NINDS (MMH NIH grant #:NS053719), Epilepsy Foundation (BSM), Autism Speaks (MMH and JGC), and the FRAXA Foundation (MMH and JGC).
REFERENCE LIST


Hays SA, Huber KM, Gibson JR. Altered Neocortical Rhythmic Activity States in Fmr1 KO Mice Are Due to Enhanced mGluR5 Signaling and Involve Changes in Excitatory Circuitry. Journal of Neuroscience 31: 14223–14234, 2011.


Semyanov A, Walker MC, Kullmann DM, Silver RA. Tonically active GABA A


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| K-Gluc + TTX | wild-type    | 10-90% rise time | 1.00 ± 0.03    | 0.91 ± 0.03   | 0.06     | 6  |
|              |              | weighted tau    | 13.04 ± 0.88   | 13.43 ± 0.81  | 0.32     | -  |

| CsCl        | wild-type    | 10-90% rise time | 1.05 ± 0.12 ms | 1.20 ± 0.13 ms | 0.08     | 8  |
|              |              | weighted tau    | 17.43 ± 1.02   | 18.08 ± 0.99  | 0.02*    | -  |

| CsCl + TTX  | wild-type    | 10-90% rise time | 1.38 ± 0.10 ms | 1.37 ± 0.11 ms | 0.53     | 9  |
|              |              | weighted tau    | 18.73 ± 0.62   | 20.32 ± 0.69  | 0.01*    | -  |

Table 1. Summary of α5ia induced changes.
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<td>Fmr1 KO</td>
<td>baseline</td>
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<td>α5ia</td>
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<td>α5ia</td>
<td>0.31 ± 0.02</td>
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Table 2. Total conductance and excitatory/inhibitory components are not significantly different between WT and Fmr1 KO slices and α5ia does not affect conductance in either genotype.
**FIGURE LEGENDS**

**Figure 1.** *Fmr1* KO principal neurons of the BLA have reduced δ-subunit mediated tonic GABAergic currents. (A,B) Representative current clamp traces showing typical responses of principal neurons to depolarizing (+150 pA) and hyperpolarizing (-100 pA) current injections (600 ms). (Aii,Bii) 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, 1 μM. (*V*<sub>hold</sub> = -60 mV) 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 at 1 μM concentrations of THIP. *p* < 0.05.

**Figure 2.** *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) (*V*<sub>hold</sub> = -70 mV). 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.

**Figure 3.** 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 $\alpha$5ia ($V_{\text{hold}} = -60$ mV). (C) In the presence of $\alpha$5ia 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), $\alpha$5ia (red solid); Fmr1 KO: right, baseline (gray dotted), $\alpha$5ia (red dotted)) show slight but significant increases in decay constant $\tau$ (Table 2). *$p < 0.05$.

**Figure 4.** Recording IPSCs with CsCl-based pipette solution occludes increases in inhibitory efficacy in response to $\alpha$5-GABA$_A$ receptor blockade. (A) Application of $\alpha$5ia (1.5 $\mu$M) 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 $\alpha$5ia (left). (C) In both sIPSC (left) and mIPSC (right) recordings the decay constant $\tau_D$ increases slightly and significantly in response to $\alpha$5ia application (Table 2). *$p < 0.05$.

**Figure 5.** The presence of $\alpha$5-GABA$_A$R activity affects evoked response kinetics and synaptic balance. (A,B) Representative conductance measurements ($G_{\text{tot}}$, black; $G_e$, blue; $G_i$, 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 $\alpha$5-GABA$_A$ receptor blockade ($\alpha$5ia, 1.5 $\mu$M) in WT (Aiii) and Fmr1 KO (Biii) cells. (C) Conductance measurements of WT and Fmr1 KO cells reveal no significant differences in $G_{\text{tot}}$, $G_e$, or $G_i$ between genotypes or conditions (baseline or $\alpha$5ia) (conductance density (nS/pF)). (D) In addition overall E/I balance is similar among genotypes and conditions (Conductance Area: $G_e$ (nS/ms)/$G_i$).
(nS/ms)). (E) 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). (F) 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). (G) 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). (H), Summary data from all cells indicate that Ge latency (G) and E/I ratio at peak Ge (F) are negatively correlated (linear regression, r = -0.587, p < 0.0001).