

**Decreased Surface Expression of the δ Subunit of the GABA_A Receptor Contributes to Reduced
Tonic Inhibition in Dentate Granule Cells in a Mouse Model of
Fragile X Syndrome**

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Abstract – 390 Words

While numerous changes in the GABA system have been identified in models of Fragile X Syndrome (FXS), alterations in subunits of the GABA_A receptors (GABA_ARs) that mediate tonic inhibition are particularly intriguing. Considering the key role of tonic inhibition in controlling neuronal excitability, reduced tonic inhibition could contribute to FXS-associated disorders such as hyperactivity, hypersensitivity, and increased seizure susceptibility. The current study has focused on the expression and function of the δ subunit of the GABA_AR, a major subunit involved in tonic inhibition, in granule cells of the dentate gyrus in the *Fmr1* knockout (KO) mouse model of FXS. Electrophysiological studies of dentate granule cells revealed a marked, nearly four-fold, decrease in tonic inhibition in the *Fmr1* KO mice, as well as reduced effects of two δ subunit-preferring pharmacological agents, THIP and DS2, supporting the suggestion that δ subunit-containing GABA_ARs are compromised in the *Fmr1* KO mice. Immunohistochemistry demonstrated a small but statistically significant decrease in δ subunit labeling in the molecular layer of the dentate gyrus in *Fmr1* KO mice compared to wildtype (WT) littermates. The discrepancy between the large deficits in GABA-mediated tonic inhibition in granule cells in the *Fmr1* KO mice and only modest reductions in immunolabeling of the δ subunit led to studies of surface expression of the δ subunit. Cross-linking experiments followed by Western blot analysis demonstrated a small, non-significant decrease in total δ subunit protein in the hippocampus of *Fmr1* KO mouse, but a six-fold decrease in surface expression of the δ subunit in the *Fmr1* KO mice. No significant changes were observed in total or surface expression of the $\alpha 4$ subunit protein, a major partner of the δ subunit in the forebrain. Postembedding immunogold labeling for the δ subunit demonstrated a large, three-fold, decrease in the number of symmetric synapses with immunolabeling at perisynaptic locations in *Fmr1* KO mice. While $\alpha 4$ immunogold particles were also reduced at perisynaptic locations in the *Fmr1* KO mice, the labeling was increased at synaptic sites. Together these findings suggest that, in the dentate gyrus, altered surface expression of the δ subunit, rather than a decrease in δ subunit expression alone, could be limiting δ subunit-mediated tonic inhibition in this model of FXS. Finding ways to increase

surface expression of the δ subunit of the GABA_AR could be a novel approach to treatment of hyperexcitability-related alterations in FXS.

Key words: GABA receptor, Tonic inhibition, Dentate gyrus, *Fmr1* gene, Fragile X mental retardation protein, Fragile X Syndrome

Highlights

Tonic inhibition is reduced in the dentate gyrus in a model of Fragile X Syndrome.

Surface expression of GABA_AR δ subunits is significantly reduced in this FXS model.

Labeling of the δ subunit is reduced at perisynaptic locations in the *Fmr1* KO mouse.

Labeling of the $\alpha 4$ subunit is increased at synaptic locations in the *Fmr1* KO mouse.

Decreased surface expression of the δ subunit limits tonic inhibition in this model.

Introduction

Fragile X syndrome (FXS) is the most common form of inherited cognitive impairment in humans and results from loss of function of the *Fmr1* gene that encodes the fragile X mental retardation protein (FMRP) (Kooy et al., 2000). This RNA-binding protein has many functions that include regulation of translation and transport of a subset of mRNAs into the dendrites and, through such functions, influences synapse development and plasticity (Bassell and Warren, 2008; Pfeiffer and Huber, 2009) for reviews). Loss of FMRP function affects multiple neurotransmitter and signaling systems, including the GABA system (D'Hulst and Kooy, 2009; Santoro et al., 2012). While numerous types of alterations in the GABA system have been reported (D'Hulst et al., 2009; Paluszkiwicz et al., 2011) for reviews, a reduction in GABA_A receptor (GABA_AR)-mediated tonic inhibition is particularly intriguing, both as a basic functional change in FXS and as a target for treatment. Tonic inhibition provides a powerful control of neuronal excitability (Brickley and Mody, 2012; Otis et al., 1991; Semyanov et al., 2004), and a decrease in tonic inhibition could contribute to the increased network excitability that is often observed in models of FXS (Gibson et al., 2008; Goncalves et al., 2013) and thus be associated with behavioral changes such as hyperactivity, hypersensitivity to sensory stimuli, and increased seizure susceptibility (Contractor et al., 2015).

Although several GABA_AR subunits can be involved in tonic inhibition, the δ subunit is a critical subunit in several major brain regions, including the dentate gyrus, and expression of the δ subunit conveys special properties (Brickley and Mody, 2012). δ Subunit-containing GABA_ARs are located at extra- and perisynaptic sites, where they are ideally positioned to respond to ambient levels of GABA within the extracellular space or to spillover at the synapse; they have a high affinity for GABA and slow rates of desensitization; and, importantly, they are extremely sensitive to endogenous compounds such as neuroactive steroids (Saxena and Macdonald, 1994; Wei et al., 2003; Wohlfarth et al., 2002). As a result of these properties, δ subunit-containing GABA_ARs can be modulated continuously to help maintain the general level of excitability of neuronal networks (Belelli et al., 2009; Olsen and Sieghart, 2008; Sun et

al., 2004; Wei et al., 2002). Interestingly, the δ subunit mRNA was one of the first mRNAs to be identified as a direct target of FMRP (Miyashiro et al., 2003). If lack of FMRP leads to altered translation or transport of δ subunit mRNA or dysregulation of δ subunit expression and function, such changes could be associated with deficits in tonic inhibition and potentially constitute a key alteration in FXS.

Despite considerable interest in GABA_ARs in FXS, GABA-mediated tonic inhibition has been studied in only a limited number of brain regions, such as the subiculum and basolateral nucleus of the amygdala (Curia et al., 2009; Olmos-Serrano et al., 2010). Although tonic inhibition is prominent and mediated primarily by δ subunit-containing GABA_ARs in several major forebrain regions such as the dentate gyrus, alterations of δ -mediated tonic inhibition have been studied in FXS. Altered tonic inhibition in the dentate gyrus could be quite important in FXS as this region serves as a gateway to the hippocampus and regulates the large amount of incoming information from the entorhinal cortex. Reduced control of granule cell activity in the dentate gyrus can lead to increased excitability throughout the hippocampal circuit and contribute to deficits in learning and memory as well as increased seizure susceptibility, conditions that are found in children with FXS (Berry-Kravis, 2002; Hagerman and Stafstrom, 2009; Musumeci et al., 1999).

The major goals of this study were to determine if tonic inhibition is impaired in dentate granule cells in the *Fmr1* KO mouse model of FXS and if such changes are accompanied by alterations in the expression and localization of the δ subunit of the GABA_AR in the dentate gyrus, using immunolabeling and biochemical methods. The findings suggest that decreased surface expression of the δ subunit, rather than a decrease in δ subunit expression alone, is likely limiting tonic inhibition in the dentate gyrus and add to the growing evidence for deficits in δ subunit-mediated tonic inhibition in FXS. Preliminary reports have been presented previously (Houser et al., 2014; Zhang et al., 2016).

Methods

Animals

Fmr1 KO male mice on a C57BL/6 background and their male wild-type (WT) littermates were used for all experiments. *Fmr1* KO mice were originally obtained from Dr. William Greenough (University of Illinois at Urbana-Champaign) and bred at the University of California, Los Angeles. Genotypes were determined by PCR analysis of DNA extracted from tail samples. After weaning, mice were housed in mixed genotype groups of the same sex under standard laboratory conditions with a 12:12 h light-dark cycle. Animals for immunohistochemical studies were studied from postnatal day (PN) 14-60, and those for electrophysiological and biochemical studies were studied at 2-3 months of age. Data from 34 *Fmr1* WT and 35 *Fmr1* KO littermates were used in the study, and animal numbers are indicated for each experiment. All animal use protocols conformed to National Institutes of Health guidelines and were approved by the University of California, Los Angeles, Chancellor's Animal Research Committee.

Immunohistochemistry

Tissue preparation for light microscopy. All mice used for light microscopic immunohistochemical studies were deeply anesthetized with sodium pentobarbital (90 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.3. After 1 h at 4°C, brains were removed and postfixed for 1 h. After rinsing, brains were cryoprotected in a 30% sucrose solution overnight at 4°C and frozen in cryo embedding compound on dry ice. Forebrain blocks containing the hippocampus were sectioned coronally at 30 μ M with a cryostat.

Antisera and immunohistochemical methods. The following GABA_AR subunit-specific antisera were used to localize δ and $\alpha 4$ subunits in the immunohistochemical studies: rabbit anti δ subunit (1:000; Millipore AB9752 or PhosphoSolutions 868-GDN, no longer commercially available) and rabbit anti $\alpha 4$ subunit (1:1000; Millipore AB5457). The specificity of each antiserum was confirmed by a lack of immunohistochemical labeling in tissue from δ and $\alpha 4$ subunit KO animals, respectively (Peng et al., 2002; Peng et al., 2014). Prior to immunohistochemistry, free-floating sections were incubated in 1%

H₂O₂ for 30 min and then processed with a water bath heating antigen-retrieval method to reduce endogenous peroxidase-like activity and enhance specific labeling of the receptor subunits (Peng et al., 2002). Briefly, the sections were heated to 90°C for 70 min in sodium citrate solution (pH 8.6). After cooling and rinsing in 0.1 M Tris buffered saline (TBS, pH 7.3), sections were processed for immunohistochemistry with standard avidin-biotin-peroxidase methods (Vectastain Elite ABC; Vector Laboratories) as described in detail previously (Peng et al, 2002; Peng pilo 2004).

Densitometry methods. The intensity of immunolabeling was analyzed with an Axioplan 2 microscope equipped with an AxioCam digital camera system and AxioVision 4.6 software. (Zeiss). To evaluate possible differences in density of GABA_AR subunit labeling in the *Fmr1* WT and KO mice, sections from each animal at comparable levels of the hippocampus were processed in the same experimental run with identical conditions for each subunit. Linear black and white digital images of immunolabeling in the dentate molecular layer, from each side of the brain, were obtained under identical conditions on the same day with stabilized light levels for densitometric analysis (n=2-5 animals per group at each age; 4-6 samples per animal for each subunit) . The entire molecular layer of the dentate gyrus was outlined in each image, and the densities of labeling (grey values) within this region were determined with morphometric AxioVision software (version 4.6; Zeiss). Data were analyzed with Student's *t*-test, and *p* < 0.05 was considered statistically significant.

Immunogold Labeling for Electron Microscopy

Tissue preparation. Three pairs of *Fmr1* WT and KO animals were prepared for postembedding immunogold labeling for the $\alpha 4$ and δ subunits of GABA_ARs, as described previously (Zhang et al., 2007). Following perfusion of the animals with 4% paraformaldehyde and 0.1% glutaraldehyde, brain sections were cut coronally at 0.5–1 mm with a razor blade, and small blocks of the dentate gyrus were trimmed from these sections. Specimens were cryoprotected, frozen at –190°C in a cryofixation unit (EM CPC; Leica), and then transferred to a cryosubstitution unit (EM AFS; Leica), which was programmed for all subsequent steps (Zhang et al., 2007). Specimens were immersed in 4% uranyl acetate (Electron Microscopy Sciences), dissolved in anhydrous methanol for 24 h at –90°C, rinsed in methanol at –45°C,

and infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences) for 48 h at -45°C . The resin was polymerized with ultraviolet light (360 nm) for 24 h at -45°C and then warmed in 4°C steps to 0°C .

Immunogold labeling. Ultrathin sections were cut on a microtome (Reichert-Jung, Vienna, Austria), picked up on nickel mesh grids, and processed for immunogold labeling with previously described methods (Peng et al., 2014; Zhang et al., 2007). After appropriate pre-treatment, sections were incubated in primary antiserum, rabbit anti- δ subunit (1:100; AB9752, Millipore or 868-GDN, PhosphoSolutions, no longer available) or rabbit anti- $\alpha 4$ subunit (1:200; AB5457, Millipore) in TBS containing 2% human serum albumin (HSA) for 18–24 h at room temperature. Sections were then incubated for 2.5 h in the secondary antisera, goat anti-rabbit IgG or F(ab')₂ fragment of goat-anti-rabbit IgG (Aurion; distributed by Electron Microscopy Sciences) conjugated to 10 nm colloidal gold particles, diluted 1:30 in 0.05 M TBS, pH 8.0, containing 2% HSA. After immunogold processing, sections were stained with uranyl acetate for 40 min and lead citrate for 4 min.

Quantitative analysis. Randomly selected series of $\alpha 4$ or δ subunit-labeled synaptic profiles within the molecular layer of the dentate gyrus were photographed with a JEOL 100CX II electron microscope at a primary magnification of 19,000x. The localization of colloidal gold particles was determined for each symmetric synapse in the photomicrographs. Symmetric synaptic contacts were operationally defined as regions with close apposition between an axon terminal and putative granule cell dendrites at which the presynaptic and postsynaptic membranes were precisely parallel. Such contacts generally included a thin postsynaptic density and some electron-dense material in the cleft between the membranes.

For analysis of δ subunit labeling in the *Fmr1* WT and KO animals, synapses were classified as labeled (immunogold particles at perisynaptic or nearby extrasynaptic sites) or unlabeled (no immunogold labeling near the synapse). For analysis of $\alpha 4$ labeling, gold particle positioning along the synaptic membranes was classified as either perisynaptic or synaptic. Labeling was operationally defined as perisynaptic if the gold particles were located either directly at the ends of the synaptic contact, within 30 nm of the ends of the synaptic contact, or along the extrasynaptic membranes that extended up to 100 nm

beyond the end of the synapse. Gold particles that were located at extrasynaptic sites farther than 100 nm from the ends of a synaptic contact were not included in this analysis. Labeling was classified as synaptic if gold particles were located directly at synaptic contacts, excluding the perisynaptic sites indicated above. Synaptic labeling was further described as within the center third or outer thirds of the synaptic density.

Electrophysiology

Tonic inhibition in granule cells of the dentate gyrus was studied with in vitro whole-cell recording in young adult (2 month) *Fmr1* WT and KO littermates. To determine the contributions of the δ subunit to GABA-mediated tonic inhibition in the dentate granule cells, the effects of the δ subunit-selective agonist THIP (1 μ M) and a δ subunit selective allosteric modulator DS2 (5 μ M) were studied.

Brain slice preparation. Hippocampal slices were prepared from 2 month-old *Fmr1* WT and KO littermates. To prepare slices, animals were deeply anesthetized and decapitated, and brains were placed in ice-cold, modified artificial cerebrospinal fluid (aCSF) containing 65 mM sucrose, 82.7 mM NaCl, 2.4 mM KCl, 0.5 mM CaCl₂, 6.8 mM MgCl₂, 1.4 mM NaH₂PO₄, 23.8 mM NaHCO₃ and 23.7 mM D-glucose and saturated with 95% O₂/5% CO₂. This solution also served to cut 300 μ m-thick coronal or horizontal slices containing hippocampus and cortex using a VT-1000 vibratome (Leica). Brain slices were allowed to equilibrate for 30 min at 35°C and then switched to 21–23 °C (RT) in normal aCSF containing 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, 2 mM MgCl₂, 26 mM NaHCO₃ and 10 mM D-glucose, continuously bubbled with a mixture of 95% O₂/5% CO₂ gas.

Drug applications. The drugs used in the electrophysiology studies included SR95531, THIP and DS2 (all from Tocris Bioscience) and kynurenic acid (Sigma Aldrich). SR95531, THIP and DS2 were dissolved in DMSO and diluted 1:1000 into aCSF before use. For kynurenic acid, we added the powder into the fresh recording buffer at 2 mM working concentration each time.

Electrophysiological recording from brain slices. For in vitro slice studies of tonic inhibition, dentate gyrus granule cells were recorded in whole-cell mode using pipettes with a typical resistance of

4–5 M Ω when filled with internal solution containing 140 mM CsCl, 4 mM NaCl, 1 mM MgCl₂, 1 mM QX-314, 10 mM HEPES, 0.1 mM EGTA, 2 mM Mg-ATP, 0.3 mM Na-GTP with pH set to 7.3. The initial studies of tonic current were performed in the absence of added GABA, since ambient GABA in healthy slice preparations was sufficient to evoke a distinct tonic baseline shift that could be detected when GABA_ARs were blocked with SR95531. Such conditions were considered the most physiological and avoided the possibility that adding GABA to the bath would desensitize the high affinity δ subunit-containing receptors (Bright and Smart, 2013). For subsequent pharmacological experiments, we added GABA (5 μ M) to the bath in order to increase the size of the tonic current sufficiently to allow comparison of the effects of δ subunit-selective modulators in the *Fmr1* WT and KO mice.

Neurons in all recordings were visualized with an iXon EMCCD camera (Andor Technology) and infrared optics on an upright epifluorescence microscope (Axioskop 2 FS, Zeiss). pCLAMP 8.2 software and an Axopatch-1D amplifier were used for electrophysiology (Axon Instruments). Data were filtered at 2 kHz and acquired at a sampling rate of 20 kHz. Solutions were continuously perfused at a rate of 2 ml/min. All the drugs used bath applications for at least 5 min to ensure the effect on the recorded slice.

Analysis of electrophysiological data. Tonic GABA_AR-mediated current was defined as the steady-state current blocked by saturating concentrations of SR95531, and its magnitude was calculated by plotting all-point histograms of relevant 30 sec segments of data. Graphs for all studies were created in OriginPro 8 and assembled in CorelDraw 12. Normally distributed data were analyzed using unpaired Student's two tailed *t* tests, with significance declared at $p < 0.05$. Nonparametric Mann-Whitney rank sum test was used to assess the statistical significance of data deviating from normality. Data are presented as mean \pm s.e.m.

Immunoblotting

Measurement of surface receptor subunit expression by cross-linking. To determine cell surface protein levels from *Fmr1* WT vs KO mice, cross-linking experiments followed by Western blot analysis were performed, as described previously (Grosshans et al., 2002). Briefly, 400 μ m thick coronal

hippocampal slices were prepared using a tissue chopper (VT1200S, Leica). Slices were incubated either in ice-cold ACSF composed of 124 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 1 mM KH₂PO₄, 10 mM D-glucose, 26 mM NaHCO₃ and oxygenated with 95%O₂/5% CO₂, pH 7.4), or ACSF containing 1 mg/ml bis(sulfosuccinimidyl)suberate (BS³) (Pierce) at 4°C for 45 min, for measurements of total or internal protein, respectively. The cell-impermeable BS³ bi-functionally cross-links all surface proteins leading to large molecular weight aggregates which do not reliably migrate in the gel. Therefore, only internal proteins show up in the gel of the BS³ sample. The cross-link reaction was quenched with 20 mM Tris wash buffer, pH 7.6, and the slices were homogenized in homogenizing buffer containing 10 mM Tris, pH 8.0, 1 mM EDTA and 1% SDS. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce).

SDS-PAGE and Western blot analysis. Proteins were separated on SDS-polyacrylamide gels (Bio-Rad) using the Bio-Rad Mini-Protean 3 cell system. Proteins were transferred on a PVDF membrane (Bio-Rad) and blocked with 4% nonfat dry milk in TTBS. Blots were incubated overnight at 4°C with primary polyclonal rabbit antibodies against the following: δ (aa1-44) (Jones et al., 1997); $\alpha 4$ (aa379-421) (Ebert et al., 1996); (both at 1 μ g/ml, gift of W. Sieghart); $\alpha 4$ (1:1000; Millipore, AB5457); or mouse monoclonal β -actin (1:1000; Sigma, A2228), followed by HRP-conjugated secondary antibodies (1:5000; Rockland) for 2 h at room temperature. Bands were detected using ECL detection kit (GE Healthcare) and exposed to X-ray films (MidSci).

Data analysis. Protein signals were analyzed by densitometry using ImageQuant5.2 (Molecular Dynamics). For cross-linking experiments, the signal intensity of bands was normalized to the corresponding β -actin signal after background subtraction. Surface protein levels were calculated by subtraction of the internal protein signal (from BS³-treated slices) from the respective total protein signal (from untreated slices). Statistical comparisons were made by Student's unpaired t-test and the family-wise error was controlled by the Bonferroni correction for t tests. $p < 0.0125$ was considered statistically significant. All values are shown as mean \pm SEM with n representing the number of experiments.

Results

δ Subunit labeling is slightly lower in the dentate gyrus of Fmr1 KO mice

Patterns of δ subunit immunoperoxidase labeling in the dentate gyrus were compared in *Fmr1* WT and KO littermates at postnatal days (PN) 14, 21, 28, 35 and 60. In WT animals, the normal pattern of δ subunit labeling in the hippocampus was evident as early as PN14, although the immunoreactivity appeared slightly lower at the young ages and progressively increased at older ages (Fig. 1). Labeling was highest in the molecular layer of the dentate gyrus, where the δ subunit is normally located on dendrites of dentate granule cells (Fig. 1). Very low levels of labeling were found in the hilus and CA3 region, with slightly higher labeling in CA1.

The broad patterns of labeling were similar in WT and KO mice at all ages studied (illustrated for PN 14, 21, 35 and 60 in Fig. 1). However, the level of labeling in the molecular layer of the dentate gyrus appeared consistently lower in the KO animals when compared to their WT littermates, although the differences were slight (Fig. 1). Densitometric analyses of the immunohistochemical labeling in the mice at PN 35 (n = 4 WT and 4 KO) and PN 60 (n = 3 WT and 4 KO) confirmed lower levels of labeling in the *Fmr1* KO animals compared to their WT littermates (PN 35, 15.1% lower, $p < 0.05$; PN 60, 15.2% lower, $p < 0.05$) (Fig. 1I).

Immunolabeling for the $\alpha 4$ subunit in WT animals closely resembled that of the δ subunit in the hippocampus (Fig. 2; compare with Fig. 1). No differences in the patterns or levels of $\alpha 4$ labeling were observed in WT and *Fmr1* KO mice, and densitometric analysis revealed no significant differences (Fig. 2).

These immunohistochemical findings raised questions about the functional effects of the limited decreases in δ subunit expression on tonic inhibition in dentate granule cells.

δ subunit-containing GABA_AR-mediated tonic inhibition is severely reduced in Fmr1 KO mice.

To determine if tonic inhibition was altered in the dentate granule cells, we made whole-cell recordings from these neurons in young adult (2 month) *Fmr1* WT and KO littermates. We found

significantly lower GABA_AR-mediated tonic currents in *Fmr1* KO mice (31.1 ± 2.4 pA in WT mice vs 6.7 ± 1.4 pA in KO littermates ($p < 0.0001$) (Fig. 3A,B). Similar differences were found in tonic inhibitory conductance (11.4 ± 1.3 S/F in WT mice and 2.4 ± 0.5 in KO mice; $p < 0.0001$) (Fig. 3B). These marked differences in tonic current and conductance were observed without any change in membrane properties as no differences were observed in either membrane resistance or membrane capacitance in the two groups of mice (Fig. 3C).

To further probe the function of δ subunit-containing GABA_ARs in these animals, we next tested the efficacy of THIP, a δ subunit-preferring agonist at low concentrations (Jia et al., 2005; Meera et al., 2011). In WT animals, THIP (1 μ M) substantially increased tonic current, as expected in animals with normal levels of δ subunit-containing receptors (Fig. 4). In contrast, in *Fmr1* KO mice, THIP-activated currents were small, consistent with low levels of δ subunit-containing GABA_ARs (-19.8 ± 1.8 pA in WT and -8.5 ± 2.0 pA in *Fmr1* KO mice, $p = 0.0007$) (Fig. 4). Normalized to baseline tonic current, the THIP-activated current was approximately 2.2-fold larger in WT than in *Fmr1* KO mice (84% enhancement in WT vs 38% enhancement in KO mice; $p = 0.0154$) (Fig. 4).

To further evaluate the δ subunit contribution to the lower tonic current in the *Fmr1* KO mice, we studied the effects of the δ subunit-selective GABA_AR positive allosteric modulator DS2 (Jensen et al., 2013; Wafford et al., 2009). The DS2-enhanced current was significantly larger in the WT than in the *Fmr1* KO mice (-19.0 ± 3.2 pA in WT and -9.3 ± 2.0 pA in KO mice; $p = 0.019$) (Fig. 5). This represented a 3-fold greater enhancement in the WT compared to that in the *Fmr1* KO mice (95% enhancement in WT vs 32% enhancement in KO mice; $p = 0.0126$) (Fig. 5).

Together these electrophysiological findings suggest that δ subunit-containing GABA_AR-mediated tonic inhibition is severely reduced in dentate granule cells in the *Fmr1* KO mice compared to that in WT littermates. The substantial decrease in tonic inhibition differed markedly from the limited decreases in δ subunit expression in the immunohistochemical experiments. These discrepant findings suggested that additional alterations, beyond a basic reduction in the δ subunit protein, could be contributing to the

functional changes in tonic inhibition. This led to immunoblotting experiments to evaluate potential differences in both total and surface protein levels in WT and *Fmr1* KO littermates.

*Cell surface protein levels of the δ subunit are significantly reduced in *Fmr1* KO mice*

To determine the total and surface expression of the δ subunit, we conducted cross-linking experiments followed by Western blot analysis. We found a 31.4% but non-significant ($p = 0.0726$, unpaired t-test) decrease in total δ subunit protein in the *Fmr1* KO mouse (Fig. 6). However, a four-fold decrease in surface expression of the δ subunit was found in the *Fmr1* KO mice ($p < 0.0032$, unpaired t-test) (Fig. 6). Because the $\alpha 4$ subunit is a major partner of the δ subunit in the forebrain (including dentate granule cells), the levels of $\alpha 4$ subunit were also studied. The level of total $\alpha 4$ protein in the KO animals did not differ significantly from that in the WT mice ($p = 0.971$, unpaired t-test) (Fig. 6). Likewise, no significant differences were found in levels of $\alpha 4$ subunit surface protein between WT and KO mice ($p = 0.981$, unpaired t-test) (Fig. 6). These findings suggested that the differences in δ subunit-mediated tonic inhibition could be related to reduced surface expression of the δ subunit in the *Fmr1* KO mouse, and this led to questions about the subcellular localization of both the δ and $\alpha 4$ subunits.

*Immunogold labeling of the δ subunit is severely reduced near synaptic contacts on dentate granule cells in *Fmr1* KO mice*

Light microscopic studies did not allow a distinction between localization of the subunits within the cytoplasm or near synaptic sites on the surface of the granule cell dendrites. Postembedding immunogold labeling was thus used to determine if there were alterations in the subcellular localization of the δ and $\alpha 4$ subunits. After immunolabeling for the δ subunit, symmetric synapses in the dentate molecular layer were identified, and the presence or absence of immunogold particles was determined at each symmetric synapse that exhibited a clear postsynaptic density. In WT littermates ($n = 3$ mice; 183 total synapses), immunogold particles were found in perisynaptic locations in 83.8% of the synapses, while 16.2% of symmetric synapses were unlabeled (Fig. 7). In contrast, in the *Fmr1* KO mice ($n = 3$ mice; 212 total

synapses), only 24.6% of the symmetric synapses showed immunogold labeling along the plasma membrane at perisynaptic sites, and 75.4% of the symmetric synapses showed no immunogold labeling either at or near the synaptic contacts (Fig. 7). Differences in numbers of labeled and unlabeled synapses in the two groups of animals were highly significant (Student's t-test, $p < 0.001$).

These findings are consistent with the biochemical findings of significantly lower surface expression of the δ subunit in the *Fmr1* KO mice and demonstrate that a reduction occurs at perisynaptic sites where δ subunit-containing receptors normally mediate tonic inhibition.

*Immunogold labeling of the $\alpha 4$ subunit is retained at the cell surface but is altered in synaptic location in *Fmr1* KO mice*

The $\alpha 4$ subunit is a major partner of the δ subunit in dentate granule cells, and the severe decrease in surface labeling of the δ subunit led to questions about the localization of the $\alpha 4$ subunit. Thus similar immunogold labeling was used to determine the localization of the $\alpha 4$ subunit. In *Fmr1* WT littermates, 78.8% of the symmetric synapses ($n = 3$ mice; 101 total synapses) showed immunogold labeling at perisynaptic or extrasynaptic locations, while 21.2% exhibited labeling directly at the synaptic contacts, with labeling at either the center third (19.5%) or outer third (1.7%) (Fig. 8). In contrast, in the *Fmr1* KO mouse, 80.3% of the symmetric synapses ($n = 3$ mice; 116 total synapses), showed $\alpha 4$ subunit labeling directly at the synaptic contacts, at either the center third (71.9%) or outer third (8.4%) (Fig. 8). Labeling at perisynaptic or nearby extrasynaptic locations was found in only 19.7% of the total symmetric synapses in *Fmr1* KO mice (Fig. 8). These findings suggest that, while surface expression of the δ subunit is altered in the *Fmr1* KO mouse, surface labeling of the $\alpha 4$ subunit is maintained, but the location of the $\alpha 4$ subunit is altered, possibly due to lack of δ subunit partnership at perisynaptic sites.

Discussion

This study provides the first evidence for decreased neuronal cell surface expression of the δ subunit of the GABA_AR in a model of FXS. The major findings of the study are that 1) δ subunit-mediated tonic inhibition in dentate granule cells is substantially lower in *Fmr1* KO mice than in WT littermates; 2) surface expression in the δ subunit is reduced in the dentate gyrus of the *Fmr1* KO mice, as demonstrated by both biochemical and immunogold labeling studies; and 3) the substantial decrease in δ subunit surface expression contrasts with the limited decrease in total δ subunit protein and immunolabeling in the dentate molecular layer. Together the findings suggest that the decrease in tonic inhibition in the dentate gyrus is due primarily to a decrease in surface expression of δ subunit-containing receptors rather than a reduction in δ subunit protein alone.

The marked reduction in tonic inhibition in dentate granule cells in this model of FXS could have important functional effects as the granule cells control the massive input from the entorhinal cortex and can thus limit the information that reaches the hippocampus (Dengler and Coulter, 2016; Leutgeb et al., 2007). This filtering function of the dentate gyrus provides a critical early step in information processing within the hippocampal formation. Reduced tonic inhibitory control of dentate granule cells could negatively impact learning and memory as well as increase seizure susceptibility and levels of anxiety (Lee et al., 2016; Maguire et al., 2005; Whissell et al., 2013). A decrease in tonic inhibition has been found previously in other brain regions in mouse models of FXS, including the subiculum (Curia et al., 2009), basolateral nucleus of the amygdala (Martin et al., 2014; Olmos-Serrano et al., 2010), and the CA1 region of the hippocampus (Sabanov et al., 2016). The current findings support the suggestion that a decrease in tonic inhibition could be a common finding in FXS, occurring across multiple brain regions where the deficits could contribute to several behavioral features of FXS, including hyperexcitability, hypersensitivity, and increased seizure susceptibility (Martin et al., 2014; Paluszkiewicz et al., 2011; Whissell et al., 2015). The current electrophysiological studies also confirm that the altered tonic inhibition in dentate granule cells of the *Fmr1* KO mouse results from deficits in δ subunit-containing

GABA_ARs, as demonstrated by reduced responses to both THIP, a δ subunit-preferring agonist at low (μ M) concentrations (Jia et al., 2005; Meera et al., 2011), and DS2, a δ subunit-selective positive allosteric modulator of GABA_ARs (Jensen et al., 2013; Wafford et al., 2009).

Alterations in the δ subunit of the GABA_AR in FXS has been suspected for some time, following the discovery that the δ subunit mRNA is one of the direct targets of FMRP, as demonstrated initially by antibody-positioned RNA amplification methods (Miyashiro et al., 2003) and subsequently by electrophoretic mobility shift assays (Braat et al., 2015). Lower levels of δ subunit mRNA have also been found in *Fmr1* KO mice in several forebrain regions, including the cerebral cortex and subiculum (Braat and Kooy, 2015; D'Hulst et al., 2006; Gantois et al., 2006).

Descriptions of decreased δ subunit mRNA have led to the frequent assumption that the δ protein is also decreased. However, studies of the δ subunit protein have been limited, and the findings have differed among brain regions and ages. Decreases in the δ subunit protein in *Fmr1* KO mice have been found in preparations of the entire forebrain at PN12, but not at PN5 or in adults (Adusei et al., 2010), in the subiculum in young adult animals (Curia et al., 2009), and in the hippocampus at PN22 (Sabanov et al., 2016).

No detailed immunohistochemical studies of δ subunit expression in the *Fmr1* KO mouse have been published, but, based on previous biochemical studies and the electrophysiological findings in the current study, a substantial decrease in δ subunit labeling was expected. Surprisingly, δ subunit labeling in the dentate gyrus was only slightly, though consistently, lower in the *Fmr1* KO mice when compared with WT littermates. Likewise, only a small, statistically nonsignificant, decrease in total δ subunit protein was detected in the current biochemical studies.

A previous study of tonic inhibition and δ subunit expression in the subiculum of *Fmr1* KO mice found remarkably similar discrepancies between a very large decrease in tonic inhibition in subicular neurons (~91% lower than in control mice) and a more limited underexpression of the δ protein in this region (~28% lower) (Curia et al., 2009). Surface expression of the δ subunit was not determined in the

previous study. However, based on the similarities to the present findings, it is plausible that a decrease in surface expression of the δ subunit also occurs in the subiculum of *Fmr1* KO mice and, potentially, other brain regions where δ subunit-containing GABA_ARs mediate tonic inhibition.

Significantly lower δ subunit protein expression was recently found in the hippocampus of *Fmr1* KO mice at PN22 (Sabanov et al., 2016), and it is likely that the brain samples included the dentate gyrus. δ subunit levels were determined in a Triton-insoluble fraction which could have been enriched in membranes, allowing detection of significant differences between KO and WT mice. However, total and surface protein levels were not distinguished.

The lower surface expression of the δ subunit in the *Fmr1* KO mice led to questions about the expression and localization of the $\alpha 4$ subunit of the GABA_AR, as the $\alpha 4$ subunit is the normal partner of the δ subunit in dentate gyrus granule cells and is also normally localized primarily at perisynaptic locations on granule cell dendrites (Zhang et al., 2007). No differences were observed in light microscopic immunohistochemical labeling of the $\alpha 4$ subunit, and no significant changes in $\alpha 4$ subunit levels were found in biochemical studies of either total or surface expression of this subunit in the hippocampus of the *Fmr1* KO mice. Consistent with such findings, immunogold labeling was evident along the plasma membrane of granule cell dendrites. However, the location of the $\alpha 4$ subunit was altered in the *Fmr1* KO mouse. Rather than being localized at the normal perisynaptic location in dentate granule cells, immunogold labeling of the $\alpha 4$ subunit was found directly at synaptic contacts. A similar change in $\alpha 4$ subunit localization was found previously in a rat hyperexcitability model of alcohol withdrawal syndrome in which δ subunit-mediated tonic inhibition was also reduced (Liang et al., 2006). Such changes in $\alpha 4$ subunit localization, presumably related to the reduced surface expression of the δ subunit, may reflect a change in $\alpha 4$ subunit partnership within GABA_ARs, from predominant partnership with the δ subunit at perisynaptic locations to increased partnership with the $\gamma 2$ subunit directly at synaptic contacts.

This idea is based on previous suggestions that, during development, the δ and $\gamma 2$ subunits compete with each other for partnership with $\alpha 4$ in GABA_ARs (Tretter et al., 2001). In the forebrain, including the dentate gyrus, partnership of the δ and $\alpha 4$ subunits normally predominates (Sur et al., 1999), and the two subunits have very similar regional and cellular localizations (Peng et al., 2002; Pirker et al., 2000). However, when the δ subunit is deficient, as in the δ subunit KO mouse, expression of the $\gamma 2$ subunit increases (Peng et al., 2002), consistent with the formation of $\alpha 4/\beta/\gamma 2$ GABA_ARs. Also, in a mouse model of epilepsy in which δ subunit expression is decreased during the chronic period, the levels of both $\alpha 4$ and $\gamma 2$ are increased (Peng et al., 2004; Rajasekaran et al., 2010), possibly resulting in increased partnership of these subunits. Similar decreases in δ subunit expression and associated increases in $\alpha 4$ and $\gamma 2$ expression have been observed following acute and chronic alcohol administration in rodent models of alcohol withdrawal (Lindemeyer et al., 2014; Shen et al., 2011). In all of these models in which the δ and $\alpha 4$ subunits are altered, network excitability was increased (Houser et al., 2012; Olsen and Spigelman, 2012; Peng et al., 2004; Spigelman et al., 2003)

The basic mechanisms underlying the decrease in surface expression of the δ subunit are unknown. The reduced surface expression could be related to deficits in activity-dependent transport of FMRP and its δ subunit mRNA cargo to local translation sites near GABAergic synapses. Indeed, the δ subunit mRNA was one of the select FMRP targets for which stimulus-induced dendritic mRNA transport was impaired in cultured hippocampal neurons from *Fmr1* KO mice (Dictenberg et al., 2008). Alternatively, the lack of surface expression could reflect either a deficit in trafficking of the δ subunit-containing receptors to the dendritic surface or increased endocytosis of these receptors (Gonzalez et al., 2012; Shen et al., 2011). While the basic mechanisms could be related directly to the δ subunit, alterations in associated subunits, such as altered phosphorylation of the $\beta 3$ subunit, as observed in *Fmr1* KO mice (Vien et al., 2015), could also play a role. The possibility of identifying the underlying mechanisms and finding ways to increase surface expression of δ subunit-containing GABA_ARs is particularly intriguing since the current findings indicate that a substantial amount of δ subunit protein is present in the neurons

but fails to reach its normal localization at the dendritic surface. Interestingly, there are other examples of decreased surface expression of membrane-associated proteins in *Fmr1* KO mice, including the AMPA receptor subunit GluR1 in the lateral amygdala, in this case possibly related to excessive mGluR signaling (Suvrathan et al., 2010).

The identification of deficits in tonic inhibition in an additional brain region provides further support for the development of pharmacological agents that could selectively enhance tonic inhibition. Despite lower δ subunit expression in the dentate gyrus, a δ subunit-preferring agonist, THIP, and a δ subunit-selective positive allosteric modulator, DS2, increased tonic currents in dentate granule cells of *Fmr1* KO mice, although the enhancement was less than that in WT granule cells. Despite the reduced surface pool of δ subunit-containing GABA_ARs, THIP augmented tonic inhibition and reduced hyperexcitability of principal cells of the basolateral nucleus of the amygdala of *Fmr1* KO mice in brain slices (Olmos-Serrano et al., 2010), and also reduced some behavioral deficits, including hyperexcitability, *in vivo* (Olmos-Serrano et al., 2011). At low (micromolar) concentrations, THIP could be exerting its effects primarily at remaining δ subunit-containing nonsynaptic receptors to increase tonic inhibition. However, at higher concentrations, THIP could be activating other GABA_ARs that do not contain the δ subunit.

Neurosteroids are also major positive allosteric modulators of GABA_ARs and many, such as tetrahydrodeoxycorticosterone (THDOC), have high efficacy at nonsynaptic δ subunit-containing receptors (Stell et al., 2003; Wohlfarth et al., 2002). However, all neuroactive steroids, including allopregnanolone and a synthetic analog, ganaxolone, can modulate both nonsynaptic and synaptic GABA_ARs (Carter et al., 1997; Reddy and Estes, 2016). In previous behavioral studies in *Fmr1* KO mice, acute administration of ganaxolone prevented audiogenic seizures (Heulens et al., 2012) and reduced repetitive, perseverative behavior (Braat et al., 2015). Enhanced tonic inhibition could have contributed to the behavioral changes, but increased phasic inhibition at synaptic receptors could also play a role. Ganaxolone is currently in a phase II clinical trial for treatment of children with Fragile X

(ClinicalTrials.gov Identifier NCT01725152) while THIP is in a trial for Angelman Syndrome (ClinicalTrials.gov Identifier [NCT02996305](#)).

The search for synthetic neurosteroids with selective actions at nonsynaptic GABA_ARs has led to the development of a new positive allosteric modulator of GABA_ARs, SGE-812 (Martin et al., 2016). This compound is highly selective for $\delta/\alpha 4$ subunit-containing GABA_ARs at low concentration and increased tonic inhibition in principal neurons of the basolateral nucleus of the amygdala in slices from *Fmr1* KO mice. Whether such synthetic neuroactive steroids are acting at remaining perisynaptic $\alpha 4/\delta$ -expressing GABA_ARs or potentially at other $\alpha 4$ -containing receptors is unclear (Martin et al., 2016). However, such GABA_AR modulators hold promise for the treatment of FXS by increasing tonic inhibition at δ or $\alpha 4$ subunit receptors and thus providing a more limited and targeted approach to GABA_AR-mediated treatment.

Beyond the direct effects of neuroactive steroids as positive allosteric modulators of GABA_ARs, some neurosteroids such as THDOC can increase the trafficking and membrane insertion/stabilization of $\alpha 4$ subunit-containing GABA_ARs in hippocampal neurons apparently through protein kinase C (PKC)-dependent phosphorylation of the $\alpha 4$ and $\beta 3$ subunits (Abramian et al., 2014; Abramian et al., 2010; Adams et al., 2015). Recently, allopregnanolone and another new synthetic neuroactive steroid, SGE-516, were also found to increase the surface expression of $\alpha 4/\beta/\delta$ subunit-expressing receptors in dentate granule cells, possibly involving PKC-dependent phosphorylation of the $\beta 3$ subunit (Modgil et al., 2017). Thus some endogenous and synthetic neurosteroids (although not ganaxolone) could enhance tonic inhibition by both direct modulatory effects on nonsynaptic GABA_ARs and enhanced trafficking and surface expression of these receptors (Modgil et al., 2017). Considering the current findings of lower surface expression of the δ subunit in *Fmr1* KO mice, it would be particularly interesting to learn if such compounds can increase δ subunit-containing GABA_ARs at nonsynaptic sites along the membrane and enhance tonic inhibition in this mouse model of FXS.

Findings of this study suggest that altered surface expression of the δ subunit of the GABA_AR, rather than a decrease in δ subunit expression alone, is likely limiting tonic inhibition in the dentate gyrus in this model of FXS. Whether deficits in surface expression are confined to the δ subunit or could be found for additional receptor proteins that require trafficking to and insertion at the membrane surface in FXS remains to be determined. The findings add to the growing number of GABA system alterations that have been identified in models of FXS and suggest novel treatment approaches for this disorder.

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Figure Legends

Fig. 1. Immunolabeling of the δ subunit in the dentate gyrus is slightly lower in *Fmr1* KO mice than in WT littermates at PN14-PN60. (A,C,E,G) In WT animals, the δ subunit is strongly expressed in the molecular layer of the dentate gyrus (M). Labeling is very low in the hilus (H) and CA3, but slightly higher in CA1. Similar patterns of labeling are evident from PN14 to PN60, but the level of δ subunit labeling in the molecular layer increases throughout this period and labeling of interneurons appears strongest at PN14. (B,D,F,H) In *Fmr1* KO animals, the patterns of δ subunit labeling closely resemble those of the WT littermates at each age. However, slightly lower levels of labeling are found in the dentate molecular layer in the KO animals. (I) Densitometry confirmed small but significant differences in labeling intensity in the molecular layer at PN35 and PN60. Mean intensities of labeling for WT and KO littermates at four ages are included in the bar graph. However, only data from animals at PN35 and PN60 were analyzed statistically due to limited numbers of littermates at younger ages. $*p < 0.001$. Scale bar = 100 μ M (A-H).

Fig. 2. Immunolabeling of the $\alpha 4$ subunit in the dentate gyrus is similar in *Fmr1* WT and KO mice at PN35. (A,B) $\alpha 4$ labeling is highest in the molecular layer (M) of the dentate gyrus in WT and KO mice, and the pattern of $\alpha 4$ labeling closely resembles that of the δ subunit (see Fig. 1E,F). The level of $\alpha 4$ labeling in the molecular layer is similar in WT and KO littermates. (C) Densitometry demonstrated no significant difference in the mean intensity of $\alpha 4$ labeling in the molecular layer of WT and KO littermates. Scale bar = 100 μ M (A,B). NOTE, “C” is not visible in Figure.

Fig. 3. GABA_AR-mediated tonic current in dentate granule cells is significantly lower in *Fmr1* KO mice than in WT littermates. (A) Example traces from WT (top) and *Fmr1* KO (bottom) dentate gyrus granule cells demonstrate a large tonic current that is sensitive to the GABA_AR antagonist SR 95531 in WT but

not in the KO cell. To the right are all points histograms of the current levels prior to (black) and following application of SR 95531 (grey). (B) Summary of the mean \pm s.e.m. levels of SR 95531-sensitive tonic current (left) and conductance (right) in WT and KO conditions. Significant reductions in current and conductance were observed in the KO relative to WT cells. (C) No significant differences were observed in basic membrane properties of membrane resistance (left) or capacitance (right) when comparing WT and KO granule cells.

Fig. 4. Activation of GABA_AR-mediated current by the δ subunit-selective agonist THIP is significantly decreased in *Fmr1* KO mice. Example traces shown on the left indicate the current activated by application of 1 μ M THIP, an agonist selective for δ subunit-containing GABA_ARs. A recording from a WT granule cell is shown on the top and a KO granule cell on the bottom. To the upper right are summaries of the levels of current activated by THIP in each condition (mean \pm s.e.m.). Open symbols indicate individual cells. On the lower right is a summary of the amount, expressed as a percentage, of additional current activated by THIP relative to the SR 95531-sensitive tonic current level prior to THIP.

Fig. 5. Enhancement of GABA-mediated tonic current by the δ subunit-selective positive allosteric modulator DS2 is significantly decreased in *Fmr1* KO mice. Example traces shown on the left indicate the current activated by application of DS-2, a positive modulator selective for δ subunit-containing GABA_ARs. A recording from a WT granule cell is shown on the top and a KO granule cell on the bottom. To the upper right are summaries of levels of current increase observed in DS-2 in each condition (mean \pm s.e.m.). Open symbols indicate individual cells. On the lower right is a summary of the percent increase in SR 95531-sensitive tonic current following DS2 application.

Fig. 6. Surface expression of the GABA_AR δ subunit is decreased in *Fmr1* KO mice. (A) Representative Western blots for δ (left panel) and $\alpha 4$ (right panel) after cell surface cross-linking (t = amount of total protein; int = amount of internal protein). The respective β -actin signals are shown below. (B) Quantification of the total and surface δ subunit levels. Surface protein levels were calculated by subtracting internal protein signal from the total protein signal. A four-fold decrease in surface expression of the δ subunit was found in the KO mice. (C) Quantification of the total and surface $\alpha 4$ subunit levels of WT vs KO. No differences in total or surface expression of $\alpha 4$ subunit protein were found in WT and KO mice. Data are mean \pm SEM, n = 5-8 mice/group. * $p < 0.0125$, unpaired t-test between KO and WT.

Fig. 7. Immunogold labeling of the δ subunit is decreased along the dendritic plasma membrane in *Fmr1* KO mice. (A,C) In *Fmr1* WT mice, δ subunit immunogold labeling is localized predominantly at perisynaptic locations (arrows) near symmetric synapses (open arrowheads; T = axon terminal). (B,D) In *Fmr1* KO littermates, many symmetric synapses (open arrowheads) lack δ subunit immunogold labeling at perisynaptic sites. Immunogold particles were occasionally observed within the cytoplasm of granule cell dendrites (arrow in panel D). (E) Quantitative analysis in WT animals demonstrated δ subunit immunogold labeling at perisynaptic locations in a high percentage of symmetric synapses (identified as labeled synapses). In contrast, a high percentage of symmetric synapses in *Fmr1* KO mice lacked labeling at the synaptic membrane (identified as unlabeled synapses). Scale bars = 0.2 μ M (A-D).

Fig. 8. Immunogold labeling of the $\alpha 4$ subunit is decreased at perisynaptic locations but increased at synaptic sites in *Fmr1* KO mice. (A,C) In WT mice, labeling for the $\alpha 4$ subunit was found primarily at perisynaptic (arrow in panel A) or extrasynaptic (arrow in panel C) locations near symmetric synapses (open arrowheads). (B,D) In *Fmr1* KO animals, $\alpha 4$ subunit immunogold labeling (arrows) was

frequently found directly at synaptic contacts (open arrowheads). (E) Quantitative analysis of $\alpha 4$ labeling in WT animals demonstrated immunogold particles directly at synaptic contacts in a high percentage of symmetric synapses. (F) Immunogold labeling of the $\alpha 4$ subunit is found predominantly at perisynaptic locations in *Fmr1* WT animals but near the center of symmetric synapses in *Fmr1* KO animals. Lower percentages of synapses were labeled at other synaptic or nonsynaptic locations in both groups of animals. Scale bars = 0.1 μ M (A-D)

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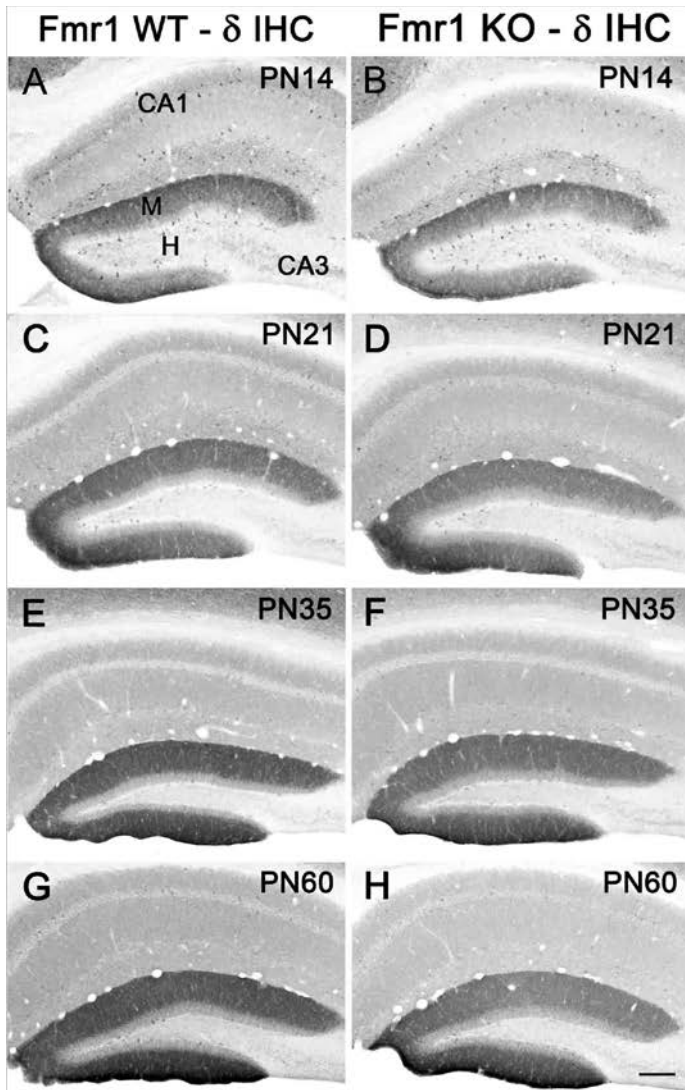


Fig.1 – 1 column

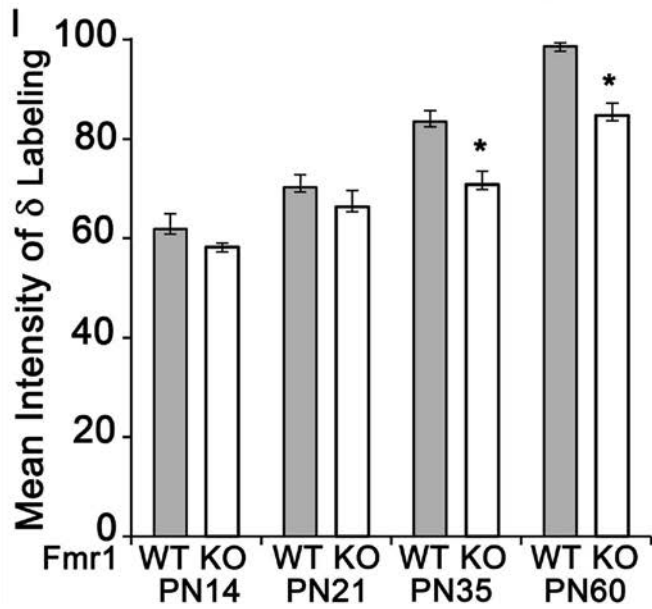


Fig. 2. 1.5 column

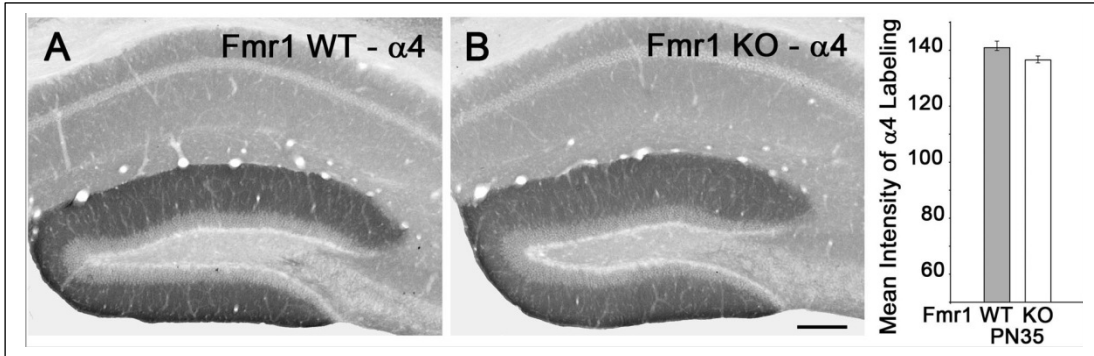
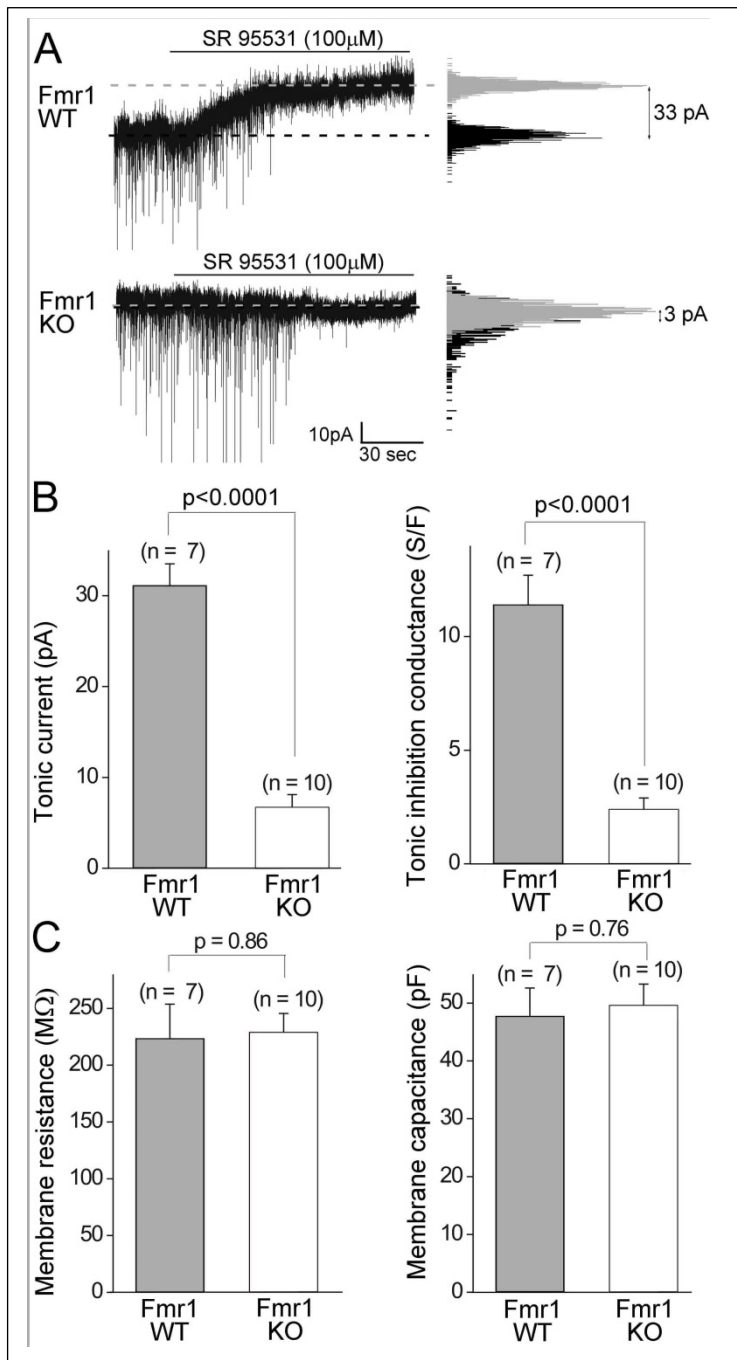


Fig. 3. 1 column (Revised color – grey. See next page for original and explanation)



(Alternate Fig. 3)

Fig. 3. 1 column (As original, with red) If used in paper would be red online but only B&W in printed version. Not sure if the difference in red and black will show up properly when changed to B&W. See previous page with grey used instead of red. *XP & TO - Input needed.*

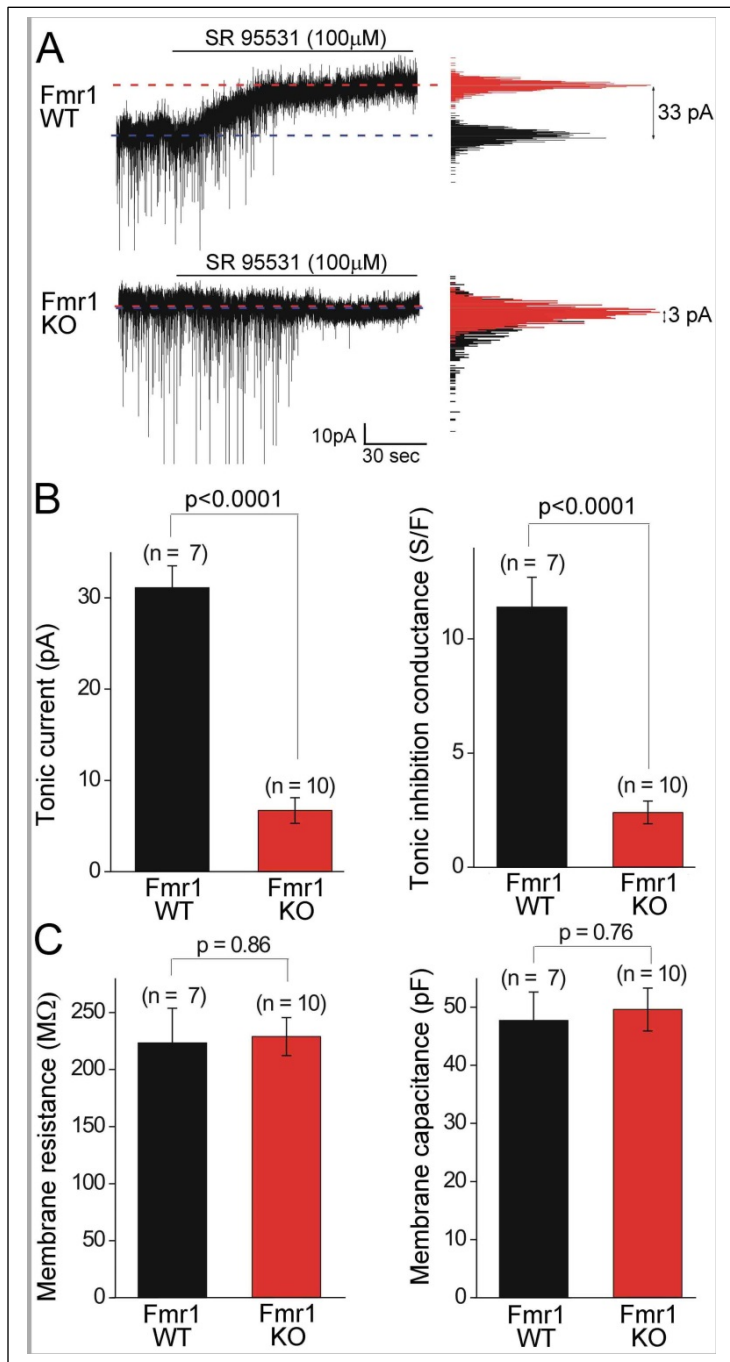


Fig. 4 – 1 column

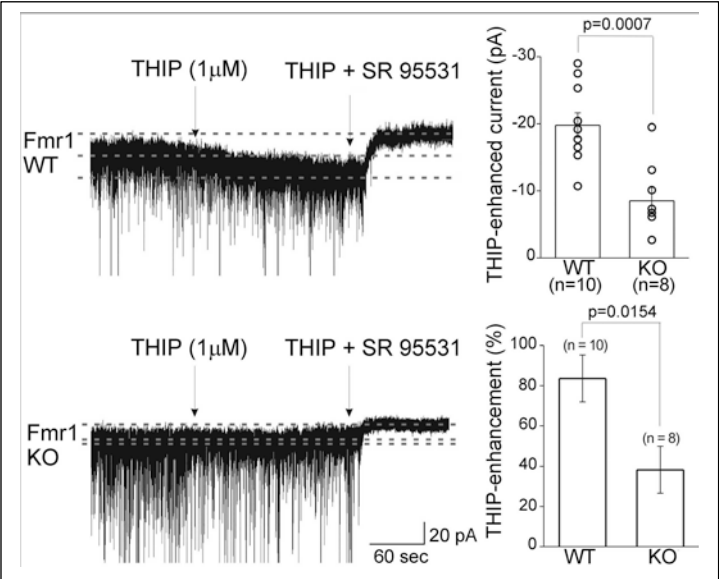


Fig. 5 – 1 column

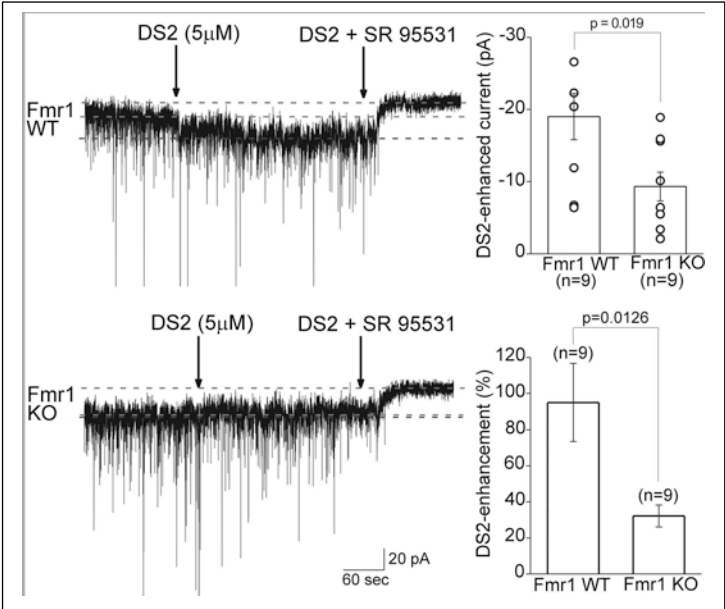


Fig. 6. 1 column

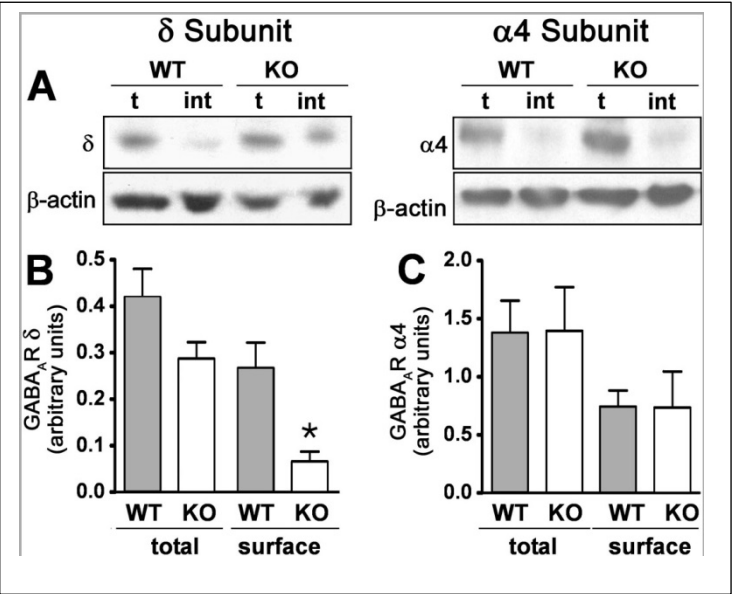


Fig. 7 – 1.5 column

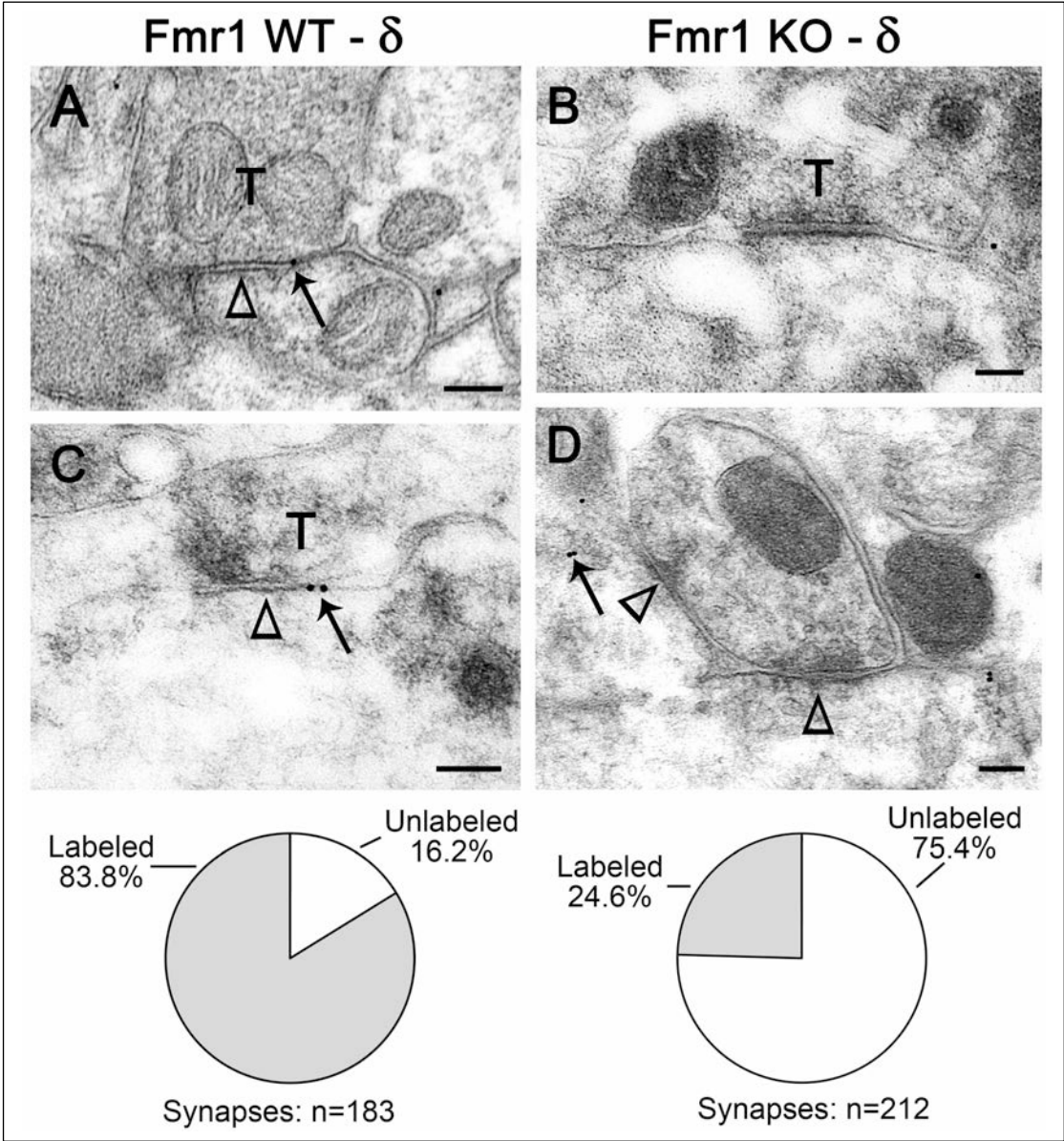


Fig. 8 – 1 column (or more likely 1.5, as for Fig. 7)

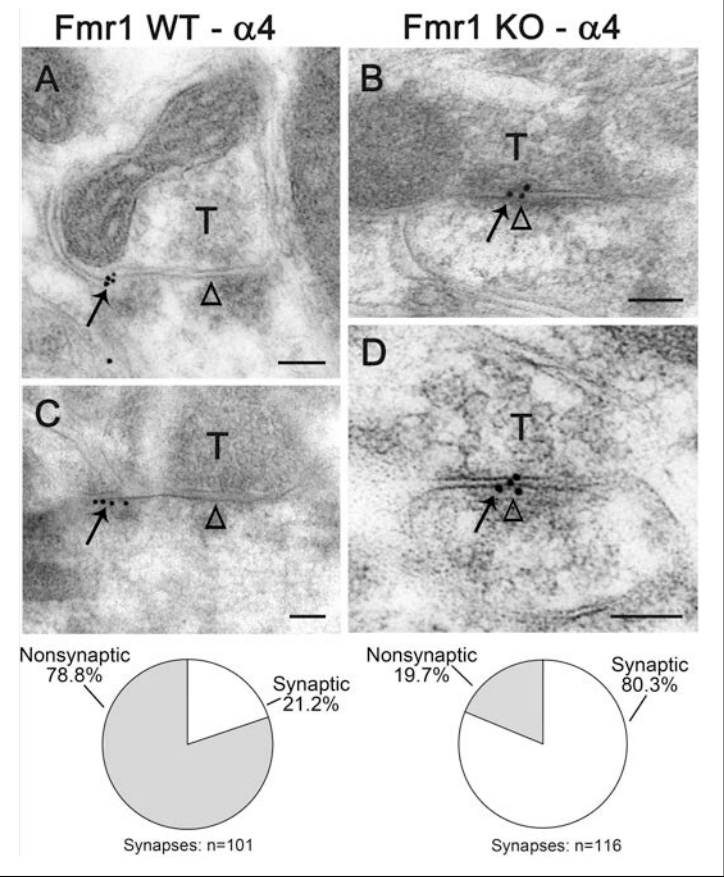


Fig. 9 – 1 column

