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Research Paper

# 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

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 ($GABA_A$Rs) 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 $GABA_A$R, 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 $GABA_A$Rs 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 mice, but a four-fold decrease in surface expression of the δ subunit in these mice. No significant changes were observed in total or surface expression of the α4 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 α4 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 $GABA_A$R could be a novel approach to treatment of hyperexcitability-related alterations in FXS.

## 1. 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

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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 (Braat and Kooy, 2015b; D'Hulst et al., 2009; Paluszkiewicz 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; Mtchedlishvili and Kapur, 2006; Olsen and Sieghart, 2008; Sun et al., 2004). 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 brain regions such as the dentate gyrus, alterations of δ-mediated tonic inhibition have not been studied in FXS in these regions. 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 (Heinemann et al., 1992). 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 et al., 2010; 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).

## 2. Methods

### 2.1. 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 a colony established 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 groups of the same sex under standard laboratory conditions with a 12:12 h light-dark cycle. Animals for immunohistochemistry were studied from postnatal day (PN) 14–60, and those for electrophysiological, biochemical and electron microscopic 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.

### 2.2. Immunohistochemistry

#### 2.2.1. 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 in situ at 4 °C, brains were removed and postfixed for 1 h. After rinsing, brains were cryoprotected in a 30% sucrose solution overnight and frozen in cryo-embedding compound on dry ice. Forebrain blocks containing the hippocampus were sectioned coronally at 30 μm with a cryostat.

#### 2.2.2. Antisera and immunohistochemical methods

The following $GABA_AR$ subunit-specific antisera were used to localize δ and α4 subunits in the immunohistochemical studies: rabbit anti-δ subunit (1:1000; Millipore AB9752 or PhosphoSolutions 868-GDN, no longer commercially available) and rabbit anti-α4 subunit (1:1000; Millipore AB5457). The specificity of each antiserum was confirmed by a lack of immunohistochemical labeling in tissue from δ and α4 subunit KO animals, respectively (Peng et al., 2002; Peng et al., 2014). Prior to immunohistochemistry, free-floating sections were incubated in 1% $H_2O_2$ 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 et al., 2004).

#### 2.2.3. Densitometry methods

The intensity of immunolabeling was analyzed with an Axioskop 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. Data were analyzed with a nonparametric Wilcoxon Rank Sum Test, and $p < 0.05$ was considered statistically significant.