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GABAergic circuit dysfunction in the *Drosophila* Fragile X syndrome model ☆

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ABSTRACT

Fragile X syndrome (FXS), caused by loss of *FMR1* gene function, is the most common heritable cause of intellectual disability and autism spectrum disorders. The *FMR1* protein (FMRP) translational regulator mediates activity-dependent control of synapses. In addition to the metabotropic glutamate receptor (mGluR) hyperexcitation FXS theory, the GABA theory postulates that hypoinhibition is causative for disease state symptoms. Here, we use the *Drosophila* FXS model to assay central brain GABAergic circuitry, especially within the Mushroom Body (MB) learning center. All 3 $GABA_A$ receptor ($GABA_AR$) subunits are reportedly downregulated in *dfmr1* null brains. We demonstrate parallel downregulation of glutamic acid decarboxylase (GAD), the rate-limiting GABA synthesis enzyme, although GABAergic cell numbers appear unaffected. *M*osaic *a*nalysis with a *r*epressible *c*ell *m*arker (MARCM) single-cell clonal studies show that *dfmr1* null GABAergic neurons innervating the MB calyx display altered architectural development, with early underdevelopment followed by later overelaboration. In addition, a new class of extra-calyx terminating GABAergic neurons is shown to include MB intrinsic α/β Kenyon Cells (KCs), revealing a novel level of MB inhibitory regulation. Functionally, *dfmr1* null GABAergic neurons exhibit elevated calcium signaling and altered kinetics in response to acute depolarization. To test the role of these GABAergic changes, we attempted to pharmacologically restore GABAergic signaling and assay effects on the compromised MB-dependent olfactory learning in *dfmr1* mutants, but found no improvement. Our results show that GABAergic circuit structure and function are impaired in the FXS disease state, but that correction of hypoinhibition alone is not sufficient to rescue a behavioral learning impairment.

Introduction

GABAergic dysfunction is implicated in a range of neurodevelopmental disorders, including autism, epilepsy, Rett syndrome, and Fragile X syndrome (FXS) (Coghlan et al., 2012; D'Hulst and Kooy, 2007, 2009; Gatto and Broadie, 2010; Paluszkiewicz et al., 2010; Pizzarelli et al., 2011). In mouse and *Drosophila* FXS disease models, GABAergic deficiencies include depressed *glutamic acid decarboxylase* (*gad*) mRNA (D'Hulst et al., 2009) and $GABA_A$ receptor ($GABA_AR$) mRNA/protein levels (Adusei et al., 2010; Curia et al., 2009; D'Hulst et al., 2006; Gantois et al., 2006; Idrissi et al., 2005). GABAergic functional defects also occur in *Fragile X mental retardation 1* (*FMR1*) knockout mice. In the hippocampus, there is decreased GABAergic tonic inhibition (Curia et al., 2009). In the amygdala, both tonic and phasic inhibitory currents are reduced (Olmos-Serrano et al., 2010). Striatal spiny neurons display increased spontaneous inhibitory current frequency, with reduced paired-pulse ratio of evoked inhibitory currents (Centonze et al., 2008), consistent with reported fronto-striatal circuit disruption in clinical FXS (Menon et al., 2004). Together, these findings indicate depressed GABAergic function in the FXS disease state (Paluszkiewicz et al., 2010), suggesting GABAergic therapeutic treatment potential. The first such trial was conducted in the *Drosophila* FXS model, with a chemical screen demonstrating that feeding GABA or GABA reuptake inhibitors prevents a host of *Drosophila FMR1* (*dfmr1*) null phenotypes (Chang et al., 2008).

With only 3000 GABAergic neurons in the genetically-tractable *Drosophila* brain (Buchner et al., 1988; Chiang et al., 2011), this disease model provides an accessible system for testing GABAergic circuit

Abbreviations: 2iL, 2nd instar larval; AEL, after egg lay; ANOVA, analysis of variance; APL, anterior paired lateral; BSA, bovine serum albumin; ChAT, choline acetyltransferase; CS+, conditioned stimulus; CS−, control stimulus; dBrainbow, *Drosophila* Brainbow; *dfmr1*, *Drosophila FMR1*; dFMRP, *Drosophila* Fragile X mental retardation protein; DSHB, Developmental Studies Hybridoma Bank; FasII, Fasciclin II; FLP, flippase; *FMR1*, *Fragile X mental retardation 1*; FMRP, Fragile X mental retardation protein; FRT, FLP recombination target; FXS, Fragile X syndrome; $GABA_AR$, $GABA_A$ receptor; GAD, glutamic acid decarboxylase; HITS-CLIP, high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation; HS, heat-shock; KC, Kenyon Cell; LI, learning index; MARCM, *m*osaic *a*nalysis with a *r*epressible *c*ell *m*arker; MB, Mushroom Body; MCH, 4-methylcyclohexanol; mGluR, metabotropic glutamate receptor; NGS, normal goat serum; NipA, nipecotic acid; OCT, 3-octanol; P1, pupal day 1; PBS, phosphate buffered saline; PBST, 0.2% triton X-100 in PBS; PN, projection neuron; RDL, resistance to dieldrin.

☆ Conflict of interest: The authors declare the absence of any commercial or financial relationships that could be possibly construed as a potential conflict of interest.

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impairments. A key focus is the central brain Mushroom Body (MB) olfactory learning and memory center, in which the *resistance to dieldrin* (RDL) $GABA_AR$ in MB-intrinsic Kenyon Cell (KC) neurons is involved in synaptic integration within the MB calyx (Enell et al., 2007; Leiss et al., 2009; Liu et al., 2007; Su and O'Dowd, 2003). MB-targeted RDL knockdown elevates calcium responses to odor presentation and increases olfactory learning acquisition, although not memory stability (Liu et al., 2007). Importantly, we have shown that *dfmr1* null mutants display altered KC architecture (Pan et al., 2004; Siller and Broadie, 2011; Tessier and Broadie, 2008) and striking olfactory learning and memory impairments (Bolduc et al., 2008; Coffee et al., 2012). We therefore hypothesized that loss of *Drosophila* Fragile X mental retardation protein (dFMRP) may cause GABAergic circuitry defects in the MB underlying disruption of learning and memory behaviors. In this study, we assay GABAergic system expression, structure, and function in the well-characterized *Drosophila* FXS model (Coffee et al., 2010; Gatto and Broadie, 2011; Repicky and Broadie, 2008; Siller and Broadie, 2011; Tessier and Broadie, 2011). We report GAD depression with normal GABAergic neuron numbers, developmental stage-specific MB GABAergic neuron structural defects, a novel subclass of MB-intrinsic GABAergic KCs, and functional alterations in GABAergic neuron calcium signaling dynamics. However, efforts to pharmacologically augment GABAergic signaling, as done previously (Chang et al., 2008), failed to restore MB-dependent learning defects in *dfmr1* mutants. These findings reveal MB GABAergic circuitry defects in the *Drosophila* FXS disease model, but suggest that restoring inhibitory balance alone is insufficient to correct learning.

Materials and methods

Drosophila genetics

Drosophila stocks were maintained on standard cornmeal/agar/molasses food in humidity-controlled incubators with a 12 hour light:dark cycle at 25 °C. Two independent *dfmr1* null alleles ($dfmr1^{50M}$ (Zhang et al., 2001) and $dfmr1^{2}$ (Dockendorff et al., 2002)) were compared to w^{1118} genetic background control. The A9.9 GAD1-Gal4/CyO driver line (Gero Misenböck, University of Oxford, Oxford, UK (Ng et al., 2002)) was used for GABAergic neuron labeling and transgene expression. The OK107-Gal4 driver line was used for MB KC labeling and transgene expression. Transgenes included: UAS-mCD8::GFP, -DenMark, -dBrainbow, -GFP.nls (Bloomington *Drosophila* Stock Center, Bloomington, IN), and the UAS-GCaMP1.3 Ca^{2+} reporter (Nakai et al., 2001). For comparisons of control and *dfmr1*, standard genetic techniques were used to introduce all transgenic elements into both backgrounds. For pharmacological modulation of GABAergic signaling, animals were raised throughout development and into adulthood on standard food supplemented with either 40 μM GABA or 40 μM nipecotic acid (NipA) (Chang et al., 2008). As previously, heated molten food was cooled to 50 °C before drugs were added and then thoroughly mixed to achieve even distribution.

Drosophila Brainbow and MARCM

Drosophila Brainbow (dBrainbow) was used to clonally subdivide the GAD-Gal4 GABAergic neuron population (Hampel et al., 2011). For Cre-mediated recombination, y^1w^{67c23} P{Crey}1b; sna^{Sco}/CyO; Dr^1/TM3, Sb^1 (Bloomington) was crossed to GAD-Gal4/CyO and GAD-Gal4; $dfmr1^{50M}$/TM6. F1 offspring harboring recombinase and driver were then crossed to w^{1118}; P{UAS-Brainbow}attP40 (Bloomington) and P{UAS-Brainbow}attP40/CyO; $dfmr1^{50M}$/TM6, respectively. Experimental animals were females of the genotypes: y^1w^{67c23} P{Crey}1b/w^{1118}; GAD-Gal4/P{UAS-Brainbow}attP40 and y^1w^{67c23} P{Crey}1b/+; GAD-Gal4/P{UAS-Brainbow}attP40; $dfmr1^{50M}$. *M*osaic *a*nalysis with a *r*epressible cell *m*arker (MARCM) clonal analyses were then employed to further dissect the GABAergic neuron population (Lee and Luo, 2001; Wu and Luo, 2006). GAL80 inhibits GAL4 transcription factor activity, and following temperature-sensitive flippase (FLP) recombinase/FLP recombination target (FRT)-mediated mitotic recombination, the GAL80 transgene is removed from one of the daughter cells providing for expression of a GAL4-driven reporter gene specifically in this daughter cell and its progeny. For control and *dfmr1* clone induction, UAS-mCD8::GFP, hsFLP; FRT82b, tubGal80 was crossed to either GAD-Gal4; FRT82b or GAD-Gal4; FRT82b, $dfmr1^{50M}$/TM6. FLP/FRT-mediated recombination was then induced with 37 °C heat-shock (HS) at indicated times; 1 hour HS at 2 days after egg lay (AEL) was used for most examinations. Clones generated were of the following genotypes: UAS-mCD8::GFP, hsFLP/+; GAD-Gal4/+; FRT82b and UAS-mCD8::GFP, hsFLP/+; GAD-Gal4/+; FRT82b, $dfmr1^{50M}$. Structural analyses were done using the ImageJ/FIJI (NIH, Bethesda, MD, http://rsb.info.nih.gov/ij/) Simple Neurite Tracer segmentation tool to generate overlaid, skeletonized outlines for quantification (Longair et al., 2011).

Immunocytochemistry

Studies were performed essentially as previously described (Gatto and Broadie, 2008, 2009, 2011). Briefly, staged brains were dissected in phosphate buffered saline (PBS) and then fixed for 30 min with 4% paraformaldehyde/4% sucrose in PBS (pH 7.4) at room temperature or with ice-cold Bouin's fixative (GAD only (Kolodziejczyk et al., 2008)). Preparations were rinsed with PBS, then blocked and permeablized with 0.2% triton X-100 in PBS (PBST) supplemented with 1% bovine serum albumin (BSA) and 0.5% normal goat serum (NGS) for 1 h at room temperature. Primary and secondary antibodies were diluted in PBST with 0.2% BSA and 0.1% NGS and incubated overnight at 4 °C and 2–4 h at room temperature, respectively. Antibodies used included: anti-FasII (1:10; 1D4, mouse, Developmental Studies Hybridoma Bank (DSHB), University of Iowa), anti-GAD (1:500; 819, rabbit (Featherstone et al., 2000)), anti-GFP (1:2000; ab290, rabbit, AbCAM Inc., Cambridge, MA), anti-GFP-FITC (1:200; ab6662, goat, AbCAM), anti-myc (1:50; 9E10, mouse, DSHB), anti-HA (1:100; 3F10, rat, Roche Applied Science, Indianapolis, IN), anti-RFP (1:2000; 600-401-379, rabbit, Rockland Immunochemicals Inc., Gilbertsville, PA), anti-ChAT (1:100; ChAT4B1, mouse, DSHB), anti-dFMRP (1:500; 6A15, mouse, Sigma, St. Louis, MO), and anti-GABA (1:500; A2052, rabbit, Sigma). Alexa-568/633-phalloidin (1:25; Invitrogen-Molecular Probes, Carlsbad, CA) was used for F-actin visualization. Alexa-Fluor secondaries (1:250; Invitrogen-Molecular Probes) were used throughout. All specimens were mounted in Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA), and fluorescent images were collected using a ZEISS LSM 510 META laser scanning confocal microscope.

Neuronal cell counts

To examine the cell number and organization of GABAergic neurons, a nucleus-targeted UAS-GFP.nls (Bloomington) transgene driven by GAD-Gal4 was introduced into both control and *dfmr1* null backgrounds. Brains from 1 day old adult animals were dissected and processed following the immunocytochemical methods described above. Confocal imaging was done of whole brains, with a particular focus on the posterior aspect of the MB calyx region. Maximum intensity projections were generated from Z-stacks to quantify GFP-positive nuclei within the area adjacent to the F-actin delineated MB calyx. From the center of the calyx, a 50 μm diameter circle was drawn and all marked nuclear profiles in this brain region counted in control and *dfmr1* null animals.

MB structural assays

Staged brains from 1 day old adults were dissected, fixed, and labeled with anti-FasII (DSHB), as described above, in order to examine MB structural phenotypes. Controls and *dfmr1* null animals were examined with and without constant 40 μM GABA feeding (Chang et al.,