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Acamprosate rescues neuronal defects in the *Drosophila* model of Fragile X Syndrome

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ABSTRACT

Aims: Several off-label studies have shown that acamprosate can provide some clinical benefits in youth with Fragile X Syndrome (FXS), an autism spectrum disorder caused by loss of function of the highly conserved FMR1 gene. This study investigated the ability of acamprosate to rescue cellular, molecular and behavioral defects in the *Drosophila* model of FXS.
Main methods: A high (100 μM) and low (10 μM) dose of acamprosate was fed to *Drosophila* FXS (*dfmr1* null) or genetic control (w^{1118}) larvae and then analyzed in multiple paradigms. A larval crawling assay was used to monitor aberrant FXS behavior, overgrowth of the neuromuscular junction (NMJ) was quantified to assess neuronal development, and quantitative RT-PCR was used to evaluate expression of deregulated *cbp53E* mRNA.
Key findings: Acamprosate treatment partially or completely rescued all of the FXS phenotypes analyzed, according to dose. High doses rescued cellular overgrowth and dysregulated *cbp53E* mRNA expression, but aberrant crawling behavior was not affected. Low doses of acamprosate, however, did not affect synapse number at the NMJ, but could rescue NMJ overgrowth, locomotor defects, and *cbp53E* mRNA expression. This dual nature of acamprosate suggests multiple molecular mechanisms may be involved in acamprosate function depending on the dosage used.
Significance: Acamprosate may be a useful therapy for FXS and potentially other autism spectrum disorders. However, understanding the molecular mechanisms involved with different doses of this drug will likely be necessary to obtain optimal results.

1. Introduction

Fragile X Syndrome (FXS) is the most common cause of inherited intellectual disability [1]. Common symptoms of this broad spectrum disorder include severe intellectual developmental delays, hyperactivity, increased anxiety, hypersensitivity to stimuli, and autism [2–7]. FXS is caused by loss of function of the fragile x mental retardation 1 (FMR1) gene which encodes the RNA-binding fragile x mental retardation protein (FMRP) [8,9]. FMRP regulates the translation, editing, and stability of hundreds of different mRNAs [10–13]. The misregulation of these RNAs leads to characteristic overgrowth of neuronal processes and hyperactive synaptic signaling [14–17]. Potential therapies for FXS are therefore aimed at modulating the diverse mechanisms underlying this disease.

Recent work has focused on the utility of the drug acamprosate to alleviate symptoms of FXS [18,19]. Acamprosate was approved by the FDA in 2004 to treat relapse from alcohol withdrawal. Though the mechanism of acamprosate function is not well understood, it is believed to antagonize glutamatergic signaling which is overactive during periods of alcohol withdrawal [20–22]. Since similar hyperactivity is present in FXS, acamprosate was used in several small clinical studies to treat both FXS and autism. In each case, significant improvements were seen in the behavioral assessment scores from patients treated with the drug. Moreover, potential molecular biomarkers of the disease also showed improved changes in expression after treatment with acamprosate [23,24]. Thus, the clinical benefits of this compound to treat diseases other than alcoholic relapse are promising.

Analysis of acamprosate's function was also carried out in the mouse model of FXS [25]. The results of these studies were somewhat mixed. For example, the duration of spontaneous synaptic firing, or UP states, is prolonged in FMR1 knockout (KO) mice. While this electrophysiological phenotype was rescued by acamprosate treatment, the behavioral response of FMRP KO mice to audiogenically induced seizures was not. Similarly, the use of an open elevated zero maze showed

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that acamprosate treatment exacerbates the already reduced anxiety exhibited by FMR1 KO animals. Locomotor activity, however, is elevated in this FXS model and acamprosate was able to reduce activity in FMR1 knockouts. Additionally, the potential biomarker and signaling molecule phospho-ERK is increased in the FXS mouse brain and acamprosate restored the ratio of phospho-ERK/total ERK to normal levels. Thus some FXS mouse phenotypes are affected by acamprosate treatment while others are not. Therefore, the extent of acamprosate's effects on pathoneurological parameters remains to be elucidated.

We exploited the *Drosophila* FXS model to expand upon the role of acamprosate in treating FXS symptoms. Human FMR1 is well conserved in the *Drosophila* dFMR1 gene and the *dfmr1* null genetic model has consistently mimicked the behavioral, cellular, and molecular, symptoms of FXS [26,27]. Specifically, *dfmr1* null animals exhibit defects in larval locomotion including reduced transposition and increased turning [28,29]. At the cellular level, *dfmr1* null larval NMJs are overgrown as characterized by increased neuronal branching, synaptic bouton formation, and synaptic active zone marker expression [27,30]. In fact, many genes are either up or down regulated with the loss of *dfmr1* including *cbp53E*, which is a calcium-binding protein potentially involved in regulating neuronal physiology [12,31–33]. We analyzed each of these parameters and similar to studies in mice, have characterized both complete and incomplete recovery of FXS phenotypes in *dfmr1* mutant *Drosophila* treated with acamprosate. Behavioral changes as a result of acamprosate administration in control animals also indicate that this drug can function regardless of dFMR1 expression. Since our results indicate that some phenotypes can only be affected by a specific concentration of acamprosate, these data may be important to future preclinical and clinical studies.

2. Materials and methods

2.1. Drosophila stocks

The genetic control strain was w^{1118} and the $w;dfmr1^{50M}/TM6GFP$ stock was used to collect homozygous $w;dfmr1^{50M}$ null animals for all analyses. Except for acamprosate treated animals, all *Drosophila* stocks were maintained on standard cornmeal molasses agar at 25 °C with 12 h light/dark cycling.

2.2. Acamprosate administration

Acamprosate (a kind gift from Dr. Craig Erickson) was made as a 100 mM stock solution, aliquoted, and stored at − 20 °C until needed. Since acamprosate is generally stable at high temperatures, standard cornmeal molasses agar was cooled to 40 °C and the drug was added to final concentrations of 10 μM and 100 μM respectively [34,35]. Adult animals were placed in vials with acamprosate treated food and allowed to lay eggs. Only first generation, third-instar larvae were selected for experimentation.

2.3. Immunohistochemistry

Wandering third-instar larvae were dissected in 1 × phosphate buffer saline (PBS) and fixed in a 1 × PBS, 4% paraformaldehyde, 4% sucrose solution for 30 min at 25 °C. All larvae were then washed three times in wash buffer (1 × PBS; 1% BSA; 0.2% Triton X-100) for 30 min each at 25 °C and incubated in the following primary antibodies for 12–16 h at 4 °C: rabbit anti-horseradish peroxidase (1:200, Jackson Laboratories), mouse anti-Discs Large (1:200, 4F3, Developmental Studies Hybridoma Bank), and mouse anti-NC82/Bruchpilot (1:200, Developmental Studies Hybridoma Bank). Samples were washed three times in wash buffer for 30 min each before application of the following secondary antibodies for 1–2 h at 25 °C (all at 1:2000 dilutions): Alexa Fluor 488 goat anti-mouse IgG (Jackson Laboratories), Alexa Fluor 647 goat anti-mouse IgG (Invitrogen), and Alexa Flour 488 goat anti-rabbit IgG (Invitrogen). Finally, all samples were washed three times for 30 min each at 25 °C before mounting in Fluoromount G (eBioscience, Inc.).

2.4. Confocal microscopy

Z-stacks were obtained on a Zeiss LSM 710 confocal microscope under 40 × (1.3 na) and 63 × (1.4 na) magnifications. Imaris 8 (Bitplane Inc.) and ImageJ were used to analyze maximum intensity projections. NMJs were quantified for neuronal complexity as previously described [26,32]. Briefly, NMJs of abdominal segment 3, muscle 4 were analyzed under all treatment conditions. Values from the two paired muscle segments for each animal were averaged to produce a single data point. Branches were defined as having at least two boutons connected to the NMJ arbor. Bruchpilot NMJ quantification was manually calculated from maximum z-projections and puncta per NMJ were divided by number of boutons. Bruchpilot positive puncta per bouton values were averaged between the two paired muscles of a single larva to produce a single data point.

2.5. Larval movement analysis

Larval locomotion was analyzed similar to previous methods [29]. Wandering third-instar larvae were placed on a 10 cm plate containing 1.5% agar. The plate was placed on a level environment inside a small box with holes for two small LED lights to illuminate the larvae, and a hole at the top to accommodate a camera. The larvae were allowed to adjust for 5 min to this environment. Following the adjustment period, larval movement was filmed for 2 min. Videos of 10 frames per second at a resolution of 640 × 480 pixels were used to produce larval path traces in ImageJ. A custom program was written to analyze the distance travelled between frames and the angle of the turn made between frames. The threshold for what was considered a turn was set at 20° to allow for analysis of wider movements.

2.6. Quantitative reverse transcription PCR

RNA was extracted from whole wandering third-instar larvae using Trizol (Invitrogen) according to the manufacturing instructions. Five larvae were pooled per condition, and complete sets of 6 different conditions were extracted at the same time. One complete set equal to one biological replicate consisted of the following: control untreated, control 10 μM acamprosate treated, control 100 μM acamprosate treated, *dfmr1* null untreated, *dfmr1* null 10 μM acamprosate treated and *dfmr1* null 100 μM acamprosate treated. RNA was treated with DNAase Turbo (Ambion) and quantified on a SpectraMax M3 plate reader (Molecular Devices). 1 μg of RNA was used to make cDNA using SuperScript III reverse transcriptase and random hexamer primers (Invitrogen). 1 μL of cDNA was then used for qPCR reactions using Power Sybr Green Master Mix (Applied Biosystems) and performed on a BioRad CFX Connect Real Time System thermocycler. Each sample for each primer set was run in triplicate and a melt profile was observed for each primer set to ensure single product amplifications. PCR cycling parameters were as follows: 96 °C—10 min (94 °C—30 s, 60 °C—30 s, 72 °C—20 s) × 40 cycles, 72 °C—5 min. Primer sets for each target were as follows: *cbp53E* forward: 5′-GCCAGTGTTAGCAGAATCATGGTCAGTT-3′; *cbp53E* reverse: 5′-GCCAACAAGGACGGACGTCTGCAGTTGTC-3′; *alpha tubulin* forward: 5′-ACGTTTGTCAAGCCTCATAGC-3′; *alpha tubulin* reverse: 5′-GAGATACATTCACGCATATTGAGTT-3′. ΔΔCt calculations were performed using *alpha tubulin* as the reference to determine fold change of the target transcript.

2.7. Statistical analysis

For larval movement and NMJ analyses two-way ANOVAs were performed on all data sets followed by a Tukey's post hoc multiple