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ORIGINAL ARTICLE

Climbing as a measurement of locomotion ability in the *Drosophila* model of fragile X syndrome

Vedrana Makević[1], Maja Stojković[2], Marko Biorac[1], Sara Milojević[2], Maria Capovilla[3], Dragana Protić[2,4]

[1]University of Belgrade, Faculty of Medicine, Department of Pathophysiology, Belgrade, Serbia

[2]University of Belgrade, Faculty of Medicine, Department of Pharmacology, Clinical Pharmacology and Toxicology, Belgrade, Serbia

[3]UMR7275 CNRS-INSERM-UniCA, Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne Sophia Antipolis, France

[4]Fragile X Clinic, Special Hospital for Cerebral Palsy and Developmental Neurology, 11000 Belgrade, Serbia

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Correspondence to:
Dragana Protic,

Department of Pharmacology, Clinical Pharmacology and Toxicology, Faculty of Medicine, University of Belgrade,

1, Dr Subotića Starijeg Street, 11000 Belgrade, Serbia

E-mail: dragana.protic@med.bg.ac.rs

Summary

Introduction: Fragile X syndrome (FXS) is the most common monogenetic cause of intellectual disability (ID) and autism spectrum disorder (ASD) in humans. The *Drosophila melanogaster* model of FXS (*dFMR1* mutants) is an excellent model for research in the field of FXS. The aim of this study was a comprehensive investigation of climbing abilities, as a measurement of locomotion, in the $dFMR1^{B55}$ line as a *Drosophila* model of FXS.

Methods: In this study, control w^{1118} and $dFMR1^{B55}$ lines of fruit flies were used. The climbing performance of flies was examined using a climbing performance assay for groups of flies as well as for individual flies. Parameters that represent climbing ability, speed and endurance were determined. Females and males were analyzed separately.

Results: This study revealed the following: (i) worse climbing performance of $dFMR1^{B55}$ males in comparison to w^{1118} males; (ii) worse climbing success of $dFMR1^{B55}$ females in comparison to w^{1118} females; (iii) better climbing performance of top performer males in comparison to top performer females in the group climbing test in both $dFMR1^{B55}$ and w^{1118} groups; (iv) better, but not statistically significant, climbing performance (based on the time needed for 50% of flies to complete the task), and a higher success rate in $dFMR1^{B55}$ females in comparison to $dFMR1^{B55}$ males. **Conclusion**: According to the results of the current study, climbing impairment was proved only in $dFMR1^{B55}$ males, while $dFMR1^{B55}$ females had climbing abilities similar to control w^{1118} females.

Keywords: fragile X syndrome, *Drosophila melanogaster*, climbing assay

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INTRODUCTION

Drosophila melanogaster as a model organism has contributed enormously to the field of biomedical research, especially to genetics (1). The genome homology between fruit flies and humans is approximately 60% for all genes and 75% for the disease-causing genes (2, 3). Classes of neurotransmitters, molecular pathways, synaptic plasticity, and neuronal signaling are highly conserved in fruit flies (4-6). In addition, some human body structures have counterparts in *Drosophila,* such as brain parts involved in learning and memory, like the human hippocampus and the *Drosophila* mushroom bodies (7). The ease of maintenance, small size, short generation time and life span, large progeny, fewer ethical concerns, and the availability of genetic tools make *Drosophila melanogaster* an excellent model organism for neurobehavioral investigations (1, 8, 9). Numerous *Drosophila* models have been developed primarily for the exploration of genes related to human diseases (8, 10-12). An example of such disorders is fragile X Syndrome (FXS) (13, 14).

With a prevalence of 1:5000 in males and 1:8000 in females, FXS is the most common hereditary cause of intellectual disability (ID) and autism spectrum disorder (ASD) in humans (15). Expansion of CGG trinucleotides repeats (more than 200 CGG repeats) within the *Fragile X Messenger Ribonucleoprotein 1* (*FMR1*) gene, which is located at Xq27.3, its hypermethylation and transcriptional silencing lead to deficiency or absence of the encoded *FMR1* protein (FMRP). This protein is an RNA-binding protein which is involved in the regulation of numerous mRNAs in postsynaptic neurons (16). Moreover, FMRP is essential for neural development and it is implicated in post-transcriptional regulation and in the microRNA and Piwi-interacting RNA pathways. Additionally, it is a part of P-bodies and stress granules (reviewed in (17)). Deficiency of FMRP is the main cause of the clinical features in individuals with FXS. Core symptoms associated with the absence of functional FMRP in affected individuals (18, 19) reviewed in (20) are: ASD (presented in almost 60% of males (21)), shyness, abnormal eye contact and social anxiety, attention deficit hyperactivity disorder (ADHD), sensory hyperarousal, aggressive behavior, sleep problems, repetitive behaviors, and hand flapping. In addition, physical characteristics including elongated faces, prominent ears, joint hypermobility, soft skin, flat feet, high palate, and macroorchidism are also observed in these individuals (16, 22-24). Motor impairments, like delayed motor development and atypical motor behaviors, common in FXS, usually represent the first signs of impaired development in affected children (25-28).

Various animal models of this syndrome have been developed, including the *Fmr1* knock-out (KO) mice (29), zebrafish (30) and the *Drosophila melanogaster* model of FXS (14, 31, 32). *Fmr1* (FBgn0028734); (33), the *Drosophila* homolog of the human *FMR1* gene herein called *dFMR1,* is highly conserved with 35% identity and 60% similarity in two KH domains (34) (reviewed in: (17)). Thus, the fruit fly model of FXS (*dFMR1* mutants) is an excellent model for research in the field of FXS. Namely, this model of flies exhibits altered sleep and circadian rhythm (13, 14, 35-37), defects in learning, memory (38-40) and locomotion (35, 41-43), and changes in social interactions (13, 44) and repetitive behavior (45). Defects in locomotor activity include alternated larva crawling (42, 43), impairment in climbing (45, 46) and in flight (41). As mentioned above, genotype/phenotype overlap makes the *dFMR1* mutants an excellent model for studying FXS and a great tool for pharmacological research in this field. However, there are several different *dFMR1* mutant lines, but the exact differences among their behavioral characteristics have not been clarified yet and only very few studies have been conducted on molecular or phenotypic sex-differences in FXS model organisms.

The aim of this study was a comprehensive investigation of climbing abilities, as a measurement of locomotion, in the $dFMR1^{B55}$ line as a *Drosophila* model of FXS, analyzing females and males separately. Current research will enable more intensive use of this specific model in future preclinical research in the field of FXS.

MATERIALS AND METHODS

Flies

In this study, the $dFMR1^{B55}$ mutant (FX group) was used for researching climbing ability. This line was generated by Inoue and his group (2002) by imprecise excision of the EP(3)3422 P-element that caused a deletion of *dFMR1* genomic DNA containing exons 2, 3, and 4 and creating a protein null allele (14). As control, wild type w^{1118} flies were employed (WT group).

All experimental groups of flies were grown on standard cornmeal/agar/molasses medium at 25°C with 60% humidity under a 12-h light cycle which starts at 7 am and a dark cycle starting at 7 pm. Both sexes of seven-day-old virgin flies, were used separately for all experiments. All assays were performed in the dark under a dim red light to avoid the phototaxis effect (47), between noon and 3 pm to prevent potential circadian rhythm effect on climbing performance.

Climbing Performance Assay for Groups of Flies

For climbing trials, seven-day-old virgin male and female flies from the $dFMR1^{B55}$ and w^{1118} stocks previously separated by sex and genotype were transferred into empty tubes that contained 10 flies each (48). The tubes were constructed from two vials joined at their openings and connected with clear tape in order to make them longer (49). Flies were accustomed to dark conditions for an hour before the experiment.

Table 1. Results of analyses of climbing in the groups of wild-type flies and *dFMR1* mutants

Group climbing						
	Males			Females		
	WT	FX	p value	WT	FX	p value
N1	11	11		11	10	
Failure rate (%)	18.2	27.3	0.500[¥]	9.1	0.0	0.524[¥]
N2	9	8		10	10	
t1 (s) mean ± SD	6.557 ± 1.580	7.250 ± 0.886	0.290	8.908 ± 2.038	9.450 ± 1.072	0.467
t2 (s) mean ± SD	16.213 ± 4.351	35.281 ± 20.480	**0.015**	17.308 ± 10.820	24.292 ± 13.199	0.212
SR mean ± SD (%)	79.167 ± 10.607	64.375 ± 4.580	**0.002**	85.583 ± 8.231	74.015 ± 14.649	**0.043**

Student's t-test is used unless indicated otherwise. [¥]Chi square test. The failure rate was calculated as percentage of cases that were excluded from further analysis because half of a tested population failed to reach the goal of 17.5 cm during three minutes of observation in three or more experiments.

Abbreviations: WT, wild-type; FX, fragile X; N1, total number of experimental groups; N2, number of experimental groups used in analyses after exclusion of experimental groups in which half of the flies in the group did not pass the 17.5 cm mark in three minutes in three out of four trials; t1(s) , the time, in seconds, needed for the first fly in the group to pass the mark drawn at 17.5 cm from the bottom of the tube; t2(s), the time, in seconds, needed for half of the flies in the group (5 flies) to pass the 17.5 cm mark; SR, Success Rate represents the percentage of flies that passed the 17.5 cm mark within three minutes. Bold: statistically significant p values.

Each tube was gently tapped on a soft surface making flies fall to the bottom and initiate the negative geotaxis reflex (disturbed flies start to climb opposite to the gravitation vector (47)). The following parameters were recorded: (1) the time for the first fly (top performer) in the group to pass a mark drown at 17.5 cm from the bottom of the tube; (2) the time for half of the flies in the group (5 flies) to pass the 17.5 cm mark; (3) the percentage of experiments in which half of the flies in the group did not climb to the 17.5 cm mark within three minutes; (4) the percentage of flies that passed the 17.5 cm mark within three minutes (success rate). The whole procedure was repeated four times for each group, with three-minute intervals between measurements, and their average was taken for statistical analysis. At least 10 samples per genotype were analyzed. Experiments in which half of the flies in the group did not pass the 17.5 cm mark in three minutes in three out of four trials were excluded from data analyses. This number is represented as the parameter 'failure rate'(46).

Climbing Performance Assay for Individual Flies

An assay for individual climbing performance of flies was used to measure climbing speed and endurance (50). Individual flies were transferred in the modified serological pipette with marks at distances of nine and 27 cm from the bottom. Flies were knocked down to the bottom of the tube to initiate the negative geotaxis reflex. The time that a fly took to reach the 9 cm mark was recorded and used for the estimation of climbing speed. For the estimation of endurance, we measured the distance that the fly climbed within 15 s (27 cm was considered maximal distance and we stopped the time when the fly reached it). Average of three measurements per tube were used for statistical analysis. In the analysis, we entered just the cases in which the fly passed the 9 cm mark (50).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA). All data are shown as mean +/- SD or median (range). Student's t-test or Mann Whitney U test were used for analyzing differences between the compared groups depending on the normality of the distribution, except for the failure rate for which the Chi square test was used. This failure rate was calculated as percentage of cases that were excluded from further analysis because half of the tested population failed to reach the goal of 17.5 cm during the period of three minutes of observation in three or more experiments. $p<0.05$ was considered statistically significant.

RESULTS

Results of Climbing Performance for Groups of Flies

The results of climbing performance for groups of flies are shown in **Table 1** and **Table 2**. In both sexes, the first fly of the wild-type (WT) group climbed the assigned height of 17.5 cm faster than the first fly of the FX group, but this difference was not statistically significant (males p=0.290, females p=0.467, **Table 1**). However, the first half of WT males (five flies) reached 17.5 cm significantly faster than first half of FX males (p=0.015, **Table 1**). Results for females were in the same direction but without statistical significance (p=0.212, **Table 1**). The flies were observed for 3 minutes and the percentage of flies that succeeded to climb 17.5 cm during that period (success rate) was significantly lowerin the FX group compared to the WT group in both sexes (males p=0.002, females p=0.043, **Table 1**).

Table 2. Sex comparison of climbing in the groups of wild-type flies and *dFMR1* mutants

Group climbing sex comparison						
	WT			FX		
	Males	Females	p value	Males	Females	p value
N1	11	11		11	10	
Failure rate (%)	18.2	9.1	0.534[¥]	27.3	0.0	0.074[¥]
N2	9	10		8	10	
t1 (s) mean ± SDI	6.556 ± 1.580	8.908 ± 2.038	**0.013**	7.250 ± 0.886	9.4500 ± 1.0728	**<0.001**
t2 (s) median	16.750	14.0000	0.487[§]	31.292	19.917	0.076[§]
t2 (s) range	(10.25 - 24.00)	(9.00 - 46.75)	NA	(16.50 - 83.00)	(13.00 - 49.25)	NA
SR mean ± SD (%)	79.167 ± 10.607	79.167 ± 8.231	0.157	64.375 ± 4.581	74.015 ± 14.649	0.075

Student's t-test is used unless indicated otherwise. [§]Mann Whitney U test was used; [¥]Chi square test. The failure rate was calculated as percentage of cases that were excluded from further analysis because half of a tested population failed to reach the goal of 17.5 cm during three minutes of observation in three or more experiments.

Abbreviations: WT, wild-type; FX, *dFMR1*^{B55}; N1, total number of experimental groups; N2, number of experimental groups used in analyses after exclusion of experimental groups in which half of the flies in the group did not pass the 17.5 cm mark in three minutes in three out of four trials; t1(s), the time, in seconds, needed for the first fly in the group to pass the mark drown at 17.5 cm from the bottom of the tube; t2(s), the time, in seconds, needed for half of the flies in the group (5 flies) to pass the 17.5 cm mark; SR, Success Rate represents the percentage of flies that passed the 17.5 cm mark within three minutes. Bold: statistically significant p values.

The comparison between sexes revealed that the first male fly was significantly faster than the first female fly in both groups (WT group: p=0.013, FX group: p<0.001, **Table 2**). Conversely, the first half of the female group came to the goal faster than the first half of the male group in WT flies and especially in FX flies. Although in this case the difference between WT and FX females and males was not significant, a positive trend is observed in the FX group (p=0.487 and p=0.076, respectively, **Table 2**). Furthermore, FX females had higher success rate than FX males (p=0.075, **Table 2**). This failure rate was consistent among all investigated groups, with the exception of FX males that failed much more than FX females, but without statistical significance (p=0.074, **Table 2**).

Results of Climbing Performance for Individual Flies

As described above, in the next climbing assay, every fly is tested separately and climbing speed and endurance of an individual fly are obtained. The results are represented in **Tables 3** and **4**. The results undoubtedly show that FX males are slower and less endurant than WT males (p=0.018, p=0.001; respectfully, **Table 3**). However, WT and FX females had similar speed and endurance (p>0.05, both). Further, WT and FX males were faster and more endurant than females (p<0.001, all. **Table 4**).

DISCUSSION

In the current investigation, *dFMR1*^{B55} mutant climbing abilities were examined in groups of flies, as presented in previous studies (45, 46, 51). Furthermore, this study described, for the first time, climbing abilities in *dFMR1*^{B55} mutants using individual flies. This research, for the first time, compared *dFMR1*^{B55} mutant climbing performance in females and males separately, and provided information about *dFMR1*^{B55} mutant endurance. Finally, this study revealed the following: (i) worse climbing performance of *dFMR1*^{B55} males in comparison to w^{1118} males; (ii) worse climbing success of *dFMR1*^{B55} females in comparison to w^{1118} females; (iii) better climbing performance of top performer males in comparison to top performer females in the group climbing test in both *dFMR1*^{B55} and w^{1118} groups; (iv) better, but not statistically significant, climbing performance (based on the time needed for 50% of flies to complete the task) and higher success rate in *dFMR1*^{B55} females in comparison to *dFMR1*^{B55} males.

Table 3. The results of analyses of climbing of individual wild-type flies and *dFMR1* mutants

Individual climbing						
	Males			Females		
	WT	FX	p value	WT	FX	p value
N	29	33		18	22	
speed	1.865 ± 0.372	1.614 ± 0.431	**0.018**	1.238 ± 0.396	1.256 ± 0.299	0.867
endurance	22.138 ± 3.592	18.581 ± 4.341	**0.001**	14.880 ± 3.091	14.712 ± 2.271	0.845

Student's t-test is used. Speed (cm/s) was calculated based on time in seconds that a fly took to reach the 9 cm mark; endurance (cm), the distance a fly climbed within 15 s.

Abbreviations: N, number of individual flies; WT, wild-type; FX, *dFMR1*^{B55} mutants. Bold: statistically significant p values.

Table 4. Sex comparison of climbing of individual wild-type flies and *dFMR1* mutants

Individual climbing sex comparison						
	WT			FX		
	Males	Females	p value	Males	Females	p value
N	29	18		33	22	
speed	1.864 ± 0.372	1.238 ± 0.396	**<0.001**	1.614 ± 0.431	1.256 ± 0.299	**0.001**
endurance	22.138 ± 3.592	14.879 ± 3.090	**<0.001**	18.580 ± 4.341	14.712 ± 2.271	**<0.001**

Student's t-test is used. Speed (cm/s) was calculated based on time in seconds that a fly took to reach the 9 cm mark; endurance (cm), the distance a fly climbed within 15 s.

Abbreviations: WT, wild-type; FX, *dFMR1*^{B55} mutants; N, number of individual flies. Bold: statistically significant p values.

Our study is in accordance with the results of other studies that investigated the motor capabilities of *dFMR1* mutants and also found a motor impairment. They investigated motor capability like flight (41), larva crawling (42, 43), and average motor activity (35). On the other hand, the study conducted by Dockendorff *et al.* (2002) found that *dFMR1* fly total motor activity observed during nine days in the dark was similar with motor activity in control WT flies. However, this discrepancy could be explained by different conditions/methods which were used in this study such as dark during nine days of experiments (13). Interestingly, *Fmr1*-KO male mouse performance on standard motor tests (including climbing) was similar to their WT counterparts with an exception of the raised-beam test in which *Fmr1* mice performed worse (52). However, motor learning is proven to be impaired in *Fmr1* mice (53).

Additional studies examined this ability in *dFMR1* mutants only in males (45, 46). Martinez and colleagues (2007) found that the first *dFMR1* male in the group climbed with a similar speed as the first male in the control group but the first half of *dFMR1* males was slower than their WT counterpart and the success rate of *dFMR1* was decreased compared to Canton S controls, but not to the Oregon red control line (46). The study of Tauber and colleagues (2011) obtained similar results, but they found differences in favor of controls even between the fastest male climbers in the two groups (45). However, this study used genetically rescued mutant flies as controls.

The current study investigated climbing ability of both sexes in *dFMR1*^{B55} mutants. Two assays used in the current study consistently showed that *dFMR1*^{B55} males are poorer climbers in comparison to WT. However, apart from the lower success rate in group climbing, *dFMR1*^{B55} females did not show differences in climbing ability compared with control flies. Therefore, the current research revealed that the mutation affects climbing abilities in a sex-specific manner in the *dFMR1*^{B55} mutants. Similarly, a recent study described that lead (plumb, Pb) exposure worsened climbing abilities of *Drosophila* Oregon-R in a sex-dependent manner. Namely, Pb provoked climbing impairment in both sexes, but climbing ability in male flies was more affected than in females. It is important to mention that Pb also induced other human-autistic-like behavior in fruit flies which is similar to the features of *dFMR1* mutants which were used in our study (9). Niveditha et al. (2017) found that females had lower reactive oxygen species levels and higher antioxidant levels than males (54). Thus, we could suggest that better oxidative stress handling in fly females might be responsible for their better climbing performance in general (54).

In conclusion, according to the results of the current study, climbing impairment was proved only in *dFMR1*^{B55} males, while *dFMR1*^{B55} females had similar climbing abilities to control WT. Thus, we could recommend that *dFMR1*^{B55} male mutants might present an excellent model for further research of locomotion impairment in the field of FXS. Also, *dFMR1*^{B55} male mutants could be an important 'tool' for pharmacological research and investigations of pharmacological agent effects to this kind of behavior. On the other hand, investigation of different aspects of climbing performance in *dFMR1*^{B55} female mutants could be unreliable. In addition, we can suggest always using both assays ('group' and 'individual') based on different parameters that can be obtained. Briefly, the current study demonstrates that *dFMR1*^{B55} male mutants are a very useful tool for research on locomotion and motility in the field of fragile X.

Conflicts of interest

None to declare

Ethical approval

This study includes research only on alternative model, i. e. Drosophila fragile X model. According to the National Centre for the Replacement Refinement & Reduction of Animal in Research, partial replacement includes the use of some animals that, based on current scientific thinking, are not considered capable of experiencing suffering. This includes invertebrates such as Drosophila, nematode worms and social amoebae, and immature forms of vertebrates (https://www.nc3rs.org.uk/the-3rs). According to the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (https://eur-lex.europa.eu/eli/dir/2010/63/oj) and Serbia Law on animals' welfare (Sl. list RS 41/09 at: https://

www.paragraf.rs/propisi/zakon_o_dobrobiti_zivotinja.html), ethical review permissions are not needed for scientific research which include alternative models such as Drosophila.

Author Contributions

V.M. - acquisition, analysis, and interpretation of data, preparing the draft of the manuscript, M. S. - acquisition, analysis, and interpretation of data, M. B. - preparing the draft of the manuscrip, S. M. - acquisition and analysis of data, M. C. - interpretation of revised version of manuscript, D. P. - conception and design of the work and interpretation of revised version of manuscript.

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TEST PENJANJA KAO MERA LOKOMOTRONE SPOSOBNOSTI NA MODELU VINSKE MUŠICE ZA FRAGILNI X SINDROM

Vedrana Makević[1], Maja Stojković[2], Marko Biorac[3], Sara Milojević[2], Maria Capovilla[4], Dragana Protić[2]

Sažetak

Uvod: Fragilni X sindrom (FXS) je najčešći monogenetski uzrok intelektualne zaostalosti i poremećaja iz spektra autizma kod ljudi. Odličan model za istraživanje u ovoj oblasti je *Drosophila melanogaster* model FXS (*dFMR1* mutanti). Cilj ove studije bio je sveobuhvatno istraživanje penjanja, kao mere lokomocije, linije *dFMR1*^{B55}, koja je model FXS kod vinske mušice.

Metode: U ovoj studiji korišćene su linije vinskih mušica *w*^{1118} i *dFMR1*^{B55}. Sposbnost penjanja muva ispitana je testovima penjanja za grupe muva i za pojedinačne muve. Određivani su parametri koji se odnose na sposobnost penjanja, brzinu i izdržljivost muva. Ženke i mužjaci su zasebno analizirani.

Rezultati: Ova studija je pokazala: (i) lošiju sposobnost penjanja *dFMR1*^{B55} mužjaka u poređenju sa *w*^{1118} mužjacima, (ii) slabiju stopu uspeha u penjanju *dFMR1*^{B55} ženki u poređenju sa *w*^{1118} ženkama, (iii) bolju penjačku sposobnost mužjaka u odnosu na ženke u obe ispitivane linije pokazanu u individualnom i grupnom testu penjanja u poređenju najbržih muva, (iiii) bolju sposobnost penjanja (baziranu na vremenu potrebnom da 50% muva izvrši zadatak) i veću stopa uspeha *dFMR1*^{B55} ženki u poređenju sa *dFMR1*^{B55} muškarcima, premda ove razlike nisu bile statistički značajne.

Zaključak: U ovoj studiji, poremećaj penjanja je dokazan samo kod *dFMR1*^{B55} mužjaka, dok su *dFMR1*^{B55} ženke imale slične sposobnosti penjanja sa kontrolnim *w*^{1118}.

Ključne reči: fragilni X sindrom, *Drosophila melanogaster*, test penjanja

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