Fly 4:3, 216-225; July/August/September 2010; © 2010 Landes Bioscience

An assay for social interaction in Drosophila fragile X mutants

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Key words: Drosophila, Fragile X, autism, social behavior, novel assay

We developed a novel assay to examine social interactions in Drosophila and, as a first attempt, apply it here at examining the behavior of Drosophila Fragile X Mental Retardation gene (*dfmr1*) mutants. Fragile X syndrome is the most common cause of single gene intellectual disability (ID) and is frequently associated with autism. Our results suggest that *dfmr1* mutants are less active than wild-type flies and interact with each other less often. In addition, mutants for one allele of *dfmr1*, *dfmr1*^{B55}, are more likely to come in close contact with a wild-type fly than another *dfmr1*^{B55} mutant. Our results raise the possibility of defective social expression with preserved receptive abilities. We further suggest that the assay may be applied in a general strategy of examining endophenoypes of complex human neurological disorders in Drosophila, and specifically in order to understand the genetic basis of social interaction defects linked with ID.

Introduction

Social behavior is typically defined as the exchange of stimuli to initiate, maintain and terminate interaction between two or more individuals.[1] The study of innate social behavior has a long history; even in the canonical model organism Drosophila, interest in these behaviors can be traced as far back as Sturtevant's early work.[2] More recently, a tremendous amount of information has been gained with regard to the social behaviors of aggression[3-7] and courtship[8,9] in flies. In each case, genetic dissection and detailed behavioral descriptions have elucidated the molecular basis of these complex phenotypes. Drosophila's genetic tractability has also been advantageous in understanding the molecular basis for memory defects observed in intellectual disability (ID) syndromes such as mental retardation autosomal recessive 1 (*neurotrypsin*),[10] Fragile X syndrome (*dfmr1*)[11,12] and Angelman syndrome (*dube3a*).[13] These successes cement the utility of Drosophila as a model organism for studying human neurological disease, and highlight the importance of using flies for gaining a deeper biological insight into the underlying components of complex phenotypes. As an example of importance to this paper, studying Drosophila social behavior may yield a more thorough understanding of the molecular basis of defective social function linked to ID, and in turn, may elucidate complex phenotypic diseases like Autism Spectrum Disorder (ASD), of which social deficits are one aspect of the phenotype.

The complexity of the phenotypes observed in such disorders makes the study of their neural basis difficult. Several researchers have suggested a "splitting" approach where specific behavioral traits are examined as opposed to a "lumping" approach where a mixture of various phenotypic presentations is studied as a group (reviewed in ref. 14). The splitting approach corresponds to studying the specific "endophenotype," or sub-behavior with given individual features. For example, many autistic patients show impairment in the use of facial expression, body posture and gestures. These impairments are instances of the endophenotype that psychologists have termed motor dyspraxia, which is defined as a partial loss of the ability to perform skilled, coordinated movements in the absence of any associated defect in motor or sensory functions.[15] Because these impairments often result in a failure to regulate social interactions, understanding the biological basis of motor dyspraxia could be helpful at understanding the social deficits observed in ASD.

On the molecular side, an increasing number of single gene mutations have now been linked to ASD[16-19] and offer the possibility of genotype-phenotype correlations. Among the single-gene diseases most commonly associated with autism is Fragile X Mental Retardation syndrome, which is caused by a mutation in or, more frequently, transcriptional silencing of the Fragile X mental retardation gene (FMR1). This leads to the absence of normal Fragile X mental retardation protein (FMRP),[20-22] an RNA-binding protein involved in translational control.[23-26] There is high prevalence of ASD among Fragile X patients (up to 30%),[27,28] and these patients share similar social impairments. Furthermore, social defects have been recently observed in an FMR1 knockout mouse, suggesting a general phenotypic contribution of this gene. Drosophila's genome, too, contains an orthologue of FMR1, *dfmr1*. Importantly, mutants for *dfmr1* have

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Submitted: 09/15/09; Revised: 04/06/10; Accepted: 05/05/10
Previously published online: www.landesbioscience.com/journals/fly/article/12280

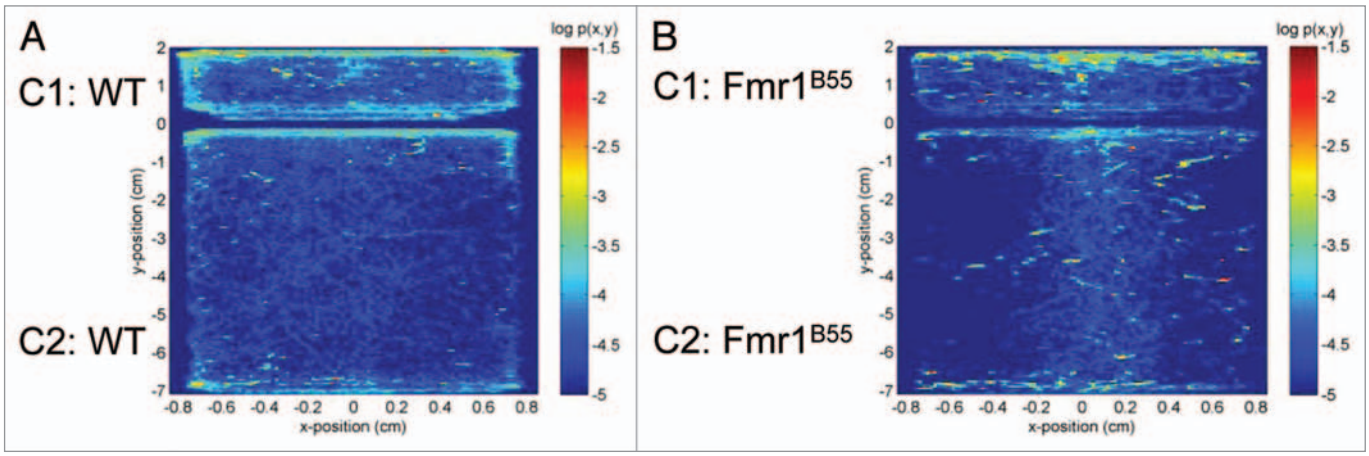

Figure 1 (continued on next page). Pair-wise Fragile X mutant *dfmr1*^{B55} social behavior differs from wild-type. (A and B) Joint spatial distributions *p(x,y)* for wild-type (A) and *dfmr1*^{B55} flies (B). A single fly is placed in each chamber for each test, and the experiment is performed for N pairs of flies (N = 7 pairs for WT and N = 9 pairs for *dfmr1*^{B55}). The small chamber is designated as Chamber 1 (C1) and the long chamber is designated as Chamber 2 (C2). The divider between boundaries is at y = 0 cm.

circadian rhythm defects,[29,30] neuropathological anomalies[31-33] and memory dysfunction,[11,12] phenotypes which are also observed in human Fragile X syndrome patients.

In this paper, we present an assay for assessing social interaction in flies, adapted from an assay for social interaction in mice.[34-36] We demonstrate the utility of the assay by examining the behavior of two mutant alleles for the Drosophila Fragile X syndrome orthologue gene, *dfmr1* and suggest this assay and analyses therein may be useful for elucidating endophenotypes of complex human neurological disorders. Here we show that Drosophila *dfmr1* mutants appear to have social defects which manifest as an increase in interfly distance. In addition, we show modification of this behavior when the *dfmr1* mutant is exposed to a wild-type fly. This may suggest that, as in Fragile X patients (where dyspraxia are observed), d*fmr1* mutants fail to initiate social interaction, perhaps due to impairment of motor signals. This example demonstrates a strategy for the examination of human neurological disorder endophenotypes, using Drosophila as the model system.

Results

Differential exploratory behavior in drosophila fragile X mutants. We began by studying the baseline locomotor and exploratory performance of single wild-type and homozygous *dfmr1*^{B55} Fragile X mutant females (using 3-day-old flies raised individually) in our chamber (**Suppl. Fig. 1**). Analysis of the trajectories of these flies (**Suppl. Fig. 2A and B**) shows that both groups share an attraction for the boundaries of the test chamber, spending equal amounts of time near both the top and bottom boundaries. This tendency to stay at the periphery of the test chamber is a common characteristic of fly exploratory behavior.[37,38] Furthermore, the top of the chamber presented an area of increased interest for both groups in comparison to the bottom of the chamber, possibly due to the presence of a fine mesh which separated this chamber from a second chamber (see Methods and **Suppl. Fig. 1**). There were also distinctive differences in the behavior of each group. Most importantly, wild-type flies displayed higher locomotor activity compared to the *dfmr1*^{B55} mutant flies, which can be seen in the spatial probability distribution as a fairly uniform coverage of space (**Suppl. Fig. 2B**) and a significantly higher value for a line-crossing metric (**Suppl. Fig. 2C**). Wild-type flies dart across the chamber, whereas *dfmr1*^{B55} mutants exhibit a greater probability of stopping for long lengths of time in the chamber interior, covering less overall area in the process. These stops are marked by the large, isolated peaks in the joint distribution, *p(x,y)* (**Suppl. Fig. 2B**). The abnormal locomotor activity of *dfmr1* mutants is consistent with several studies in the literature. Tracking of *dfmr1* larvae[39] showed a decrease in the amount of time that these larvae spend crawling. In addition, initial studies of circadian activity observed that *dfmr1* flies were more likely to have erratic short bouts of activity when compared to wild-type control flies.[29,30] Dockendorff indeed observed an erratic locomotor activity pattern (which he called arrhythmic phenotype) in absence of motor or locomotor defects, which could be interpreted as a basic form of dyspraxia.

Pair-wise behavior of drosophila fragile X mutants. Having established a baseline for solitary behavior, we studied the effect on the behavior of wild-type and *dfmr1*^{B55} mutants when a second, isogenic fly is placed in an adjacent chamber. We observed that the spatial probability distributions differed between wild-type and *dfmr1*^{B55} mutant flies in this case (**Fig. 1A and B**); however, the locomotor activity in each chamber remained consistent with the solitary behavior of **Supplementary Figure 1**. In the small chamber (designated as Chamber 1: C1), the wild-type flies were probabilistically located nearer the divider than the *dfmr1*^{B55} flies (peak at y≈0.25 cm in **Fig. 1C**). On the other hand, the *dfmr1*^{B55} mutants had a tendency to stay farther from the divider in this chamber (peak at y≈0.5 cm in **Fig. 1D**). Both groups spent an equivalent amount of time near the top of C1; the cause of the distribution is unclear, but is likely a property of the test chamber.

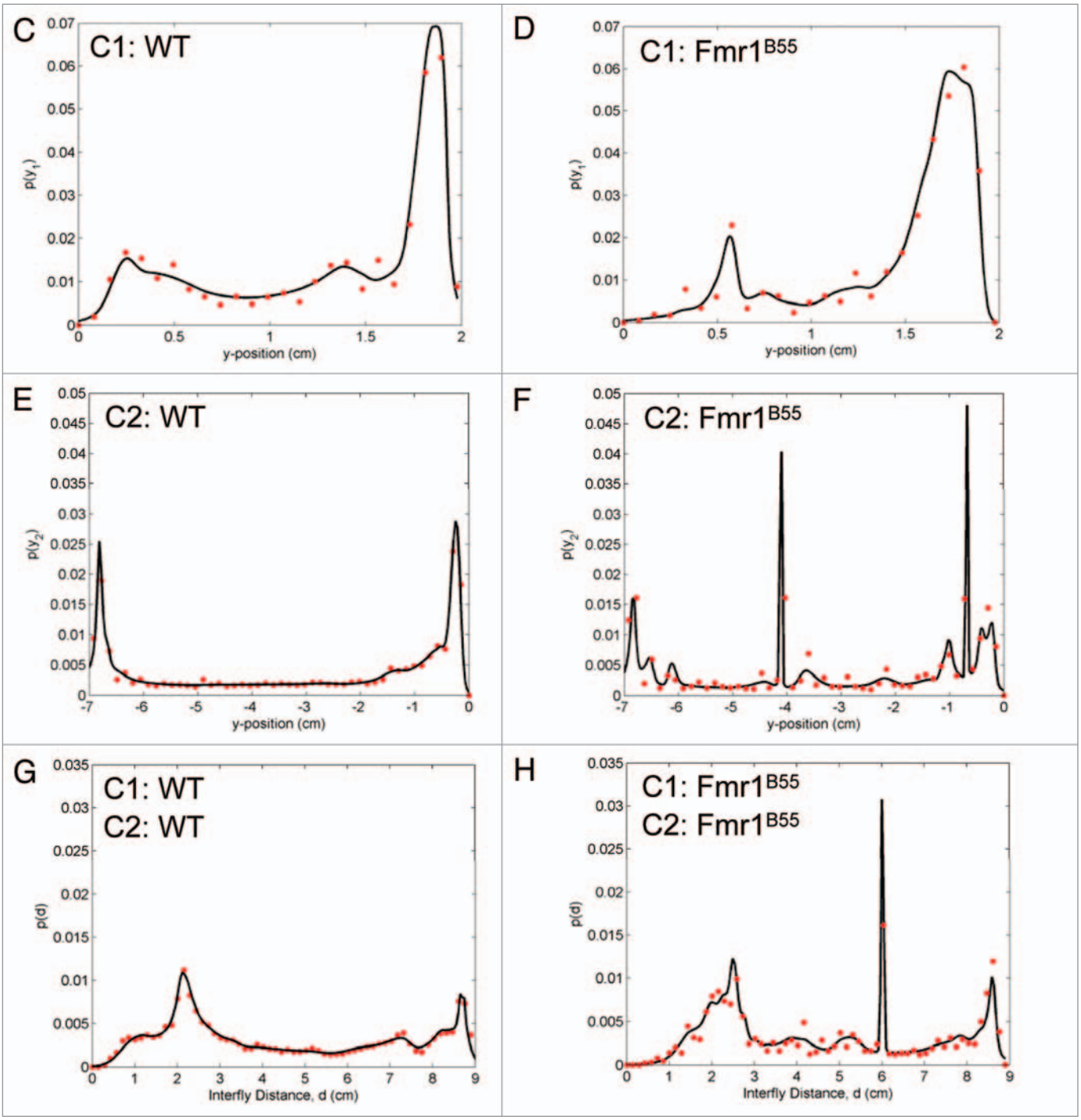

Figure 1 (continued from previous page). Pair-wise Fragile X mutant *dfmr1*^{B55} behavior differs from wild-type. (C and D) Marginal probability distribution *p(y)* of wild-type (C) and *dfmr1*^{B55} (d) flies in CI. (E and F) Marginal distribution of wild-type (E) and *dfmr1*^{B55} (F) flies in C2. (G and H) Interfly distance distribution *p(d)* for wild-type (G) and *dfmr1*^{B55} (H) flies.

To examine the consistency of the phenotype and its association with the *dfmr1* locus, we tested a second published allele previously used for circadian rhythm, olfactory and courtship memory, as well as neuroimaging: the homozygous *dmfr1*^{3} allele (**Fig. 2**). As opposed to *dfmr1*^{B55}, no transcript is possible for *dfmr1*^{3}, as the transcriptional start site is completely removed. Indeed, *dfmr1*^{B55} have been shown to produce minimal amount of dFmrp in testes.[40] For *dfmr1*^{3} in C1, we observed a greater stopping tendency than wildtype, similar to that of *dfmr1*^{B55}, and an even more uniform spatial preference than *dfmr1*^{B55} (**Figs. 1A and B** to **2A**). Therefore, flies with this null mutation show a phenotype that seems to exist on a scale somewhere between that of the *dfmr1*^{B55} mutants and wild-type flies.

In the long chamber (designated as Chamber 2: C2) wild-type flies (**Fig. 1A**) showed more of a distributed interest (spatially speaking) in the chamber division, and a uniform exploration of the entire available space. *dfmr1*^{B55} mutants, in contrast, spent more time stopped in the chamber interior (**Fig. 1B and F**). It is difficult to ascertain whether the frequent and long stops made by the *dfmr1*^{B55} flies are an indication of small obstacles in the vial (scratches, dust, etc.,); however, the large spikes in the average distributions suggest that there may be common points of interest characteristic to the vial that were present on repeated sessions separated by several days. Otherwise, one would expect the stop-spikes to average out across days. This is not the case (c.f. **Figs. 1 and 3** and **Suppl. Fig. 2**). Interestingly, these same points do not seem to affect wild-type behavior, or at least not to the same extent. This could suggest an increased attraction to obstacles for *dfmr1*^{B55} over wild-type flies, but that hypothesis is unable to be examined with these data and the current experimental setup. Nevertheless, mutant flies in both chambers showed much less exploration, which is consistent with previous observations, at least for larval behavior.[39] *dfmr1*^{3} homozygous mutant flies were also tested in the same situation. Again, flies in the long chamber (C2) shared phenotypic characteristics with both *dfmr1*^{B55} and wild-type flies, having an increased number of interior stops like *dfmr1*^{B55}, yet with a more uniform spatial distribution (**Fig. 2A, C and E**).

Detailed examination of boundary preferences between strains. Any social interactions in this assay are forced to occur at the boundary between chambers. To begin dissecting the possibility of social interaction from a mere boundary preference, we further quantified the likelihood for flies to be located in a 5 mm zone from each boundary in each genotype (**Fig. 4**). This zone was defined by the peak widths in the marginal spatial distributions for wild-type flies near the division boundary (**Fig. 1C and E, Suppl. Fig. 2B**) and corresponds to approximately two body lengths. Furthermore, to investigate the possibility that any difference in possible social interactions (defined below) depend on the receptive or expressive skills of flies, as in autistic patients, we studied the behavior of *dfmr1*^{B55} mutants in C2 in presence of either a wild-type fly (**Fig. 3B**) or another *dfmr1*^{B55} mutant in C1 (**Figs. 3A,** which is a replicate experiment of **1D–F**).

First, all groups spent equivalent amounts of time within 5 mm of the chamber extremes, both at the top of C1 (**Fig. 4A**) and the bottom of C2 (**Fig. 4D**). Behavior near the chamber division, however showed more variety. In C1, wild-type flies near the divider showed similar behavior when exposed to either a wild-type or a *dfmr1*^{B55} mutant fly (**Fig. 4B**). In contrast, there was a significant increase in *dfmr1*^{B55} localization at the divider in C2 when these flies were exposed to a wild-type fly in C1 (**Fig. 4C**). This "normalization" of behavior (i.e., compared to wild-type) did not appear to be the result of a general change in locomotor activity (**Fig. 4F, Suppl. Fig. 3**). *dfmr1*^{3} mutants were tested in a similar paradigm and obtained a similar qualitative reduction in probability of flies being distal to the border (**Fig. 2E and F**; although see caveats in Methods). For *dfmr1*^{3} flies in C2, there was no significant change in the time spent in within 5 mm of any boundary (including the division), whether the fly in the adjacent chamber was wild-type or *dfmr1*^{3} (**Suppl. Fig. 4**).

Likelihood of social interaction as measured by the interfly distance. Congregation near the chamber divider suggests the possibility for social interaction. In order to examine whether this corresponded to social interactions or simply to boundary visits at different times, we examined the interfly distance distributions for wild-type (**Fig. 1G**), *dfmr1*^{B55} (**Fig. 1H**) and *dfmr1*^{3} (**Fig. 2G**) mutant flies. Co-location at the arena boundary corresponds to small values for *d*. While co-location does not necessarily ensure that a true social interaction is taking place, it can serve as a measure for interaction likelihood. To assess the presence of a true social interaction, tracking resolution higher than that available to our current system is required; further studies would need to be performed at higher magnification to observe any stereotyped behavior.

The distance distributions for all groups are quite similar, sharing expected broad peaks near the locations where flies are located at chamber boundaries (~2.5 cm, ~7 cm, ~9 cm). Direct social interactions would likely be restricted to $d < 1.5$ cm, a distance which corresponds to the flies being located at the center divider but at opposite ends. As a conservative estimate of the social interaction possibility and for consistency with our analysis above, we examined the probability that flies are found within 5 mm of each other. Wild-type flies spent significantly more time within 5 mm of each other than *dfmr1*^{B55} mutants (**Fig. 4E and Table 1**). The observation that when exposed to a wild-type fly in C1, a *dfmr1*^{B55} mutant fly in C2 is found with greater probability closer to the divider (cf. **Fig. 3E and F**) is also reflected in the differences between the interfly distance distributions (**Figs. 3G and H, 4E**). To wit, these flies are also found closer together. *dfmr1*^{3} mutants, too, show little likelihood of being within 5 mm of each other; in fact, such little likelihood as to result in nearly no data for this measure (**Suppl. Fig. 4E**). Hence, the normalization of interfly distance seen in *dfmr1*^{B55} mutants is not seen in *dfmr1*^{3} mutants when they are exposed to a wild-type fly in C1.

A possible hypothesis for the behavioral renormalization of the *dfmr1*^{B55} flies' behavior in the presence of a wild-type partner is that that these flies have a relatively normal receptive response to interaction initiation but may fail to display appropriate motor output to encourage continued interaction from the opposing fly. Another possibility is that *dfmr1*^{B55} flies failed to emit some "chemo-attractant cues" and/or possessed less receptor for it. Since the divider was made of a plastic mesh, we cannot rule out the possibility of a hydrocarbon based attraction and the fact that flies could have still perceived it through gustatory modality (as is the case for pheromone).[41,42] Therefore, a wild-type fly could "attract" a *dfmr1*^{B55} mutant fly. One may suggest that more severe defects observed in *dfmr1*^{3} may be related to a total absence of *dfmr1* transcription due to the nature of the genetic lesion. Clearly, a higher-magnification behavioral study associated with an impermeable divider would be required to properly investigate these hypotheses, and so we merely present this as an observation.

Discussion

A key step to future treatments for complex neurological diseases, such as ASD, is in understanding the neurobiological basis of the

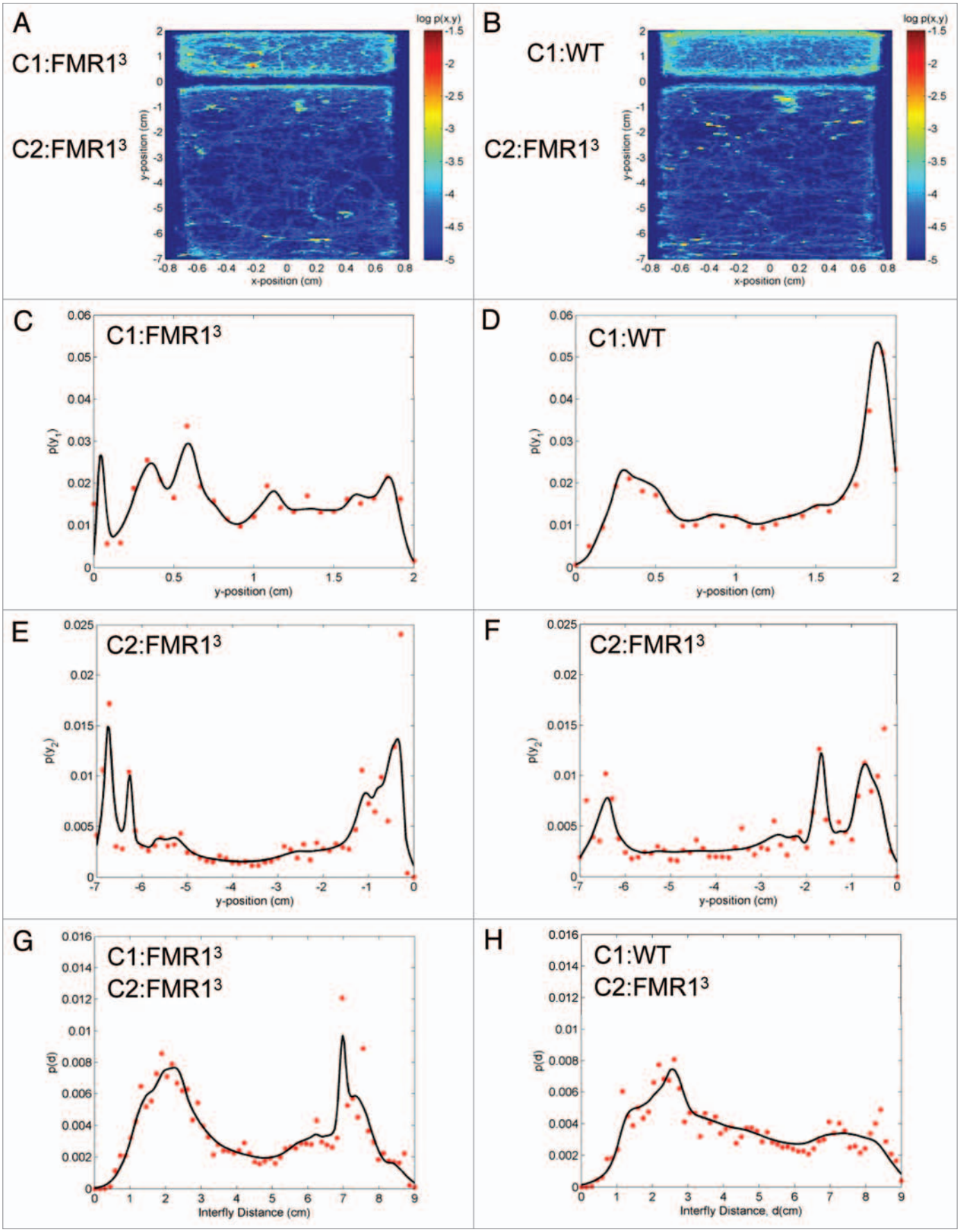
A
C1:FMR1³
C2:FMR1³
log p(x,y)
y-position (cm)
x-position (cm)
B
C1:WT
C2:FMR1³
log p(x,y)
y-position (cm)
x-position (cm)
C
C1:FMR1³
p(y1)
y-position (cm)
D
C1:WT
p(y1)
y-position (cm)
E
C2:FMR1³
p(y2)
y-position (cm)
F
C2:FMR1³
p(y2)
y-position (cm)
G
C1:FMR1³
C2:FMR1³
p(d)
Interfly Distance (cm)
H
C1:WT
C2:FMR1³
p(d)
Interfly Distance, d(cm)

Figure 2 (See previous page). Pair-wise Fragile X *dfmr1*^{3} mutant social behavior. (A and B) Joint spatial distributions *p(x,y)* with a *dfmr1*^{3} mutant in each chamber or with wild-type fly in C1 and a *dfmr1*^{3} fly in C2 (B). (C and D) Marginal probability distribution *p(y)* of *dfmr1*^{3} mutant (C) and wild-type (D) in C1. (E and F) Marginal distribution of *dfmr1*^{3} mutant in C2 when exposed to wild-type (E) or mutant *dfmr1*^{3} (f) in C1. (G and H) Interfly distance distribution *p(d)* when *dfmr1*^{3} are exposed to *dfmr1*^{3} (G) or wild-type flies (H). N = 10 pairs for *dfmr1*^{3}-*dfmr1*^{3} and N = 10 pairs for *dfmr1*^{3}-wild-type.

Figure 3 (See next page). Modification of *dfmr1*^{B55} mutant social behavior. (A and B) Joint spatial distributions *p(x,y)* with a *dfmr1*^{B55} mutant in each chamber or with wild-type fly in C1 and a *dfmr1*^{B55} fly in C2 (B). (C and D) Marginal probability distribution *p(y)* of *dfmr1*^{B55} mutant (C) and wild-type (D) in C1. (E and F) Marginal distribution of *dfmr1*^{B55} mutant in C2 when exposed to wild-type (E) or mutant *dfmr1*^{B55} (F) in C1. (G and H) Interfly distance distribution *p(d)* when *dfmr1*^{B55} are exposed to *dfmr1*^{B55} (G) or wild-type flies (H). N = 9 pairs for *dfmr1*^{B55}-*dfmr1*^{B55} and N = 10 pairs for *dfmr1*^{B55}-wild-type.

resulting behavioral phenotypes. Although a reductionist strategy, development of an assay focusing on a specific, restricted feature of the disease (or endophenotype) in an animal model will be crucial in understanding the fundamental genetic mechanisms underlying the disease. Along these lines, we developed a simple single fly assay to study social interaction, which can be further adopted for high-throughput studies, thus taking full advantage of Drosophila genetics. Here, we used two Drosophila Fragile X mutants, which had previously been shown to overlap with human in terms of neuropathology, circadian rhythm defects and memory dysfunction as an example of this strategy. Previous studies focusing on courtship behaviors had already revealed a of lack of sustained interest in social interaction for males courting female and immature males.[29] Our results suggest that *dfmr1*^{B55} and *dfmr1*^{3} mutants were less likely to interact than wild-type flies. In addition, using homo and heterogenotypic combinations we showed that the defect in *dfmr1*^{B55} mutant may be the result of a failure to generate motor gestures that lead to initiation of social interaction. Interestingly, an increasing bulk of clinical evidence supports a role for motor dyspraxia in explaining autistic behavior. Nevertheless, future investigation needs to be performed to verify this result and dissect, for example, which subsets of neurons mediate this behavior or whether orthologs of other ASD candidate genes have similar effects on social behavior. The aim of this paper was to present a plausible strategy by which to do this, and along with a growing compendium of studies in Drosophila, the data presented here show the possibilities of examining "autistic" behaviors in simple animal models to dissect the sensory and motor components of the disease, thus gaining insight into this complex disease phenotype.

Summary Methods

Flies were raised and disposed of as per Cold Spring Harbor Laboratory regulations under the supervision of Dr. Tim Tully. The *dfmr1*^{B55} mutant[30] was obtained from the Broadie laboratory (Vanderbilt U). The *dfmr1*^{3} mutant was obtained from Dr. Tom Jongens (University of Pennsylvania). Flies were outcrossed for six generations to *w*^{1118}*(isoCJ1)* control flies to equilibrate genetic backgrounds. Drosophila virgin female were isolated 1/vial for three days and then used for behavior. The testing chamber was a cylindrical cast acrylic vial with two adjacent, but physically separate, chambers. The interior of chamber one was 2 cm long and 1.5 cm in diameter; the interior of chamber 2 was 7 cm long and 1.5 cm in diameter. To acquire videos, we used a Basler A622f progressive scan monochrome camera with a custom video acquisition program developed in the Mitra lab. Tracking of fly trajectories was performed using a modification of FTrack, a Matlab toolbox for trajectory tracking and analysis.[38]

Methods

Drosophila stocks. Flies were raised and disposed of as per Cold Spring Harbor Laboratory regulations under the supervision of Dr. Tim Tully. The *dfmr1*^{B55} mutant[30] was obtained from the Dr. Kendal Broadie laboratory (Vanderbilt University). It was previously generated from an imprecise excision of EP(3)3422 P element inserted within *dfmr1* by Inoue for the study of circadian rhythm and other behavior.[30] The deletion removes 2.5 Kb of genomic DNA including exons 2, 3 and 4. The *dfmr1*^{3} allele was generated from the imprecise excision of EP(3)3517 P Element by Dockendorff.[29] The *dfmr1*^{3} mutant was obtained from Dr. Tom Jongens (University of Pennsylvania). Flies were outcrossed for six generations to *w*^{1118}*(isoCJ1)* control flies to equilibrate genetic backgrounds.

Behavioral analysis. Drosophila female were collected within 3 hours of birth and placed in regular food vials. One fly was placed in each vial. Flies were stored at 23°C and tested at three days of life. Flies were placed in the environmental room for 1 hour prior to testing.

The assay was always performed in a controlled environment room at 25°C with 30% humidity with light. The experiments were also always performed at the same time (18–21 h) on flies maintained on 12:12 LD cycles. It should be noted, however, that the *dfmr1*^{3}-*dfmr1*^{3} and *dfmr1*^{3}-WT experiments were performed in a different lab (U. Alberta vs. CSHL) on a different date, but in an identical environment room. The chambers were cleaned between each genotype with 50% liquinox.

The testing chamber was a custom-made cylindrical cast acrylic vial with two chambers separated by a plastic mesh. The interior of chamber 1 was 2 cm long and 1.5 cm in diameter; the interior of chamber 2 was 7 cm long and 1.5 cm in diameter. The two chambers were divided by a thin plastic mesh mounted on a circular ring fitted tightly inside the cast acrylic vial. The fly in the smaller chamber was always loaded laterally first, followed by the second fly (in the larger chamber). Light was present above the testing chamber and the camera. To acquire videos, we used a Basler A622f progressive scan monochrome camera with a custom video acquisition program developed by one of the authors (DV). Tracking of fly trajectories was performed blind to genotype using a modification of FTrack, a Matlab toolbox for trajectory tracking and analysis.[38] Each video captured 9,000

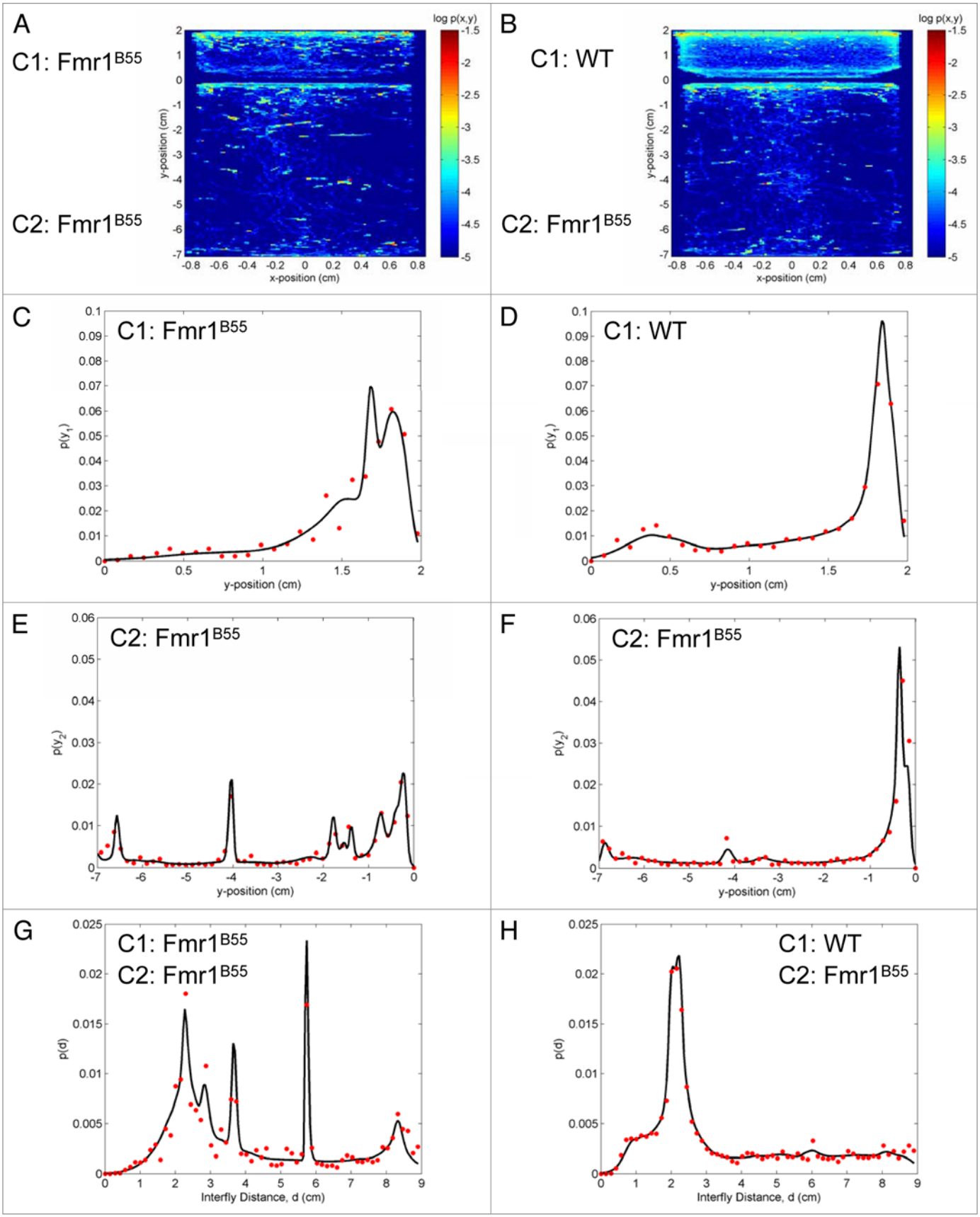

Figure 3 (See previous page for legend).

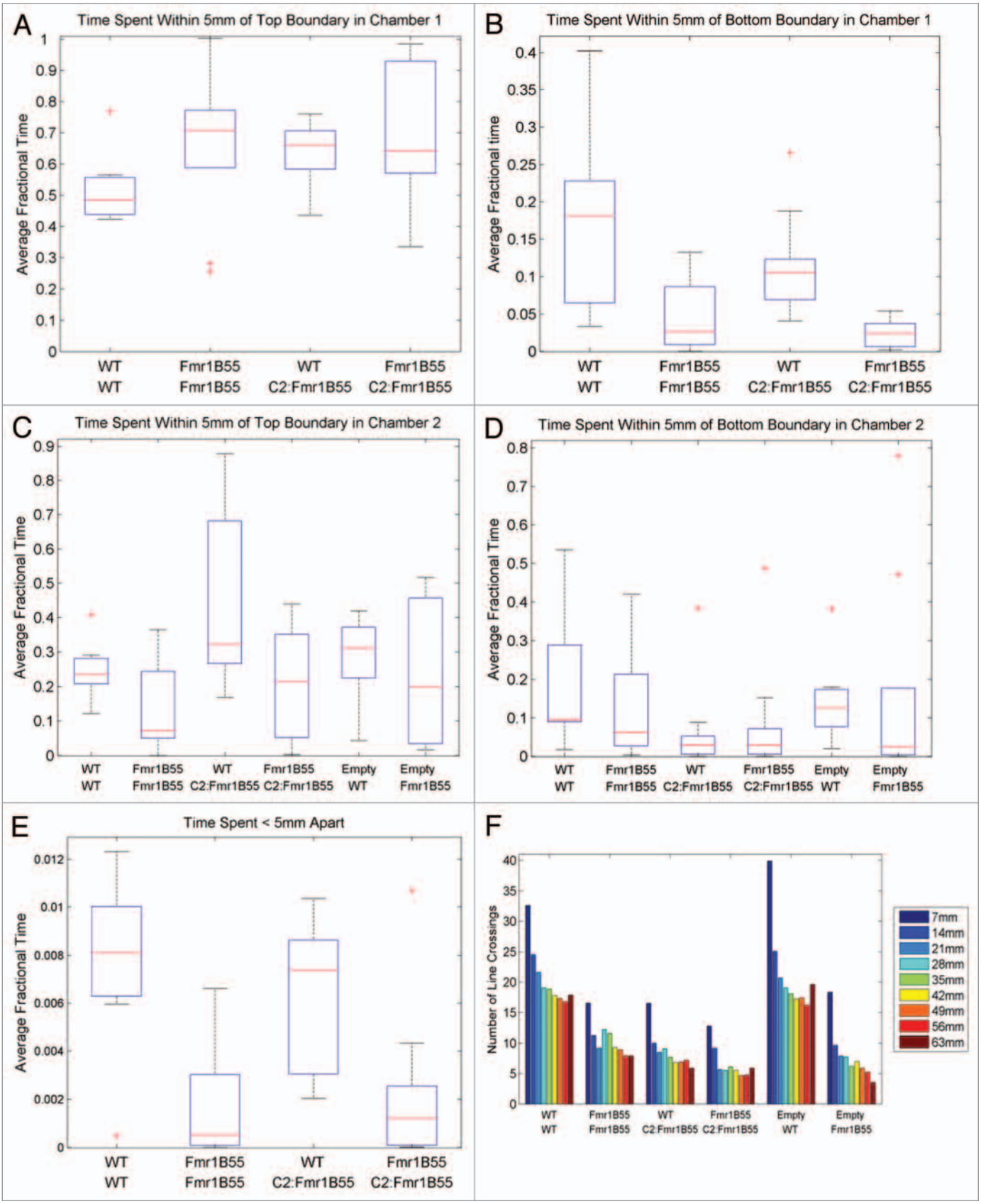

Figure 4 (See next page for legend).

Figure 4 (See previous page). Fragile X mutant behavior modification is influenced by behavior of both flies involved. (A) Tukey box-plots of the average fractional time spent by flies in chamber 1 at the top of the chamber for each genotype pair. In this plot, and all subsequent boxplots, the + symbols are outlier points, defined as having values greater than 1.5 times the interquartile range from the ends of the box. The *dfmr1*^{B55}-*dfmr1*^{B55} and *dfmr1*^{B55}-C2: *dfmr1*^{B55} groups are identical genotype pairs, but were tested on separate days and are presented as separate groups in the plots. *dfmr1*^{B55}/*dfmr1*^{B55} correspond to the data in Figure 1, whereas *dfmr1*^{B55}/C2: *dfmr1*^{B55} correspond to the data in Figure 3. (B) Average fractional time spent by fly in chamber 1 at the bottom of the chamber for each genotype pair. (C) Average fractional time spent by fly in chamber 2 at top of the chamber for each genotype pair. (D) Average fractional time spent by fly in chamber 2 at the bottom of the chamber for each genotype pair. (E) Average fractional time spent at an interfly distance less than 5 mm for each genotype pair. (F) Number of crossings of an imaginary line at various distances from the wall dividing chamber 1 and chamber 2 for each genotype. Statistical comparison summary for (A–E) is shown in Table 1.

Table 1. ANOVA comparisons for Figure 4

Figure	ANOVA p value	ANOVA F statistic	Permutation ANOVA p value	Pairs sig. @ ≤ 0.05	Pairs sig. @ ≤ 0.01
4A	0.288	1.31	0.278	-	-
4B	0.0014	6.64	0.0014	A–B A–D C–D	A–D
4C	0.0117	3.33	0.0114	B–C	B–C
4D	0.669	0.642	0.667	-	-
4E	6.6×10^{-4}	7.48	5×10^{-4}	A–B A–D C–B C–D	A–B

A, WT-WT; B, *dfmr1*^{B55}-*dfmr1*^{B55}; C, WT-C2: *dfmr1*^{B55}; D, *dfmr1*^{B55}-C2: *dfmr1*^{B55}. Pairwise significance assessed with a post-hoc Tukey-Kramer test at the values specified in the Table.

frames at 40 frames per second, for a total of 3.75 minutes (the *dfmr1*^{3}-*dfmr1*^{3} and *dfmr1*^{3}-WT experiments were acquired at 29 frames per second for the same length of time). This time was arbitrarily chosen, as it appeared to be long enough so as to sufficiently characterize the behavior. Recording began 5 seconds after the introduction of the second fly.

For analysis, all trajectories were normalized to a common coordinate space. This was performed by manually selecting several landmarks on the vial in each video and then rotating the raw coordinates onto a space in which the vial boundary defined the x-axis (y = 0 cm). This procedure aligns the coordinates to within an error of ~0.5 mm (2 pixels).

Probability distribution histograms (red dots on probability plots) were calculated as described in ref. 41. Estimates of the probability density (black lines) were obtained using Locfit, a local likelihood and regression model.[43] Model parameters were chosen visually by trial-and-error to obtain reasonably smooth estimates of the underlying histograms. In cases such as **Figures 1F, H and 3E, G** the estimates were intentionally undersmoothed in order to best capture the large likelihood of stopping for the Fmr1 mutant flies—a behavior not exhibited by the wild-type flies. Smoothing away these stops causes an unrepresentative description of fly locomotor behavior. In each case, distributions are averages of N flies (N values reported in figure captions). All subsequent quantification and statistics were calculated using the density estimates. Both FTrack and Locfit are publically available as part of Chronux, a Matlab toolbox for the analysis of neural data (www.chronux.org/).

Statistical analysis. For the statistical analysis of time spent near boundaries and interfly distances, comparison of multiple groups was done in two ways. First, it was not immediately obvious that the assumptions of normality and equal variance would hold for this data. This would preclude a proper analysis of variance (ANOVA). Due to the small number of samples, these assumptions were difficult to check; therefore, a permutation-style ANOVA test was performed in each case. For this test, the ANOVA F-statistic was calculated for 10^4 permutations of the class labels. The empirical distributions obtained in this method and the p-values were nearly identical to that expected from traditional one-way ANOVA (an *F*-distribution with the proper degrees of freedom). Therefore, it was assumed that the assumptions of ANOVA were not violated and a multiple comparisons test was done using the Tukey-Kramer method to assess pairwise differences based on the one-way ANOVA results. In the cases where only two groups were compared (**Suppl. Fig. 1**), Wilcoxon rank sum tests were used to assess significance. Because the *dfmr1*^{3}-*dfmr1*^{3} and *dfmr1*^{3}-WT experiments were performed in a different lab on a different date, no statistics were done to quantitatively compare these data to the other groups, and comparison to those groups should thus be considered only qualitatively.

Acknowledgements

We would like to thank Mr. Robert Eifert at Cold Spring Harbor for manufacturing the testing chamber to our specification. Financial support for this project was from the William R. Miller Fellowship and CCHCSP career development award to Francois V. Bolduc. and Dart Neuroscience LLC and NIH to Tim Tully. Partha P. Mitra was supported by the Crick-Clay Professorship.

Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/BolducFLY4-3-Sup.pdf

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