Neuron, Vol. 34, 973–984, June 13, 2002, Copyright ©2002 by Cell Press

Drosophila Lacking *dfmr1* Activity Show Defects in Circadian Output and Fail to Maintain Courtship Interest

Thomas C. Dockendorff,[1,8] Henry S. Su,[2,7] Sean M.J. McBride,[3,7,9] Zhaohai Yang,[4] Catherine H. Choi,[3] Kathleen K. Siwicki,[5] Amita Sehgal,[2,4] and Thomas A. Jongens[1,6]

[1]Department of Genetics
[2]Department of Neuroscience
[3]Department of Physiology
[4]HHMI
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania 19104
[5]Department of Biology
Swarthmore College
Swarthmore, Pennsylvania 19081

Summary

Fragile X mental retardation is a prominent genetic disorder caused by the lack of the *FMR1* gene product, a known RNA binding protein. Specific physiologic pathways regulated by *FMR1* function have yet to be identified. Adult *dfmr1* (also called *dfxr*) mutant flies display arrhythmic circadian activity and have erratic patterns of locomotor activity, whereas overexpression of dFMR1 leads to a lengthened period. *dfmr1* mutant males also display reduced courtship activity which appears to result from their inability to maintain courtship interest. Molecular analysis fails to reveal any defects in the expression of clock components; however, the CREB output is affected. Morphological analysis of neurons required for normal circadian behavior reveals subtle abnormalities, suggesting that defects in axonal pathfinding or synapse formation may cause the observed behavioral defects.

Introduction

Fragile X syndrome is caused by transcriptional silencing or loss of function of the *FMR1* gene (de Vries et al., 1998; Jin and Warren, 2000). The frequency with which this disorder appears (1/5000 births), along with its global distribution, make it one of the most prominent human genetic disorders. In addition to mental retardation, other clinically relevant behavioral symptoms include hyperactivity, attention deficit disorder, autism, sleep disorders, and memory deficits (Reiss et al., 1995; Hagerman, 1996; de Vries et al., 1998; Gould et al., 2000; Jin and Warren, 2000). Associated physical abnormalities include maxillofacial abnormalities, macroorchidism in male patients, and alterations in dendritic spine morphology (de Vries et al., 1998; Jin and Warren, 2000). The diverse phenotypes suggest that FMR1 protein acts in several physiological processes. Mice homozygous for a disruption of their *FMR1* homolog share many of the above phenotypes (Dutch-Belgian Fragile X Consortium, 1994; Comery et al., 1997).

Biochemical studies have established that the *FMR1* gene and its relatives *(FXR1, FXR2)* encode RNA binding proteins that copurify with each other, as well as with 60S ribosome subunits (Siomi et al., 1993, 1995, 1996; Zhang et al., 1995). The RNA binding capacity of FMR1 protein is selective and is mediated by two KH domains in the protein (Ashley et al., 1993; Siomi et al., 1994). FMR/FXR proteins are widely expressed in developing and adult tissues of mammals, but are at elevated levels in neurons (Bakker et al., 2000). Immuno-gold labeling has localized FMR1 protein to dendrites and dendritic spines of neurons (Feng et al., 1997). *FMR1* message has been found in synaptoneurosomes, and this message is translated in response to neurotransmitter action (Weiler et al., 1997). The observation that FMR1 protein binds specific RNAs, is associated with ribosomes, and is present at synapses has prompted speculation that FMR1 protein may play a role in synaptic maturation by regulating the ability of certain transcripts to serve as templates for translation (Weiler et al., 1997). Nonetheless, specific RNA substrates for FMR/FXR proteins and the physiological consequences and significance of such interactions remain largely unknown.

We have previously reported that *Drosophila* has a single gene (*dfmr1*, or also called *dfxr*) whose product shares sequence identity and biochemical properties with the FMR/FXR protein family (Wan et al., 2000). In this study, we report the identification and characterization of *dfmr1* loss-of-function alleles. Analysis of these alleles has revealed that the *dfmr1* gene is required for normal circadian behavior and courtship activity. The mutants are arrhythmic and have an erratic pattern of locomotor activity with periods of hyperactivity. Molecular analysis of the clock genes and of a known output of the clock has revealed that *dfmr1* activity is required downstream of the clock, and is required for normal cAMP response element binding protein (CREB) activity which is modulated by the clock (Belvin et al., 1999). Analysis of courtship behavior in the *dfmr1* mutants has revealed a defect in overall courtship activity. The mutants initiate normal numbers of courtship attempts, but are unable to maintain courtship interest. Analyses of adult brain structure reveal no apparent defects in mutant mushroom bodies, but abnormal axon branching and an overgrowth of terminal arborizations in individual neurons required for circadian behavior. These results are similar to defects observed at the neuromuscular junction (NMJ) (Zhang et al., 2001) and suggest that the observed behavioral defects may be due to growth and maturation defects of neurons. However, since the circadian and associated locomotor defects are not rescued by the introduction of a *futsch* loss-of-function mutation into the *dfmr1* mutant background, these behavioral defects are not due to the same synaptic defects caused

[6]Correspondence: jongens@mail.med.upenn.edu
[7]These authors contributed equally to this work.
[8]Present address: Department of Zoology, Miami University, Oxford, Ohio 45056.
[9]Present address: Section of Molecular Cardiology, Departments of Medicine and Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461.

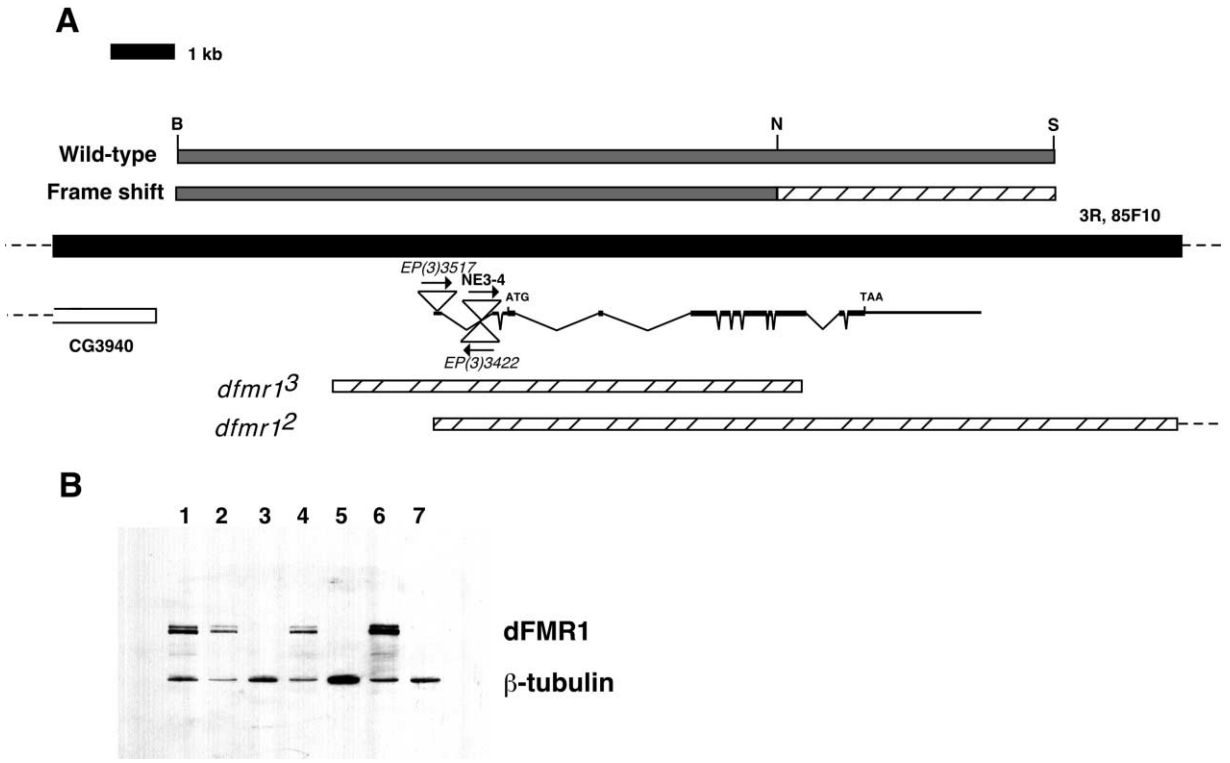

Figure 1. Molecular Characterization of *dfmr1* Alleles Obtained from a P Element Imprecise Excision Screen

(A) The *dfmr1* locus maps to 85F10 of the *D. melanogaster* cytogenetic map. The transcribed region of *dfmr1* encompasses about 8.5 kb. An imprecise excision screen was conducted using a P element stock with an insertion that maps to the *dfmr1* gene *[EP(3)3517]*. Two alleles were identified where most or all of the *dfmr1* open reading frame was removed by imprecise excision of the P element. Hatched boxes delineate the extent of deleted DNA. The distal breakpoint for $dfmr1^2$ has not yet been mapped. Two genomic rescue fragments have been cloned and introduced into flies via P element transformation. Both are cloned as 14 kb BamHI/StuI fragments. One encodes a wild-type *dfmr1* gene and the other has a frameshift introduced at an NcoI site to disrupt *dfmr1* translation. CG3940 is a predicted gene encoding carbonic anhydrase (Berkeley *Drosophila* Genome Project). Only the upstream regulatory sequence of this gene is present in the genomic rescue fragments. *EP(3)3422* and *NE3-4* are two additional EP P element insertions in the *dfmr1* gene. The arrows associated with each P element insertion indicate the orientation of the element with respect to its ability to direct overexpression. (B) Western blotting of protein extracts from adult flies. Lane 1: w^{1118}; Lane 2: $dfmr1^2$ heterozygote; Lane 3: $dfmr1^2$ homozygote; Lane 4: $dfmr1^3$ heterozygote; Lane 5: $dfmr1^3$ homozygote; Lane 6: $dfmr1^3$ homozygote with one copy of wild-type rescue; Lane 7: $dfmr1^3$ homozygote with one copy of FS rescue. Western blotting was done using the anti-dFMR1 and anti-β-tubulin antibodies (Wan et al., 2000).

by FUTSCH misregulation at the NMJ and eye (Zhang et al., 2001). The similarities in the biochemical properties of *dfmr1* and FMR1 (Wan et al., 2000) and their loss-of-function phenotypes suggest that these two proteins have conserved function in similar behavioral processes.

Results

Isolation of *dfmr1* Null Alleles

A P element insertion *(P[EP]3517)* was found to map within the 5′ UTR of the *dfmr1* locus (Figure 1A). Flies homozygous for this insertion are viable and have no obvious visible phenotypes. A screen to obtain stocks with deletions of *dfmr1* via imprecise excision of the P element was conducted. No excision lines were identified that were lethal in *trans* to a deficiency which uncovers the *dfmr1* locus, *Df(3R)by62*, suggesting that loss of *dfmr1* is not a lethal event. Thus, from each excision line, pupae or adults that were homozygous for the excision chromosome were analyzed by Western blotting with an anti-dFMR1 antibody to identify lines lacking dFMR1 expression. Two such stocks were identified and the extent of the deletions within the *dfmr1* locus were delimited by Southern hybridizations (Figure 1A). Both $dfmr1^2$ and $dfmr1^3$ are molecular null alleles. Flies homozygous for either allele are viable and appear to be morphologically normal in external appearance. $dfmr1^3$ was used for further analyses given that it does not appear to disrupt the function of any adjacent genes. Two genomic rescue fragments were constructed and transformed to flies. Both are 14 kb fragments that span the *dfmr1* transcriptional unit (Figure 1A). One encodes a wild-type *dfmr1* gene (WT rescue) and is used to demonstrate that phenotypes observed in the mutants are caused by the lesion generated by excision of the P element. The second rescue fragment (FS rescue) has a frameshift mutation introduced in the *dfmr1* open reading frame and is used to show that the function of the *dfmr1* ORF is responsible for any phenotypic rescue.

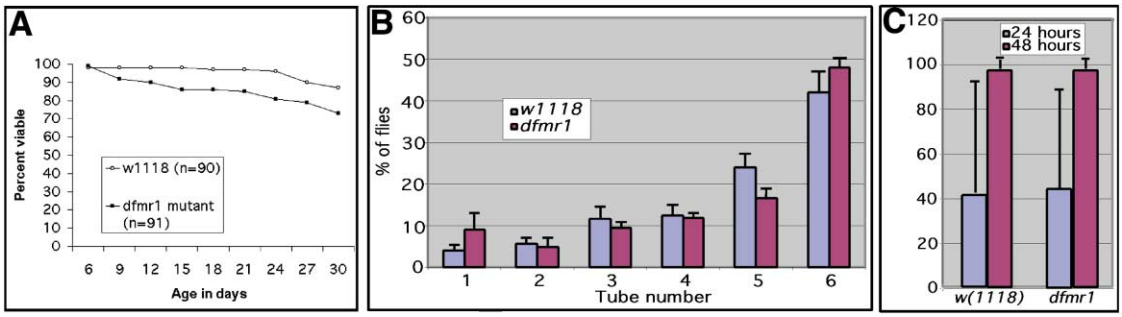

Figure 2. Analysis of the Lifespan, Phototactic, and Chemotactic Capabilities of the *dfmr1*^{3} Allele

(A) Viability curve of *w*^{1118} and *dfmr1*^{3} flies. Flies of each genotype were placed in fresh food vials (20 per vial/5 vials each genotype) and monitored for viability every 3 days for 30 days. (B) Countercurrent phototactic results using a six trial countercurrent apparatus with 20 s trials (Balinger and Benzer, 1988). Results show the average percentage of flies in each tube obtained in three separate trials of 50 male flies of *w*^{1118} or *dfmr1*^{3} flies. Error bars indicate SEM. (C) Chemoattraction results obtained from a trap assay (Orgad et al., 2000) using yeast paste as an attractant. Percentage of flies trapped are indicated for 24 and 48 hr intervals and error bars indicate SEM.

These rescue fragments were then crossed into the *dfmr1* mutant background to create isogenic stocks that differ only in their ability to express wild-type dFMR1 protein (Figure 1B).

dfmr1 Mutant Flies Are Arrhythmic

Flies homozygous for a *dfmr1* deletion were found at expected Mendelian ratios and have near normal fitness as determined in a standard viability assay (Figure 2A). Analysis of simple behaviors revealed that the mutant flies have normal phototactic and chemotactic abilities in standard assays (Figures 2B and 2C). However, in collecting virgin flies for various studies, we observed that the majority of *dfmr1* mutant flies failed to eclose soon after daylight, suggesting a defect in their circadian system (Konopka and Benzer, 1971; Sehgal et al., 1994). To examine this possibility, we examined the eclosion profile of a population of *dfmr1*mutant flies. Control (*w*^{1118}) and *dfmr1* mutant larvae were entrained to a 12 hr light: 12 hr dark (LD12:12) cycle for at least 5 days, then pupae were collected, loaded into eclosion monitors, and placed in constant darkness for 6 days. The eclosion monitors determine the number of flies eclosing relative to time of day. Analysis of the eclosion profiles revealed that the majority of the control flies eclosed in the early morning hours ("the circadian gate") with a period of 23.5 hr (n = 501, FFT = 0.184; Figure 3A). The *dfmr1* mutant flies, on the other hand, did not show a tendency to eclose in the gate and eclosed with a broadened delayed peak and reduced amplitude of rhythm (n = 298, FFT = 0.068). Thus the *dfmr1* mutant flies display reduced strength and altered phase with respect to time of eclosion.

Since the *dfmr1* mutant flies displayed a circadian defect with respect to eclosion, we investigated the possibility that they might also display such defects in locomotor activity. We examined the rest:activity rhythms of *dfmr1* mutant flies by entraining both mutant and control flies to an LD 12:12 cycle for 5 days prior to eclosion, aged them 2 additional days in identical conditions, then placed them in activity monitors that quantify the frequency with which a fly crosses an infrared beam that is passed through the chamber (Yang and Sehgal, 2001). The activity of the flies was monitored for 9 days in constant darkness, which allows for a determination of free running rest:activity rhythms. Examination of actograms and periodogram analysis showed that while control *w*^{1118} flies have a normal 23–24 hr circadian cycle, most *dfmr1* mutants are arrhythmic (Table 1A and Figure 3B). The wild-type *dfmr1* rescue fragment (WT rescue) restores rhythmicity to mutants, whereas the rescue fragment containing the frameshift mutation (FS rescue) does not. Representative actograms show that *w*^{1118} flies and *dfmr1* mutant flies with one copy of WT rescue (*dfmr1*; WT rescue/+) have a circadian pattern of about 12–14 hr of activity followed by about 10–12 hr of rest (Figure 3B). In contrast, *dfmr1* mutants and *dfmr1*; FS rescue/+ mutants have erratic activity patterns that are characterized by occasional brief bouts of relatively high activity (Figure 3B).

The erratic locomotor activity patterns of the *dfmr1* mutants is not necessarily a consequence of the flies simply being arrhythmic since null mutants for *timeless* (*tim*) or *period* (*per*) are also arrhythmic, but maintain a relatively constant level of activity under similar experimental conditions (Konopka and Benzer, 1971; Sehgal et al., 1994). To determine if this erratic behavior could be due to locomotor defects, we examined the overall activity of the *dfmr1* mutant flies. Quantification of total activity over the course of 9 days in constant darkness fails to reveal any significant overall difference between control and *dfmr1* mutant flies (764 ± 264 counts per day for *w*^{1118} and 884 ± 541 counts per day for *dfmr1,* p = 0.32). The similarity in batch activity between mutant and control flies, as well as the occasional bouts of high activity observed in the *dfmr1* mutants, suggest that motor function and locomotor abilities are not impaired in these animals and thus not the cause of the arrhythmic phenotype.

Mutants that lack circadian function are unable to sustain rhythmicity in constant darkness, but can be driven to display activity rhythms in the presence of light:dark cycles. To determine if this was the case for *dfmr1* mutants, we exposed mutant and control flies to

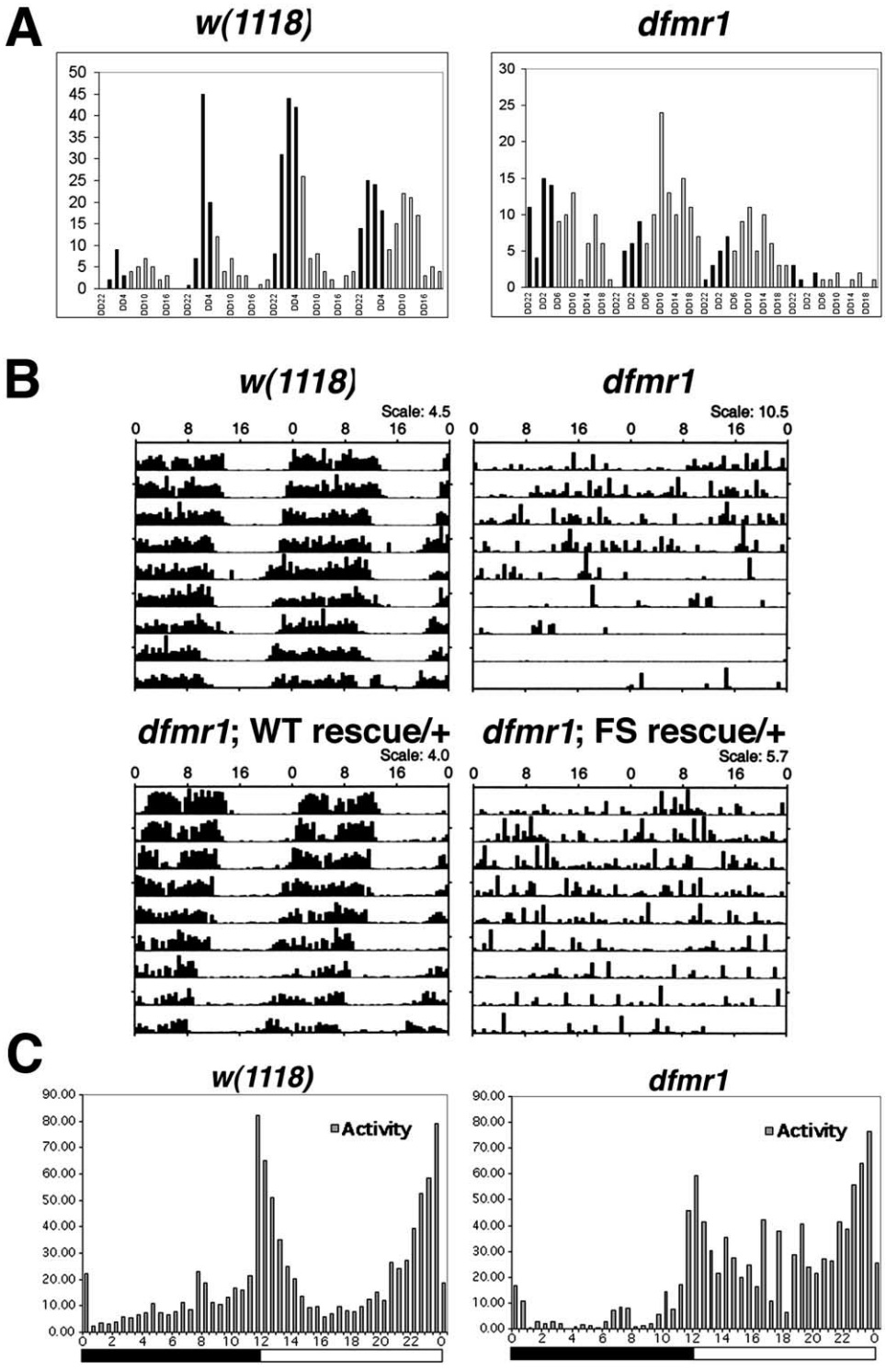

Figure 3. Rest Activity of *dfmr1* Mutant and Control Flies

(A) Eclosion timing of w^{1118} (n = 501) and *dfmr1* (n = 298) flies in DD. Pupae from larvae that had been entrained in LD for five days were placed in eclosion monitors in constant darkness for several days. The number of flies eclosing relative to the time of day were plotted. The dark bars indicate the "circadian gate," the time period each day when most wild-type flies eclose. Although the majority of w^{1118} flies eclosed during the gate, the *dfmr1* mutant flies eclosed over an extended period of time. (B) Representative actograms from flies of the genotypes indicated above each actogram. Flies that had been entrained to a light:dark cycle were placed in the activity monitors in constant darkness and their activity was recorded for 9 days. Flies expressing *dfmr1* have rhythmic patterns of rest and activity, while flies lacking wild-type *dfmr1* have erratically timed short bouts of relatively high activity. (C) Locomotor activity of w^{1118} (n = 19) and *dfmr1* (n = 14) flies averaged over 9 days in LD. Mean activity levels are reported across flies for each time point (zeitgeber time). The black bars across the bottom of each plot indicate the 12 hr of darkness and the open bars indicate the hours of light. The activity profiles of both w^{1118} and *dfmr1* were similar

Table 1. Circadian Phenotypes in Constant Darkness

Genotype	n	% Rhythmic	Average Period[a]	FFT[b]
(A)				
w^{1118}	27	100	23.4 ± 0.4	0.100 ± 0.044
dfmr1^{3}	21	14[c]	23.3 ± 0.3	0.014 ± 0.015
dfmr1^{3}; WT rescue/+	22	100	23.5 ± 0.5	0.099 ± 0.046
dfmr1^{3}; FS rescue/+	21	14[c]	24.2 ± 0.3	0.014 ± 0.014
(B)				
TM3, Sb/NE3-4	32	100	23.1 ± 0.1	0.127 ± 0.044
tim-Gal4/+; *NE3-4*/+	43	49	25.9 ± 0.1	0.129 ± 0.067
EP3517/+	18	100	23.8 ± 0.1	0.213 ± 0.064
tim-Gal4/+; *EP3517*/+	27	100	25.4 ± 0.1	0.136 ± 0.043
tim-Gal4/+; *EP3422*/+	19	100	23.8 ± 0.1	0.122 ± 0.045

Circadian and activity studies were done using monitors from Trikinetics and data were analyzed using the ClockLab software from MatLab as described in Yang and Sehgal (2001).

[a] Average period and standard deviation of flies judged to be rhythmic. Rhythmicity was based upon actogram and periodogram and on FFT value.

[b] Fast Fourier Transforms are a measure of the strength of the rhythmicity and were averaged for all flies tested of a given genotype.

[c] These flies were judged to be weakly rhythmic, with an average FFT value of 0.043 ± 0.010 for the *dfmr1* flies and a value of 0.042 ± 0.006 for *dfmr1*; FS rescue/+ mutants.

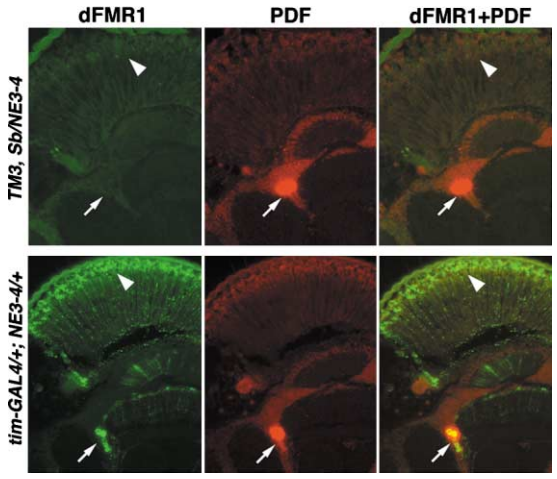

Figure 4. Overexpression of dFMR1 Protein in the Lateral Neurons as a Result of Combining the *NE 3-4* EP Insertion with the *tim-GAL4* Driver

Head sections of adults containing the *NE3-4* insertion alone (*TM3, Sb/NE3-4*) or in a genetic background containing a *tim*-GAL4 driver (*tim-GAL4/+;NE3-4*) were stained with anti-dFMR1(green) and anti-PDF (red) antibodies. The anti-PDF staining indicates the position of the lateral neurons (arrow). The position of photoreceptor nuclei is indicated with arrowheads. Elevated levels of dFMR1 protein are observed in the lateral neurons and photoreceptors, as well as other cells in the brain and optic lobe when the *NE3-4* insertion is in a genetic background containing the *tim-GAL4* driver.

an LD 12:12 cycle for 5 days prior to eclosion, aged them 2 additional days in identical conditions, then placed them in activity monitors as described above. We found that most of the *dfmr1* mutant flies were rhythmic and displayed a very similar activity profile to that of *w*^{1118} (Figure 3C). Both *w*^{1118} and *dfmr1* mutants displayed heightened levels of activity in anticipation of lights turning on and off. The anticipatory activity is indicative of underlying clock function, which was further supported by analysis of clock proteins in *dfmr1* mutants (discussed below).

Overexpression of dFMR1 Leads to a Lengthened Period

The specific requirement of *dfmr1* for normal circadian behavior is reinforced by additional evidence that overexpression of dFMR1 protein also leads to altered circadian behavior. In an independent screen for genes whose overexpression leads to a circadian defect, we identified the *dfmr1* gene. Using the EP P element system (Rørth, 1996), novel insertion lines were generated and crossed into a genetic background containing a *tim-GAL4* driver, then assayed for changes in circadian behavior. NE 3-4 was identified as an insertion that gives rise to arrhythmicity and a lengthened period in a *tim-GAL4*-dependent manner (Table 1B). Molecular characterization of this line revealed that the EP P element insert is in the first intron of the *dfmr1* gene in an orientation that directs the overexpression of *dfmr1* in cells expressing GAL4 (Figure 1A). In fact, when the NE 3-4 line was crossed to a *tim-GAL4* line, the progeny were observed to overexpress dFMR1 protein in cells that express *tim*, including the lateral neurons, the site of the circadian clock, as well as other neurons in the brain and optic lobe (Figure 4). Since two other EP P element insertions near the 5′ end of the *dfmr1* gene were previously identified by the Berkeley *Drosophila* Genome Project (BDGP), we also tested whether these lines would lead to altered circadian patterns when introduced into background containing *tim-GAL4*. We found that the EP3517 insertion also resulted in a lengthened period, but the EP3422 insertion, which is in the opposite orientation of NE3-4 and EP3517, did not (Figure 1A; Table 1B). Thus not only is *dfmr1* activity required for normal circadian behavior, but elevation in dFMR1 protein levels lengthens the period.

The Clock Genes Cycle Normally in *dfmr1* Mutants

The circadian clock is regulated by an autoregulatory feedback loop in which the transcription of *timeless* (*tim*) and *period* (*per*) mRNA is repressed by an increase in PER and TIM protein concentration in the nucleus, leading to molecular oscillations of *tim* and *per* mRNA and resultant proteins (reviewed by Dunlap, 1999; Williams and Sehgal, 2001). To determine if the loss of *dfmr1*

in that both displayed two daily peaks of activity, anticipatory behavior at the light and dark transitions, and a steady increase in activity during the later half of the day. Some of the *dfmr1* were arrhythmic (FFT < 0.040) in LD as 26% (5/19) lacked significant 24 hr rhythms. The histograms shown are derived only from rhythmic flies.

activity leads to defects in the molecular oscillations of the clock components, we first examined the levels of *tim* and *per* mRNA by performing RNase protection assays. In this assay, the circadian oscillations of *tim* and *per* mRNA in the *dfmr1* mutant background were indistinguishable from those of the w^{1118} control flies (Figure 5A).

Next, we examined the molecular oscillations of PER and TIM proteins in the lateral neurons of the *dfmr1* mutant brains by whole-mount immunostaining. No difference in the cycling of the two proteins was observed between the *dfmr1* mutants and control flies. PER protein staining was strongest at CT 2, low or undetectable at CT 8 and CT 14, and detectable again at CT 20 in both the mutant and control brains (Figure 5B), suggesting that PER protein oscillations are normal in the *dfmr1* mutant flies. Examination of TIM protein levels in similarly prepared samples also failed to reveal any differences between *dfmr1* mutant and control flies (not shown). Taken together, our results suggest that the loss of *dfmr1* activity does not affect circadian clock function at the molecular level, but affects the pathway that translates signals from the clock and produces rhythmic rest:activity. Thus the mutation must have a strong effect on a pathway that is downstream from the endogenous clock signal.

A Known Output of the Clock Is Affected by Loss of *dfmr1* Activity

One known clock-controlled gene in *Drosophila* is the cAMP response element binding protein (CREB) (Belvin et al., 1999). To determine if the circadian oscillation of this protein is affected in the *dfmr1* mutant flies, we monitored control and *dfmr1* mutant flies carrying the CRE-luciferase (CRE-luc) reporter gene in a luminometer continuously in DD for up to 4 days. Although cycling of the CRE-luc reporter is detected in the *dfmr1* mutant background, the amplitude of the oscillations is clearly reduced compared to the oscillations in the w^{1118} control background (Figure 5C). This result indicates that *dfmr1* affects a known molecular output of the clock.

Another known output from the clock is the neuropeptide pigment dispersing factor (PDF) (Park et al., 2000) which is specifically expressed in certain ventral lateral neurons (LN_v) that control circadian rhythms (Helfrich-Forster, 1995, 1998). The small lateral neurons (s-LN_v) project into the dorsal protocerebrum, and daily rhythms in PDF levels have been observed in their terminal arborizations (Park et al., 2000). We examined this cycling in brains of mutant and control flies that had been entrained in LD 12:12 for 5 days, by immunostaining them with anti-PDF (Helfrich-Forster and Homberg, 1993). We observed normal cycling of PDF levels in the termini of the small lateral neurons in the *dfmr1* mutant brains (not shown). Thus this output of the clock is not affected, at the normal site of its release, providing further evidence for normal central clock functioning in the *dfmr1* mutant flies.

dfmr1 Mutants Display a Defect in Courtship Behavior

Several mutations that disrupt output in the circadian system in *Drosophila* are known to affect multiple complex behaviors. For example, PKA, CREB, and NF1 mutants exhibit both learning and clock output phenotypes (Skoulakis et al., 1993; Bourtchuladze et al., 1994; Yin et al., 1994; Majercak et al., 1997; Guo et al., 2000; Williams et al., 2001). Moreover, the multiple cognitive deficits and psychiatric diagnoses of fragile X patients led us to expect that *dfmr1* might influence multiple complex behaviors in *Drosophila*. To this end, we evaluated *dfmr1* mutant males in a courtship assay. Courtship in *Drosophila* involves a complex series of sensory and behavioral interactions between male and female. As such, it provides a sensitive assay for behavioral abnormalities. Male-specific behaviors include orientation toward and following of a female, tapping her with his forelegs, extending and vibrating one wing, licking her genitalia and attempting copulation (Hall, 1994; Greenspan and Ferveur, 2000). In 10 min courtship assays with virgin females, 5-day-old males of mutant genotypes (*dfmr1* and *dfmr1*; FS rescue/+) spent significantly less time engaged in active courtship than did w^{1118} or *dfmr1*; WT rescue/+ control males (Figure 6A). Most control males directed sustained bouts of wing vibration toward the virgin, and many also attempted to copulate (Figure 6B). In contrast, nearly 80% of *dfmr1* males and >50% of *dfmr1*; FS rescue/+ males failed to advance beyond the initial stages of courtship (following and tapping). Thus the mutants clearly failed to activate advanced stages of courtship in response to the courtship-stimulating cues of virgin females.

A Failure to Maintain Courtship Interest Is Observed in *dfmr1* Mutant Flies

To ask whether the reduced courtship represented a defect in central courtship-activation systems or a specific sensory deficit, similar courtship assays were performed in the presence of immature males, which possess different pheromonal profiles than virgin females, but also stimulate older males to court (reviewed by Hall, 1994). Similar to results obtained with virgin female objects, *dfmr1* mutant males displayed significantly less courtship activity than control males toward immature male objects (Figure 6C), and largely failed to proceed to the more advanced stages of courtship exhibited by controls (Figure 6D). The results indicate that *dfmr1* mutants display the same lack of interest in courting two anatomically and pheromonally distinct objects, suggesting that this behavioral phenotype is not likely a result of a specific sensory deficit. This interpretation is supported by results of analyzing the fine structure of courtship behavior. The duration of individual bouts of courtship varied greatly in control males, but was consistently very short in the mutants. Indeed, only 7% of courtship bouts with immature males lasted longer than a single sampling interval in *dfmr1* and *dfmr1*; FS rescue/+ mutant males (the average interval sampling time was 11 s), while w^{1118} and *dfmr1*; WT rescue/+ males averaged 2.6 and 1.6 sampling intervals per bout, respectively ($p < 0.01$; *dfmr1*; WT rescue/+ males versus *dfmr1* and *dfmr1*; FS rescue/+ males, Mann-Whitney U tests). During pairings with immature males, *dfmr1* and *dfmr1*; FS rescue/+ males initiated courtship just as often as w^{1118} males, averaging 4–5 bouts of courtship in 10 min. *dfmr1*; WT rescue/+ males initiated slightly

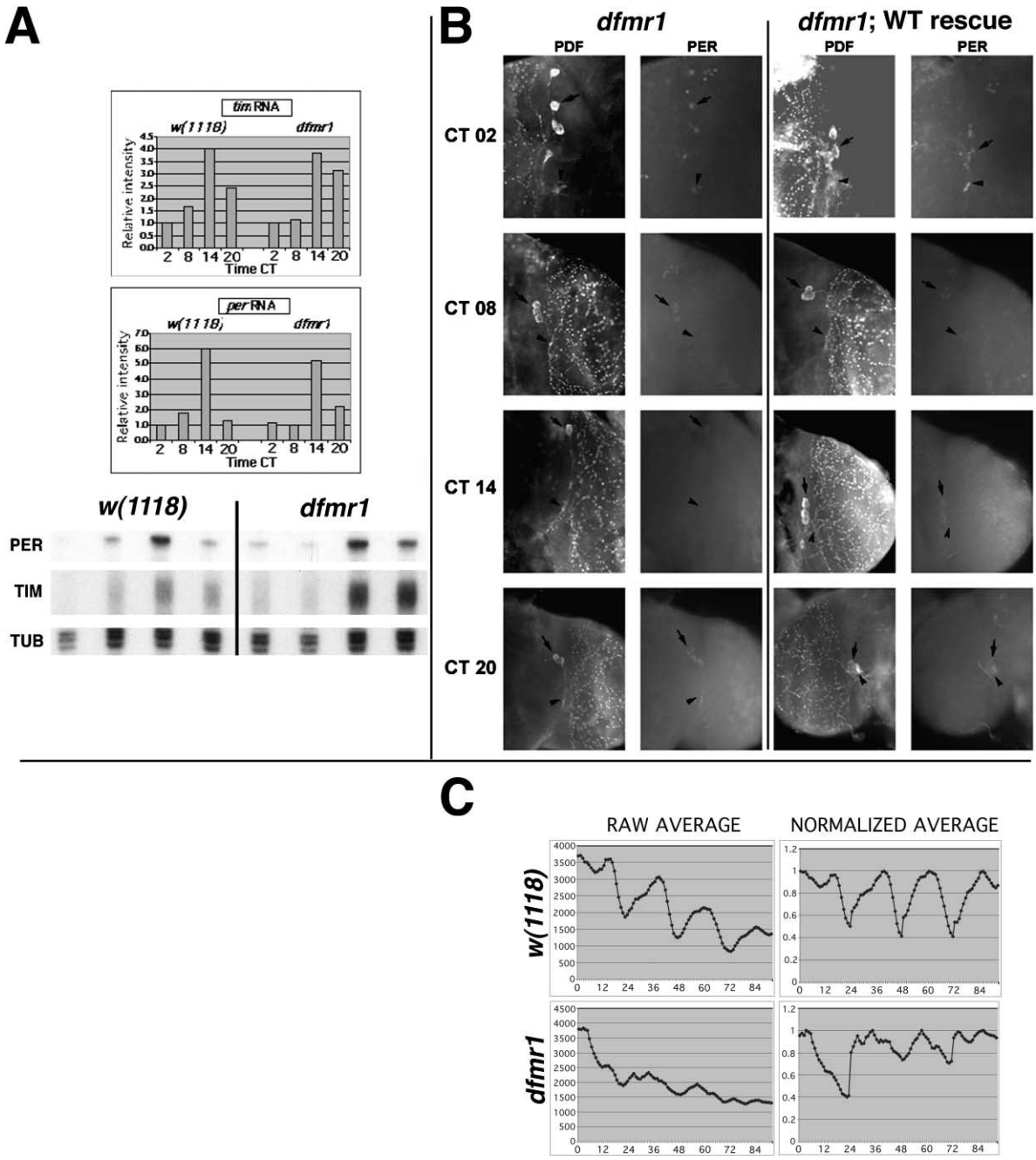

Figure 5. Effect of the *dfmr1* Mutation on Circadian Clock Gene Expression

(A) RNase protection assays (RPAs) were performed to measure the abundance of *per* and *tim* mRNA relative to a control, *tubulin* (*tub*), at various time points in constant darkness (Williams et al., 2001). Data presented are representative of three independent trials. The actual RPA data from this experiment are shown below the two plots. (B) Whole-mount immunostainings of adult brains from *dfmr1* mutant and *dfmr1*; WT rescue/+ flies were double labeled for PER and PDF[22]. For each genotype the left panels show the staining for PDF. The right panels show the staining pattern for PER. Time for each sample is indicated on the left. (C) CRE-luc reporter activity for w^{1118} (n = 58) and *dfmr1* (n = 48) flies was obtained for flies entrained in 12:12 LD cycles for 5 days, then placed in DD and monitored for a minimum of 3 days. Raw data were averaged across flies and plotted in the left panels. Data were normalized to remove both linear and nonlinear trends due to depletion of the luciferin substrate (right panels). Significant oscillations can be detected for both genotypes; however, the *dfmr1* mutants clearly have a reduced oscillation amplitude.

more frequently, averaging 6.6 bouts/10 min. These results indicate that the reduced courtship phenotype of *dfmr1* mutant males is largely the result of a failure to engage in sustained bouts of courtship.

Morphological Analysis of the Lateral Neurons Reveals Subtle Defects in *dfmr1* Mutant Flies

Given the rather ubiquitous expression pattern of dFMR1 during development (Wan et al., 2000), the erratic locomo-

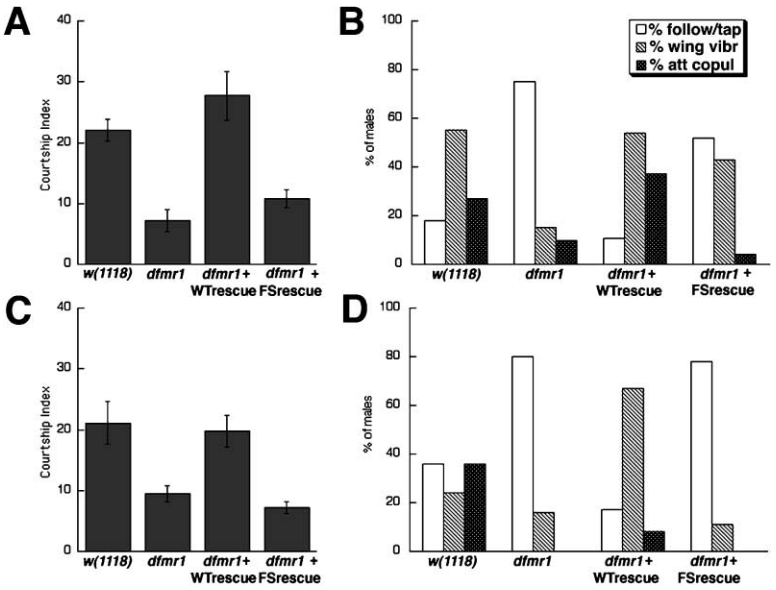

Figure 6. Courtship Studies of *dfmr1* Mutants and Controls

(A) *dfmr1* mutant males court virgin females less actively than control males. The Courtship Index (CI) of 5-day-old males was measured as the percent of time they engaged in courtship behavior during 10 min pairings with individual *XX*, *yf* virgin females. Courtship assays and data processing were performed as described in McBride et al. (1999). The average CI of *dfmr1* males was suppressed relative to w^{1118} controls ($p < 0.0001$ by ANOVA of arcsin-transformed CIs). This phenotype was rescued in mutant males with one copy of wild-type rescue, but was not rescued in mutant males with one copy of FS rescue. Error bars indicate SEM, n = 19–22 males for all groups except n = 41 for mutants with FS rescue. (B) The courtship of *dfmr1* mutant males toward virgin females differs qualitatively from that of control males. The percent of males that reached different levels of courtship during 10 min pairings with virgin females is plotted for each genotype. Most mutant males (*dfmr1* and *dfmr1*; FS rescue/+) failed to proceed past the initial stages of courtship (following and tapping). Most control males (w^{1118} and *dfmr1*; WT rescue/+) exhibited higher levels of courtship and 27%–37% attempted copulation. (C) *dfmr1* mutant males court less actively than controls during 10 min pairings with immature CS males ($p < 0.0001$ by ANOVA of arcsin-transformed CIs). This courtship phenotype is rescued by WT rescue, but not FS rescue. Error bars indicate SEM, n = 24–27 males for all groups. (D) Qualitative analysis of courtship with immature males. The percent of males that reached different levels of courtship during 10 min pairings with immature males is plotted for each genotype. Most mutant males (*dfmr1* and *dfmr1*; FS rescue/+) failed to proceed past the initial stages of courtship (following and tapping). Most control males (w^{1118} and *dfmr1*; WT rescue/+) exhibited higher levels of courtship (wing vibration and/or copulation attempts).

tor activity, as well as the defects in circadian function and courtship behavior, could be due to abnormal neural development in *dfmr1* mutants. The morphology of the circadian system was examined in *dfmr1* mutants by staining of adult brains with the anti-PDF antibody and comparing them to similarly stained control brains. Both mutant and control brains contained identical numbers of small and large ventral lateral neurons (s-LN_V and l-LN_V, respectively). The axon projections from the l-LN_V to the contralateral optic lobes via the posterior optic tract (POT) and those from the s-LN_V to the ipsilateral dorsal protocerebrum were present in mutant brains (Figure 7A versus 7B). However, close examination revealed a high frequency of collateral branches emanating from these axons in the *dfmr1* mutant brains (Figure 7B). Both the s-LN_V and l-LN_V projections into the central brain were examined at high magnification for collateral branches. In 17 control brain hemispheres examined, we found three projections with collateral branches from either the s-LN_V or l-LN_V projections (17.6%), whereas in 23 *dfmr1* brain hemispheres examined, we found 18 (78.2%) with collateral branches ($p < 0.001$ chi-square analysis). Furthermore, comparison of the s-LN_V termini in mutant and control brains revealed more extensive arborizations in the *dfmr1* mutant brains (Figures 7C and 7D versus 7E and 7F). To quantitate this difference, confocal stacks were made of mutant and control s-LN_V termini, and the number of branches at the termini were counted. For control s-LN_V termini, the average number of branches was 4.2 ± 0.47, n = 17. For 23 mutant hemispheres examined, reliable counts of terminal branches could only be made in 17 with an average of 6.9 ± 0.59 ($p = .0011$, unpaired t test). For the termini in the other six hemispheres, the branching was too complex or disorganized to count reliably (Figures 7E and 7F). These results clearly show that the axon projections of the lateral neuron projections in the *dfmr1* mutant flies are abnormal.

In *Drosophila*, mushroom bodies (MBs) have been identified as structures involved in short-term and long-term memory (McBride et al., 1999; Dubnau et al., 2001; McGuire et al., 2001; Pascual and Preat, 2001), as well as normal locomotor behavior (Martin et al., 1998), and courtship (Ferveur et al., 1995). Central complex regions also modulate locomotor behavior (Martin et al., 1999). When these and other structures were examined in brain sections of control (*dfmr1*; WT rescue/+) and *dfmr1* mutant (*dfmr1*; FS rescue/+) male flies, no specific abnormalities were detected in the neuropil regions and fiber tracts of mutant brains (not shown). To look at the structure of the MBs in more detail, we stained both mutant control brains with an antibody raised to Fasicilin II (ID4), which has been shown to specifically label a subset of the MB (Jefferies et al., 2002). The peduncle, α, and β lobes of MBs in mutant and control brains were found to be very similar in structure and shape (Figures 7G and 7H). Measurements of the diameters and lengths of the α and β lobes of ten MBs each showed no difference between the mutant and control. Thus the MBs of *dfmr1* mutant brains do not appear to be different from those in control brains.

The Circadian Defect of *dfmr1* Mutant Flies Is Not Rescued by the Introduction of *futsch* Loss-of-Function Mutation

A recent report on *dfmr1* (Zhang et al., 2001) indicates a genetic interaction of dFMR1 with the MAP1B homolog FUTSCH. A *dfmr1*/*futsch* double mutant combination is

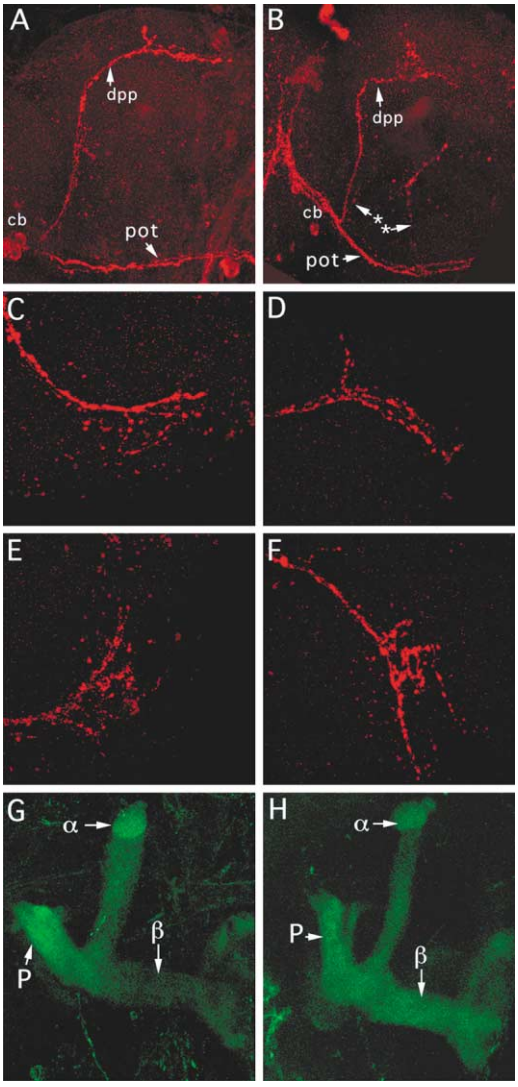

Figure 7. Morphological Analysis of Lateral Neurons and Mushroom Bodies in the Brains of Control and *dfmr1* Flies

(A), (C), (D), and (G) are *dfmr1*; WT rescue/+ brains and (B), (E), (F), and (H) are *dfmr1* mutant brains. (A and B) Anti-PDF staining to detect lateral neurons and their projection patterns. "cb" indicates position of the cell bodies of PDF-staining lateral neurons. "dpp" indicates the positions of the dorsal protocerebral projections coming from the s-LN_v. "pot" indicates the positions of the projections into the posterior optic tract emanating from the l-LN_v. The asterisks in (B) indicate the position of collateral projections. (C–F) Termini of control (C and D) and *dfmr1* mutant (E and F) dorsal protocerebral projections. The mutant termini are more extensively branched. (G and H). Mushroom bodies of control (G) and H) *dfmr1*mutant brains stained with anti-Fas II antibody. The positions of the α-lobe (α), β-lobe (β), and peduncle (P) are indicated.

reported to rescue all reported synaptic defects detected at the neuromuscular junction (NMJ) and in the eye of a *dfmr1* mutant, prompting a hypothesis that *futsch* misregulation is likely a key factor in pathologies associated with fragile X mental retardation (Zhang et al., 2001). To extend upon these observations, we generated *dfmr1/futsch* double mutants using the *futsch*^{N94}allele (Hummel et al., 2000) to see if the circadian defect observed in the *dfmr1* mutants would be similarly rescued. Viable *futsch*^{N94}; *dfmr1* double mutants were entrained to a light:dark cycle in a manner identical to the previous *dfmr1* circadian studies and placed in activity monitors. The *futsch*^{N94} allele and *dfmr1* mutants were used as controls. We found that *futsch*^{N94}mutants have no detectable circadian defect and that the *dfmr1*^{3}/*futsch*^{N94} showed no change in locomotor activity profiles or strength of rhythmicity when compared to *dfmr1* mutants (FFT = .007 ± 0.005, n = 9 versus FFT = .008 ± 0.004, n = 12, respectively). Thus, the circadian phenotype exhibited by *dfmr1* mutants is not largely a consequence of the same *futsch* misregulation reported at the NMJ and eye (Zhang et al., 2001).

Discussion

We have shown that although *dfmr1* mutant flies are indistinguishable from wild-type controls in overall locomotor activity and ability to perform simple chemotactic and phototactic behavior, they have significant circadian and courtship behavioral phenotypes. The circadian system appears to be disrupted downstream of the molecular pacemaker, as molecular analysis of the clock genes failed to reveal any defect in the oscillations of their mRNAs and resultant proteins. CREB protein activity has been shown to be a regulated output of the clock (Belvin et al., 1999). We found that the circadian oscillations of a CRE-luciferase reporter were significantly reduced in amplitude in the *dfmr1* mutant background. These results indicate that *dfmr1* gene activity is required for this known molecular output. Due to effects of feedback on the clock, at this time it is unknown if *dfmr1* acts upstream, downstream, or parallel to CREB-mediated transcription. Also, whether this is the only output pathway affected is not known. Clearly PDF appears to cycle normally at the termini of the s-LN_v, but given the structural defects observed in the lateral neurons, it is conceivable that PDF release is affected in ways that we did not observe. For example, PDF release may be more diffuse in the *dfmr1* mutants due to the increased arborization at the lateral neuron termini. Therefore, the arrhythmicity observed in the *dfmr1* mutants could be due to effects on multiple output pathways, or due to unique effects caused by a reduction in the oscillation amplitude of CREB protein activity. In addition to arrhythmic behavior, we observed a very erratic pattern of locomotor activity with brief periods of hyperactivity. This erratic pattern of activity has not been noted in other arrhythmic mutants (J. Hendricks, personal communication). It could result from a unique effect of *dfmr1* activity on circadian output or possibly from defects in another system that regulates locomotor activity.

In addition to the circadian defects, the *dfmr1* mutant males exhibited abnormal courtship behavior. *dfmr1* mutant males displayed reduced overall courtship activity toward two independent targets, virgin females and immature males, which are anatomically distinct and have different pheromonal profiles (Hall, 1994). This result suggests a basic defect in courtship rather than a failure to recognize or respond to a specific cue. This defect, however, was not seen in the number of initiations of courtship attempts, but rather in their duration,

suggesting that *dfmr1* mutants are unable to maintain courtship interest. The behavioral phenotypes of the *dfmr1* mutant place it in a growing class of *Drosophila* genes that affect both circadian output and other complex behaviors. The *pka*, *CREB*, and *Nf1* genes, which were originally shown to be mutants affecting learning and/or memory, have since been shown to affect circadian rhythms and more specifically output from the clock (Skoulakis et al., 1993; Bourtchuladze et al., 1994; Yin et al., 1994; Majercak et al., 1997; Belvin et al., 1999; Guo et al., 2000; Williams et al., 2001). The evidence suggests a basic link between the circadian modulation of activity and integrative and associative behaviors.

What is the cellular basis for the behavioral phenotypes produced by dFMR1/FMR1 loss of function? Anatomical analyses in both vertebrate and *Drosophila* models suggest that regulation of axon growth and/or synapse formation may be affected (Comery et al., 1997; Zhang et al., 2001; this study). Because *dfmr1* is widely expressed in the developing central nervous system (Wan et al., 2000; Zhang et al., 2001), the inappropriate collateral branching and terminal arborization we observe in the projections of the ventral lateral neurons are likely to be present in other neurons as well. Thus, while subtle defects in the projections of these specific neurons may not be sufficient to explain the behavioral phenotypes of *dfmr1* mutants (Renn et al., 1999; Helfrich-Forster et al., 2000), similar abnormalities in the wiring of many neurons throughout the brain could result in a selective disruption of complex behaviors.

A current model holds that dFMR1 negatively regulates the translation of the MAP1B homolog *futsch*. This model is supported by biochemical interaction between dFMR1 protein and *futsch* mRNA, a dependence of FUTSCH proteins levels on *dfmr1* expression levels, and an observation that a double mutant combination of *dfmr1* and a hypomorphic allele of *futsch* ameliorates the larval NMJ and photoreceptor phenotypes reported in *dfmr1* mutants (Zhang et al., 2001). By contrast we did not find that the introduction of a *futsch* loss-of-function mutant into the *dfmr1* mutant background rescued the circadian phenotype. Therefore, while dFMR1 protein may regulate the form of terminal arborizations in both central and peripheral neurons, these effects may involve the regulation of different transcripts during the development of different synapses.

The recent use of biochemical screens, coupled with microarray analysis, to search for RNA substrates bound by FMR1 protein, has identified several dozen candidate transcripts that may be regulated by FMR1 (Brown et al., 2001; Darnell et al., 2001). One interesting candidate to note is the transcript for *SCOP*, a gene conserved between mammals and insects, whose transcript is expressed in a circadian manner in the suprachiasmatic nucleus of rats (Shimizu et al., 1999; Berkeley *Drosophila* Genome Project). Although the functions for SCOP and its necessity for circadian function are not known, our results that demonstrate a lack of circadian output in *dfmr1* mutants provide a measure of validation that substrates identified in the biochemical screens have a substantial likelihood of having an in vivo association with FMR1 protein function.

It is interesting to note that the defects observed in the *dfmr1* mutant flies for circadian and courtship behavior have similarities to behavioral phenotypes observed in patients with fragile X syndrome. Fragile X patients commonly have abnormal sleep patterns with shortened periods of sleep and longer wake episodes (Hagerman, 1996), suggesting defects in their circadian systems. Consistent with this observation, many fragile X patients have been found to have an altered melatonin profile, a well-known output of the circadian clock (Gould et al., 2000). Cognitive analysis of fragile X patients has identified mild to severe mental retardation, associated with specific weaknesses in short-term memory, sequential information processing, and other more complex abilities (Hagerman, 1996; Backes et al., 2000). Psychiatric diagnoses are also common among fragile X patients, with a high rate of attention deficit hyperactivity disorder (Hagerman, 1996; Backes et al., 2000). The similarities between the human and fly mutant phenotypes and the shared biochemical properties of the two proteins (Wan et al., 2000) suggest that studies of *dfmr1* will be a useful model to identify physiological pathways and substrates affected by the *FMR1* gene.

Experimental Procedures

Generation of *dfmr1* Alleles

The P element of stock *P[EP]3517* is inserted within the first exon of the *dfmr1* locus. Flies homozygous for this P insertion are viable, fertile, and have no visible abnormalities. To assess the effects of a deletion allele, an imprecise excision screen was conducted, using the Δ2–3 transposase to mobilize the P element, using previously described methodologies (Faulkner et al., 1998). Approximately 270 stocks that had lost the w^+ marker associated with the P element were generated. None of these stocks failed to complement a deficiency *[Df(3R)by62]* that uncovers the *dfmr1* locus. Thus, anti-dFMR1 antibody (Wan et al., 2000) was used to screen stocks homozygous for the excision chromosome for reduction or loss of dFMR1 protein. Two stocks were identified where most or the entire *dfmr1* open reading frame had been deleted as judged by Southern hybridization. These stocks were backcrossed to w^{1118}, the parent stock of the *EP* lines (Rørth, 1996), then balanced using the *TM6C Tb Sb* balancer chromosome.

Measurement of Time of Eclosion

Larvae were entrained to a 12:12 light:dark cycle (lights on:ZT 0, lights off: ZT12) at 25°C, 5 days after parent crosses were set up. On the tenth day post-cross, pupae were taped to eclosion monitors (Trikinetics) using double-sided tape. After lights off, monitors were transferred into constant darkness at 25°C. Eclosion activity was collected in 30 min bins. Eclosion histograms were generated from pooled sets of data and the data were pooled into 2 hr bins. The period was set at 24 hr and Fast-Fourier Transform values were calculated as a measure of rhythmicity. All analysis was done using Clocklab software (Actimetrics, Inc).

Measurement of Rest:Activity Rhythms

Flies were entrained on standard cornmeal, agar-molasses-yeast medium at 25°C. For activity assays, male flies were entrained to a 12:12 hr light:dark cycle for a minimum of 5 days prior to loading in glass tubes for activity monitoring using a Trikinetics (Waltham, MA) system interfaced with an Apple computer. Data were subsequently analyzed using the Clocklab (Actimetrics) software package. Rhythmicity was determined by visual inspection of the actograms, periodograms, and FFT analysis.

RNase Protection Assays

Flies, entrained in light:dark cycles for 3 days, were collected during the first 24 hr period of constant darkness. Total RNA was extracted from heads using the Biotecx (Houston, TX) reagent using manufacturer's suggested procedures. RNase protection assays were performed as previously described (Williams et al., 2001), using probes

for *tim, per*, and *tubulin* mRNA simultaneously. After electrophoresis of the Rnase-treated samples, the signal for each of the three mRNAs was quantitated using a phosphoimager (Molecular Dynamics). Relative abundance of *tim* and *per* mRNA was defined as a ratio with *tub*, e.g., *per/tub* and *tim/tub*, and normalized to the highest time point value for each experiment.

Luciferase Assays

Male flies carrying CRE-luc (785-9) in the w^{1118} or *dfmr1* mutant background were entrained for 5 days in a 12:12 LD cycle at 25°C. On the day prior to initiating testing, flies were anaesthetized and loaded into 96 well opaque culture plates containing a sucrose-agar medium mixed with 100 μM luciferin (Biomol). Each plate was covered with a ventilated clear plastic sticker and placed in the incubator for adaptation. The following day, plates were placed in a luminometer (Hewlett Packard) at the time of lights off for continuous monitoring of luciferase activity over several days. Data from flies that did not survive the duration of the experiment were discarded. Since the depletion of luciferase substrate, during the course of an experiment, causes a decrease in amplitude, data were normalized in 24 hr intervals, by determining the ratio of each time point in that 24 hr period to the highest luciferase activity value obtained in that interval. These ratios are plotted with respect to time.

Immunohistochemistry

Whole-mount immunostaining of adult brains was performed as described in Helfrich-Forster and Homberg (1993), except that anti-PDF was used at a 1:1000 dilution. Anti-fascilin II antibody (ID4) was used at a 1:5 dilution. Secondary antibodies were obtained from Jackson Immunologicals. Images were captured using a Leica scanning laser confocal microscope.

Longevity and Simple Behavior Assays

Phototaxis assays were performed as described in Balinger and Benzer (1988), except that free-running phototaxis intervals were 20 s and a six tube apparatus was used. Flies were counted in each tube after six consecutive trials were performed. Tests for chemotaxis/olfaction were performed using a trap assay with yeast paste as an attractant (Orgad et al., 2000). Longevity assays were performed by placing 20 control or *dfmr1* mutant adults in fresh food vials and transferring the flies to fresh vials every 3 days. Viable flies were counted at each transfer.

Screen for EP Lines with Altered Rhythms

The genetic screen was modified from the EP screen described previously (Rørth, 1996; Rørth et al., 1998). Briefly, X chromosome EP lines EP443, EP446, or EP448 virgin female flies (BDGP) were crossed to *yw*, *Ki*, Δ2-3/*TM3*, *Sb* males to generate new EP insertions. Male progeny were crossed to virgin *yw*, *tim-GAL4* females. In the next generation, males that bore both *tim-GAL4* and a new EP insertion were tested for locomotor activity rhythm. Individual lines were established from flies that displayed arrhythmic activity patterns or abnormal period length.

Courtship Assays

Assays were performed as described in McBride et al.(1999). Briefly, test males were collected using ether anesthesia within 4 hr of eclosion, and reared in individual food vials at 25°C in 12:12 LD for 4–6 days. Virgin XX, *yf* females were reared in groups of 10–20 per vial. Immature Canton-S males were collected within 30 min of eclosion, and aged less than 3 hr in food vials. All experiments were conducted during light phase.

Courtship assays were performed by aspirating an unanesthetized male subject and either a virgin female or an immature male into a porcelain or glass mating chamber of 25 mm diameter and 10 mm depth. The experimenter was blind to the genotype of the test males. Courting pairs were observed over the course of 10 min, and the courtship index (CI) was determined as the proportion of time spent by the male in active courtship. Specific courtship behaviors (following, tapping, wing vibration, licking, and copulation attempts) were recorded during this period. In tests with immature males, these behaviors were noted systematically at regular intervals (average sampling interval = 11 s), from which data the frequency and duration of individual bouts of courtship were determined.

Acknowledgments

We thank M. Fortini and H. Kazazian for critical comments on the manuscript. Special thanks go to the Sehgal laboratory for their discussions, interest, and help with experiments, especially Julie Williams, Edith Myers, and Sriram Sathyanarayanan. We would also like to thank Christian Klambt for *futsch* alleles. T.C.D. was supported in part by a postdoctoral fellowship from the FRAXA Foundation. This work was supported by NIH grants to T.A.J. and A.S. A.S. is an Associate Investigator of the Howard Hughes Medical Institute.

Received: November 27, 2001
Revised: April 17, 2002

References

Ashley, C.T., Jr., Wilkinson, K.D., Reines, D., and Warren, S.T. (1993). FMR1 protein: conserved RNP family domains and selective RNA binding. Science *262*, 563–566.

Backes, M., Genc, B., Schreck, J., Doerfler, W., Lehmkuhl, G., and von Gontard, A. (2000). Cognitive and behavioral profile of fragile X boys: correlations to molecular data. Am. J. Med. Genet. *95*, 150–156.

Bakker, C.E., de Diego Otero, Y., Bontekoe, C., Raghoe, P., Luteijn, T., Hoogeveen, A.T., Oostra, B.A., and Willemsen, R. (2000). Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse. Exp. Cell Res. *258*, 162–170.

Balinger, D.G., and Benzer, S. (1988). Photophobe (Ppb), a Drosophila mutant with a reversed sign of phototaxis; the mutation shows a allele-specific interaction with sevenless. Proc. Natl. Acad. Sci. USA *85*, 3960–3964.

Belvin, M.P., Zhou, H., and Yin, J.C. (1999). The Drosophila dCREB2 gene affects the circadian clock. Neuron *22*, 777–787.

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., and Silva, A.J. (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell *79*, 59–68.

Brown, V., Jin, P., Ceman, S., Darnell, J.C., O'Donnell, W.T., Tenenbaum, S.A., Jin, X., Feng, Y., Wilkinson, K.D., Keene, J.D., et al. (2001). Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell *107*, 477–487.

Comery, T.A., Harris, J.B., Willems, P.J., Oostra, B.A., Irwin, S.A., Weiler, I.J., and Greenough, W.T. (1997). Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits. Proc. Natl. Acad. Sci. USA *94*, 5401–5404.

Darnell, J.C., Jensen, K.B., Jin, P., Brown, V., Warren, S.T., and Darnell, R.B. (2001). Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. Cell *107*, 489–499.

de Vries, B.B., Halley, D.J., Oostra, B.A., and Niermeijer, M.F. (1998). The fragile X syndrome. J. Med. Genet. *35*, 579–589.

Dubnau, J., Grady, L., Kitamoto, T., and Tully, T. (2001). Disruption of neurotransmission in Drosophila mushroom body blocks retrieval but not acquisition of memory. Nature *411*, 476–480.

Dunlap, J.C. (1999). Molecular bases for circadian clocks. Cell *96*, 271–290.

Dutch-Belgian Fragile X Consortium (1994). *Fmr1* knockout mice: A model to study fragile X mental retardation. Cell *78*, 23–33.

Faulkner, D., Dockendorff, T.C., and Jongens, T.A. (1998). Clonal analysis of cmp44E, which encodes a putative transmembrane protein, indicates a requirement for cell viability in Drosophila. Dev. Genet. *23*, 264–274.

Feng, Y., Gutekunst, C., Eberhardt, D.E., Yi, H., Warren, S.T., and Hersch, S.M. (1997). Fragile X mental retardation protein: Nucleocytoplasmic shuttling and association with somatodendritic ribosomes. J. Neurosci. *17*, 1539–1547.

Ferveur, J.-F., Stortkuhl, K.F., Stocker, R.F., and Greenspan, R.J.

(1995). Genetic feminization of brain structures and changed sexual orientation in male Drosophila. Science *267*, 902–905.

Gould, E.L., Loesch, D.Z., Martin, M.J., Hagerman, R.J., Armstrong, S.M., and Huggins, R.M. (2000). Melatonin profiles and sleep characteristics in boys with fragile X syndrome: a preliminary study. Am. J. Med. Genet. *95*, 307–315.

Greenspan, R.J., and Ferveur, J.F. (2000). Courtship in Drosophila. Annu. Rev. Genet. *34*, 205–232.

Guo, H.F., Tong, J., Hannan, F., Luo, L., and Zhong, Y. (2000). A neurofibromatosis-1-regulated pathway is required for learning in Drosophila. Nature *403*, 895–898.

Hagerman, R.J. (1996). Physical and behavioral phenotype. In Fragile X Syndrome. Diagnosis, Treatment, and Research, R.J. Hagerman and A. Cronister, eds. (Baltimore, MD: Johns Hopkins University Press), pp. 3–87.

Hall, J.C. (1994). The mating of a fly. Science *264*, 1702–1714.

Helfrich-Forster, C. (1995). The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA *92*, 612–616.

Helfrich-Forster, C. (1998). Robust circadian rhythmicity of Drosophila melanogaster requires the presence of lateral neurons: a brain-behavioral study of disconnected mutants. J. Comp. Physiol. *182*, 435–453.

Helfrich-Forster, C., and Homberg, U. (1993). Pigment-dispersing hormone-immunoreactive neurons in the nervous system of wild-type Drosophila melanogaster and of several mutants with altered circadian rhythmicity. J. Comp. Neurol. *337*, 177–190.

Helfrich-Forster, C., Tauber, M., Park, J.H., Muhlig-Versen, M., Schneuwly, S., and Hofbauer, A. (2000). Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in Drosophila melanogaster. J. Neurosci. *20*, 3339–3353.

Hummel, T., Krukkert, K., Roos, J., Davis, G., and Klämbt, C. (2000). *Drosophila* Futsch/22C10 is a MAP1B-like protein required for dendritic and axonal development. Neuron *26*, 357–370.

Jefferies, G.S., Marin, E.C., Watts, R.J., and Luo, L. (2002). Development of neuronal connectivity in Drosophila antennal lobes and mushroom bodies. Curr. Opin. Neurobiol. *1*, 80–86.

Jin, P., and Warren, S.T. (2000). Understanding the molecular basis of fragile X syndrome. Hum. Mol. Genet. *9*, 901–908.

Konopka, R.J., and Benzer, S. (1971). Clock mutants of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA *68*, 2112–2116.

Majercak, J., Kalderon, D., and Edery, I. (1997). Drosophila melanogaster deficient in protein kinase A manifests behavior-specific arrhythmia but normal clock function. Mol. Cell. Biol. *17*, 5915–5922.

Martin, J.-R., Ernst, R., and Heisenberg, M. (1998). Mushroom bodies suppress locomotor activity in Drosophila melanogaster. Learn. Mem. *5*, 179–191.

Martin, J.-R., Raabe, T., and Heisenberg, M. (1999). Central complex substructures are required for the maintenance of locomotor activity in Drosophila melanogaster. J. Comp. Physiol. *185*, 277–288.

McBride, S.M., Giuliani, G., Choi, C., Krause, P., Correale, D., Watson, K., Baker, G., and Siwicki, K.K. (1999). Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in Drosophila melanogaster. Neuron *24*, 967–977.

McGuire, S.E., Le, P.T., and Davis, R.L. (2001). The role of Drosophila mushroom body signalling in olfactory memory. Science *293*, 1330–1333.

Orgad, S., Rosenfeld, G., Greenspan, R.J., and Segal, D. (2000). courtless, the Drosophila UBC7 homolog, is involved in male courtship behavior and spermatogenesis. Genetics *155*, 1267–1280.

Park, J.H., Helfrich-Forster, C., Lee, G., Liu, L., Rosbash, M., and Hall, J.C. (2000). Differential regulation of circadian pacemaker output by separate clock genes in Drosophila. Proc. Natl. Acad. Sci. USA *28*, 3608–3613.

Pascual, A., and Preat, T. (2001). Localization of long-term memory within the Drosophila mushroom body. Science *294*, 1115–1117.

Reiss, A.L., Abrams, M.T., Greenlaw, R., Freund, L., and Denckla, M.B. (1995). Neurodevelopmental effects of the FMR-1 full mutation in humans. Nat. Med. *1*, 159–167.

Renn, S.C., Park, J.H., Rosbash, M., Hall, J.C., and Taghert, P.H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. Cell *99*, 791–802.

Rørth, P. (1996). A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. Proc. Natl. Acad. Sci. USA *93*, 12418–12422.

Rørth, P., Szabo, K., Bailey, A., Laverty, T., Rehm, J., Rubin, G.M., Weigmann, K., Milan, M., Benes, V., Ansorge, W., and Cohen, S.M. (1998). Systematic gain-of-function genetics in Drosophila. Development *125*, 1049–1057.

Sehgal, A., Price, J.L., Man, B., and Young, M.W. (1994). Loss of circadian behavioral rhythms and per RNA oscillations in the Drosophila mutant timeless. Science *263*, 1603–1606.

Shimizu, K., Okada, M., Takano, A., and Nagai, K. (1999). SCOP, a novel gene product expressed in a circadian manner in rat suprachiasmitc nucleus. FEBS Lett. *458*, 363–369.

Siomi, H., Siomi, M.C., Nussbaum, R.L., and Dreyfuss, G. (1993). The protein product of the fragile X gene, *FMR1*, has characteristics of an RNA-binding protein. Cell *74*, 291–298.

Siomi, H., Choi, M., Siomi, M.C., Nussbaum, R.L., and Dreyfuss, G. (1994). Essential role for KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. Cell *77*, 33–39.

Siomi, M.C., Siomi, H., Sauer, W.H., Srinivasan, S., Nussbaum, R.L., and Dreyfuss, G. (1995). FXR1, an autosomal homolog of the fragile X mental retardation gene. EMBO J. *14*, 2401–2408.

Siomi, M.C., Zhang, Y., Siomi, H., and Dreyfuss, G. (1996). Specific sequences in the fragile X syndrome protein FMR1 and the FXR proteins mediate their binding to 60S ribosomal subunits and the interactions among them. Mol. Cell. Biol. *16*, 3825–3832.

Skoulakis, E.M.C., Kalderon, D., and Davis, R.L. (1993). Preferential expression in mushroom bodies of the catalytic subunit of protein kinase A and its role in learning and memory. Neuron *11*, 197–208.

Wan, L., Dockendorff, T.C., Jongens, T.A., and Dreyfuss, G. (2000). Characterization of dFMR1, a Drosophila homolog of the fragile X mental retardation protein. Mol. Cell. Biol. *20*, 8536–8547.

Weiler, I.J., Irwin, S.A., Klintsova, A.Y., Spencer, C.M., Brazelton, A.D., Miyashiro, K., Comery, T.A., Patel, B., Eberwine, J., and Greenough, W.T. (1997). Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. Proc. Natl. Acad. Sci. USA *94*, 5395–5400.

Williams, J.A., and Sehgal, A. (2001). Molecular components of the circadian system in Drosophila. Annu. Rev. Physiol. *63*, 729–755.

Williams, J.A., Su, H.S., Bernards, A., Field, J., and Sehgal, A. (2001). A circadian output in Drosophila mediated by neurofibromatosis-*1* and Ras/MAPK. Science *293*, 2251–2256.

Yang, Z., and Sehgal, A. (2001). Role of molecular oscillations in generating behavioral rhythms in Drosophila. Neuron *29*, 453–467.

Yin, J.C., Wallach, J.S., Del Vecchio, M., Wilder, E.L., Zhou, H., Quinn, W.G., and Tully, T. (1994). Induction of a dominant negative CREB transgene specifically blocks long-term memory in Drosophila. Cell *79*, 49–58.

Zhang, Y., O'Connor, J.P., Siomi, M.C., Srinivasan, S., Dutra, A., Nussbaum, R.L., and Dreyfuss, G. (1995). The fragile X mental retardation syndrome protein interacts with novel homologs FXR1 and FXR2. EMBO J. *14*, 5358–5366.

Zhang, Y.Q., Bailey, A.M., Matthies, H.J.G., Renden, R.B., Smith, M.A., Speese, S.D., Rubin, G.M., and Broadie, K. (2001). *Drosophila* Fragile X-related gene regulates the MAP1B homolog futsch to control synaptic structure and function. Cell *107*, 591–603.