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Substitution of Critical Isoleucines in the KH Domains of Drosophila Fragile X Protein Results in Partial Loss-of-Function Phenotypes

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ABSTRACT

Fragile X mental retardation proteins (FMRP) are RNA-binding proteins that interact with a subset of cellular RNAs. Several RNA-binding domains have been identified in FMRP, but the contribution of these individual domains to FMRP function in an animal model is not well understood. In this study, we have generated flies with point mutations in the KH domains of the *Drosophila melanogaster* fragile X gene (*dfmr1*) in the context of a genomic rescue fragment. The substitutions of conserved isoleucine residues within the KH domains with asparagine are thought to impair binding of RNA substrates and perhaps the ability of FMRP to assemble into mRNP complexes. The mutants were analyzed for defects in development and behavior that are associated with deletion null alleles of *dfmr1*. We find that these KH domain mutations result in partial loss of function or no significant loss of function for the phenotypes assayed. The phenotypes resulting from these KH domain mutants imply that the capacities of the mutant proteins to bind RNA and form functional mRNP complexes are not wholly disrupted and are consistent with biochemical models suggesting that RNA-binding domains of FMRP can function independently.

THE fragile X mental retardation protein (FMRP) is an RNA-binding protein necessary for normal neuronal development and behavior in all species where its function has been examined. A general model for FMRP function is that it regulates nucleocytoplasmic transport, subcellular localization, and translation of select RNA transcripts (reviewed by Bardoni and Mandel 2002; Jin and Warren 2003; Jin *et al.* 2004a; Bagni and Greenough 2005). Biochemical analyses have uncovered several RNA-binding motifs associated with FMRP function, including two KH domains (hnRNP-*K h*omology) and an arginine and glycine-rich motif (RGG box) that are common to RNA-binding proteins (Ashley *et al.* 1993; Siomi *et al.* 1993). The highly conserved N termini of FMRPs have RNA-binding capacity as well (Adinolfi *et al.* 1999, 2003). The N-terminal 110 amino acids of FMRPs are similar to Tudor/Agenet domains and are members of an extended family that is referred to as the Tudor domain "royal family" (Maurer-Stroh *et al.* 2003). This domain family is related to methyl-substrate-binding proteins that are implicated in regulation of chromatin structure and includes the chromodomain.

RNA substrates for FMRP have conserved elements in primary sequence and/or higher-order structures that interact with the aforementioned RNA-binding domains. A G-quartet structure within RNA interacts with the RGG box (Darnell *et al.* 2001; Schaeffer *et al.* 2001), and the second KH domain recognizes a loop–loop pseudoknot RNA structure referred to as a kissing complex (Darnell *et al.* 2005). A stem-loop structure within *BC1* RNA is reported to interact specifically with the N-terminal 217 amino acids of FMRP (Zalfa *et al.* 2005; but see Wang *et al.* 2005 for an opposing view). These studies demonstrate that individual RNA-binding domains of FMRP have distinct substrates with which they interact and that the ability of these domains to bind substrates is not dependent upon other FMRP RNA-binding domains. Loss of function of any of these domains might then result in only a subset of RNA substrates losing the ability to bind FMRP.

Expansion of a CGG trinucleotide repeat in the 5′-UTR of the *FMR1* gene, followed by methylation and transcriptional silencing, is the basis for the vast majority of fragile X cases in humans (see O'Donnell and Warren 2002 for a review of human fragile X inheritance patterns). Other alleles result from deletions or nonsense codons, and thus little structure–function information has been obtained from analysis of human *FMR1* alleles. One significant exception is the substitution

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of a highly conserved isoleucine residue in KH domains to asparagine (I304N) within the second KH domain of human FMRP that is associated with unusually severe fragile X phenotypes (De Boulle *et al.* 1993). Until recently, models to explain the effects of the I304N substitution have been enigmatic. Defects in RNA binding have been proposed on the basis of the finding that the I304N protein is impaired in binding RNA homopolymers under high salt concentrations (Siomi *et al.* 1994) and the analysis of a co-crystal structure of a KH domain and RNA substrate (Lewis *et al.* 2000). Although the I304N protein can bind bulk poly(A) RNA (Feng *et al.* 1997), in contrast to wild-type FMRP, it does not associate with polyribosomes (Feng *et al.* 1997) or with itself (Laggerbauer *et al.* 2001). These results suggest that the inability to form proper messenger ribonucleoprotein (mRNP) complexes is a significant factor contributing to the I304N phenotype and have prompted suggestions that the severity of phenotypes associated with the mutation arise from dominant negative or antimorphic effects (Feng *et al.* 1997). The above biochemical studies have been reconciled by the findings that the second KH domain of FMRP binds kissing complex RNAs, the I304N substitution abolishes this association, and the kissing complex RNAs can compete FMRP off polyribosomes (Darnell *et al.* 2005). These results imply that FMRP association with polyribosomes is dependent upon an interaction of the second KH domain with RNAs containing a kissing complex structure.

Many studies demonstrate that the Drosophila fragile X protein shares biochemical functions with its vertebrate counterparts and regulates similar neural and behavioral functions (reviewed by Gao 2002; Jin and Warren 2003; Dölen and Bear 2005; Zhang and Broadie 2005). Previously existing alleles of *dfmr1* are strong or null alleles resulting from imprecise *P*-element excisions or nonsense codons (Zhang *et al.* 2001; Dockendorff *et al.* 2002; Lee *et al.* 2003). Although KH domain mutations exist for *dfmr1* as cDNA constructs, the misexpression or overexpression of these alleles in neural and muscle tissues via the *GAL4-UAS* system results in physiologic abnormalities or cell death (Wan *et al.* 2000; Zhang *et al.* 2001). Starting with a genomic rescue fragment encompassing *dfmr1*, we have created derivatives of this rescue fragment where conserved isoleucine residues in the KH domains have been mutated as a means to assess the importance of these domains in an animal model. These *P*-element-borne transgenes have been recombined onto a chromosome deleted for *dfmr1* to produce animals that express only mutant forms of dFMR1 protein. Our analyses of flies with the KH domain mutations show that they result in either partial or no loss of function of the behavior and developmental phenotypes that were examined. These findings show that the mutant proteins retain a significant degree of function *in vivo* and are consistent with biochemical models that predict FMRP RNA-binding domains as having some independent functions. These alleles of *dfmr1* will be useful tools for both genetic and biochemical screens in identifying RNAs and proteins that interact with the fragile X protein.

MATERIALS AND METHODS

Generation of KH domain mutants: A subclone of a 14-kb *Bam*HI–*Stu*I genomic fragment spanning the *dfmr1* locus was subjected to site-directed mutagenesis via the mega-primer technique, using a proofreading polymerase (methods compiled in Sambrook and Russell 2001). All PCR-amplified fragments were sequenced to confirm the presence of the desired mutation and the absence of secondary mutations resulting from base misincorporations during amplification. Mutant DNA was then substituted for the corresponding wild-type fragment and the resulting mutant rescue fragments were cloned into pCaSpeR-4 (Pirrotta 1988) for subsequent transformation. Transformations were done under conditions described in Spradling and Rubin (1982). We found the transformation efficiency to be very low, with one transformed fly appearing in ~300 G_0 crosses for each of the mutant transgenes. Both mutant transgenes mapped to the third chromosome, and thus the transgene insertions were recombined onto a chromosome harboring the $dfmr1^3$ allele, which removes the *dfmr1* open reading frame (Dockendorff *et al.* 2002; Pan *et al.* 2004). These transgenes were judged to map within two recombination units of the endogenous *dfmr1* locus on the basis of the frequency with which recombination of the two loci occurred. The resulting stocks are of the following genotypes: $P[dfmr1^{I244N}]w^+$ $dfmr1^3/TM6C$ *Tb Sb* and $P[dfmr1^{I307N}]w^+$ $dfmr1^3/TM6C$ *Tb Sb*. These recombinant stocks were then crossed to flies with the $dfmr1^3$ allele to produce animals heterozygous for the transgene insertion and homozygous for the $dfmr1^3$ allele. Thus, the only dFMR1 protein produced in such animals is from the mutant allele. For clarity, throughout the text and figures these stocks will be referred to simply by the nature of the KH domain substitution. To test for effects of an increased dosage, we crossed the stocks with the transgenes recombined onto the $dfmr1^3$ null chromosome to a stock expressing the Δ2-3 transposase. Flies were selected that had enhanced expression of the *mini-white* marker and of mutant dFMR1 protein, indicative of a replicative transposition event. For both transgenes, the second copy of the insertion mapped to the third chromosome, and these stocks were balanced using *TM6C Tb Sb*. To differentiate these stocks from those with the single copy of the transgene, we refer to them in figures and text with the suffix "2X."

Fly stocks, genetics, and culture: All *dfmr1* mutant stocks in this study were derived from a w^{1118} background and maintained on a yeast–cornmeal–molasses medium at 25°. The third chromosome balancer *TM6C Tb Sb* was used to maintain *dfmr1* alleles.

Courtship and circadian behavior analyses: For courtship behavior testing, males of the appropriate genotypes were collected within 2 hr of eclosion and kept in isolation prior to testing. Female targets were of the genotype *XX, y, f* (attached X) and collected as virgins for courtship testing. All flies were kept in 12:12 light/dark (LD) cycles at 25° and 70–75% relative humidity and were aged for 4 days prior to analysis. For the naive courtship analysis, the 4-day-old male and female were transferred via aspiration to a mating chamber 20 mm in diameter and 5 mm deep. These chambers were kept in humidified conditions throughout the assay. Transferred males were given a 5-min recovery period prior to addition of the female target. All assays were performed within 30 min of

the change in light cycle. Males were monitored for courtship activity that included following of the female, wing extension and vibration, tapping of the female with his foreleg, and attempted copulation for a period of 10 min or until copulation occurred. The percentage of time that the male spent in active pursuit of the female was recorded as the courtship index. A minimum of 25 animals was tested for each genotype.

Circadian behavior was tested as described in Dockendorff *et al.* (2002). Flies were entrained to a 12:12 light/dark cycle, placed into activity monitors (Trikinetics, Waltham, MA), maintained in light/dark cycles, and then placed under constant darkness. Locomotion activity was collected in 30-min bins. The percentage of flies judged to be rhythmic was assessed by Clocklab software (Actimetrics, Evanston, IL) as follows: Using a confidence level of 0.025, batch analyses were performed for the genotypes tested, monitoring the locomotion activity in constant darkness over 7 days. The difference between the power (1) and significance (1) values was calculated for each fly, and a value of <10 was the basis for judging an arrhythmic phenotype. Visual analyses of periodograms and actograms were also conducted to confirm the results.

Antibodies and immunocytochemistry: Larval neuromuscular junction (NMJ) type I boutons were detected by staining third instar larval fillets with antihorseradish peroxidase (Cappel, Aurora, OH) at a dilution of 1:200. Mushroom bodies were visualized by staining whole mounts of brains with anti-FasII at a 1:10 dilution (mAb 1D4 obtained from University of Iowa Developmental Studies Hybridoma Bank). Secondary antibodies conjugated to either HRP or fluorochrome were obtained from Jackson ImmunoResearch (West Grove, PA) and used at a 1:200 dilution. Confocal images were collected on an Olympus FV500 microscope. Western blots were performed as described in Wan *et al.* (2000) using anti-dFMR1 antibody 5A11 at a 1:1000 dilution and anti-β-tubulin mAb E7 (both from the University of Iowa Developmental Studies Hybridoma Bank) at a 1:400 dilution.

Statistical analyses: Courtship indexes were arcsin transformed and then analyzed by one-way ANOVA, followed by a Tukey–Kramer post-test. NMJ bouton counts were analyzed by one-way ANOVA with a Tukey–Kramer post-test or by a Kruskal–Wallis test, followed by a Dunn post-test. The analyses of courtship indexes and NMJ bouton counts were conducted using InStat software from GraphPad (San Diego). Comparisons of mushroom-body (MB) axon midline crossings and circadian rhythmicity were made by a chi-square test for homogeneity.

RESULTS

Generation of point mutations in KH domains of *dfmr1*: A 14-kb genomic rescue fragment has previously been shown to rescue all known behavioral and developmental phenotypes associated with *dfmr1* loss of function (Dockendorff *et al.* 2002; Lee *et al.* 2003; Michel *et al.* 2004; Costa *et al.* 2005). Subclones of this fragment were subjected to site-directed mutagenesis to convert highly conserved isoleucine residues in the two KH domains to asparagines (I244N, I307N for the Drosophila protein; Figure 1A). These substitutions are predicted to strongly inhibit binding of cognate RNA substrates (Lewis *et al.* 2000; Darnell *et al.* 2005) and may interfere with folding of the KH domain

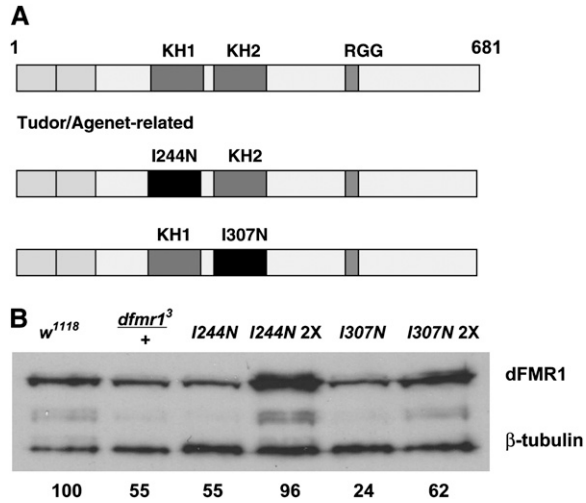

Figure 1.—Schematic of fragile X protein and expression analysis of *dfmr1* KH domain alleles. (A) RNA-binding domains of FMRP. Two KH domains, an RGG box, and two tandem copies of a Tudor/Agenet-related domain have all been demonstrated to bind RNA. KH domains with conserved isoleucine residues mutated to asparagine for this study are depicted, (B) Western blot of total fly extracts from wild-type, *dfmr1*^{3} heterozygote, and flies expressing one or two (2X) copies of a transgene harboring a *dfmr1* genomic rescue fragment coding for either an I244N or an I307N substitution in the KH domains. Extracts were prepared from males aged 2–3 days. Signals were scanned and quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA), and average expression levels compared to a *w*^{1118} control from six independent blots are given.

(Musco *et al.* 1996, 1997). Upon reconstruction of mutated sequences to the rescue fragment, the mutant rescue fragments were introduced to flies via *P*-element transformation and then recombined to chromosomes harboring the *dfmr1*^{3} allele, a deletion null allele where the entire open reading frame of *dfmr1* is removed (Dockendorff *et al.* 2002; Pan *et al.* 2004). Crossing such chromosomes to the *dfmr1*^{3} chromosome results in flies heterozygous for the *P*-element transgenes and that express only the mutant alleles under control of the endogenous *dfmr1* promoter. Since it might be expected that an *I244N I307N* double mutant could have an additive effect on any phenotypes observed with the single KH domain mutants, we attempted to obtain stocks with such an allele. Despite extensive efforts, we failed to obtain transgenic animals that harbored an *I244N I307N* double mutation of *dfmr1* that was expressed via its endogenous promoter even in an otherwise wild-type background. Although *UAS-GAL4* overexpression of a *dfmr1* cDNA with the *I244N I307N* double mutation in the developing eye fails to induce a rough eye phenotype (Wan *et al.* 2000), we have observed that overexpression of the same transgene by *myosin heavy chain-GAL4* can be lethal to pupae, indicating that such an allele can have dominant deleterious effects in specific tissues (T. C. Dockendorff, unpublished observations).

To discern the level of dFMR1 protein expression from the transgenes, Western blotting was performed on male flies harboring the mutant KH domain transgenes as the sole allele of *dfmr1*. These studies show that flies with a single copy of the transgene with the *I244N* allele express the mutant protein at a level very similar to that seen with a dfmr1 heterozygote. The single copy of the I307N transgene had ~25% the expression of dFMR1 protein present in a control w^{1118} background (Figure 1B). To create stocks with increased doses of each transgene, the *P* elements were mobilized and stocks that had undergone replicative transposition resulting in elevated levels of the mutant proteins were selected. These stocks were judged to express mutant proteins at 97 and 62% of the level seen with w^{1118} for the I244N and I307N substitutions, respectively (Figure 1B). To control for dosage effects, *dfmr1* heterozygotes are included in all of the following analyses. Since the human I304N substitution has been hypothesized to exert dominant effects, we examined our KH domain allele stocks for dominant phenotypes through analysis of flies that expressed both a mutant and wild-type allele of *dfmr1*. These stocks are noted in figures with both the KH domain allele designation and a "+" for the wild-type allele.

The NMJ bouton overgrowth phenotype associated with null alleles of *dfmr1* is not observed with the I244N or I307N KH domain substitutions: Strong or null alleles of *dfmr1* result in an overgrowth of larval neuromuscular junction boutons (Zhang *et al.* 2001; Jin *et al.* 2004b). To assess the impact of the KH domain mutations on this phenotype, we analyzed the numbers of type I boutons at larval NMJs using flies with wild-type and null alleles of *dfmr1* as controls. We examined muscles 4, 6/7, and 12 from segment A3 for the analysis of larval NMJs. Figure 2 shows that, in all muscles examined, there is no significant increase in bouton number over wild type or *dfmr1* heterozygote controls from larvae with a single copy of a transgene expressing either I244N or I307N substitutions as the sole source of dFMR1 protein, while there is the expected pronounced overgrowth of boutons from larvae homozygous for the null allele of *dfmr1*. These results indicate that neither of the Ile → Asn substitutions in the KH domains affects the ability of dFMR1 to regulate larval NMJ bouton numbers and thus suggests that other domains of FMRP play a more vital role in this process.

Analysis of midline crossing frequency in mushroom-body β-lobe neurons: Axon development defects have been reported in the central nervous system of flies homozygous for null alleles of dfmr1. The ventral lateral neurons, dorsal cluster neurons, and neurons of the mushroom body all have visible defects in branching, neurite extension, and/or guidance (Dockendorff *et al.* 2002; Morales *et al.* 2002; Michel *et al.* 2004; Pan *et al.* 2004; Reeve *et al.* 2005). A significant fraction of flies homozygous for strong or null alleles of *dfmr1*

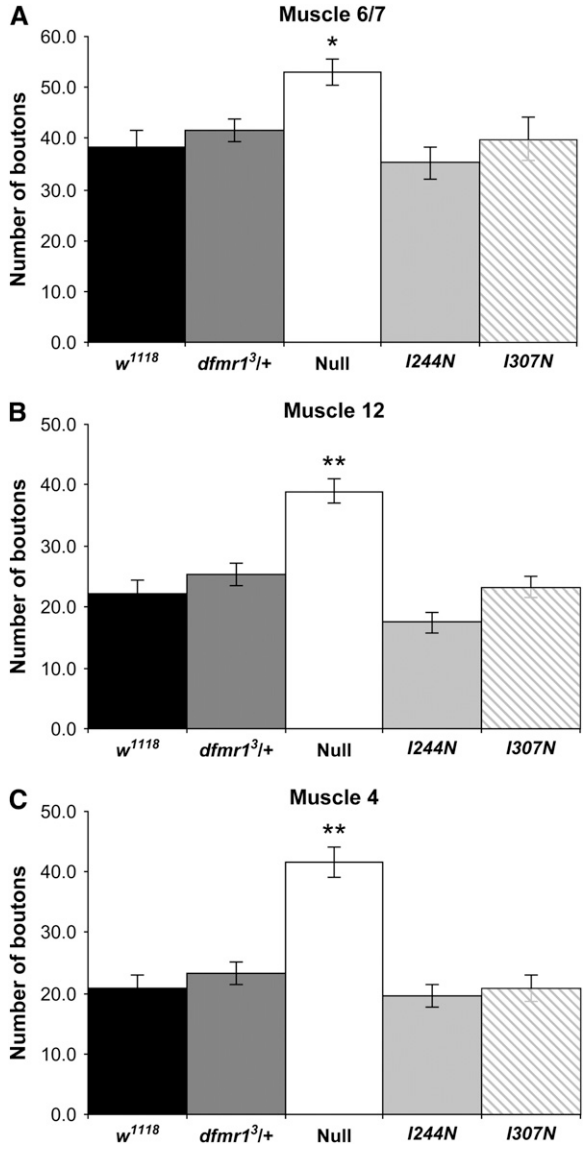

Figure 2.—Numbers of larval NMJ boutons are not increased in flies expressing KH domain I244N or I307N substitutions as a sole source of dFMR1 protein. Third instar larvae were dissected and probed with antibodies against horseradish peroxidase to assess numbers of type I NMJ boutons, which are increased in flies homozygous for strong or null alleles of *dfmr1* (Zhang *et al.* 2001; Jin *et al.* 2004b). Analyses of several muscle types from segment A3 show that type I boutons are significantly increased in all muscle types examined from animals homozygous for a null allele of *dfmr1* compared to all wild-type and KH domain alleles examined ($P < 0.001$ for muscle 4, Kruskal–Wallis test and Dunn post-test; $P < 0.01$ for muscle 6/7, one-way ANOVA, followed by a Tukey–Kramer post-test; $P < 0.001$ for muscle 12, Kruskal–Wallis test and Dunn post-test). There are no significant changes in type I bouton numbers when wild-type controls are compared with either of the two KH domain mutants. The allele designations denote the sole source of dFMR1 protein. Results are from analysis of at least 20 hemi-segments for each genotype.

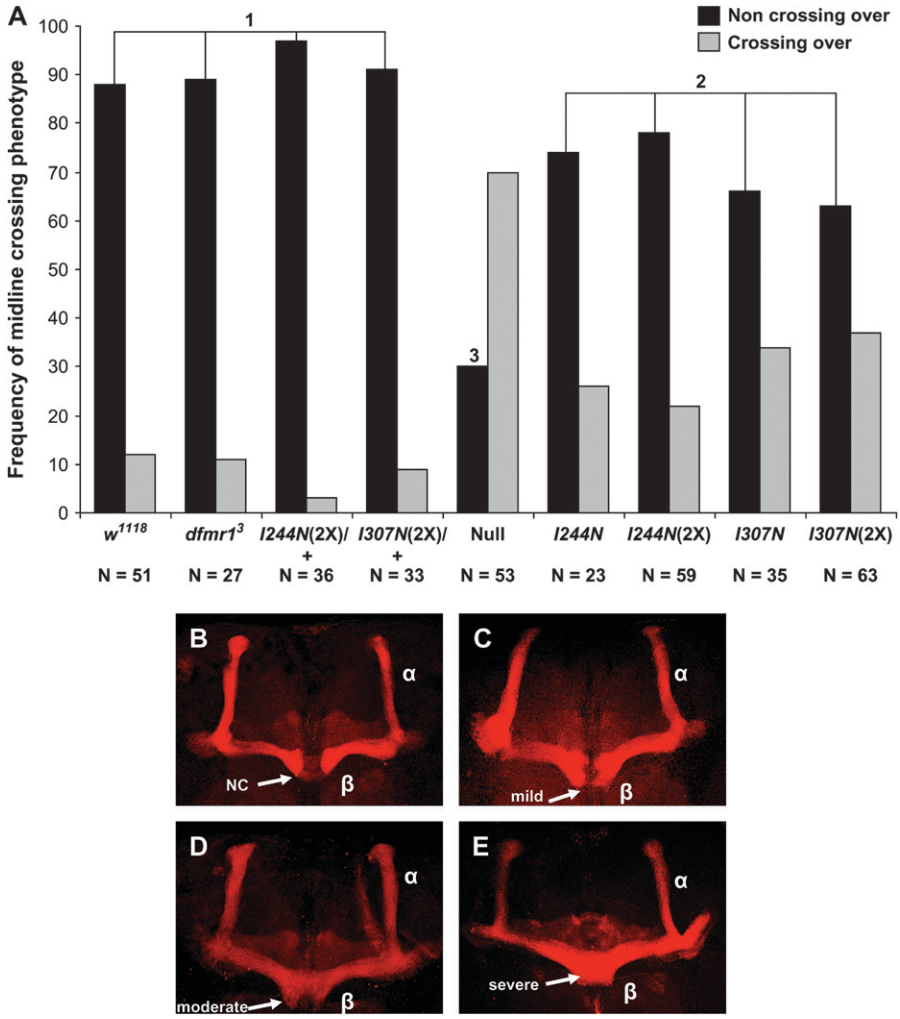

FIGURE 3.—MB β-lobe phenotypes of flies with *dfmr1* KH domain alleles. (A) Representation of the frequency with which a midline crossing of β-lobe neurons was observed. Anti-FasII staining of MBs from 2-day-old flies shows that the I244N and I307N substitutions result in a frequency of midline crossing phenotypes intermediate to flies with a wild-type allele of *dfmr1* and to those homozygous for a *dfmr1* null allele. The genotypes denote the allele of *dfmr1* being expressed, while the presence of a wild-type allele to test for dominant effects of the mutant allele is denoted by a "+." The frequency with which a no-crossing phenotype occurred did not change upon an increase in dosage of either mutant KH domain protein. Expression of either mutant KH domain transgene in a background with a wild-type copy of *dfmr1* present has no effect on midline crossing frequency, indicating that the transgene insertions and mutant proteins do not elicit a detectable dominant effect. Genotypes grouped under a common numerical designation do not differ from each other in percentage of brains observed with a midline crossing of β-lobe axons, while those under different numerical designations differ from each other as judged by chi-square tests of homogeneity. KH domain alleles differ from the null allele in frequency of midline crossing ($P < 0.0001$) and from flies with a wild-type allele of *dfmr1* ($P = 0.0152$). (B–E) Representative examples of MB morphology illustrating the variety of midline crossing phenotypes observed. α- and β-Lobes are noted, while arrows point to the midline where crossovers of the β-lobe neurons may occur. NC, no crossover.

have midline crossings of MB β-lobe neurons (MICHEL *et al.* 2004). To monitor the effects of the KH domain alleles on axon development, we used the mushroom-body β-lobe phenotype described by MICHEL *et al.* (2004), visualizing MBs in whole-brain mounts from 2-day-old animals via anti-FasII immunostaining. Figure 3 shows that, when compared to a wild-type allele control, flies expressing dFMR1 with the I244N or I307N substitution had a significant increase in the frequency of midline crossings compared to controls where a wild-type allele of *dfmr1* was present, but this frequency was not as great as that observed in brains from flies homozygous for a null allele of *dfmr1* (Figure 3). Thus, the KH domains of dFMR1 play a role in regulating processes that contribute to normal axon development, and the other dFMR1 domains must contribute functions as well. We also examined the midline crossing phenotype in brains from flies where the dosage of either KH domain allele was increased. The frequency of midline crossings did not change significantly from what was observed with the single dose, indicating that the Ile → Asn substitutions have a strong effect on the function of the KH domains and that other domains of dFMR1 are not able to compensate for the defects.

The KH domain mutations display a partially penetrant circadian phenotype in constant darkness: Flies with null alleles of *dfmr1* fail to retain circadian rhythmicity when transferred into constant darkness (DD) with ~80% penetrance (DOCKENDORFF *et al.* 2002; INOUE *et al.* 2002; MORALES *et al.* 2002). The KH domain mutants had circadian behavior examined by monitoring rest/activity rhythms in both LD and DD. Flies with either mutant allele are capable of responding to light, as judged by their rhythmic locomotion activity (T. DOCKENDORFF and J. PARK, unpublished observations). As was seen with the MB β-lobe phenotype, both KH domain substitutions resulted in a statistically significant increase in the percentage of flies that fail to retain rhythmic locomotion activity in constant darkness when compared to flies with a wild-type allele of *dfmr1* (Figure 4). Likewise, the percentage of KH domain mutants lacking rhythmic activity is not as great as that observed

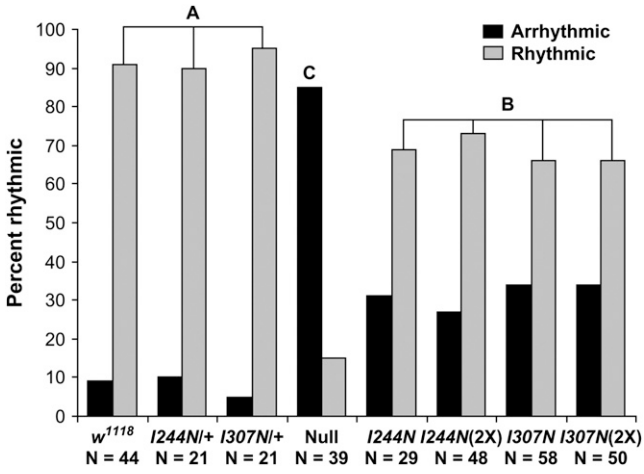

Figure 4.—Analysis of circadian locomotion activity of flies expressing dFMR1 with mutant KH domains in constant darkness. An assignment of rhythmic *vs.* arrhythmic activity for individual flies was determined using ClockLab software as described in materials and methods. The percentage of flies from each genotype judged to be rhythmic was compared by a chi-square test for homogeneity. Genotypes that are grouped by a common number do not have any significant difference between them in the percentage of animals displaying a rhythmic locomotion phenotype, while separate groups differ to a confidence level of <0.0001. Increasing the dose of mutant dFMR1 protein had no significant effect on the percentage of animals judged to have maintained rhythmic locomotion activity, indicating that other RNA-binding domains of the mutant proteins are unable to compensate for the defects in the KH domains. The genotypes denote the allele of *dfmr1* that is the sole source of dFMR1 protein, while the presence of a wild-type allele to test for dominant effects of the mutant allele is denoted by a "+." Flies that express both a mutant and wild-type allele of *dfmr1* resemble wild-type flies in the percentage of animals judged to be rhythmic, demonstrating that the mutant allele and transgene insertion do not have a detectable dominant effect.

for flies homozygous for the $dfmr1^3$ null allele, demonstrating that the KH domain alleles result in partial loss of function. For mutant flies determined to have retained rhythmic locomotion activity in constant darkness, the DD period of such flies did not significantly differ from flies with a wild-type *dfmr1* allele (not shown). Flies expressing both the mutant transgene allele and a wild-type allele of *dfmr1* do not differ from wild-type controls in the percentage of animals judged to have retained rhythmic locomotion, demonstrating that the insertions and mutant alleles have no dominant effect. An increase in dosage of either KH domain allele had no effect on the percentage of flies judged to be arrhythmic, again demonstrating a strong loss-of-function effect on the KH domains and the inability of other dFMR1 domains to provide compensatory function for this phenotype.

Naive courtship activity is reduced in flies with either KH domain mutation: Courtship in Drosophila consists of stereotyped behaviors by males toward receptive females (reviewed by Hall 1994; Greenspan and Ferveur 2000). The courtship process has thus been used as an ethologically relevant measure of Drosophila behavior. Flies with a null allele of *dfmr1* have deficits in naive courtship activity (Dockendorff *et al.* 2002), which is measured as the percentage of time that a male fly spends in courtship activity toward a receptive virgin female during a given period of time and defined as the courtship index. We analyzed the naive courtship activity of flies with the KH domain alleles, measuring the time spent by the male in following the female target, wing extension and vibration, tapping with foreleg, and attempted copulation (Figure 5). Flies that harbor both a copy of the transgene bearing either of the KH domain alleles and a wild-type allele of *dfmr1* do not significantly differ from a wild-type control in the amount of time engaged in courtship behavior, showing that these insertions and alleles do not result in any dominant effect that contributes to the phenotype (Figure 5, A and B). Our analyses of courtship activity in flies where the KH domain allele is the only source of dFMR1 show that the Ile → Asn substitutions in either KH domain has a significant adverse effect on the courtship index when compared to flies with a wild-type allele. The values for the courtship index of both KH domain mutants are also significantly different from those seen with the null allele of *dfmr1*, indicating that dFMR1 proteins with the KH domain point mutations still have activity and behave as partial loss-of-function alleles. An increase in the dosage of either KH domain allele results in only a small, statistically insignificant increase in the courtship index. This increase could be accounted for by elevated expression of the *mini white* marker associated with the second copy of the *P*-element vector, since the *white* gene is known to positively influence courtship activity (reviewed by Hall 1994). Thus, as was seen with the morphology of mushroom bodies and circadian locomotion, an increased dosage of the mutant KH domain alleles did not provide rescue of this phenotype.

DISCUSSION

The vast majority of human fragile X cases arise through the expansion of a trinucleotide repeat, resulting in silencing of the *FMR1* gene. Thus, relatively little insight into structure–function relationships has been gained from analysis of human *FMR1* alleles. Biochemical and cell-culture-based studies of fragile X proteins have uncovered several RNA-binding domains that likely contribute to their *in vivo* function. The amenability of *Drosophila melanogaster* to transgenics provides an opportunity to conduct structure–function studies of FMRP in the context of an intact animal. To this end, we have generated flies expressing *dfmr1* alleles where a codon for a highly conserved isoleucine residue in each of the two KH domains was mutated to code for asparagine. These mutations are predicted to result in a

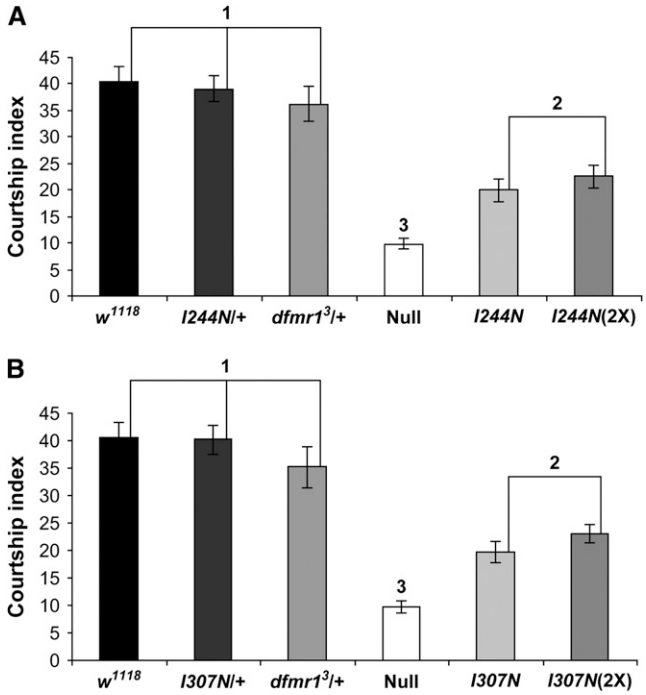

FIGURE 5.—Analysis of naive courtship activity of flies expressing dFMR1 KH domain mutations. Naive courtship was analyzed as described in MATERIALS AND METHODS. At least 25 flies of each genotype were tested. For each mutant KH domain transgene, expression in a background with a wild-type allele of *dfmr1* does not result in a phenotype differing from wild type, indicating that the transgene insertion and mutant protein do not induce a detectable dominant effect. (A) Flies expressing dFMR1 with the I244N substitution as the sole source of dFMR1 protein have a significant decrease in naive courtship activity compared to flies with a wild-type allele of *dfmr1*, but the decrease in courtship activity is not as strong as is observed in flies homozygous for a *dfmr1* null allele. Increasing the dosage of the *I244N* allele does not result in a significant increase in courtship activity. Courtship indexes were arcsin transformed and the data were analyzed by a one-way ANOVA, followed by a Tukey–Kramer post-test. The *P*-value for the ANOVA is <0.0001. Genotypes under the same numerical heading do not vary from each other to a significant extent, while genotypes under different numerical headings are significantly different from each other ($P < 0.05$). (B) The I307N substitution results in a significant decrease in naive courtship activity compared to flies expressing wild-type dFMR1, but not to the degree observed with flies homozygous for a null allele of *dfmr1*. As was seen with the *I244N* allele, an increase in dosage of the *I307N* allele does not produce a significant increase in naive courtship activity. The data for these genotypes were processed in the same manner as the *I244N* flies, and the *P*-value for the ANOVA is <0.0001. Genotypes under the same numerical grouping do not differ from each other in courtship activity to a significant extent, while genotypes under different numerical groupings have a significant variation in the courtship index ($P < 0.01$).

strong loss of affinity for specific RNA ligands as judged from structural (LEWIS *et al.* 2000; RAMOS *et al.* 2003) and biochemical studies (DARNELL *et al.* 2005) and may interfere with the ability of FMRP to interact with other proteins as well (FENG *et al.* 1997; LAGGERBAUER *et al.* 2001; RAMOS *et al.* 2003). We then examined the effects of these mutations on neural development and behavior phenotypes that are associated with null alleles of *dfmr1*. Several conclusions can be made from these results. Neither of the KH domain alleles produced a phenotype that matched the degree of severity seen with the *dfmr1* deletion null allele. For all phenotypes analyzed, the KH domain alleles were recessive to the wild-type *dfmr1* allele. The failure of increased dosage of the mutant proteins to provide any significant measure of rescue indicates that the Ile → Asn substitutions are strong loss-of-function mutations in the KH domains, which is consistent with past biochemical and biophysical analyses of KH domains (LEWIS *et al.* 2000; RAMOS *et al.* 2003; DARNELL *et al.* 2005). These results suggest that the ability of the mutant proteins to bind certain RNA species *in vivo* is lost and that other RNA-binding domains of dFMR1 cannot compensate for the defect. Prior studies have shown that the individual FMRP RNA-binding domains can bind RNA as a discrete unit *in vitro* (ADINOLFI *et al.* 1999; DARNELL *et al.* 2001, 2005; SCHAEFFER *et al.* 2001), and our results are consistent with the observations from these studies. The partial loss-of-function phenotypes resulting from these KH domain alleles demonstrate that the mutant proteins retain function and must be able to bind RNA and assemble into at least some mRNP complexes *in vivo*.

KH domain phenotypes and roles for other RNA-binding domains of FMRPs: Why is there a difference between the lack of phenotype seen with the larval NMJ bouton numbers and the partial loss of function observed for other phenotypes analyzed? It is possible that regulation of a different subset of RNAs is involved in larval NMJ bouton development and that these RNAs are not reliant upon interaction with dFMR1 KH domains to conduct their functions and be appropriately regulated. A number of possibilities can explain the partial loss-of-function phenotypes associated with mushroom-body development, courtship behavior, and circadian locomotion activity. It could be that dFMR1 regulates the activity of multiple genes involved in these processes and that the KH domains are responsible for modulating the activity of a subset of these genes. Multiple RNA-binding domains in dFMR1 could also regulate the activity of any one transcript, and loss of KH domain function could lead to a partial degree of misregulation for such transcripts that result in the degree of phenotype observed.

That we did not observe a null phenotype with either of the KH domain alleles makes it rather probable that the other RNA-binding domains of FMRP significantly contribute to its *in vivo* function. Along with the G-quartet binding RGG box, the N-terminal 217 amino acids of human FMRP also binds RNA (ADINOLFI *et al.* 1999, 2003; ZALFA *et al.* 2005). The N-terminal amino acids of FMRP are well conserved between mammals and insects (WAN *et al.* 2000) and are related to methyl-substrate-binding domains that are associated with

chromatin regulation (Maurer-Stroh *et al.* 2003). This is of interest because FMRP can be detected in the nucleus and has biochemical and genetic interactions with Argonaute proteins (Verheij *et al.* 1993; Caudy *et al.* 2002; Ishizuka *et al.* 2002; Jin *et al.* 2004b; Xu *et al.* 2004). Mutations of RNA interference (RNAi) components affect silencing of heterochromatin in *D. melanogaster* (Pal-Bhadra *et al.* 2004; see Lippman and Martienssen 2004; Matzke and Birchler 2005; Wassenegger 2005 for reviews of RNAi-based regulation of chromatin). Indeed, it has recently been reported that a null allele of *dfmr1* affects *white* gene expression in centromeric heterochromatin (Deshpande *et al.* 2006). If an FMRP is part of a complex that modulates chromatin structure via RNAi-based mechanisms, the loss of such activity via a null mutation could conceivably affect the expression of many genes that might be part of the fragile X pathway, including those that may contribute to the phenotypes examined in this study.

Comparing phenotypes of the human I304N and Drosophila I307N substitutions: The failure of the I304N protein to bind certain RNAs and its abnormal mRNP fractionation profile have been suggested as the basis for the unusually severe fragile X phenotypes observed with a patient expressing the I304N substitution (Feng *et al.* 1997; Darnell *et al.* 2005) and have been hypothesized to arise from a dominant effect exerted by the I304N protein (Feng *et al.* 1997). The studies here provide an opportunity to make comparisons between the human I304N and Drosophila I307N phenotypes. Given the severity of fragile X phenotypes associated with the I304N substitution, our findings of relatively modest phenotypes associated with the analogous I307N substitution in the Drosophila model may seem surprising. The failure to observe a phenotype with the I307N protein that matches the strength of a *dfmr1* null allele means that the I307N protein must be able to bind RNA and to assemble into at least a subset of the mRNP complexes that the wild-type protein does. We feel that several hypotheses are plausible for explaining the differences observed between the human I304N and the Drosophila I307N phenotypes. Subtle differences in substrate binding by these KH domains is one possibility, with the human I304N protein unable to bind certain critical RNA substrates, while the binding of orthologous substrates (if they exist) to the Drosophila I307N protein is impaired to a lesser degree. In vertebrates, a FMRP interacts with FXR1 and FXR2 proteins that are not present in *D. melanogaster* (Zhang *et al.* 1995). Since the I304N protein can interact with both FXR1 and FXR2 *in vitro* (Laggerbauer *et al.* 2001; Mazroui *et al.* 2003), it is possible that I304N FMRP could induce a deleterious gain-of-function effect on either FXR protein that enhances the severity of the phenotype. Alternatively, since not all I304N protein cofractionates with FXR2 (Feng *et al.* 1997), the I304N protein may acquire a deleterious function when not in complex with FXR2. Another consideration is the possibility of unusual contributions from the genetic background of the I304N individual that could enhance the fragile X phenotypes. To recapitulate the I304N substitution in the mouse model could be helpful in discerning whether these final two hypotheses have validity. Finally, none of the above explanations are mutually exclusive, and thus any combination of these scenarios might contribute to the severity of the fragile X phenotypes observed with the I304N patient.

The KH domain alleles as probes for FMRP functions: What RNAs do fragile X proteins bind to? What is the composition of a fragile X protein-containing mRNP particle? These are questions that still dominate research on FMRP. Biochemical screens utilizing microarrays, along with yeast two-hybrid screens and proteomics approaches, have been undertaken to address these questions (Ceman *et al.* 1999; Brown *et al.* 2001; Darnell *et al.* 2001; Schenck *et al.* 2001; Ishizuka *et al.* 2002; Miyashiro *et al.* 2003; Costa *et al.* 2005; Zarnescu *et al.* 2005) and have uncovered several interacting proteins and dozens of RNA species identified as candidate ligands. *In vivo* genetics-based analyses of these interactions will be necessary for their final validation. Drosophila has proven to be a significant model for study of fragile X protein function owing to the similarities with vertebrate models in biochemical properties and loss-of-function phenotypes (Wan *et al.* 2000; Zhang *et al.* 2001; Dockendorff *et al.* 2002; McBride *et al.* 2005). Thus, the tools of Drosophila genetics will be instrumental in identifying and ordering the physiologic pathways that fragile X protein regulates. In addition to their value as stocks with sensitized backgrounds for probing genetic interactions, these stocks with the KH domain mutations will serve as useful comparative tools for biochemical and genetic studies in identifying the RNAs regulated by the fragile X protein and the protein interactions needed for its role in neural function.

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