Fragile X-Related Proteins Regulate Mammalian Circadian Behavioral Rhythms

Jing Zhang,[1] Zhe Fang,[1] Corinne Jud,[2] Mariska J. Vansteensel,[3] Krista Kaasik,[4] Cheng Chi Lee,[4] Urs Albrecht,[2] Filippo Tamanini,[5] Johanna H. Meijer,[3] Ben A. Oostra,[6] and David L. Nelson[1,*]

Fragile X syndrome results from the absence of the fragile X mental retardation 1 (*FMR1*) gene product (FMRP). *FMR1* has two paralogs in vertebrates: *fragile X related gene 1* and *2* (*FXR1* and *FXR2*). Here we show that *Fmr1/Fxr2* double knockout (KO) and *Fmr1* KO/*Fxr2* heterozygous animals exhibit a loss of rhythmic activity in a light:dark (LD) cycle, and that *Fmr1* or *Fxr2* KO mice display a shorter free-running period of locomotor activity in total darkness (DD). Molecular analysis and in vitro electrophysiological studies suggest essentially normal function of cells in the suprachiasmatic nucleus (SCN) in *Fmr1/Fxr2* double KO mice. However, the cyclical patterns of abundance of several core clock component messenger (m) RNAs are altered in the livers of double KO mice. Furthermore, FXR2P alone or FMRP and FXR2P together can increase PER1- or PER2-mediated BMAL1-Neuronal PAS2 (NPAS2) transcriptional activity in a dose-dependent manner. These data collectively demonstrate that *FMR1* and *FXR2* are required for the presence of rhythmic circadian behavior in mammals and suggest that this role may be relevant to sleep and other behavioral alterations observed in fragile X patients.

Introduction

Fragile X syndrome (FXS [MIM #300624]) is the most common form of inherited mental retardation, with an estimated prevalence of 1 in 4000 males and 1 in 8000 females.[1] Typical fragile X patients display physical and behavioral abnormalities, such as a long, narrow face, large ears, macroorchidism, cognitive impairment, hyperactivity, and autistic behaviors.[2] The common form of this syndrome is caused by an expansion mutation of a CGG triplet repeat in the 5′ UTR of the *FMR1* gene, which transcriptionally silences the gene and leads to loss of FMRP.[3] FMRP is an RNA-binding protein that associates with translating polyribosomes.[4–6] *Fmr1* mRNA and FMRP are expressed ubiquitously, particularly the brain and testes.[7]

FMRP (MIM *309550), FXR1P (MIM *600819), and FXR2P (MIM *605339) compose a small family of RNA-binding proteins (fragile X-related protein family; *FXR* family).[8,9] These three proteins share >60% amino acid identity and are able to form homo- and heterotypic interactions with each other.[9,10] It has been postulated that the presence of FXR1P and FXR2P could partially compensate for the loss of FMRP in the fragile X syndrome. *Drosophila* has a single gene, *Drosophila* FMR1 (*dfmr1*) or *Drosophila* Fmr1-related gene (*dfxr*), whose product shares sequence identity and biochemical properties with the fragile X-related protein family.[11] To unravel the molecular and cellular bases of fragile X syndrome and to explore therapeutic strategies, both *Drosophila* and mouse models have been produced. We and others tested *dfmr* loss-of-function flies and found altered circadian behavior without detectable alteration in central clock components.[12,13] The disturbed activity-rest cycle of the fly, together with the observed sleep disorders in fragile X patients,[2,14] led us to investigate circadian behavior in mice with viable mutations in members of the *FXR* family.[15,16]

In this study, we demonstrate that mice lacking FMRP or FXR2P display a shorter free-running period of locomotor activity in total darkness. In the absence of both proteins, completely arrhythmic activity was observed in animals maintained in a light:dark cycle. This arrhythmic phenotype is unique among circadian defects described in mouse models to date. Interestingly, in the absence of FMRP/FXR2P, the cyclical patterns of abundance of several core clock component mRNAs are altered in the liver, which contains a major peripheral clock. By using an in vitro luciferase transfection assay, we observed that FXR2P alone or FMRP and FXR2P together can increase PER1- or PER2-mediated BMAL1-NPAS2 transcriptional activity in a dose-dependent manner. These data collectively demonstrate that *FMR1* and *FXR2* are required for the presence of behavioral circadian rhythms and indicate that this role may be relevant to the behavioral alterations observed in fragile X patients.

Material and Methods

Locomotor Assays

All experiments were performed with male C57BLl/6J mice of the following genotypes: *Fmr1* KO, *Fxr2* KO, *Fmr1/Fxr*2 double KO, *Fmr1* KO/*Fxr2* heterozygous, and their nonmutant littermates as controls (6- to 10-month-old). Animals were individually housed in standard cages equipped with running wheels within ventilated, light-tight chambers with time-controlled lighting.

[1]Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA; [2]Department of Medicine, Division of Biochemistry, University of Fribourg, 1700 Fribourg, Switzerland; [3]Department of Molecular Cell Biology, Leiden University Medical Center, Postal Zone S5-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands; [4]Department of Biochemistry and Molecular Biology, University of Texas Health Science Center, Houston, TX 77030, USA; [5]MGC, Department of Genetics, [6]Department of Clinical Genetics, Erasmus University Medical Center, 3000 CA Rotterdam, The Netherlands
*Correspondence: nelson@bcm.tmc.edu
DOI 10.1016/j.ajhg.2008.06.003.

Experimental animals were continuously provided with food and water. Wheel-running activity was recorded by a computer data-acquisition system (Chronobiology kit, Stanford Software Systems). Animals were maintained on a 12:12 light/dark cycle for 2 weeks and then were transferred to constant darkness for at least 2 weeks. The raw activity data can be visualized and analyzed with a χ^2 periodogram (Chronobiology kit). The data were plotted in double plot activity histograms. The free-running period, τ, and total activity were analyzed with the Chronobiology kit.

In Situ Hybridization

Adult wild-type (6- to 10-month-old), *Fmr1* mutant, *Fxr2* mutant, and *Fmr1/Fxr2* double mutant mice were maintained in 12:12 LD cycle for 2 weeks and then released into DD. At 36^{th}, 42^{nd}, 48^{th}, and 54^{th} hour in the constant darkness, the mice were sacrificed for the experiments. Specimen preparation and in situ hybridization were carried out as described previously.[17] In brief, the [^{35}S]UTP (1250 Ci/mmol, PerkinElmer)-labeled riboprobes were synthesized with the RNAMaxx High Yield transcription kit (Stratagene) according to manufacturer's protocol. The *mBmal1* probe was made from a cDNA corresponding to nucleotides (nt) 654–1290 (accession number AF015953), *mCry1* corresponds to nt 190–771 (AB000777), *mPer1* to nt 620–1164 (AF022992), and *mPer2* to nt 229–768 (AF036893). 7 μm thick paraffin sections were dewaxed, rehydrated, and fixed in 4% paraformaldehyde. Sections were then permeabilized with a proteinase K (Roche) digestion before they were fixed again and acetylated. After serial dehydration, hybridization was performed overnight at 55°C in a humid chamber. Stringency washes were carried out at 63°C. Slides were subjected to a ribonuclease A (Sigma) digestion and then dehydrated in graded ethanol series. Quantification was performed by densitometric analysis (GS-700 or GS-800, BioRad) of autoradiography films (Amersham Hyperfilm) with the Quantity One software (BioRad). Data from the SCN were normalized by subtracting the optical density measured in the lateral hypothalamus next to the SCN. For each experiment, 2 animals per genotype were used and 4 to 9 adjacent SCN sections per animal were analyzed. Relative RNA abundance values were calculated by defining the highest wild-type mean value of each experiment as 100%. For statistical analysis, two-way ANOVA with Bonferroni post-test (GraphPad Prism software) was performed.

In Vitro Electrophysiology

The multiunit electrical activity rhythms of SCN neurons were recorded as described previously.[18] In short, brains of WT, *Fmr1* KO, and *Fmr1/Fxr*2 double KO mice were rapidly dissected from the skull at the same Zeitgeber times (1.7 hr to 2.4 hr after lights on). Coronal hypothalamic slices that contained the SCN were sectioned and transferred to an interface chamber. Slices were oxygenated with humidified 95% O_2/5% CO_2 and perfused with artificial cerebrospinal fluid (ACSF) at 35°C. We used one slice per animal and attempted simultaneous recordings of multiunit neuronal activity from the dorsal and ventral SCN with two stationary electrodes.

Northern Blot Analysis

Liver total RNA was isolated by guanidinium isothiocyanate extraction and CsCl centrifugation[19] from adult wild-type (6- to 10-month-old), *Fmr1* mutant, *Fxr2* mutant, and *Fmr1/Fxr2* double mutant mice. These animals were maintained in 12:12 LD cycle for 2 weeks and then released into DD for at least 24 hr. For all northern blot analyses, 25 μg of total RNA was separated on 1.2% agarose-formaldehyde gels and transferred to nylon membranes. The UV crosslinked membranes were hybridized with ^{32}P-labeled cDNA probes for *Bmal1, Clock, Per1, Per2*, *Cry1, Npas2*, and *Actin* according to the instructions of the manufacturer (BD Biosciences). To calculate relative RNA abundance, optical densities of *Bmal1, Clock, Per1, Per2, Cry1*, and *Npas2* hybridization were divided by densities from *Actin* hybridization to the same blot. Normalized values were then averaged for the blots prepared from different sets of RNA samples.

Real-Time PCR

The same RNA isolated for northern blot analysis was also used for real-time PCR analysis. To prepare cDNA, 1–3 μg total RNA was reverse-transcribed with random primers at a final concentration of 3 mM $MgCl_2$, 75 mM KCl, and 50 mM Tris-HCl (pH 8.3), 1 mM each of dNTPs, 10 units of RNase inhibitor, and 100 units of Superscript II Rnase H$^-$reverse transcriptase (Invitrogen). The reaction mixture was incubated at 37°C for 60 min. Real-time PCR was performed with the ABI PRISM 7000 Sequence Detector System (SDS) (Applied Biosystems) and MicroAmp optical 96-well plates (Applied Biosystems). Gene expression assays were performed with commercially available Pre-made TaqMan Gene Expression assays for eight genes. The specific Pre-made TaqMan Gene Expression assays used in this study were: *Bmal1* (Applied Biosystems, Assay ID: Mm00500226_m1), *Clock* (Mm00455950_m1), *Per1* (Mm00501813_m1), *Per2* (Mm00478113_m1), *Cry1* (Mm00514392_m1), and *Npas2* (Mm00500848_m1). Eukaryotic 18S rRNA (Applied Biosystems 4333760T) was used as endogenous control. The thermocycling profile for all PCR reactions began with 1 cycle of 2 min at 50°C and 10 min at 95°C and was followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All samples were run in triplicate.

Dual Luciferase Reporter Assays

Transient transfection was carried out with the Lipofectamine^{2000} reagent (Invitrogen) according to the manufacturer's instructions. In brief, 1×10^5 HeLa cells were seeded in each well of a 24-well tissue culture plate. The cells were incubated until 85%–90% confluence. Cells in each well were transfected with 10 ng of the *mPer1-luc* or 40 ng *mPer2-luc* reporter construct. Expression vectors for BMAL1, CLOCK, NPAS2, PER1, PER2, CRY1, FMR1, and FXR2 were cotransfected respectively. The renilla luciferase reporter pRL-TK plasmid (Promega) was cotransfected as an internal control. The pcDNA3.1 (Invitrogen) vector was used to bring the total amount of plasmid DNA to 0.8 μg/well. Transfection was done with Lipofectamine^{2000} reagent. After transfection for 18 hr, cells were collected and lysed and the firefly and renilla luciferase activity were measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions with a TD-20e luminometer (Turner designs).

Results

Fmr1 or *Fxr2* KO Mice Display a Shorter Free-Running Period of Locomotor Activity in DD

To determine whether mutations in the fragile X-related protein family alter circadian behavior, we measured the circadian wheel-running activity of *Fmr1* knockout (*Fmr1* KO) mice, *Fxr2* knockout (*Fxr2* KO) mice, and of their

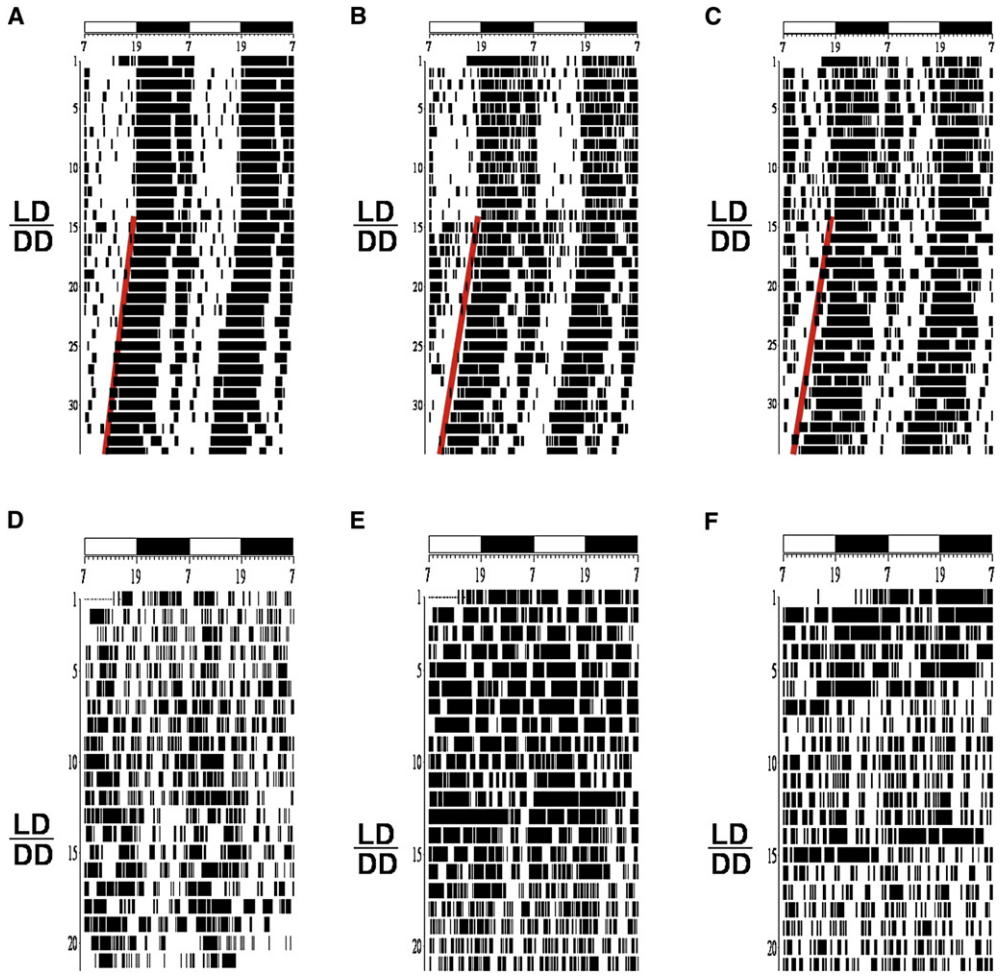

Figure 1. Representative Locomotor Activity Records of Wild-Type, *Fmr1* KO, *Fxr2* KO, and *Fmr1/Fxr2* Double KO Mice

(A) Representative locomotor activity records of WT mice.

(B) Representative locomotor activity records of *Fmr1* KO mice.

(C) Representative locomotor activity records of *Fxr2* KO mice.

(D–F) Representative locomotor activity records of *Fmr1/Fxr2* double KO mice. Activity records are double-plotted, so that 48 hr is shown on each horizontal trace with a 24 hr day presented both beneath and to the right of the preceding day. Times of activity are indicated by black vertical marks. All records show activity in wheel running activity during exposure to a 12:12 light/dark cycle and after release into constant darkness (as indicated by the LD/DD in the margin). The slope of the line (in red) aligned with the points of onset of activity on a 24 hr scale plot reflects the period length in DD. The open and dark rectangles at the top of the figure indicate the LD cycle during entrainment.

wild-type (WT) littermates individually in circadian activity-monitoring chambers. Mice were initially maintained in a 12 hr light:12 hr dark (12:12 LD) cycle for 2 weeks to establish entrainment. After release in constant darkness (DD), the WT animals showed a free-running period (tau or τ) of slightly less than 24 hr ($\tau = 23.88 \pm 0.05$; $n = 12$) (Figure 1A). *Fmr1* and *Fxr2* knockout mice displayed significantly shorter circadian periods than their WT littermates in DD ($\tau = 23.71 \pm 0.06$, $p < 0.01$; $n = 18$; and $\tau = 23.56 \pm 0.05$, $p < 0.01$; $n = 28$; respectively) (Figures 1B and 1C; for all tau values, see Figure S1A available online). These findings suggest that FMRP and FXR2P affect the period length of the circadian system. Under LD conditions, *Fmr1* and *Fxr2* knockout mice were entrained by light (Figures 1B and 1C), suggesting that a deficiency in either gene alone does not produce a detectable loss of entrainment of locomotor activity.

Fmr1/Fxr2 Double KO and *Fmr1* KO/*Fxr2* Heterozygous Mice Exhibit a Loss of Rhythmic Activity in Both LD and DD

Because *Fmr1* knockout mice express FXR2P at normal levels (and vice versa), functional redundancy may blur phenotypic outcomes in single KO animals. It was of interest, therefore, to examine *Fmr1/Fxr2* double knockout mice, which are viable and do not exhibit major physical defects.[20] Unexpectedly, the locomotor activity patterns of these double mutant animals not only exhibited a striking and complete loss of circadian rhythm in constant darkness but were also arrhythmic under LD cycles (Figures

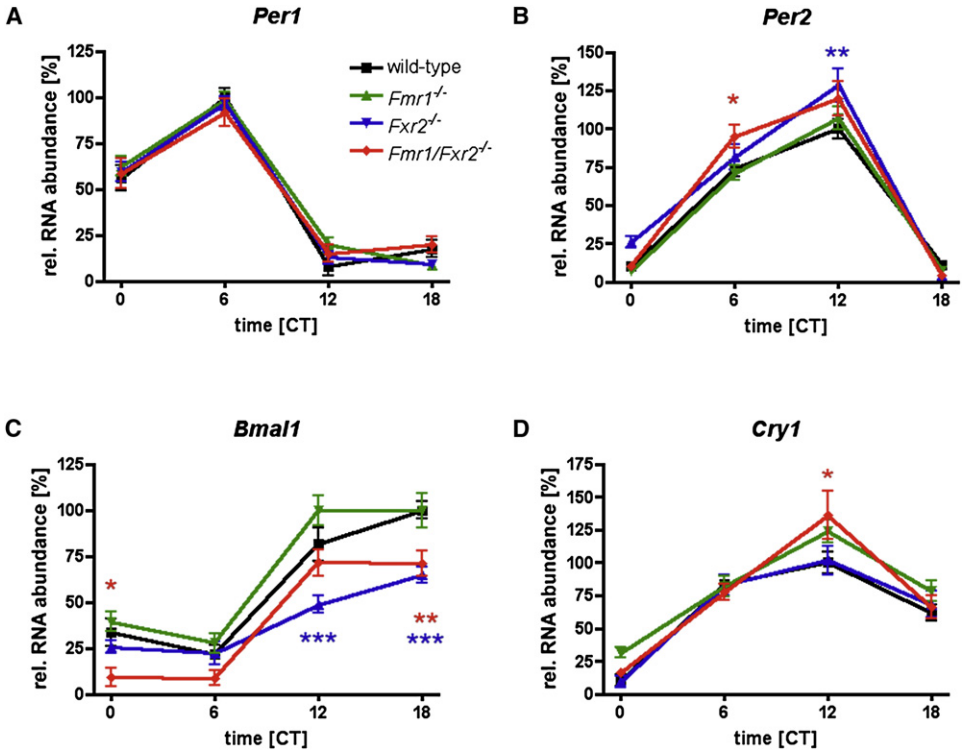

Figure 2. Expression Analysis of Wild-Type, *Fmr1* KO, *Fxr2* KO, and *Fmr1/Fxr2* Double KO under DD Conditions in SCN
In situ hybridization results for brains isolated from WT and the three knockout genotypes sacrificed at four different time points for *Per1* (A), *Per2* (B), *Bmal1* (C), and *Cry1* (D) mRNA levels of *Per1, Per2, Bmal1*, and *Cry1* were quantified and normalized. Graphs illustrate the relative transcript level of these four genes. The largest value of WT is normalized to 100%. Error bars indicate the SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. CT, circadian time.

1D–1F, n = 12; for more samples from double KO mice under LD conditions, see Figures S2A–S2D). Chi-square periodogram analysis in LD for double knockout mice showed no measurable circadian rhythm (Figures S2E–S2H). Interestingly, *Fmr1* knockout mice that were heterozygous for the *Fxr2* mutation (n = 8) also demonstrated wheel-running patterns that were largely comparable to double-knockout mice (Figures S2I–S2P), with weakly cyclical activity in LD that does not appear to be synchronized to the 24 hr period. Apparently, in the absence of FMR1, half normal levels of FXR2 are inadequate to establish entrainment in the LD cycle. Because the average levels of wheel-running activity did not differ significantly ($p > 0.05$ by one-way ANOVA) among the four genotypes in DD (wheel rotations per day for WT, *Fmr1*, *Fxr2*, and double knockout mice are 26,512 ± 2,898; 27,924 ± 2,378; 26,735 ± 1,836; and 23,980 ± 2,798, respectively), the absence of a circadian rhythm cannot be explained by a change in total activity.

Our behavioral experiments demonstrate that disruption of either of the two fragile X mental retardation genes results in alterations of circadian period, and combined disruption of both results in arrhythmicity in behavior. Light and visual perception in double KO mice are normal[20] and no abnormalities could be detected in the eyes of these animals by histology (data not shown). These results indicate that FXRPs function in the mammalian circadian clock or act downstream in the control of activity.

Molecular Analysis and In Vitro Electrophysiological Studies Suggest Normal SCN Function in the *Fmr1/Fxr2* Double KO Mice

To investigate whether the behavioral differences between the three knockout strains and their WT littermates might be due to a defect in the central circadian pacemaker, we examined clock gene expression in the suprachiasmatic nucleus (SCN) of the brain. Figure 2 illustrates expression of clock gene mRNAs (*Per1, Per2, Bmal1*, and *Cry1*) in the mouse SCN by in situ hybridization. The expression of *Per1, Per2, Bmal1*, and *Cry1* was rhythmic in the SCN over 24 hr in constant darkness (Figure 2). Significant quantitative differences were observed between genotypes. For example, *Fmr1/Fxr2* double knockout animals showed an increased level of *Cry1* expression at CT12, which coincides with the predicted time of light off (Figure 2D). Nonetheless, the overall pattern of clock gene expression in the SCN appears unchanged between animals of different genotypes. We also investigated the electrical activity of the SCN pacemaker of WT, *Fmr1* KO (n = 6), and *Fmr1/Fxr2* double KO (n = 7) mice. Electrical activity is controlled by the core clock and serves as a major output signal of the SCN. We found that the SCN electrical activity shows high amplitude circadian rhythmicity in WT (data not

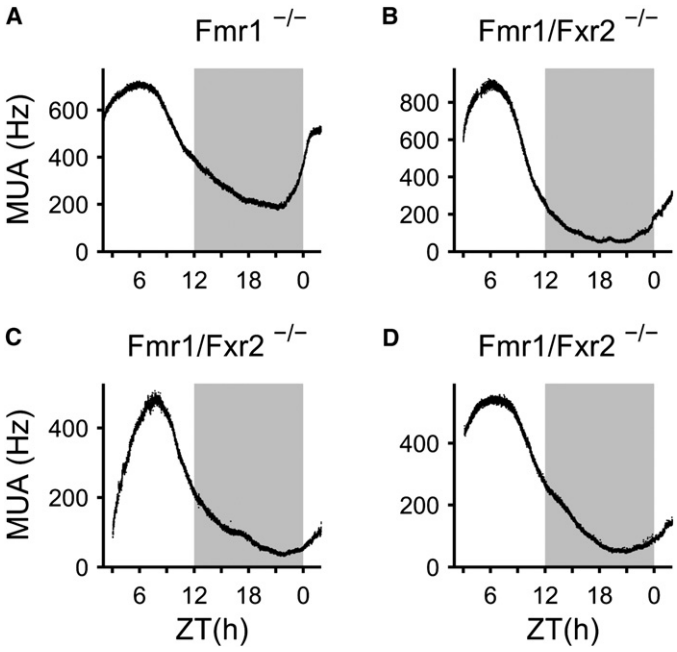

Figure 3. Representative Recordings of SCN Electrical Activity in *Fmr1* KO and *Fmr1/Fxr2* Double KO Mice

(A) Representative recordings of SCN electrical activity in *Fmr1* KO mice.

(B–D) Representative recordings of SCN electrical activity in *Fmr1/Fxr2* double KO mice. The electrical activity traces are individual examples of neuronal ensemble recordings. The open and dark rectangles at the top of the figure indicate the LD cycle to which the animals were entrained before slice preparation, and time is indicated on the *x* axis. The electrical activity is counted per 10 s and presented on the *y* axis. MUA, multiunit electrical activity.

shown), *Fmr1* KO (Figure 3A), and *Fmr1/Fxr2* double KO (Figures 3B–3D) mice, with no significant differences in peak time ($p > 0.2$). Preparation of slices 12 hr out of phase from *Fmr1/Fxr2* double KO animals results in similar peak time (data not shown). Our molecular and in vitro electrophysiological data suggest that the SCN pacemaker is intact in knockout animals and that behavioral differences observed between wild-type and *Fmr1* KO, *Fxr2* KO, and *Fmr1/Fxr2* double KO animals are unlikely to result from a deficiency in the SCN but could be caused by a deficiency downstream of the SCN pacemaker.

The Cyclical Patterns of Abundance of Several Core Clock Component mRNAs Are Altered in the Liver of Double KO Mice

When we examined the mRNA levels in the liver, which is probably the best described peripheral clock in mammals, we observed a substantial change in clock gene expression in knockout animals. Expression of *Bmal1* in the livers of double knockout mice is decreased at CT0 (Figures 4A, 4B, and 5A; Figures S3A and S3G). Compared to WT mice, *mPer1* expression in the liver of *Fxr2* and *Fmr1/Fxr2* double mutants is significantly increased at CT6 ($p < 0.01$ for both genotypes, Figures 4A, 4B, and 5C; Figures S3C and S3G). It is also notable that the distributions of levels of *mPer1* expression are much wider at CT6 and CT12 in the liver of *Fxr2* and *Fmr1/Fxr2* double knockout mice (Figure 5C; Figure S3C). *mPer2* expression in the liver peaks approximately 6 hr earlier in *Fmr1/Fxr2* double mutants compared to *Fmr1* knockout or WT animals (Figures 4A, 4B, and 5D; Figures S3D and S3G). Similar to *mPer1*, the variations of *mPer2* expression levels at CT6 in the liver of *Fxr2* and *Fmr1/Fxr2* double knockout mice are much more broad than those in WT animals (Figure 5D; Figure S3D). The expression of *Cry1* is significantly increased at CT12 in the livers of both double and either of the single KO mice (Figures 4A, 4B, and 5E; Figures S3E and S3G). *Clock*, which does not cycle in WT animals, was not significantly altered in the livers of the mutants (Figures 4A, 4B, and 5B; Figures S3B and S3G).

Npas2, a transcription factor that is highly similar to *Clock*, heterodimerizes with *Bmal1* and activates *Per* transcription.[21,22] Because *Clock* expression was indistinguishable between WT and mutants, we tested the expression of *Npas2* transcript in the liver (*Npas2* expression cannot be detected by in situ hybridization in the SCN). *Npas2* expression in *Fmr1* mutants is similar to that in WT. However, *Npas2* expression in *Fxr2* mutants is slightly increased at CT18 and significantly decreased at CT0. In double mutants, *Npas2* expression peaks at CT18, whereas it peaks at CT0 in WT controls (Figures 4A, 4B, and 5F; Figures S3F and S3G). This indicates that in the absence of *Fxr2, Npas2* expression is altered and that *Fmr1* has an additive effect on *Npas2* cycling. These data suggest that the loss of FMRP, FXR2P, or both affects peripheral circadian clock function.

FXR2P Alone or FMRP and FXR2P Together Can Increase PER1- or PER2-Mediated BMAL1-NPAS2 Transcriptional Activity in a Dose-Dependent Manner

To determine whether FMRP and FXR2P can directly regulate clock gene expression, we performed a luciferase reporter assay in HeLa cell transfection assays. Initial studies showed that addition of mPER2 enhanced BMAL1-NPAS2-dependent transcription from either *mPer1* or *mPer2* promoter as previously described.[23] Coexpression of FXR2P or the combination of FMRP and FXR2P together significantly enhanced the mPER2-induced BMAL1-NPAS2 transcriptional activity from both promoters (Figures 6A and 6B). Similarly, mPER1-induced BMAL1-NPAS2 transcription from the *mPer1* promoter is further activated by FXR2P or FMRP and FXR2P together (Figure 6C). Furthermore, this effect on transcriptional stimulation was dependent on the dose of FXRPs (Figures S5A and S5B), which agrees with the observations that FMRP functions in a dose-dependent manner in flies, mice, and human patients.[13,24,25] However, coexpression of FMRP or FXR2P or both together was not found to influence CRY1 repression of BMAL1-NPAS2 stimulation (Figures S4A and S4B). Interestingly, similar transfection studies with

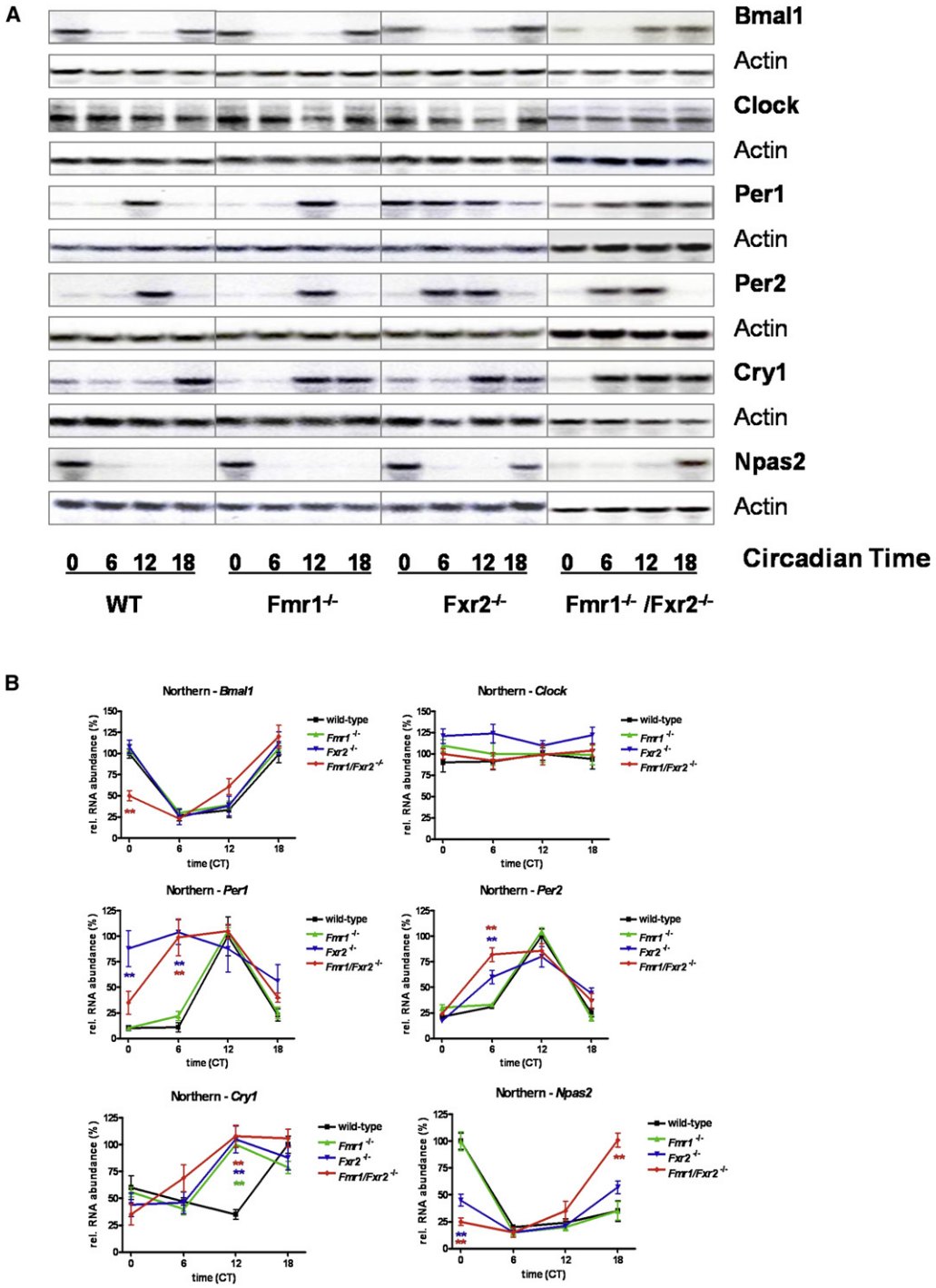

Figure 4. Expression Analysis of Wild-Type, *Fmr1* KO, *Fxr2* KO, and *Fmr1/Fxr2* Double KO Mice under DD Conditions in Liver

(A) Northern blotting results on liver tissues of WT and three different knockout mouse genotypes sacrificed at four different time points. The probes are indicated on the right. Rehybridization of the same filter with an actin probe was used to control for loading and RNA integrity. Representative results are shown from one out of four sets of WT, three sets of *Fmr1* KO, four sets of *Fxr2* KO, and five sets of *Fmr1/Fxr2* double KO mice. Each set of animals represents an independent experiment.

(B) RNA levels of *Bmal1*, *Clock*, *Per1*, *Per2*, *Cry1*, and *Npas2* were quantified and normalized in reference to actin mRNA levels. The largest value of WT is normalized to 100%. Graphs illustrate the relative transcript level of these six genes. Error bars indicate the SEM. $*p < 0.05$, $**p < 0.01$.

BMAL1-CLOCK showed no stimulation by either FMRP or FXR2P or by both together (data not shown). Our data indicate that FMRP and FXR2P have direct influence on the molecular clock feedback loop and that these effects are mediated by the BMAL1-NPAS2 rather than the BMAL1-CLOCK complex. Based on these findings and on our observations

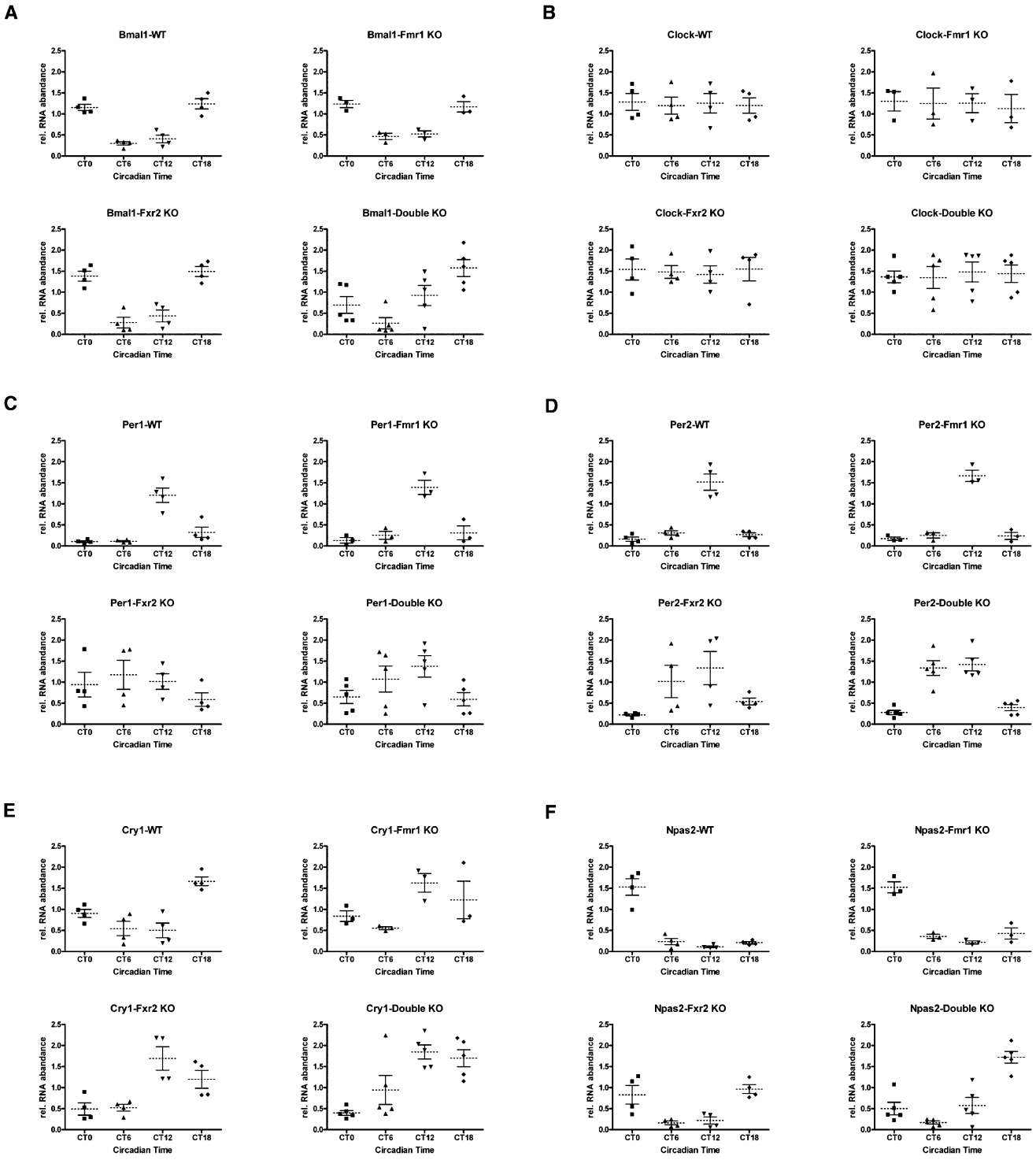

Figure 5. Expression Analysis of Four Sets of Wild-Type, Three Sets of *Fmr1* KO, Four Sets of *Fxr2* KO, and Five Sets of *Fmr1*/*Fxr2* Double KO Mice under DD Conditions in Liver

RNA levels of *Bmal1* (A), *Clock* (B), *Per1* (C), *Per2* (D), *Cry1* (E), and *Npas2* (F) were quantified and normalized in reference to actin mRNA levels. The data on relative mRNA abundance were averaged across different time points. Error bars indicate the SEM.

that *Npas2* expression is altered significantly in the livers of *Fxr* mutant animals, we propose that *NPAS2* plays an important role in the effects of the fragile X gene family on peripheral circadian oscillators and behavioral activity patterns.

Discussion

Circadian rhythmicity is a fundamental biological phenomenon in living organisms. Among animals, the fruit

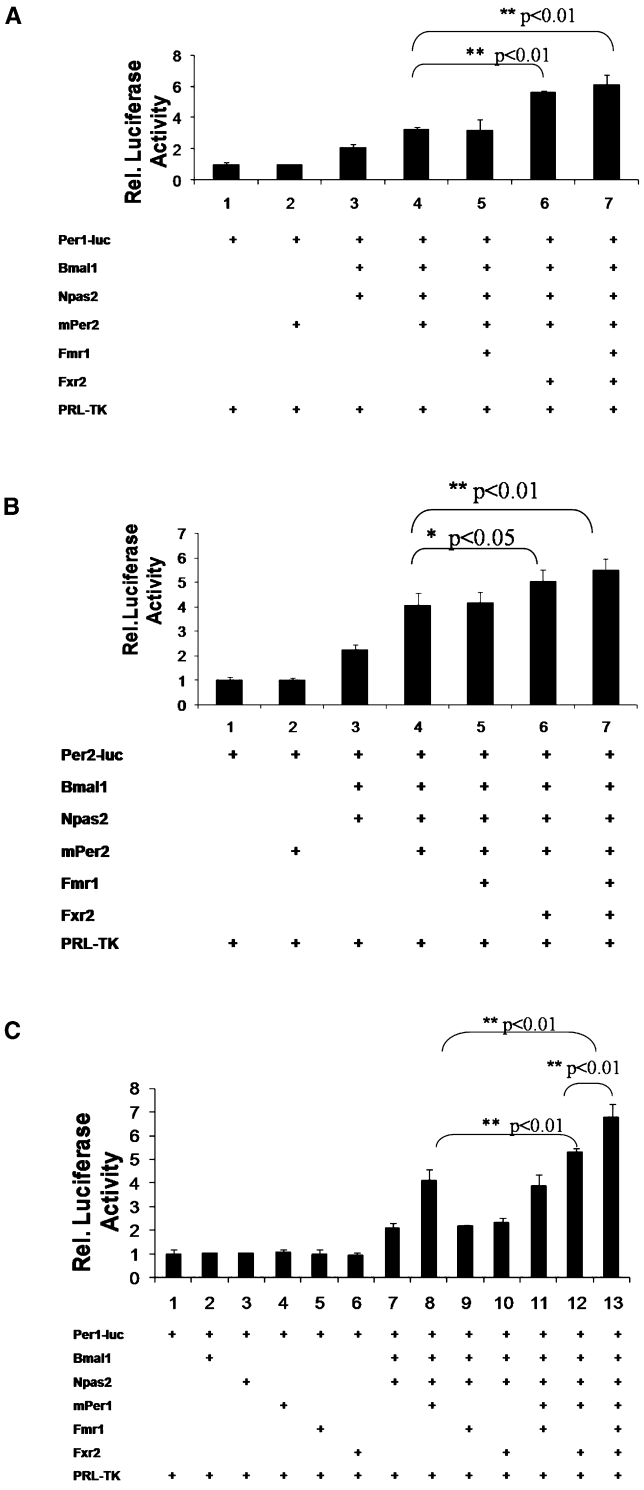

Figure 6. Upregulation of BMAL1-NPAS2-Mediated Transcription by FMR1 and FXR2 in the Presence of mPER1 or mPER2

(A) Upregulation of BMAL1-NPAS2-mediated transcription on *mPer1* promoter by FMR1 and FXR2 in the presence of mPER2. (B) Upregulation of BMAL1-NPAS2-mediated transcription on *mPer2* promoter by FMR1 and FXR2 in the presence of mPER2. (C) Upregulation of BMAL1-NPAS2-mediated transcription on *mPer1* promoter by FMR1 and FXR2 in the presence of mPER1. HeLa cells were cotransfected with the reporter construct *mPer1-luc* or *mPer2-luc* and control reporter construct Renilla luciferase encoding plasmids PRL-TK and various combinations of expression construct (Material and Methods). Luciferase activity in samples transfected with reporter constructs only is adjusted as 1.0. Error bars indicate the SD ($n = 4$).

fly and the mouse have provided the most information on the mechanism of central circadian pacemakers. These species' clocks share conserved gene products, although there are significant differences between the explanatory models for maintenance of rhythmicity.[26] In the fly, loss-of-function mutations of the *dfmr* gene have been shown to alter circadian behavior without detectably affecting the function of the central pacemaker, suggesting a role for *dfmr* in circadian output.[12,13] In the mouse, CLOCK, BMAL1, PER, and CRY are core components of the circadian oscillatory mechanisms. The active CLOCK-BMAL1 complex drives expression of numerous genes including PER and CRY, whereas PER and CRY repress CLOCK-BMAL1 transcriptional activities and therefore form a feedback regulation loop.[27,28] Regulation of CLOCK-BMAL1 activity is central to the mammalian clock. The appropriate delay between the activation and repression of transcription maintains the daily oscillations of clock proteins and gives the clock a ~24 hr period.[29] Here, we report a function for the *Fmr1* and *Fxr2* genes in circadian behavior and clock gene expression in the liver of mice. The dramatic circadian activity phenotype found in *Fmr1/Fxr2* double KO mice is unprecedented, demonstrating a significant role of those genes in clock function or output. The alterations to clock gene mRNAs in the liver, but not in the master clock, suggest that FMRP and FXR2P are acting downstream of the circadian pacemaker in mammals. CLOCK and NPAS2 have been shown to have partially redundant functions in the SCN, with CLOCK having a dominant role.[30] Our transfection data would suggest that the FXR proteins exert effects on NPAS2/BMAL1 complex rather than CLOCK/BMAL1. Thus, it is not surprising that the SCN is less affected than the liver in *Fxr* mutant animals. The differences between the SCN and liver of *Fxr* mutant mice suggest examination of the roles of FMRP and FXR2P in mice with mutations in *Clock* and/or *Npas2*. Intriguingly, animals lacking both *Clock* and *Npas2* show rhythmic activity in LD,[30] whereas *Fmr1/Fxr2* double knockouts are arrhythmic.

It is worth while to note that in addition to FMRP and FXR2P, FXR1P, another member of *FMR1* gene family, may play a compensatory role in the absence of *Fmr1*, *Fxr2*, or in *Fmr1/Fxr2* double knockout mice. Because of the early postnatal death of *Fxr1* knockout mice, a conditional *Fxr1* knockout model was produced.[31] These mice exhibit muscle defects, but the generation of *Fmr1/Fxr1/Fxr2* triple mutant mice should provide more information on potential functional interactions in circadian clock regulation and may offer a more direct comparison to the *Drosophila* loss-of-function model, where a single *FMR1*-like protein may perform many of the functions distributed among the three mammalian genes.

The neural connections between the SCN and other parts of the nervous system are important for the control of

circadian rhythms in the central nervous system.[32] Interestingly, it has been shown that the absence of FMRP causes abnormal neurotransmitter signaling, which could be responsible for neurological deficiencies in fragile X syndrome.[33,34] It is possible, therefore, that the loss of FMRP and FXR2P results in arrhythmicity resulting from inappropriate neuronal communication within the central nervous system.

A link between abnormal sleep patterns and mental retardation has been suggested from several other mental retardation disorders,[35] including Smith-Magenis syndrome,[36] where the RAI1 transcriptional activator appears to be a major contributor to phenotype. To our knowledge, our data provide the first molecular evidence to connect a protein involved in cognitive ability with the circadian system. Although sleep abnormalities are a frequent feature in males with fragile X syndrome, the level of severity varies significantly in different patients.[2,14] Our data that in the absence of *Fmr1*, losing half normal levels of *Fxr2* can result in loss of light-dark entrainment suggest that genetic variation in *Fxr2* could be responsible for the variability of sleep problems in fragile X population. Sleep abnormality could add to the phenotype; several lines of evidence suggest a role for normal sleep in learning and memory.[37] The close association between the circadian system and the timing of sleep and wakefulness together with the typical disturbances of circadian behavior and sleep in fragile X syndrome opens up a new perspective for the investigation and treatment of patients suffering from this disorder.

Supplemental Data

Five figures are available at http://www.ajhg.org/.

Acknowledgments

This work was supported in part by grant HD38038 from the US National Institute of Child Health and Human Development to D.L.N. and B.A.O., grant HD24064 from the BCM Intellectual and Developmental Disabilities Research Center, the FRAXA Research Foundation, and by Swiss National Science Foundation and EUCLOCK to U.A. The authors wish to thank Richard Paylor, Yanghong Gu, and Jianfa Zhang for their input and discussion.

Received: March 13, 2008
Revised: May 21, 2008
Accepted: June 3, 2008
Published online: June 26, 2008

Web Resources

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

References

1. Warren, S.T., and Nelson, D.L. (1994). Advances in molecular analysis of fragile X syndrome. JAMA *271*, 536–542.
2. Hagerman, R.J. (1996). Fragile X Syndrome: Diagnosis, Treatment, and Research, Second Edition (Baltimore: John Hopkins University Press), pp. 3–87.
3. Verkerk, A.J., Pieretti, M., Sutcliffe, J.S., Fu, Y.H., Kuhl, D.P., Pizzuti, A., Reiner, O., Richards, S., Victoria, M.F., Zhang, F.P., et al. (1991). Identification of a gene (*FMR-1*) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell *65*, 905–914.
4. Eberhart, D.E., Malter, H.E., Feng, Y., and Warren, S.T. (1996). The fragile X mental retardation protein is a ribonucleoprotein containing both nuclear localization and nuclear export signals. Hum. Mol. Genet. *5*, 1083–1091.
5. Corbin, F., Bouillon, M., Fortin, A., Morin, S., Rousseau, F., and Khandjian, E.W. (1997). The fragile X mental retardation protein is associated with poly(A)+ mRNA in actively translating polyribosomes. Hum. Mol. Genet. *6*, 1465–1472.
6. Feng, Y., Absher, D., Eberhart, D.E., Brown, V., Malter, H.E., and Warren, S.T. (1997). FMRP associates with polyribosomes as an mRNP, and the I304N mutation of severe fragile X syndrome abolishes this association. Mol. Cell *1*, 109–118.
7. Devys, D., Lutz, Y., Rouyer, N., Bellocq, J.P., and Mandel, J.L. (1993). The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nat. Genet. *4*, 335–340.
8. 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.
9. 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.
10. Kirkpatrick, L.L., McIlwain, K.A., and Nelson, D.L. (2001). Comparative genomic sequence analysis of the FXR gene family: *FMR1*, *FXR1*, and *FXR2*. Genomics *78*, 169–177.
11. Wan, L., Dockendorff, T.C., Jongens, T.A., and Dreyfuss, G. (2000). Characterization of dFMR1, a *Drosophila melanogaster* homolog of the fragile X mental retardation protein. Mol. Cell. Biol. *20*, 8536–8547.
12. Dockendorff, T.C., Su, H.S., McBride, S.M., Yang, Z., Choi, C.H., Siwicki, K.K., Sehgal, A., and Jongens, T.A. (2002). *Drosophila* lacking *dfmr1* activity show defects in circadian output and fail to maintain courtship interest. Neuron *34*, 973–984.
13. Morales, J., Hiesinger, P.R., Schroeder, A.J., Kume, K., Verstreken, P., Jackson, F.R., Nelson, D.L., and Hassan, B.A. (2002). *Drosophila* fragile X protein, DFXR, regulates neuronal morphology and function in the brain. Neuron *34*, 961–972.
14. 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.
15. The Dutch-Belgian Fragile X Consortium, Bakker, C.E., Verheij, C., Willemsen, R., Vanderhelm, R., Oerlemans, F., Vermey, M., Bygrave, A., Hoogeveen, A.T., Oostra, B.A., et al. (1994). *Fmr1* knockout mice: a model to study fragile X mental retardation. Cell *78*, 23–33.
16. Bontekoe, C.J., McIlwain, K.L., Nieuwenhuizen, I.M., Yuva-Paylor, L.A., Nellis, A., Willemsen, R., Fang, Z., Kirkpatrick, L., Bakker, C.E., McAninch, R., et al. (2002). Knockout mouse model for *Fxr2*: a model for mental retardation. Hum. Mol. Genet. *11*, 487–498.

17. Albrecht, U., Sun, Z.S., Eichele, G., and Lee, C.C. (1997). A differential response of two putative mammalian circadian regulators, *mper1* and *mper2*, to light. Cell *91*, 1055–1064.
18. Meijer, J.H., Schaap, J., Watanabe, K., and Albus, H. (1997). Multiunit activity recordings in the suprachiasmatic nuclei: in vivo versus in vitro models. Brain Res. *753*, 322–327.
19. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry *18*, 5294–5299.
20. Spencer, C.M., Serysheva, E., Yuva-Paylor, L.A., Oostra, B.A., Nelson, D.L., and Paylor, R. (2006). Exaggerated behavioral phenotypes in *Fmr1/Fxr2* double knockout mice reveal a functional genetic interaction between Fragile X-related proteins. Hum. Mol. Genet. *15*, 1984–1994.
21. Hogenesch, J.B., Gu, Y.Z., Jain, S., and Bradfield, C.A. (1998). The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proc. Natl. Acad. Sci. USA *95*, 5474–5479.
22. Reick, M., Garcia, J.A., Dudley, C., and McKnight, S.L. (2001). NPAS2: an analog of clock operative in the mammalian forebrain. Science *293*, 506–509.
23. Kaasik, K., and Lee, C.C. (2004). Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. Nature *430*, 467–471.
24. Peier, A.M., McIlwain, K.L., Kenneson, A., Warren, S.T., Paylor, R., and Nelson, D.L. (2000). (Over)correction of FMR1 deficiency with YAC transgenics: behavioral and physical features. Hum. Mol. Genet. *9*, 1145–1159.
25. Menon, V., Leroux, J., White, C.D., and Reiss, A.L. (2004). Frontostriatal deficits in fragile X syndrome: relation to *FMR1* gene expression. Proc. Natl. Acad. Sci. USA *101*, 3615–3620.
26. Allada, R., Emery, P., Takahashi, J.S., and Rosbash, M. (2001). Stopping time: the genetics of fly and mouse circadian clocks. Annu. Rev. Neurosci. *24*, 1091–1119.
27. Reppert, S.M., and Weaver, D.R. (2002). Coordination of circadian timing in mammals. Nature *418*, 935–941.
28. Lowrey, P.L., and Takahashi, J.S. (2004). Mammalian circadian biology: elucidating genome-wide levels of temporal organization. Annu. Rev. Genomics Hum. Genet. *5*, 407–441.
29. Gallego, M., and Virshup, D.M. (2007). Post-translational modifications regulate the ticking of the circadian clock. Nat. Rev. Mol. Cell Biol. *8*, 139–148.
30. DeBruyne, J.P., Weaver, D.R., and Reppert, S.M. (2007). CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. Nat. Neurosci. *10*, 543–545.
31. Mientjes, E.J., Willemsen, R., Kirkpatrick, L.L., Nieuwenhuizen, I.M., Hoogeveen-Westerveld, M., Verweij, M., Reis, S., Bardoni, B., Hoogeveen, A.T., Oostra, B.A., and Nelson, D.L. (2004). Fxr1 knockout mice show a striated muscle phenotype: implications for Fxr1p function in vivo. Hum. Mol. Genet. *13*, 1291–1302.
32. Schwartz, W.J., Gross, R.A., and Morton, M.T. (1987). The suprachiasmatic nuclei contain a tetrodotoxin-resistant circadian pacemaker. Proc. Natl. Acad. Sci. USA *84*, 1694–1698.
33. Bear, M.F., Huber, K.M., and Warren, S.T. (2004). The mGluR theory of fragile X mental retardation. Trends Neurosci. *27*, 370–377.
34. D'Hulst, C., De Geest, N., Reeve, S.P., Van Dam, D., De Deyn, P.P., Hassan, B.A., and Kooy, R.F. (2006). Decreased expression of the GABAA receptor in fragile X syndrome. Brain Res. *1121*, 238–245.
35. Lamont, E.W., Legault-Coutu, D., Cermakian, N., and Boivin, D.B. (2007). The role of circadian clock genes in mental disorders. Dialogues Clin. Neurosci. *9*, 333–342.
36. De Leersnyder, H., Claustrat, B., Munnich, A., and Verloes, A. (2006). Circadian rhythm disorder in a rare disease: Smith-Magenis syndrome. Mol. Cell. Endocrinol. *252*, 88–91.
37. Ji, D., and Wilson, M.A. (2007). Coordinated memory replay in the visual cortex and hippocampus during sleep. Nat. Neurosci. *10*, 100–107.