Hypnotic treatment improves sleep architecture and EEG disruptions and rescues memory deficits in a mouse model of fragile X syndrome

Highlights

- Hypnotic administration alleviates sleep alterations in a mouse model of fragile X syndrome

- NREM sleep spindles and REM theta rhythms are renormalized by hypnotic ML297

- ML297 administration rescues sleep-dependent memory consolidation in Fmr1−/y mice

- ML297 may target altered hippocampal excitatory/inhibitory balance in Fmr1−/y mice

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In brief

Using a mouse model of fragile X syndrome, Martinez et al. show that the hypnotic ML297, which partially rescues altered sleep phenotypes in mice, also reverses deficits in hippocampal memory consolidation when administered following learning experiences.
Hypnotic treatment improves sleep architecture and EEG disruptions and rescues memory deficits in a mouse model of fragile X syndrome

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SUMMARY

Fragile X syndrome (FXS) is associated with disrupted cognition and sleep abnormalities. Sleep loss negatively impacts cognitive function, and one untested possibility is that disrupted cognition in FXS is exacerbated by abnormal sleep. We tested whether ML297, a hypnotic acting on G-protein-activated inward-rectifying potassium (GIRK) channels, could reverse sleep phenotypes and disrupted memory in Fmr1−/y mice. Fmr1−/y mice exhibit reduced non-rapid eye movement (NREM) sleep and fragmented NREM architecture, altered sleep electroencephalogram (EEG) oscillations, and reduced EEG coherence between cortical areas; these are partially reversed following ML297 administration. Treatment following contextual fear or spatial learning restores disrupted memory consolidation in Fmr1−/y mice. During memory recall, Fmr1−/y mice show an altered balance of activity among hippocampal principal neurons vs. parvalbumin-expressing interneurons; this is partially reversed by ML297. Because sleep disruption could impact neurophysiological phenotypes in FXS, augmenting sleep may improve disrupted cognition in this disorder.

INTRODUCTION

Fragile X syndrome (FXS) is an X-linked neurodevelopmental disorder resulting from silencing of the FMR1 gene and loss of fragile X messenger ribonucleoprotein (FMRP). The leading cause of both heritable intellectual disability and syndromic autism spectrum disorder (ASD),1,2 FXS is characterized by altered sensory processing, hyperactivity, and cognitive impairments.3-5 Available data suggest that patients with FXS have trouble falling asleep and frequent nighttime awakenings6-9—a phenotype shared with other neurodevelopmental disorders.10-13 However, the relationship between altered sleep and the cognitive and behavioral aspects of FXS (or other ASDs) is unknown. Fmr1 knockout (Fmr1−/−) mice recapitulate behavioral phenotypes seen in people with FXS, as well as disrupted sensory cortex plasticity and hippocampus-dependent memory consolidation.14-19 However, while limited data using home-cage observational studies suggest alterations in sleep across development in Fmr1−/− mice,20,21 a full characterization of sleep behavior Fmr1−/− mice (e.g., using continuous polysomnographic recording) has not been done. Because sleep disruption can impair cognitive function and synaptic plasticity in both humans and mice,22-36 one untested possibility is that disrupted sensory processing and cognition in FXS (and other ASDs) are exacerbated by abnormal sleep. If sleep disruption is a major driver of cognitive disruption in ASD, hypnotic treatment (aimed at normalizing sleep patterns) could prove to be an important therapeutic adjuvant for treatment.33-36

Recent studies have led to the development of new classes of sleep-promoting hypnotic drugs, including orexin receptor antagonists and activators for G-protein inward-rectifying potassium (GIRK) channels.37-39 GIRK1/2-containing GIRK channels are highly expressed in regions such as the hippocampus, neocortex, and cerebellum40-42 and can be directly activated by ML297, leading to neuronal hyperpolarization.43,44 Recent studies using ML297 and other GIRK1/2 activators in rodents have shown they suppress seizure activity, act as an anxiolytic, and increase non-rapid eye movement (NREM) and rapid eye movement (REM) sleep.45,46-48 These effects may be mediated by signaling via GIRK1 (encoded by the Kcnj3 gene); Kcnj3 is highly expressed in both excitatory neurons or interneuron populations (particularly fast-spiking, parvalbumin-expressing [PV+] interneurons) in the hippocampus (particularly in dentate gyrus [DG] and CA1) and neocortex (particularly layer 6 corticothalamic neurons).49-51 Critically, GIRK1/2 channel activity is likely disrupted in the absence of FMRP, which is critical for the channels’ coupling to GABA_A receptors52,53.

We used polysomnographic recordings to fully characterize alterations to sleep architecture and cortical electroencephalogram (EEG) activity in adult Fmr1−/− mice. We found alterations to sleep amounts, architecture, and EEG oscillations, which...
are largely reversed by administration of the GIRK1/2 channel agonist ML297. ML297 administration following training on two hippocampus-mediated, sleep-dependent tasks (object location memory and contextual fear memory) led to a rescue of disrupted consolidation of memory in both tasks. These studies set the groundwork for understanding sleep as a therapeutic target for treating patients with FXS and other ASDs.

RESULTS

Fmr1<sup>−/−</sup> mice have disrupted NREM sleep architecture and altered NREM and REM EEG activity

To characterize sleep phenotypes in an FXS model, we recorded EEG/electromyography (EMG) activity continuously across the rest (light) phase in Fmr1<sup>−/−</sup> mice and male wild-type (WT) littermates. Because sleep architecture<sup>54</sup> and hippocampal plasticity<sup>60,66</sup> can vary across the estrous cycle, we focused our initial studies on male mice. To assess state-specific activity patterns at a site corresponding to disruptions in sleep-associated activity in patients with ASD<sup>57–59</sup> 4- to 5-month-old WT and Fmr1<sup>−/−</sup> mice were implanted with bilateral EEG electrodes at a posterior location over primary visual cortex (V1). For our initial studies, these mice were recorded across the 12-h light (rest) phase (ZT0–12) (Figure 1A). Fmr1<sup>−/−</sup> mice spent significantly less total time in NREM sleep, more time in wake, and equal time in REM compared to WT littermates (Figure 1B). Mean duration of NREM bouts was significantly shorter in Fmr1<sup>−/−</sup> mice (Figure 1C), and total number of NREM and wake bouts (but not REM bouts) was significantly greater in Fmr1<sup>−/−</sup> mice (Figure 1D), suggesting fragmented NREM sleep architecture.

To test whether other features of NREM were disrupted in Fmr1<sup>−/−</sup> mice, we compared EEG activity between the genotypes. Density of NREM spindles (waxing and waning 7- to 15-Hz [sigma (\(\delta\)] band) oscillations<sup>60–63</sup>, detected in the EEG using a semi-automated method, was significantly decreased in Fmr1<sup>−/−</sup> mice compared to WT littermates (Figure 1E). Spindle duration did not differ significantly between genotypes, although it tended to be modestly lower in Fmr1<sup>−/−</sup> mice (Figure 1F). With respect to overall state-specific EEG power, Fmr1<sup>−/−</sup> mice showed significantly increased \(\delta\); 0.5–4 Hz, including both 0.5- to 1-Hz slow oscillation and 1- to 4-Hz bands) and significantly reduced \(\alpha\) (7–15 Hz) activity during NREM, and significantly reduced theta (\(\theta\); 4–12 Hz, including 4- to 7-Hz low \(\theta\)) power during REM, compared to WT littermates (Figures 1G and 1H; Figures S1A and S1B). Consistent with previous findings from Fmr1<sup>−/−</sup> mice and rats, EEG gamma (\(\gamma\); 31–60 Hz) power was increased during both NREM and wake in Fmr1<sup>−/−</sup> mice (Figures S1A and S1C). The \(\delta\) power during REM and wake was similar between genotypes (Figures S1B and S1C). Moreover, NREM ripple (125–275 Hz) oscillations (measured from V1 EEG sites overlying intermediate hippocampus<sup>68–70</sup>) were altered in Fmr1<sup>−/−</sup> mice, with increased frequency of occurrence but reduced power (Figure S2). Thus, multiple state-specific cortically (e.g., slow and \(\gamma\) oscillations), thalamocortically (e.g., NREM spindles), and hippocampally (e.g., REM \(\theta\) and NREM ripples) generated oscillations<sup>23,71</sup> are altered in Fmr1<sup>−/−</sup> mice in association with disruptions in overall sleep architecture.

Administration of hypnotic compound ML297 renormalizes some aspects of NREM and REM sleep in Fmr1<sup>−/−</sup> mice

Because activation of GIRK channels (via coupling to GABA<sub>B</sub> receptors) may be disrupted in FXS due to loss of FMRP<sup>52,53</sup>, we tested whether GIRK1/2 agonist hypnotic ML297 could restore sleep in Fmr1<sup>−/−</sup> mice. We measured changes to sleep architecture and oscillations after administering ML297 or vehicle to Fmr1<sup>−/−</sup> mice and WT littermates. Mice implanted with EEG electrodes over V1 and prefrontal cortex (PFC) underwent 4-day, continuous EEG recording, consisting of a 24-h baseline (starting at ZT0) on day 1 (baseline A), a 24-h period following administration of vehicle (at ZT0) on day 2, a second 24-h baseline recording (baseline B) on day 3, and a 24-h period following administration of ML297 (30 mg/kg) on day 4 (Figure 2A).

Similar to our initial findings, baseline recordings (baselines A and B) in Fmr1<sup>−/−</sup> mice were characterized by significantly decreased NREM across the 12-h light phase, while dark (i.e., active) phase (ZT12–24) NREM was similar between genotypes (Figure 2B). Disrupted NREM architecture (decreased NREM bout durations and increased bout numbers) was evident in Fmr1<sup>−/−</sup> mice across both baseline days’ light phase but not dark phase (Figures 2C and 2D; Figures S3A–S3C). NREM phenotypes in Fmr1<sup>−/−</sup> mice continued to differ from WT littermates after vehicle administration. However, after ML297 administration, NREM sleep time, bout duration, and bout numbers in Fmr1<sup>−/−</sup> mice were similar to those of WT littermates (Figures 2B–2D). REM sleep time and architecture were comparable between genotypes across both light and dark phases and across baseline and treatment recording days (Figures 2E–2G). Latency to NREM was greater in Fmr1<sup>−/−</sup> mice than WT littermates after vehicle treatment. Surprisingly, WT littermates’ NREM latency increased (becoming similar to latency of Fmr1<sup>−/−</sup> mice) after ML297 treatment (Figure 2H). Together, these data show that several aspects of NREM sleep disruption in Fmr1<sup>−/−</sup> mice are renormalized by ML297 to match sleep patterns of WT littermates. This occurs even though latency to sleep is increased by ML297 in WT mice, and it is unchanged by ML297 in Fmr1<sup>−/−</sup> mice. Thus, the quality of sleep is changed in Fmr1<sup>−/−</sup> mice by GIRK1/2 activation, although sleep pressure is not increased. Together, these data indicate that restoration of GIRK1/2 function—thought to be disrupted in FXS<sup>52,53</sup>—can partially rescue some aspects of NREM deficits present in Fmr1<sup>−/−</sup> mice.

Cortical subregion-specific EEG changes and intracortical desynchrony are partially renormalized by ML297 in Fmr1<sup>−/−</sup> mice

Because we noted altered EEG spectral power in Fmr1<sup>−/−</sup> mice, we next assessed whether these changes were time-of-day- and brain-region specific, and how ML297 affected cortical EEG activity. In V1, across both baseline recordings (baseline A and B), we again observed increased NREM \(\delta\) and \(\gamma\) power, and reduced \(\alpha\) power, in Fmr1<sup>−/−</sup> mice—changes that were present across the light phase (Figures 3A and 3C; Figure S5A). In contrast, during the dark phase, V1 NREM \(\delta\) power remained significantly elevated, while \(\alpha\) and \(\gamma\) power no longer differed between Fmr1<sup>−/−</sup> mice and WT littermates (Figures S6A and
Finally, ripples detected in the V1 EEG during NREM again showed increased frequency of occurrence, and decreased power, in Fmr1/C0/y mice across both the light and dark phase (Figures S10, S11).

In simultaneous EEG recordings in PFC, baseline NREM power was elevated in Fmr1/C0/y mice during the light phase, but δ and σ power were comparable between WT littermates and Fmr1/C0/y mice (Figures 3B and 3D; Figure SSB). Similar trends in PFC spectral power were observed across the dark cycle (Figures S6B and S6D; Figure SSB). These regional differences in NREM δ power are consistent with reported anteroposterior axis differences in NREM slow-wave activity in children and adults with ASD.58,59

REM and wake activity alterations were also again observed in Fmr1/C0/y mice. Baseline V1 recordings again showed decreased REM θ power in Fmr1/C0/y mice compared to WT littermates across the light and dark phase (Figures 3E and 3G; Figures S6E and S6G). However, no changes in REM θ were

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**Figure 1.** Fmr1/C0/y mice exhibit disrupted NREM sleep and altered EEG activity during their rest phase

(A) Schematic of EEG/EMG recording configuration, with EEG electrodes positioned over bilateral primary visual cortex (V1) and representative 10-s epochs are shown for wake, NREM sleep, and REM sleep.

(B) Percentage recording time (for the entire 12-h light phase) spent in NREM sleep, REM sleep, and wake for WT and Fmr1/C0/y mice. Two-way repeated measures (RM) ANOVA, p(state) < 0.0001, p(genotype) = 0.19, p(state × genotype interaction) = 0.0014.

(C) Mean bout durations across the light phase for NREM, REM, and wake. Two-way RM ANOVA, p(state) < 0.0001, p(genotype) = 0.0062, p(state × genotype interaction) = 0.0003.

(D) Number of light-phase bouts of NREM, REM, and wake. Two-way RM ANOVA, p(state) < 0.0001, p(genotype) = 0.0005, p(state × genotype interaction) < 0.0001.

(E) NREM spindle density (detected events per second) during the 12-h light phase.

(F) NREM spindle duration during the 12-h light phase.

(G) NREM EEG power spectrum (left) with δ (0.5–4 Hz) and σ (7–15 Hz) frequency bands highlighted via dashed lines. Two-way RM ANOVA, p(frequency) < 0.0001, p(genotype) = 0.80, p(frequency × genotype interaction) < 0.0001. Total NREM δ-band power (middle) and total σ-band power for spindles (right) across the 12-h light phase.

(H) REM EEG power spectrum (left), showing the θ (4–12 Hz) frequency band highlighted via dashed lines. Two-way RM ANOVA, p(frequency) < 0.0001, p(genotype) = 0.10, p(frequency × genotype interaction) < 0.0001. Total REM θ-band power (right) across the 12-h light phase. Sample size: n = 8 mice/genotype. *p < 0.05, **p < 0.01, and ***p < 0.001, Sidak’s post hoc test (B–D) or two-tailed, unpaired t test (E–H). Data points and error bars indicate mean ± SEM. Symbols: filled (blue) circles and open (gray) squares = WT and Fmr1/C0/y mice, respectively.
Figure 2. ML297 administration partially renormalizes rest-phase NREM sleep architecture in Fmr1<sup>−/−</sup> mice

(A) Schematic of EEG/EMG recording configuration, with EEG electrodes positioned over primary visual (V1) and prefrontal (PFC) cortical regions (left). Experimental timeline of 4-day continuous recording in a 12:12 light-dark cycle (right). Baseline recordings were carried out on days 1 and 3; vehicle and ML297 intraperitoneal (i.p.) injections were given at lights-on (ZT0) on days 2 and 4, respectively.

(B) NREM percentage recording time for the 0- to 12 h light phase (left) and 12–24 h dark phase (right) across all 4 days for WT and Fmr1<sup>−/−</sup> mice. Two-way RM ANOVA for ZT0-12, p(condition) = 0.23, p(genotype) = 0.0009, p(condition × genotype interaction) = 0.083; and ZT12-24, p(condition) = 0.02, p(genotype) = 0.36, p(condition × genotype interaction) = 0.46.

(C) NREM bout duration for the 0- to 12 h light phase (left) and 12–24 h dark phase (right) across all 4 days for WT and Fmr1<sup>−/−</sup> mice. Two-way RM ANOVA for ZT0-12, p(condition) = 0.25, p(genotype) = 0.0009, p(condition × genotype interaction) = 0.0033; and ZT12-24, p(condition) = 0.47, p(genotype) = 0.035, p(condition × genotype interaction) = 0.63.

(D) Number of NREM bouts for the 0–12 h light phase (left) and 12–24 h dark phase (right) across all 4 days for WT and Fmr1<sup>−/−</sup> mice. Two-way RM ANOVA for ZT0-12, p(condition) = 0.09, p(genotype) = 0.0090, p(condition × genotype interaction) = 0.013; and ZT12-24, p(condition) = 0.018, p(genotype) = 0.059, p(condition × genotype interaction) = 0.029.

(E) REM percentage recording time for the 0- to 12 h light phase (left) and 12–24 h dark phase (right) across all 4 days for WT and Fmr1<sup>−/−</sup> mice. Two-way RM ANOVA for ZT0-12, p(condition) = 0.49, p(genotype) = 0.095, p(condition × genotype interaction) = 0.74; and ZT12-24, p(condition) = 0.089, p(genotype) = 0.48, p(condition × genotype interaction) = 0.021.

(F) REM bout duration for the 0- to 12 h light phase (left) and 12–24 h dark phase (right) across all 4 days for WT and Fmr1<sup>−/−</sup> mice. Two-way RM ANOVA for ZT0-12, p(condition) = 0.30, p(genotype) = 0.49, p(condition × genotype interaction) = 0.62; and ZT12-24, p(condition) = 0.94, p(genotype) = 0.16, p(condition × genotype interaction) = 0.15.

(G) Number of REM bouts for the 0–12 h light phase (left) and 12–24 h dark phase (right) across all 4 days for WT and Fmr1<sup>−/−</sup> mice. Two-way RM ANOVA for ZT0-12, p(condition) = 0.71, p(genotype) = 0.15, p(condition × genotype interaction) = 0.84; and ZT12-24, p(condition) = 0.41, p(genotype) = 0.25, p(condition × genotype interaction) = 0.63.

(H) Latency to NREM sleep after vehicle or ML297 treatment in WT and Fmr1<sup>−/−</sup> mice. Two-way RM ANOVA for NREM latency, p(treatment) = 0.023, p(genotype) = 0.043, p(treatment × genotype interaction) = 0.023; and REM latency, p(treatment) = 0.049, p(genotype) = 0.61, p(treatment × genotype interaction) = 0.10. Sample size: n = 7 mice/genotype. *p < 0.05 and **p < 0.01, Sidak’s post hoc test. Data points and error bars indicate mean ± SEM.
Figure 3. NREM and REM spectral power in V1, but not PFC, is altered in Fmr1<sup>−/−</sup> mice and normalized with ML297

(A) NREM EEG power spectra in V1 for baselines and treatment conditions during ZT0-12 (light phase) with δ (0.5–4 Hz) and θ (7–15 Hz) frequency bands highlighted via dashed lines. Two-way RM ANOVA, p(frequency) < 0.0001, p(frequency × genotype interaction) < 0.0001, for baseline A-ML297 conditions and p(genotype) = 0.085 (baseline A), = 0.003 (vehicle), = 0.057 (baseline B), and = 0.42 (ML297).

(B) NREM EEG power spectra in PFC for baselines and treatment conditions during ZT0-12 (light phase) with δ (0.5–4 Hz) and θ (7–15 Hz) frequency bands highlighted via dashed lines. Two-way RM ANOVA, p(frequency) < 0.0001, p(frequency × genotype interaction) < 0.0001, for baseline A-ML297 conditions and p(genotype) = 0.088 (baseline A), = 0.21 (vehicle), = 0.26 (baseline B), and = 0.27 (ML297).

(C) Total NREM δ-band power (left) and total θ-band power for spindles (right) in V1 during ZT0-12. Two-way RM ANOVA for total δ power, p(condition) = 0.0009, p(genotype) = 0.0052, p(condition × genotype interaction) = 0.014; and total θ power, p(condition) = 0.013, p(genotype) = 0.0049, p(condition × genotype interaction) = 0.83.

(D) Total NREM δ-band power (left) and total θ-band power for spindles (right) in PFC during ZT0-12. Two-way RM ANOVA for total δ power, p(condition) = 0.26, p(genotype) = 0.68, p(condition × genotype interaction) = 0.031; and total θ power, p(condition) = 0.062, p(genotype) = 0.10, p(condition × genotype interaction) = 0.94.

(E) REM EEG power spectra in V1 for baselines and treatment conditions during ZT0-12 (light phase) with δ (0.5–4 Hz) and θ (4–12 Hz) frequency bands highlighted via dashed lines. Two-way RM ANOVA, p(frequency) < 0.0001, p(frequency × genotype interaction) < 0.0001, for baseline A, vehicle, and baseline B conditions; ML297 condition, p(frequency) < 0.0001, p(frequency × genotype interaction) = 0.023 and p(genotype) = 0.083 (baseline A), = 0.051 (vehicle), = 0.46 (baseline B), and = 0.31 (ML297).

(F) REM EEG power spectra in PFC for baselines and treatment conditions during ZT0-12 (light phase) with δ (0.5–4 Hz) and θ (4–12 Hz) frequency bands highlighted via dashed lines. Two-way RM ANOVA, p(frequency) < 0.0001, p(frequency × genotype interaction) > 0.99, for baseline A-ML297 conditions and p(genotype) = 0.12 (baseline A), = 0.23 (vehicle), = 0.73 (baseline B), and = 0.57 (ML297).

(G) Total REM δ-band power (left) and total θ-band power (right) in V1 during ZT0-12. Two-way RM ANOVA for total δ power, p(condition) = 0.24, p(genotype) = 0.86, p(condition × genotype interaction) = 0.98; and total θ power, p(condition) = 0.54, p(genotype) = 0.0046, p(condition × genotype interaction) = 0.39.

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detectable in PFC of Fmr1<sup>−/−</sup> mice at any time of day (Figures 3F and 3H; Figures S6F and S6H). This may reflect a similar brain-region-specific change in the distribution of REM θ that has been reported in adults with ASD. In baseline wake, δ EEG power was similar between genotypes (in both cortical areas across both light and dark phases), while γ power remained elevated across both PFC and V1 in Fmr1<sup>−/−</sup> mice (Figure S7A–S7D).

Vehicle treatment had no effects on any state-specific EEG features in Fmr1<sup>−/−</sup> mice, which remained significantly different from sleep features of WT littermates (Figures 3A–3C, 3E, 3G; Figures S5A and S5B; Figures S6A, S6C, S6E, S6G; Figures S7B–S7D). However, following ML297 administration, V1 NREM δ and α power no longer differed significantly between WT and Fmr1<sup>−/−</sup> mice (Figures 3A and 3C). ML297 did not affect NREM γ power, which remained elevated in both V1 and PFC (Figures S5A and S5B), although, after ML297 administration, wake γ activity was statistically similar between WT and Fmr1<sup>−/−</sup> mice in both V1 and PFC regions across both light and dark phases (Figures S7C and S7D). REM θ power in V1 was also more similar between WT and Fmr1<sup>−/−</sup> mice after ML297 treatment (Figures 3E and 3G; Figures S6E and S6G).

Observed region-specific changes in NREM α and spindle powers in Fmr1<sup>−/−</sup> mice suggest that coordination of thalamocortical oscillations might be disrupted by loss of FMRP. Because synchronous thalamocortical activity during spindles is thought to be essential for brain development and cognitive function, we tested whether NREM spindle density, or spindle coordination between brain regions, could be altered by ML297. In V1 baseline recordings, we again observed reduced light-phase spindle density in Fmr1<sup>−/−</sup> mice compared to WT littermates (Figure 4A). In contrast, spindle density in PFC recordings differed only modestly between genotypes (Figure 4B). Vehicle administration did not affect V1 spindle density in Fmr1<sup>−/−</sup> mice, which remained significantly reduced relative to WT littermates. Following ML297 administration, V1 and PFC spindle density were similar between WT and Fmr1<sup>−/−</sup> mice (Figures 4A and 4B). Mean spindle duration was not affected by administration of either vehicle or ML297 in either V1 or PFC (Figure S9).

Because genotype-associated spindle density differences were brain-region specific (like other changes in NREM oscillations in Fmr1<sup>−/−</sup> mice), we compared simultaneously recorded NREM spindle densities in V1 vs. PFC of individual mice. Across the 24-h baseline, spindle densities between the two regions were consistently correlated in WT littermates, but not in Fmr1<sup>−/−</sup> mice (Figure 4C). When WT littermates were treated with either vehicle or ML297, the correlation between V1 and PFC spindle densities was unaffected. Vehicle administration had no effect in Fmr1<sup>−/−</sup> mice (i.e., V1 and PFC spindle densities remained uncorrelated). However, over the 24 h after ML297 treatment, spindle densities became significantly correlated between V1 and PFC EEG sites in individual mice, suggesting better coordination of thalamocortical oscillations during NREM sleep (Figure 4C).

The thalamic reticular nucleus (TRN) is a brain structure essential for both the generation of spindle oscillations and their coordination between brain structures. Critically, the activity of TRN PV+ interneurons (constituting the majority of TRN neurons)—particularly burst-mode firing in these neurons—is both necessary and sufficient for spindle generation, and dysfunction of PV+ interneurons is thought to drive cognitive disruption in FXS. Thus, we speculated that altered α activity and spindle coordination in Fmr1<sup>−/−</sup> mice could be driven by alterations in the TRN PV+ interneuron network. Using quantitative immunohistochemistry (IHC), we observed TRN structural alterations in Fmr1<sup>−/−</sup> mice, including reduced TRN PV+ interneuron density, and a trend for reduced TRN size (Figure 4D). Together, our data suggest that microcircuit-level changes in the TRN network disrupt NREM spindle generation and coordination in Fmr1<sup>−/−</sup> mice, and these spindle changes may be partially reversed by ML297 administration.

To further quantify state-specific EEG synchrony, we compared overall V1-PFC spectral coherence for Fmr1<sup>−/−</sup> mice and WT littermates. Across both baseline A and B, we found NREM V1-PFC δ-band coherence was significantly lower in Fmr1<sup>−/−</sup> mice compared to WT littermates (Figures S12A and S12C). The α-band coherence was also significantly reduced during baseline A (Figure S12C). NREM V1-PFC γ coherence (Figures S12A and S13B) and REM δ and θ coherence (Figures S12B and S12D) were similar between genotypes. Vehicle treatment did not affect these baseline differences in coherence. In contrast, ML297 administration normalized V1-PFC NREM δ coherence (Figures S12A–S12D). Together, our data suggest that coordination of NREM δ and spindle oscillations, which is disrupted in Fmr1<sup>−/−</sup> mice, can be partially renormalized by ML297.

Post-learning ML297 administration rescues deficits in sleep-dependent memory consolidation in Fmr1<sup>−/−</sup> mice

In mice, consolidation of hippocampus-dependent contextual fear memory (CFM) and object location memory (OLM) are disrupted by brief sleep deprivation (SD) in the hours immediately following training. In both CFM and OLM, Fmr1<sup>−/−</sup> mice have deficits in both CFM and OLM, but it is unclear whether the sleep disruptions we observe in Fmr1<sup>−/−</sup> mice could cause consolidation deficits for these tasks. We tested whether restoring sleep with ML297 could rescue sleep-dependent memory consolidation in Fmr1<sup>−/−</sup> mice. We first carried out single-trial conditioning (contextual fear conditioning [CFC]; placement in a novel context, paired with a foot shock) in Fmr1<sup>−/−</sup> mice and WT littermates. The two groups showed similar, very low freezing prior to shock during CFC, but, as expected, Fmr1<sup>−/−</sup> mice showed reduced freezing during CFM recall (when returned to the shock context 24 h later; Figures 5A and 5B; Figures S14A and S14B).

(H) Total REM δ-band power (left) and total θ-band power (right) in PFC during ZT0-12. Two-way RM ANOVA for total θ power, p(condition) = 0.66, p(genotype) = 0.87, p(condition × genotype interaction) = 0.86; and total δ power, p(condition) = 0.23, p(genotype) = 0.43, p(condition × genotype interaction) = 0.43. Sample size: n = 7 mice/genotype. *p < 0.05 and **p < 0.01, Sidak’s post hoc test. Data points and error bars indicate mean ± SEM.
We next tested whether partially renormalizing post-CFC sleep could improve CFM consolidation in \textit{Fmr1}\(^{-/}\) mice. Mice were administered either vehicle or ML297 immediately following CFC. To test for sleep dependence of drug administration, half of the mice within each treatment group were allowed ad \textit{libitum} sleep (SL), while the second half underwent 6 h of SD via gentle handling in their home cage (Figure 5C). Mice allowed ad \textit{libitum} sleep were visually monitored over the first 6 h post CFC to assess drug effects on sleep behavior. Consistent with our polysomnographic data, vehicle-treated \textit{Fmr1}\(^{-/}\) mice had significantly reduced post-CFC sleep time compared to those given ML297 and compared to WT littermates with either treatment (Figure 5E). In addition, consistent with the interpretation that \textit{Fmr1}\(^{-/}\) mice have reduced overall sleep drive, the number of interventions required to prevent sleep during the 6-h SD period was significantly lower in \textit{Fmr1}\(^{-/}\) mice, regardless of treatment (Figure 5E). Pre-shock freezing was similarly low between the two genotypes during CFC training (Figures 5D–5F; Figure S14C). Vehicle-treated SL \textit{Fmr1}\(^{-/}\) mice showed significantly reduced freezing during CFM testing compared with either ML297-treated SL mice or SL WT littermates. Critically, CFM performance in ML297-treated \textit{Fmr1}\(^{-/}\) SL mice was similar to WT SL mice, indicating a functional rescue of CFM consolidation by ML297 (Figures 5D–5G; Figure S14D). \textit{Fmr1}\(^{-/}\) mice and WT littermates administered ML297 showed deficits in CFM after post-CFC SD, as expected following SD, suggesting rescue of CFM consolidation in \textit{Fmr1}\(^{-/}\) mice was sleep dependent. For vehicle-treated WT mice, SD caused disruption of CFM consolidation similar to that seen in \textit{Fmr1}\(^{-/}\) mice (Figures 5D and 5G).

We next tested whether ML297 could rescue other forms of hippocampus-mediated, sleep-dependent memory in \textit{Fmr1}\(^{-/}\) mice.
mice. OLM is a form of spatial memory disrupted in Fmr1<sup>−/−</sup> mice. On the day prior to training on the OLM task (Figures 6A and S15), mice were exposed to the OLM arena for 5 min, during which open-field (OF) activity was used to measure locomotor activity and anxiety-related behavior (i.e., thigmotaxis). During the OF test (habituation period), Fmr1<sup>−/−</sup> mice and WT littermates showed similar locomotion (total travel distance) and relative time spent in outer and inner zones of the arena (Figure 6B). Mice underwent OLM training at ZT0, during which they explored two identical objects placed inside the arena for 10 min (Figure 6A); 24 h later, they returned to the arena, where one of the two objects had been displaced and the other kept in the same (familiar) location. A discrimination index, used to measure selective...
interaction with the displaced vs. familiar objects, was calculated for each mouse. As expected, discrimination indices were significantly lower during OLM testing for Fmr1<sup>-/-</sup> mice vs. WT littermates (Figure 6C). To test whether ML297 rescues OLM consolidation in Fmr1<sup>-/-</sup> mice, a second cohort of mice were administered either vehicle or ML297 immediately after OLM training (Figure 6D). While locomotion and thigmotaxis were again comparable during habituation between genotypes (Figure 6E), performance during OLM testing varied by both genotype and treatment. Fmr1<sup>-/-</sup> mice treated with ML297 showed a significant improvement in discrimination between displaced and familiar object locations compared to those treated with vehicle, suggesting at least partial rescue of OLM consolidation. However, WT littermates treated with ML297 showed no improvement in OLM consolidation, and in fact performed worse than vehicle-treated counterparts (Figure 6F). Together, these data indicate that restoration of post-learning sleep in Fmr1<sup>-/-</sup> mice with ML297 benefits both CFM and OLM consolidation and further suggest that loss of sleep could contribute to cognitive impairments associated with FXS.

**Memory consolidation improvements in Fmr1<sup>-/-</sup> mice are associated with ML297-mediated normalization of hippocampal activation patterns during recall**

Activity among hippocampal DG granule cells and interneurons plays an important role in both learning and memory retrieval.95–98 and suppression of DG activity during sleep disruption can interfere with memory consolidation.99,100 We tested whether CFM deficits in Fmr1<sup>-/-</sup> mice are associated with altered DG activity patterns during recall, and whether ML297 can affect these activity patterns. Fmr1<sup>-/-</sup> mice and WT littermates underwent CFC followed by ML297 or vehicle administration, after which they were allowed ad libitum sleep in their home cages. CFM recall was tested 24 h after CFC; 90 min after recall, mice were perfused to quantify expression patterns of the immediate-early gene (IEG) cFos, associated with DG neurons’ recall, mice were perfused to quantify expression patterns of the immediate-early gene (IEG) cFos, associated with DG neurons’ 

Figure 6. GIRK channel activation rescues disrupted sleep-dependent object location memory consolidation in Fmr1<sup>-/-</sup> mice

(A) WT and Fmr1<sup>-/-</sup> mice (n = 8/genotype) underwent habituation to the object location memory (OLM) arena (combined with open-field [OF] behavioral measurement) at ZT0; 24 h later, mice underwent OLM training with two identical objects in the arena. After an additional 24 h, mice were tested for OLM in the same arena with one object moved to a new location.

(B) Proportion of time spent in inner and outer zones of the arena (left) between WT and Fmr1<sup>-/-</sup> mice during habituation. Two-way ANOVA, p(field zone) = 0.0001, p(genotype) = 0.94, p(field zone x genotype interaction) = 0.12. Total distance traveled during habituation (right) between genotypes. Two-tailed, unpaired t test. Data shown as mean ± SEM. (C) Index for WT and Fmr1<sup>-/-</sup> mice to discriminate between displaced and non-displaced objects during OLM testing. ***p < 0.001, two-tailed, unpaired t test. Data shown as mean ± SEM.

(D) WT and Fmr1<sup>-/-</sup> mice underwent habituation to the OLM arena and OLM training as described above, followed immediately by ML297 or vehicle administration and ad libitum sleep. After an additional 24 h, mice were tested for OLM in the same arena with one object moved to a new location.

(E) OF activity (left) between WT and Fmr1<sup>-/-</sup> mice during habituation. Two-way ANOVA, p(field zone) = 0.0001, p(genotype) = 0.43, p(field zone x genotype interaction) = 0.013. Total distance traveled (right) was also similar between genotypes. Two-tailed, unpaired t test. Data shown as mean ± SEM.

(F) Index for WT and Fmr1<sup>-/-</sup> mice to discriminate between displaced and non-displaced objects during OLM testing with either vehicle or ML297 treatment. n = 8 mice/experimental condition. Two-way ANOVA, p(treatment) = 0.31, p(genotype) < 0.0001, p(treatment x genotype interaction) < 0.0001, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; Sidak’s post hoc test. Data shown as mean ± SEM.
observed in the DG of WT-vehicle mice. In contrast, ML297 significantly increased DG cFos+ neuron density compared in WT littermates (Figure 7B), consistent with recent findings.103 This suggests that overactivation of the DG network in Fmr1<sup>−/−</sup> mice disrupts CFM recall, and normalizing DG activity with post-CFM ML297 administration improves CFM recall.

Alterations in excitatory-inhibitory (E-I) balance have been proposed as a mechanism promoting hyperactivity of neural circuits Fmr1<sup>−/−</sup> mice.104,105 To test whether changes in inhibitory DG networks are associated with disrupted CFM recall, we also measured PV+ interneuron density and perineuronal net (PNN) expression in the DG following recall. DG PV+ interneuron density did not differ by genotype, regardless of treatment. However, density of DG PNNs was reduced in vehicle-treated Fmr1<sup>−/−</sup> mice. PNN density in ML297-treated Fmr1<sup>−/−</sup> mice was restored to levels observed in WT littermates (Figure 7B). We also assessed the expression of cFos among PV+ interneurons during CFM recall as a proxy measure of their activity level. Similar to overall cFos+ neuron density, the density of cFos+PV neurons was significantly increased in vehicle-treated Fmr1<sup>−/−</sup> mice compared with WT littermates (in either treatment group; Figure 7B). Similarly, the proportion of all PV+ interneurons expressing cFos (cFos+PV/PV) was significantly higher in vehicle-treated Fmr1<sup>−/−</sup> mice compared with WT littermates (in either treatment group; Figure 7B). ML297 administration to Fmr1<sup>−/−</sup> mice normalized cFos+PV density (and proportion of PV+ interneurons expressing cFos) to WT levels (Figures 7B and 7C). Finally, as a proxy measure of E-I balance, we calculated the proportion of all cFos+ neurons that were also PV+ (cFos+PV/cFos). We found that despite differences in overall cFos expression, this ratio was similar in vehicle-treated WT and Fmr1<sup>−/−</sup> mice. While ML297 significantly reduced this ratio in both genotypes, the reduction was significantly more dramatic in WT mice (Figure 7C). Overall, our data suggest overactivation of inhibitory and excitatory neuron activity in the DG network of Fmr1<sup>−/−</sup> mice is associated with disruption to CFM and that this can be attenuated with ML297 administration during CFM consolidation.

To understand the impact of ML297 treatment on recall-associated neuronal activation across the rest of the dorsal hippocampus-amygdala circuit, we also measured cFos, PV, and PNN expression within CA1, CA3, and basolateral amygdala after CFM recall. CA1 network activity was of particular interest, as it is also essential for CFM recall,106,107 and post-learning, sleep-associated activity changes are essential for CFM consolidation.108-111 Moreover, recall-associated patterns in CA1 are known to be altered in Fmr1 knockout mice.112 Vehicle-treated Fmr1<sup>−/−</sup> mice showed dramatically reduced recall-associated cFos+ neuron density within CA1 compared with WT littermates, which was restored by administration of ML297 (Figures 7D and 7E). Similar to DG, CA1 PNN density was reduced in vehicle-treated Fmr1<sup>−/−</sup> mice—an effect that was also partially reversed by ML297. PV+ and cFos+PV+ numbers were also decreased in vehicle-treated Fmr1<sup>−/−</sup> mice, relative to WT littermates. These values were not affected by ML297 administration, suggesting that post-learning ML297 treatment may alter the ratio of activated excitatory vs. inhibitory neurons in CA1 during subsequent recall in Fmr1<sup>−/−</sup> mice (Figures 7E and 7F).

In contrast to the dramatic changes in recall-associated network activity in DG and CA1 of Fmr1<sup>−/−</sup> mice, we found no change in cFos+ neuron density in CA3 (Figures S17A and S17B) and only modest reductions in amygdala (Figures S18A and S18B). While ML297 increased cFos in CA1 of Fmr1<sup>−/−</sup> mice, it had no effect in CA3 or amygdala. CA3 PV+ interneuron and PNN densities were also modestly reduced in Fmr1<sup>−/−</sup> mice; here, ML297 treatment reversed the effects of genotype on PNN density.
density. This suggests that alterations in the PV+ interneurons present throughout the hippocampus-amygdala circuit in Fmr1−/y mice are not uniformly reversed by ML297.

Taken together, these findings suggest that Fmr1−/y mice show dramatic changes to hippocampus-amygdala network activity and interneuron function, which are evident during CFM recall. Post-CFC administration of ML297 alters network activity with the DG in Fmr1−/y mice, renormalizing activity levels in subsequent recall, and has modest effects on overall cFos expression in CA1, but has few effects elsewhere in the circuit. To clarify whether these subregion-specific effects of ML297 could be driven by direct effects of the drug on GIRK1/2 channels, we immunohistochemically quantified expression of GIRK1 protein in dorsal hippocampus (Figure S16). We focused our analysis on DG and CA1 subregions, where alterations in cFos expression patterns during recall in Fmr1−/y mice appeared affected by prior ML297 administration (Figure S16A). We found relatively high levels of expression in the pyramidal cell body layer of CA1 and the granule cell body layer of DG, indicating clear expression in the principal neurons of the circuit, consistent with reports of high Kcnj3 expression in these cell types from single-cell transcriptomic analysis.50,51 Overall GIRK1 expression in CA1 oriens lacunosum-molecularare was comparatively low, with sparse labeling consistent with interneuron cell bodies. Expression of GIRK1 was highest in CA1 stratum lacunosum-molecularare (SLM), which contains a mixed population of interneurons (including somatostatin-expressing [SST+] but not PV+ interneuron types). Expression in the DG molecular layer (MDG), containing a mixed population of interneurons including PV+ interneurons,113 was similarly high with respect to the granule cell body layer. These patterns are again consistent with single-cell data indicating preferential expression of Kcnj3 among excitatory DG and CA1 neurons and fast-spiking, PV+ interneuron populations.50,51 Critically, GIRK expression levels in SLM and MDG did not differ between Fmr1−/y mice and WT littermates (Figures S16B and S16C). Taken together with our findings of altered recall-driven activation patterns in DG (and to a lesser extent CA1) of Fmr1−/y mice treated with ML297, these data suggest that GIRK1/2 activation in these regions can improve function of these circuits during sleep-dependent memory processing.

**DISCUSSION**

To examine relationships between sleep and cognitive phenotypes in FXS, we more fully characterized sleep disruptions in Fmr1−/y mice, where limited previous data suggested potential sleep disruptions comparable to patients with FXS.20,21,114 Our long-term polysomnographic recordings provide conclusive evidence that mutant mice exhibit reduced NREM sleep, fragmented NREM architecture, and brain-region-specific changes in NREM and REM sleep oscillations. These alterations are remarkably analogous to findings seen in polysomnography recordings of patients with FXS and ASD.6,6 For example, we observed increased δ power and decreased spindle density and power during NREM in Fmr1−/y mice, similar to that reported in patients with ASD and/or FXS.13,59,79,115–118 Notably, these genotype-driven EEG alterations are detected only in V1, not PFC; EEG recordings of ASD children and adults show similar spatial (i.e., anteroposterior) differences.57,59,79 These convergent findings suggest the Fmr1−/y mouse model should be useful for studying sleep phenotypes related to FXS and ASD.

Loss of Fmr1 reduces excitatory drive onto PV+ interneurons and other GABAergic interneuron subtypes while increasing the density of immature dendritic spines in neocortical and hippocampal pyramidal neurons.14,17,19,120–125 These alterations likely alter network excitability, which could underlie some of the sleep-associated EEG changes we observed in Fmr1−/y mice. Our data suggest that the TRN, populated by PV+ interneurons and essential for NREM spindle generation,26,74,126 is likely affected by more general PV+ interneuron disruption in FXS. The reduction in TRN PV+ interneuron density we observed in Fmr1−/y mice likely drives reduced sleep spindle density, and reduced spindle coordination between cortical regions, in Fmr1−/y mice. Critically, GIRK1/2 function is thought to be selectively disrupted in the absence of FMRP.52,53 We found that acute ML297 administration to Fmr1−/y mice normalized V1-PFC spindle density correlations. Available data suggest that GIRK1/2 channels are well expressed in (and modulate the physiology of) GABAergic TRN neurons.127 Thus, ML297 activation of GIRK1/2 could be directly involved in improving synchronization of spindle oscillations. Anteroposterior anatomical differences in the TRN and associated thalamocortical circuits85,87,128 may underly the site-specific EEG changes (and ML297 effects) we observe in Fmr1−/y mice and the anteroposterior differences in sleep oscillations observed in ASD. However, further study will be needed to fully understand how various microcircuit-level alterations in Fmr1−/y mice contribute to their diverse NREM spectral power and coherence changes.

Sleep is essential for cognitive functions, including memory consolidation.29,130 Given the relationship between sleep disruption and cognitive impairment, we tested whether restoration of sleep phenotypes could improve known memory consolidation deficits in Fmr1−/y mice. We found that ML297 improved consolidation of both CFM and OLM (two dorsal hippocampus-dependent, sleep-dependent forms of memory), which are normally disrupted in Fmr1−/y mice. Memory rescue of these memories by hypnotic treatment appeared to rely on sleep-dependent changes—e.g., rescue of CFM by ML297 was blocked by post-CFC SD. A parsimonious conclusion from these data is that ML297 rescues sleep-dependent memory consolidation by normalizing post-learning sleep behavior and sleep oscillations in Fmr1−/y mice.

Our current data cannot pinpoint which aspects of sleep architecture, or which state-specific oscillations, are most central to impaired sleep-dependent hippocampal processing in Fmr1−/y mice. Some features, such as sleep latency, are clearly not normalized in Fmr1−/y mice (and, indeed, sleep latency is lengthened by ML297 in WT littermates; Figure 2). Moreover, ML297 causes no apparent increase in sleep pressure (in either WT or Fmr1−/y mice) during SD, where similar numbers of interventions were required to keep mice awake, regardless of drug treatment (Figure 5). We can conclude that these features of sleep disruption are not essential to deficits in sleep-dependent memory storage in Fmr1−/y mice. On the other hand, it is worth noting that disruption of some hippocampally generated sleep features...
in Fmr1−/− mice (e.g., REM sleep) appear to be partially reversed by ML297 (Figure 3). Hippocampal δ oscillations are known to be modulated by prior learning, and to directly promote hippocampal memory consolidation, in WT mice.109,110 In contrast, alterations in NREM ripple oscillations detected in the V1 EEG of Fmr1−/− mice are largely unaffected by ML297 treatment (Figures S10 and S11). Likewise, altered cortically generated NREM slow and γ oscillations in Fmr1−/− mice are unaffected by treatment with ML297 (Figures S1, S2, S3,S4, and S5). On the other hand, altered NREM thalamocortical δ and spindle oscillations appear to be partially renormalized by ML297 administration (Figures 3 and 4; S4; S6, and S12). It is worth noting that these oscillations are tightly coordinated with one another and with the transition from NREM to REM sleep.131,132 Moreover, like hippocampally generated δ, coordinated thalamocortical spindles are thought to promote successful consolidation of hippocampally mediated memories such as CFM in WT mice.103,111 Thus, our data support a general model where both spindle-rich NREM and REM sleep work together to promote hippocampal memory storage.26,130,133 As the aforementioned features of both states are disrupted in Fmr1−/− mice, and partially restored by ML297 administration, those features may be most critical to the disruptions of sleep-dependent hippocampal memory processing we observe in our Fmr1−/− mice. Taken together, we conclude that ML297 effects on sleep-dependent memory consolidation in Fmr1−/− mice are likely due to its effects on post-learning sleep quality (continuity and oscillatory activity) rather than sleep pressure and quantity.

As described above, loss of FMRP is thought to alter the structure and function of PV+ interneurons, and our present data support this conclusion. In every brain region examined in Fmr1−/− mice, we note a reduction in the density of PV+ interneurons, and in most brain regions we observed reduced PNN ensheathment of PV+ interneurons. In general, these changes were either unaffected or only modestly changed by ML297 administration. However, after CFM recall, we noticed changes to cFos expression in control (vehicle-treated) Fmr1−/− mice that occurred uniquely in DG, where overall expression of cFos was elevated, and cFos expression in PV+ interneurons was also elevated, relative to WT littermates. While additional studies will be needed to understand whether these changes are sufficient to cause disruption of CFM recall, we found that, in ML297-treated Fmr1−/− mice (i.e., those with improved CFM consolidation), cFos expression in DG (both across DG as a whole and in PV+ interneurons) was reduced. This suggests that improved sleep-dependent memory consolidation with ML297 treatment may be associated with suppression of aberrant, elevated DG activity in Fmr1−/− mice. In contrast, overall cFos expression after recall was reduced in CA1 of Fmr1−/− mice, and ML297 administration during CFM consolidation restored cFos+ neuron numbers to levels seen in WT littermates. Together, these data suggest altered E-I balance in DG and CA1 of Fmr1−/− mice, which may be partially reversed by administration of ML297. While the precise role of altered E-I balance in disruption of memory consolidation is unclear, available data suggest that sleep itself can alter E-I balance in both cortical and hippocampal networks.134,135 Consistent with single-cell sequencing data,49–51 we also found that GIRK1 is present at high levels in the principal cell body layers of DG and CA1 and even higher levels in the SLM of CA1 (where interneurons synapse onto principal neuron dendrites) and MDG (where interneurons synapse onto granule cell dendrites). SLm interneurons are critical for gating excitatory input to CA1 pyramidal neurons from entorhinal cortex,138,139 a function that is likely critical for hippocampally mediated learning.138 MDG interneurons play a similar role in DG, gating entorhinal cortex input to granule cells, and supporting lateral inhibition among granule cells.138,140 Critically, gating of entorhinal input to both DG and CA1 is thought to be altered following loss of FMRP.101,103,104–106 Thus, ML297 treatment may also play critical roles in the hippocampus itself, to improve circuit function in the context of sleep-associated memory consolidation in Fmr1−/− mice.

**Limitations of the study**

While our present findings suggest that sleep is an important therapeutic target for FXS (and potentially other ASDs), additional work is needed to clarify the translational potential of this work. For example, it is still unclear whether hippocampal network activity changes during recall are due to prior sleep architecture changes,133 sleep-dependent oscillatory changes,108,110,144 and/or direct pharmacological effects of ML297 on hippocampal neurons.145 Future studies using hypnotics with distinct mechanisms of action, and/or local delivery of these agents, will be needed to disentangle these mechanisms. Understanding the precise microcircuit effects of GIRK1/2 activation (e.g., through a combination of pharmacologic and optogenetic manipulations, and/or single-neuron electrophysiology) may clarify the effects of ML297 on memory function. Further studies in juvenile mice will also be needed to determine our findings’ relevance to treating children with FXS. Given the role of sleep has in shaping of synaptic circuits early in life,135,136,146,147 treating sleep phenotypes in Fmr1−/− mice earlier in development may be even more beneficial for cognition. Finally, since FXS differs between men and women,148 it will be important to test how sleep, sleeping brain activity, and sleep-dependent cognitive functions are affected in female Fmr1−/− mice. Nonetheless, our initial findings provide proof of concept for targeting sleep phenotypes as a therapeutic strategy for treating cognitive aspects of FXS.

**STAR Methods**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**
- **METHOD DETAILS**
  - EEG-EMG surgical procedures and neural data acquisition
  - Pharmacological preparation and injection
  - Contextual fear conditioning (CFC)
  - Object location memory (OLM) task and open field (OF) test
  - Sleep monitoring and sleep deprivation
  - Histology and immunohistochemistry
- **QUANTIFICATION AND STATISTICAL ANALYSIS**
  - Sleep state and power spectra analysis
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2024.114266.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


STAR METHODS

KEY RESOURCES TABLE

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources, code, and reagents should be directed to and will be fulfilled by the Lead Contact, Sara J. Aton (saton@umich.edu).

Materials availability
This study did not generate unique reagents.

Data and code availability
All data reported in this paper will be shared by the lead contact upon request.
All original code has been deposited to Github at https://doi.org/10.5281/zenodo.11122261 and is publicly available as of the date of publication.
Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

We generated male Fmr1−/− mice and wild-type (WT) littermates in-house by crossing WT male mice with Fmr1 heterozygous (Fmr1+/−) female mice - both on a C57BL/6 background (The Jackson Laboratory, stock # 003025). Genotypes for mice were determined using standard PCR methods and primers described on the Jackson Laboratory website. For all experiments, 4–5 month old mice were used. Mice were housed under a 12:12 h light/dark cycle (lights on at 9:00 a.m.), provided with compressed cotton and “Enviro-dri” paper nesting and bedding material (Shepherd Specialty Papers, TN), and provided ad lib access to water and food. Mice were housed with littermates until either EEG electrode implantation surgery or behavioral studies, at which point they were single housed in standard cages with extra nesting and bedding material for enrichment. All mouse husbandry, experimental, and surgical procedures were reviewed and approved by the University of Michigan Internal Animal Care and Use Committee.

METHOD DETAILS

EEG-EMG surgical procedures and neural data acquisition

Mice were anesthetized with 1–2% isoflurane anesthesia administered ketoprofen (0.005 mg/kg; intraperitoneal injection, i.p.) prior to surgery. Each mouse received two miniature, stainless steel screw EEG electrodes (P1 Technologies, Catalog #: E362/96/1.6/SPC) positioned over primary visual cortex (V1; – 2.9 mm anterior/posterior [AP] and 2.7 mm medial/lateral [ML]) and/or prefrontal cortex (PFC; 2.8 AP, 1.8 mm ML). These sites were chosen to record at more posterior (V1) locations analogous to prior reports of disrupted sleep EEG activity in patients with ASD, and for comparison, more anterior locations.57–59 A reference screw was placed over cerebellum and a braided stainless steel wire EMG electrode was placed in the nuchal muscle. EEG electrodes were custom made from braided stainless-steel wire (Cooner Wire, Catalog No: AS636) soldered to miniature stainless steel male pins (P1 Technologies, Catalog No: 363A/PKG). The implant was secured using a pedestal (P1 Technologies, Catalog No: MS7P) bonded with super glue (Loctite). After 11 days of postoperative recovery, each mouse was moved to a new cage with an open top and habituated to tethering of flexible cables (P1 Technologies, Catalog No: 363-000) for 3 days before EEG/EMG data collection (Omniplex; Plexon Inc). Mice were recorded for four consecutive days (96 h total). Day 1 was a 24-h baseline recording (Baseline A) starting at lights-on (ZT0). After 24 h (Day 2), mice were injected with vehicle solution at lights-on and recorded. Day 3 was a second baseline recording (Baseline B). Day 4, mice were injected with ML297 (30 mg/kg) at lights-on and recorded for a final 24-h period.

Pharmacological preparation and injection

ML297 was purchased from Tocris Bioscience (Catalog #. 5380). For all experiments, ML297 was initially dissolved in DMSO and diluted with 0.5% hydroxypropyl cellulose aqueous solution. Mice were injected with a 30 mg/kg solution of ML297, dosage based on previously published studies in rodent.43,45,46 Vehicle solutions consisted of 2% DMSO in 0.5% hydroxypropyl cellulose aqueous solution.

Contextual fear conditioning (CFC)

Mice underwent single-trial CFC as previously described.31,32,46,89,150,151 Mice were handled by the experimenter for 5 min daily for 3 days prior to training. Each mouse was placed in a novel cylindrical conditioning chamber made of clear Plexiglas with a black and white checkerboard pattern and metal grid floor (Med Associates). Before each individual mouse session, the arena was cleaned with a 5% Lysol solution. Mice were allowed to freely explore for 2 min and 28 s, after which they received a 0.75 mA, 2 s foot shock through the grid floor, followed by an additional 30 s in the CFC chamber. Immediately following CFC, mice were returned to their home cage. For studies on ML297, mice were given an i.p. injection of either ML297 (30 mg/kg; Tocris) or vehicle (2% DMSO in 0.5% hydroxypropyl cellulose aqueous solution). Injections occurred within 2 min of removal from the CFC chamber. Contextual fear memory (CFM) tests were conducted 24 h later by returning mice to the CFC chamber for 5 min. Both CFC training and CFM testing began at lights-on (ZT0), and mice were video monitored continuously during both sessions.

Object location memory (OLM) task and open field (OF) test

Mice underwent a single trial OLM task as previously described.29,91,152 Here, mice are placed in a rectangular arena made of gray PVC walls, transparent PVC bottom, and the following dimensions: length of 40 cm, width of 30 cm, and height of 30 cm. Spatial cues were placed on the opposite sides of the short walls and consisted of one black and white checkerboard pattern and one black and white striping pattern. Mice were handled by the experimenter for 5 min daily for 4 days prior to training. Prior to each session, the arena and objects were cleaned with a 10% ethanol solution. Before training, mice were habituated to the arena, which consisted of 5 min of free exploration and served as the OF test. The OF test is a rapid assessment of well-defined behaviors such as anxiety-related behaviors, body activity, and locomotion that require little to no prior training to the subject mouse.153 During training, a pair of identical objects were placed symmetrically across in the middle of the arena, in identical locations for all groups. Mice were placed in the arena facing the wall and were allowed to freely explore the arena and objects for 10 min. Immediately following
training, mice were returned to their home cage. For studies on ML297, mice were given an i.p. injection of either ML297 (30 mg/kg; Tocris) or vehicle (2% DMSO in 0.5% hydroxypropyl cellulose aqueous solution). Injections occurred within 2 min of removal from the OLM arena. OLM tests were conducted 24 h later. During testing, one object was displaced (novel location) diagonally from the other object, which remained the same position (familiar location). The object that was displaced (i.e., which object was moved from its training location), and the location of the displacement, were counterbalanced during testing, to avoid confounding place and object preferences. Mice were again allowed to freely explore the arena and objects for 10 min. Both OLM training and OLM testing began at lights-on (ZT0), and mice were video monitored continuously during both sessions and the habituation (OF test) period.

Sleep monitoring and sleep deprivation
Following CFC training, mice that were either allowed ad lib sleep (Sleep) or were sleep-deprived (SD) via gentle handling over the next 6 h (ZT0-6). This method of SD was chosen based on prior work showing that the glucocorticoid response evoked by gentle handling alone is not sufficient to disrupt consolidation of CFM (and in fact may enhance consolidation). These procedures included cage tapping or shaking, and/or nest disturbance and has been previously shown to ensure above 90% wake time based on EEG/EMG validation. Following SD, all mice were allowed ad lib recovery sleep over the next 18 h prior to CFM testing. For ad lib sleep mice, sleep was quantified over the first 6 h post-CFC via visual monitoring. Every 5 min, individual mice were scored as awake or asleep, with sleep identification based on immobility, slow breathing, and presence of stereotyped (crouched) sleep postures, consistent with prior studies and has been validated with similar total sleep time in EEG/EMG implanted mice.

Histology and immunohistochemistry
To quantify hippocampal activation patterns associated with recall, 90 min following the conclusion of CFM recall tests, mice were euthanized with an overdose of sodium pentobarbital and perfused with ice-cold PBS, followed by ice-cold 4% paraformaldehyde. Brains were dissected, post-fixed, and cryoprotected in a 30% sucrose solution. 50 μm coronal dorsal hippocampal and amygdala sections were cryosectioned. To quantify parvalbumin (PV) interneuron expression in the thalamic reticular nucleus (TRN), brains were dissected, post-fixed, and rinsed in PBS. 100 μm coronal sections containing TRN were collected via a vibratome.

For all tissue, free-floating sections were washed in PBS with 0.2% Triton X-100 (PBST) three times, each for 10 min, and then incubated in Starting Block blocking buffer (Thermo Scientific) for 1 h. For fear conditioning experiments, hippocampal and amygdala sections were then incubated overnight in primary antibody at 4°C: rabbit-anti-cFos (1:1000; Abcam, ab190289), mouse-anti-PV (1:2000; Millipore, MAB1572), and wisteria floribunda agglutinin (lectin) tagged with fluorescein (WFA; 1:500; Vector Labs, FL-1351-2). TRN sections were only incubated with mouse-anti-PV (1:2000; Millipore, MAB1572). Hippocampal sections for GIRK staining were incubated with rabbit-anti-GIRK1 (1:200; Alomone Labs, APC-005). All sections were then washed in PBST, two times for 10 min and incubated with secondary antibody. For fear conditioning experiments, Alexa Fluor 647 (1:200; Invitrogen, A11032) or vehicle (2% DMSO in 0.5% hydroxypropyl cellulose aqueous solution). Injections occurred within 2 min of removal from the OLM arena. OLM tests were conducted 24 h later. During testing, one object was displaced (novel location) diagonally from the other object, which remained the same position (familiar location). The object that was displaced (i.e., which object was moved from its training location), and the location of the displacement, were counterbalanced during testing, to avoid confounding place and object preferences. Mice were again allowed to freely explore the arena and objects for 10 min. Both OLM training and OLM testing began at lights-on (ZT0), and mice were video monitored continuously during both sessions and the habituation (OF test) period.

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QUANTIFICATION AND STATISTICAL ANALYSIS

Sleep state and power spectra analysis
EEG/EMG signals (0.5–300 Hz) were amplified at 20 x, digitized, further digitally amplified at 20-100 x, and continuously recorded (with a 60-Hz notch filter applied to remove environmental noise) using Plexon Omniplex software and hardware (Plexon Inc., TX) as previously described Baseline and post-treatment recordings were scored manually in 10-s epochs as wake, NREM, or REM sleep using custom MATLAB software and previously published studies. Scorers were blind to genotype and recording day. EEG and EMG data were band-pass filtered at 0–60 Hz and 150–250 Hz, respectively, for viewing during scoring. NREM sleep was defined as synchronized, high amplitude, low frequency oscillations within the EEG and low EMG activity. REM sleep was defined as reduced, low frequency oscillations and a theta rhythm pattern (4–12 Hz) with lack of EMG activity. Wakefulness was defined as de-synchronized EEG activity with active EMG activity. For power spectral analysis, raw EEG data (0.5–300 Hz) was first filtered to 0.5–60 Hz using custom MATLAB scripts, Fast-Fourier transform (FFT) of EEG data was carried out Neuroexplorer 5 software (Nex Technologies). For comparing EEG power spectra between groups, raw power at each frequency band was quantified at each recording site as a percent of total power (from 0.5 to 300 Hz). For band-specific comparisons, percent power was summed for frequencies within the designated band.

Sleep spindle identification, ripple identification, and spectral coherence between brain regions
An automated spindle detection algorithm (custom MATLAB software) was used to identify sleep spindles in band-pass filtered EEG data (7–15 Hz), as intervals containing ≥6 successive deviations (i.e., peaks or troughs) of signal that surpassed mean signal amplitude by 1.5 standard deviations, lasting between 0.25 and 1.75 s. Spindle density was calculated as the number of specific
events detected over total NREM time (in Hz). Spindle duration was calculated using the start and end times of each detected event (i.e., beginning at the first oscillatory cycle to pass the amplitude threshold, and ending with the last successive cycle to do so). A similar method was used to detect and quantify ripples, using EEG from V1 sites (positioned just dorsal to intermediate hippocampus in all mice), data band-pass filtered at 125–275 Hz. 109,110 The coherence of raw EEG data at given frequencies was analyzed using Neuroexplorer 5 software (Nex Technologies) using previously published methods. 160,161 Coherence was calculated using a Welch taper with a 50% window overlap. Coherence generated values are expressed from 0 to 1.0, with 0 indicating no relationship between two signals (two brain regions) at a given frequency and 1.0 indicating a perfect, linear relationship. Band coherence for comparing frequency bands of interest was quantified as the average of coherence values within each designated frequency band, for each mouse.

Behavior quantification
All behavioral measurements were assessed using Ethovision XT 16 (Noldus) software with files coded for blind scoring. For CFC behavior, freezing was measured using in a semi-automated manner. Freezing was first scored based on transient periods of immobility46,162 and was verified offline based on the assessment of characteristic freezing-associated posture. 89,161 CFM-associated freezing behavior was quantified by subtracting each mouse’s % freezing time during pre-shock baseline from % freezing time across the entire CFM test. 32,46,89,150 For OF test (habituation of OLM task), the total ambulatory distance was measured to account for differences in locomotor ability. Thigmotaxis (or wall-hugging behavior), an anxiety-related behavior, was measured by comparing percentage of time spend in the outer (wall) zone vs. the inner (center) zone.153 Both locomotor ability and thigmotaxis were measured to ensure further analyses in OLM were not skewed due to inactivity instead of genotype or treatment effects. For OLM behavior, measurements were based on time mice spent exploring the familiar and displaced object. Engagement with object included directing nose to the object at no more than 1 cm and/or touching the object. A total of 10 s interaction with objects was set as a criterion for accurate calculation of object discrimination indices. The discrimination index was calculated for each mouse as (time spent with displaced/total object exploration time) to correct for mouse-to-mouse differences in total exploration time.29

Microscopy and image analysis
Images were obtained using a Leica SP8 confocal microscope with a 10× objective, to obtain z stack images (10 μm steps) for maximum projection of fluorescence signals. Identical image acquisition settings (e.g., exposure times, frame average, pixel size) were used for all sections. For analysis of hippocampal and amygdala activation patterns, three images per section of dorsal hippocampus and amygdala were taken per mouse and areas for regions of interest (ROIs) were determined using previously established methods. 89,156 cFos+, PV+, and PNN density was quantified in subregions across these ROIs (DG, CA1, and CA3). For analysis of PV + interneuron expression within the TRN, cell density was measured using the number of PV + cells counted within the area made via the ROI surrounding the TRN. Counts were made by two scorers blinded to genotype and treatment using Fiji image analysis software, with the final number computed using the average of counts made by the two scorers.

Statistical analysis
Data analyses were carried out in a blind manner; in some cases (e.g., EEG recordings and cell counts), data was consensus scored by two individuals to reduce variability. Exclusion criteria for EEG recordings were based on lack of signal in one cortical region or faulty reference electrode that prevented confirmation of recording signals. For CFC behavior, exclusion criteria consisted of freezing levels above 15% before shock administration during CFC training sessions. For OLM behavior, mice that spent less than 10 s total interacting with objects during either the 10-min training or testing session were excluded from behavioral analysis. Statistical analyses were carried out using GraphPad Prism software (Version 9.5). For each specific data set, the statistical tests and pp-values are listed within the appropriate corresponding figure legend. Data sets were tested for normality using the Shapiro-Wilk test to determine use of parametric or non-parametric tests.