Reducing brain kynurenic acid synthesis precludes kynurenine-induced sleep disturbances

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Abstract

Study Objectives

Patients with neurocognitive disorders often battle sleep disturbances. Kynurenic acid (KYNA) is a tryptophan metabolite implicated in the pathology of these illnesses. Modest increases in KYNA, an antagonist at glutamatergic and cholinergic receptors, result in cognitive impairments and sleep dysfunction. We explored the hypothesis that inhibition of the KYNA synthesizing enzyme, kynurenine aminotransferase II (KAT II), may alleviate sleep disturbances.

Methods

At the start of the light phase, adult male and female Wistar rats received systemic injections of either i) vehicle, ii) kynurenine (100mg/kg; i.p.), iii) the KAT II inhibitor, PF-04859989 (30 mg/kg; s.c), or iv) PF-04859989 and kynurenine in combination. Kynurenine and KYNA levels were evaluated in the plasma and brain. Separate animals were implanted with electroencephalogram (EEG) and electromyogram (EMG) telemetry devices to record polysomnography and evaluate vigilance states wake, rapid eye movement (REM) sleep and non-REM (NREM) sleep following each treatment.

Results

Kynurenine challenge increased brain KYNA and resulted in reduced REM duration, NREM delta power and sleep spindles. PF-04859989 reduced brain KYNA formation when given prior to kynurenine, prevented sleep disturbances, and enhanced NREM sleep.

Conclusion

REM sleep was restored when PF-04859989 was administered prior to kynurenine challenge, suggesting that reducing KYNA attenuated the kynurenine-induced decrease in REM. Further, KAT II inhibition may cause mild somnolence. Our findings suggest for the first time that reducing KYNA may serve as a potential strategy for improving sleep dynamics.

Keywords: tryptophan, REM sleep, NREM sleep, sleep spindles, neuropharmacology, α7 nicotinic acetylcholine receptor

Statement of Significance

Elevated kynurenic acid (KYNA), a tryptophan metabolite synthesized by kynurenine aminotransferase II (KAT II) from its biological precursor, kynurenine, is implicated in the pathophysiology of neurocognitive disorders and associated sleep dysfunction. We presently hypothesize that inhibition of KYNA synthesis may ameliorate sleep disturbances. Male and female rats were systemically treated with kynurenine to elevate KYNA in the brain and sleep was evaluated with polysomnography. To reduce KYNA, animals were prophylactically treated with the irreversible, brain-penetrable, KAT II inhibitor PF-04859989 prior to kynurenine challenge. KAT II inhibition, which reduced KYNA synthesis, improved sleep wake architecture and our results place new attention on a novel therapeutic avenue to treat sleep disturbances associated with elevated KYNA in neurocognitive disorders.

Introduction

Healthy sleep promotes synaptic plasticity and is necessary for the maintenance of newly formed memories.[1,2] Cortical electroencephalography (EEG) combined with electromyography (EMG) is readily employed to distinguish wake from sleep states, including non-rapid eye movement (NREM) sleep which involves high cortical delta oscillations (0.25 - 4Hz) from thalamocortical networks and rapid eye movement (REM) sleep consisting of high frequency theta oscillations (4- 8 Hz) from corticolimbic networks. NREM is largely implicated in procedural memory storage, and REM sleep in considered critical to synaptic pruning, behavioral refinement, and memory consolidation.[3-5] Patients with neurocognitive disorders often experience sleep dysregulation such as fragmented sleep, insomnia, poor quality of sleep, aberrant sleep spindles, REM sleep behavior disorders, and greater symptom severity following sleep disruption.[6-9] Clinical presentation of sleep disturbances in these patients also point to noteworthy sex differences, with men experiencing more sleep fragmentation and subsequent daytime impairments, and women experiencing more insomnia and less total sleep time.[10-13] Despite the integral relationship between sleep and cognition, there is currently a lack of FDA approved therapeutic options suitable to treat both cognitive symptoms and sleep disturbances.[14]

To investigate a novel pharmacological intervention, we presently explore the impact of inhibiting kynurenine aminotransferase II (KAT II) on sleep physiology in rodents. KAT II is the primary synthesizing enzyme for brain-derived kynurenic acid (KYNA), a metabolite of the kynurenine pathway (KP) of tryptophan metabolism (see **Figure 1A**). The essential amino acid tryptophan is pivotal to circadian rhythmicity,[15] supported via the regulatory role of serotonin and melatonin in sleep and arousal.[16,17] However, while only about 5% of tryptophan is degraded through the melatonin pathway, the vast majority is metabolized via the KP,[18,19] which generates the small neuroactive molecule KYNA, which we hypothesize also serves a critical role in modulating sleep. [20,21]

Elevations in KYNA disrupt cholinergic and glutamatergic-dependent processes, including learning and memory,[22-25] and restoring a balance in KYNA promotes cognitive function.[23,26,27] Clinical significance for studying elevations in KYNA stem from growing evidence that increased KYNA is found in the brains of individuals with neurocognitive and psychiatric disorders.[28-33] To combat cognitive dysfunction associated with these illnesses, translational studies have evaluated pharmacological approaches to reduce KYNA in the brain by inhibiting the synthesizing enzyme KAT II.[23,27,34-38]

Our present goal was to pharmacologically inhibit KAT II as a mechanistic strategy to i) reduce *de novo* KYNA production after acute treatment with kynurenine, the direct bioprecursor, and ii) overcome sleep disturbances induced by excessive KYNA.[20] We selected PF-04859989, a brain penetrable and irreversible KAT II inhibitor,[27,39] for this study and confirmed its effectiveness to reduce *de novo* KYNA formation when administered prior to kynurenine challenge. We hypothesize that KAT II inhibition with PF-04859989 attenuates dysregulated sleep-wake behavior in response to the acute kynurenine challenge in both male and female rats.

Methods

Animals

Adult male (250-350 g) and female (200 - 300 g) Wistar rats were obtained from Charles River Laboratories. Animals were kept on a 12/12 h light-dark cycle, where lights on corresponded to zeitgeber time (ZT) 0 and lights off to ZT 12 in a facility fully accredited by the American Association for the Accreditation of Laboratory Animal Care. All protocols were approved by Institutional Animal Care and Use Committees and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Chemicals

L-kynurenine sulfate ("kynurenine", purity 99.4%) was obtained from Sai Advantium (Hyperbad, India). PF-04959989 was obtained from WuXi AppTec (purity 95%; Shanghai, China). Other chemicals were obtained from various suppliers and were of the highest commercially available purity. For intraperitoneal (i.p.) injections, kynurenine was prepared daily by dissolving in phospho-buffered saline and adjusting the pH to 7.4 with 0.1N NaOH. For subcutaneous (s.c.) injections, PF-04859989 was prepared daily by dissolving in ultrapure water.

Biochemical Analysis

Male and female rats were treated acutely with vehicle (water, s.c.) or PF-04859989 (30mg/kg, s.c.) at ZT 23.5 and vehicle (saline, i.p.) or kynurenine (100 mg/kg, i.p.) at ZT 0. Animals were euthanized at ZT 2 by CO_2 asphyxiation. Whole trunk blood was collected in tubes containing 25 μl K3-EDTA (0.15%) as an anticoagulant, and centrifuged (1000 x g, 10 min) to separate plasma. The brain was rapidly removed on ice, and the following brain regions were dissected: cerebellum, brainstem, hypothalamus, basal forebrain, hippocampus, cortex, and frontal cortex. All samples were promptly snap-frozen on dry ice and stored at -80°C until biochemical analyses. Kynurenine and KYNA levels were determined by high-performance liquid chromatography (HPLC) (Wright et al. 2021; Buck et al., 2020). Briefly, plasma samples were diluted (1:10) in ultrapure water, and tissue samples were weighed and sonicated (1:5, w/v) in ultrapure water before being further diluted (final concentration: 1:10, w/v). One hundred μl of sample, either diluted plasma or tissue homogenate, was acidified with 25 μl of 6% (plasma) or 25% (tissue) perchloric acid, centrifuged (12000 x g, 10 min), and 20 μl of the supernatant was subjected to HPLC. Kynurenine and KYNA were isocratically eluted from a ReproSil-Pur C18 column (4 x 150 mm; Dr. Maisch GmbH, Ammerbuch, Germany) using a mobile phase containing 50 mM sodium acetate and 5%

acetonitrile (pH adjusted to 6.2 with glacial acetic acid) at a flow rate of 0.5 ml/min. Zinc acetate (500 mM) was delivered after the column at a flow rate of 0.1 ml/min. Kynurenine (excitation: 365 nm, emission: 480 nm, retention time ~7 minutes) and KYNA (excitation: 344 nm, emission: 398 nm, retention time ~13 minutes) were detected fluorometrically (Waters Alliance, 2475 fluorescence detector, Bedford, MA) in the eluate. Data were integrated and analyzed using Empower 3 software (Waters). The total concentration of protein in tissue samples was determined by the Lowry protein assay.[40]

Surgery

Under isoflurane anesthesia (5% induction, 2 - 3% maintenance), animals were placed in a stereotaxic frame (Stoelting, Wood Dale, IL) for the surgical implantation of EEG/EMG telemetry transmitters (PhysiolTel HD-S02; Data Science International, St. Paul, MN) according to previously published protocols.[20,21] The transmitter was inserted intraperitoneally through a dorsal incision of the abdominal region. An incision at the midline of the head was made to anchor two EEG leads to their corresponding surgical screws (Plastics One, Roanoke, VA) placed into 0.5 mm burr holes at 2.0 mm anterior/+1.5 mm lateral and 7.0 mm posterior/−1.5 mm lateral relative to bregma and secured with dental cement. Two EMG leads were inserted and sutured into the dorsal cervical neck muscle, separated by 1.0 mm. The skin on top of the head was sutured, the abdominal incision was closed with wound clips, and animals recovered for ≥ 7 days prior to experimentation.

Vaginal Lavage and Estrous Cycle Tracking

Vaginal lavages were undertaken for female subjects using transfer pipettes filled with saline. Samples were stored in microcentrifuge tubes and analyzed using a SeBa digital microscope (LAXCO Inc., Mill Creek, WA, USA) at 4X magnification within 30 minutes of sample collection.

Best efforts were made to avoid drug treatments during proestrus, wherein sleep and wake parameters are vastly different from other cycle stages for female subjects.[41,42]

Drug Treatments for Sleep Data Acquisition

Sleep Study #1: Counterbalanced vehicle (saline, i.p.) or kynurenine (100 mg/kg, i.p.) injections were given 96 h apart at ZT0. Polysomnography data were acquired for 24 h after each treatment. *Sleep Study #2:* On the first day of the experiment, animals were injected with vehicle (water, s.c.) at ZT 23.5 and vehicle (saline, i.p.) at ZT 0 to obtain baseline sleep data (denoted "vehicle" in graphs). After 96 h, animals received PF-04859989 (30 mg/kg, s.c.) at ZT 23.5 and either vehicle (saline, i.p. denoted "PF-04859989 + vehicle" in graphs) or kynurenine (100 mg/kg, i.p.; denoted "PF-04859989 + kynurenine" in graphs) at ZT 0. The experimental schedule was counterbalanced and repeated, such that each animal received PF-04859989 + vehicle and PF-04859989 + kynurenine injections. Sleep data acquisition occurred for 24 h after ZT 0 treatment. Treatments were spaced 96 h apart to record sleep within the same estrous phase for female subjects.

Sleep Data Acquisition and Analyses

Animals undergoing sleep recordings were left undisturbed in a designated room. All sleep data were recorded using Ponemah 6.10 software (DSI). All digitized signal data were scored offline with NeuroScore 3.0 (DSI) in 10-second epochs as wake (low-amplitude, high-frequency EEG combined with high-amplitude EMG), NREM (high-amplitude, low-frequency EEG with low-amplitude EMG), or REM (low-amplitude, high-frequency EEG with very low EMG amplitude). As previously described, transitions among vigilance states were scored when 2 or more 10-s epochs of the new state were noted. Parameters assessed included total vigilance state duration, number of bouts, average bout length, number of transitions between vigilance states, and spectral power frequencies during REM and NREM. Discrete fast Fourier transform (DFT) was used to calculate the EEG power spectrum for the following frequency ranges: delta (0.5 – 4 Hz), theta (4 – 8 Hz),

alpha (8 – 12 Hz), sigma (12 – 16 Hz), and beta (16 – 20 Hz). DFT power spectrum analyses were evaluated for the entire light or dark cycle. Sleep spindles were identified based on manual detection, by applying a rectified 10 - 15 Hz finite impulse response (FIR) band pass filter to the EEG signal and computing the cubed root-mean square (RMS) transform of the band pass filtered EEG signal within a 750 ms window to create a signal envelope for the accurate detection of sleep spindles. To ensure scientific rigor, all artifactual spikes with a duration of less than 0.5s were excluded from spindle analyses criteria. Sleep spindle data were analyzed for the first 4-hr period of the light phase during NREM sleep, to acquire a representative spread of NREM sleep spindles.[43,44] The DSI module for periodogram powerbands in NeuroScore was used to generate fast Fourier transform (FFT) analysis for spindle wavelet frequencies. Relative home cage activity was also recorded by Neuroscore.

Statistical Analyses

For biochemical analyses, data obtained from both sexes were combined, and comparisons were made using a 2-way analysis of variance (ANOVA) with kynurenine and/or PF-04959989 treatment as a between-subject factors. Significant main effects were followed by Bonferroni's correction with an adjusted alpha for multiple comparisons. Initial analyses of sleep architecture data (vigilance state durations, bout number, and average duration/bout) were evaluated by 2-way repeated measures (RM) ANOVA with treatment and ZT as within-subject factors in 1-hr bins, sexes combined. Subsequently, 1-hr bins, sexes combined, were analyzed separately by light and dark phases using 2-way RM ANOVA, with treatment and ZT as within-subject factors. To evaluate sex as a biological variable, data were binned into 6-hr increments, and analyzed by 3-way RM ANOVA, with treatment and time bin as within-in subject factors and sex as a between-subject factor. Subsequently, 6-hr bin data were also analyzed separately by sex, using 2-way RM ANOVA, with treatment and ZT as within-subject factors. NREM or REM power spectra, NREM delta power spectra, and REM theta power spectra were evaluated for light and dark

phases separately with 2-way RM ANOVA with frequency and treatment as within-subject factors. Spindle data (spindle density, average spindle duration, spindle amplitude) were evaluated during the 4-hr bin of analysis by 2-way RM ANOVA with treatment as a within-subject factor, and sex as a between-subject variable. Power spectra during spindles and spindle sigma power were evaluated separately by sex with 2-way RM ANOVA with frequency and treatment as within-subject factors. Relative cage activity was analyzed by 2-way RM ANOVA with sexes combined, treatment and ZT as within-subject factors. Vigilance state transitions were evaluated by 2-way RM ANOVA and divided by phase and sex. When appropriate, significant effects were evaluated with Fisher's LSD post hoc analyses. Percent of vehicle calculations (spindle density, REM duration, NREM duration, and wake duration) allowed comparisons across study #1 and study #2, thereby evaluating the impact of each drug treatment from its vehicle and comparisons across drug treatments (kynurenine, PF-04859989 + vehicle, and PF-04859989 + kynurenine treatments) with Student's t-test. All statistical analyses were performed using Prism 9.0 (GraphPad Software, La Jolla, CA, USA) and significance was defined as $P < 0.05$. All statistical effect sizes are shown in Supplemental Statistical Materials.

Results

Systemic administration of KAT II inhibitor PF-04859989 attenuates elevation in brain KYNA levels induced by acute kynurenine challenge

We first confirmed that acute systemic kynurenine treatment at ZT 0, start of the light phase, elevates plasma kynurenine levels at ZT 2 compared to vehicle treatment. Kynurenine-treated rats exhibited a 2.8-fold increase in plasma kynurenine levels compared with vehicle-treated rats (main effect of kynurenine: $F_{1,44} = 80.86$, $P<0.0001$). As kynurenine promptly enters the brain from the blood, we confirmed significant elevation in brain kynurenine levels in the cerebellum (6.2-

fold; $F_{1,43}$ = 32.95, P<0.0001), brainstem (2.3-fold; $F_{1,41}$ = 24.96, P<0.0001), hypothalamus (2.5-fold; $F_{1,43}$ = 65.02, P<0.0001), basal forebrain (3.9-fold; $F_{1,32}$ = 34.85, P<0.0001), hippocampus (5.2-fold; $F_{1,37}$ = 45.05, P<0.0001), cortex (4.1-fold; $F_{1,43}$ = 57.73, P<0.0001), and frontal cortex (3.6-fold; $F_{1,42}$ = 39.76, P<0.0001) compared to vehicle treatment. Levels of kynurenine in plasma and brain remained unchanged after pretreatment with PF-04859989 (**Supplemental Table 1**).

Upon kynurenine treatment at ZT 0, plasma KYNA increased 2.5-fold compared with plasma from vehicle-treated rats ($F_{1,43}$ = 73.71, P<0.0001) (**Figure 1B**). Across brain regions (**Figure 1C-I**), we similarly determined that kynurenine treatment elevated KYNA content in the cerebellum (3.3-fold; $F_{1,40}$ = 40.95, P<0.0001), brainstem (3.7-fold; $F_{1,37}$ = 15.74, P<0.001, hypothalamus (3.8-fold; $F_{1,41}$ = 27.20, P<0.0001), basal forebrain (2.5-fold; $F_{1,42}$ = 32.66, P<0.0001), hippocampus (5.0-fold; $F_{1,43}$ = 71.33, P<0.0001), cortex (7.0-fold; $F_{1,41}$ = 80.26, P<0.0001), and frontal cortex (9.2-fold; $F_{1,37}$ = 49.44, P<0.0001). Of significance, PF-04859989 treatment significantly impacted KYNA levels, determined peripherally in plasma ($F_{1,43}$ = 4.848, P<0.05) and also in the hypothalamus ($F_{1,41}$ = 7.497, P<0.01), basal forebrain ($F_{1,42}$ = 7.396, P<0.01), hippocampus ($F_{1,43}$ = 24.23, P<0.0001), cortex ($F_{1,41}$ = 22.71, P<0.0001), and frontal cortex ($F_{1,37}$ = 9.691, P<0.01). Pretreatment with PF-04859989 attenuated the increase in KYNA following acute kynurenine challenge, noted by a PF-04859989 x kynurenine interaction in the hypothalamus ($F_{1,41}$ = 7.076, P<0.05), hippocampus ($F_{1,43}$ = 22.00, P<0.0001), cortex ($F_{1,41}$ = 23.98, P<0.0001), and prefrontal cortex ($F_{1,37}$ = 9.248, P<0.01). A significant reduction in KYNA was determined when PF-04859989 was given prior to kynurenine compared to kynurenine alone in the hypothalamus (P<0.01), basal forebrain (P<0.05), hippocampus (P<0.0001), cortex (P<0.0001), and frontal cortex (P<0.001). PF-04859989 pretreatment did not significantly reduce KYNA formation in the cerebellum and brainstem. In summary, **Table 1** indicates the percent increase in KYNA levels from vehicle with kynurenine treatment (top panel) and the percent decrease in KYNA levels when

PF-04859989 was given prior to kynurenine challenge (bottom panel) in the plasma and individual brain regions investigated.

Kynurenine treatment reduces REM sleep and alters NREM architecture, spectral power, and spindle dynamics

Sleep Study #1 was conducted in a separate cohort of male and female rats used exclusively for sleep monitoring experiments. Initial analyses of all sleep data was conducted with both male and female subjects combined (N = 20) and evaluated in 1-hr bins. With kynurenine treatment, REM duration decreased significantly compared to vehicle treatment ($F_{1,19}$ = 18.56, P<0.001). The starkest REM duration decrease occurred in the later portion of the light phase between ZT 9 and ZT 10 (P<0.05 and P<0.01, respectively). Dark phase REM duration was unaffected by kynurenine treatment (**Figure 2A**). Subsequent analyses were conducted to interrogate the impact of sex, kynurenine treatment, and time of day. Thereby data from male (N = 8) and female (N = 12) subjects were separated and evaluated in 6-hr time bins. REM duration was significantly impacted by a kynurenine x ZT interaction ($F_{3,54}$ = 4.643, P<0.01) and a main effect of kynurenine ($F_{1,18}$ = 15.47, P<0.01). Kynurenine negatively impacted REM duration in both sexes (males: $F_{1,7}$ = 9.198, P<0.05; females: $F_{1,11}$ = 6.222, P<0.05) and a significant decrease in REM duration occurred during the latter half of the light phase (ZT 6-12, males: P<0.01, females: P<0.05; **Figure 2B**). REM bouts across the light phase were reduced with kynurenine ($F_{1,19}$ = 6.976, P<0.05; post-hoc, ZT 10: P<0.001) (**Figure 2C**). Analyses of REM bouts separated by sex in 6-hr bins revealed a significant kynurenine x ZT interaction ($F_{3,54}$ = 3.774, P<0.05) and a main effect of kynurenine ($F_{1,18}$ = 8.865, P<0.01). Kynurenine treatment reduced REM bouts in males during the latter half of the light phase (males: $F_{1,7}$ = 8.101, P<0.05; post-hoc, ZT 6-12: P<0.05), yet no significant changes in REM bout number were found in females (**Figure 2D**). Additionally, the average duration of REM bouts was not different between vehicle and kynurenine treatment across analyses (**Supplemental Figure 1A-B**).

Next, we evaluated changes in NREM. Evaluating data with sexes combined, NREM duration was impacted by a significant kynurenine x ZT interaction during the light phase ($F_{11,209}$ = 2.350, P<0.01). Post-hoc we found NREM duration increased at ZT 1 (P<0.01) and decreased at ZT 5 (P<0.001) with kynurenine treatment compared to vehicle (**Figure 2E**). Analyses separated by sex in 6-hr bins revealed no significant changes in NREM duration (**Supplemental Figure 2A**). With sexes combined, NREM bouts were impacted by a kynurenine x ZT interaction ($F_{11,209}$ = 2.387, P<0.01) and a main effect of kynurenine ($F_{1,19}$ = 7.408, P<0.05) (**Figure 2F**), with post-hoc significance determined at ZT 14 (P<0.05), ZT 21 (P<0.001) and ZT 22 (P<0.05). Analyses of NREM bout number in 6-hr bins revealed no significant impacts (**Supplemental Figure 2B**). Similarly, the average duration of each NREM bout was influenced by a kynurenine x ZT interaction during the dark phase ($F_{11,209}$ = 1.844, P<0.05), yet no differences between vehicle and kynurenine treatment were found when data were analyzed in 6-hr bins separated by sex (**Supplemental Figure 2C-2D**).

Total REM or NREM power were assessed in intervals corresponding to the light phase (ZT 0-12) or dark phase (ZT 12-24) to evaluate the impact of kynurenine treatment. During both phases, theta power during REM was increased compared with power in four other EEG frequency bands evaluated – delta, alpha, sigma, and beta (light phase: $F_{38,722}$ = 123.6, P<0.0001; dark phase: $F_{38,722}$ = 118.1, P<0.0001). During NREM, delta power was higher compared with theta, alpha, sigma, and beta (light phase: $F_{38,722}$ = 414.2, P<0.0001; dark phase: $F_{38,722}$ = 220.7, P<0.0001). Thus in our analyses, we evaluated the full power spectra from 0 – 20 Hz within each sleep state, and subsequently conducted analyses during REM within the theta range (4 – 8 Hz) during REM sleep and during NREM within the delta range (0 – 4 Hz). During both the light phase and subsequent dark phase, REM spectral power was not changed with kynurenine treatment **(Supplemental Figure 3)**. Kynurenine treatment did significantly reduce NREM spectral power (light phase, kynurenine x frequency interaction: $F_{38,722}$ = 2.667, P<0.0001; dark phase,

kynurenine x frequency interaction: $F_{38,722}$ = 2.547, P<0.0001). Consistently lower power spectra was found within the delta range during the light phase (1 Hz: P<0.0001, 1.5 Hz: P<0.0001, 2 Hz: P<0.01, 3 Hz: P<0.05) and dark phase (0.5 Hz: P<0.0001, 1.5 Hz: P<0.01, 2 Hz: P<0.01, 2.5 Hz: P<0.01, 3 Hz: P<0.01, 4 Hz: P<0.05, 11 HZ: P<0.05). A zoomed-in view of the delta range confirmed a kynurenine x frequency interaction ($F_{7,133}$ = 2.165, P<0.05), with reduced NREM spectral power at 1 Hz (P<0.001), 1.5 Hz (P<0.0001), and 2 Hz (P<0.05) during the light phase. During the dark phase, we found a similar kynurenine x ZT interaction ($F_{7,133}$ = 2.451, P<0.05), with enhanced NREM spectral power at 0.5 Hz (P<0.05), 1.5 Hz (P<0.05), 2.5 Hz (P<0.05), and 3 Hz (P<0.05) (**Figure 2G**).

NREM sleep spindles were detected between the 10 - 15 Hz frequency range for the first 4-hr portion of the light phase. Kynurenine treatment significantly reduced spindle density ($F_{1,18}$ = 23.75, P<0.0001), in a sex-dependent manner ($F_{1,18}$ = 6.410, P<0.05). The degree of decrease in spindle density following kynurenine treatment was greater in males (P<0.001) compared to females (P<0.05) **(Figure 2H)**. The frequency distribution of spindle wavelets was significantly impacted by a kynurenine x frequency interaction ($F_{39,273}$ = 2.581, P<0.0001) during the light phase. Kynurenine treatment reduced spindle power in males at frequency ranges between 9 – 10 Hz and increased spindle power between 11.5 – 15 Hz (P<0.05). In females, peak spindle frequency distribution did not differ between vehicle and kynurenine conditions **(Figure 2I)**. No differences were observed between vehicle and kynurenine treatment concerning average spindle duration and peak to peak amplitude **(Supplemental Figure 4)**.

Kynurenine challenge alters wake architecture and home cage activity

To understand how the observed changes in REM and NREM impacted arousal, we evaluated wake state architecture and general home cage activity. Comparing vehicle to kynurenine treatment with sexes combined, we determined a kynurenine x ZT interaction during the light phase ($F_{11,209}$ = 2.566, P<0.01) such that kynurenine initially reduced wake duration within the first

hour (P<0.001), but thereafter wakefulness increased (ZT 5: P<0.01) **(Supplemental Figure 5A)**. Separating analyses by sex revealed no changes in wake duration across treatments (**Supplemental Figure 5B**). The number of wake bouts, also impacted by a kynurenine x ZT interaction ($F_{11,209}$ = 2.080, P<0.05), significantly increased during the subsequent dark phase ($F_{1,19}$ = 11.45, P<0.01) (**Supplemental Figure 5C**). Similarly, 6-hr bin analyses separated by sex revealed a main effect of kynurenine in females ($F_{1,11}$ = 4.953, P<0.05) (**Supplemental Figure 5D**). We also evaluated the duration of each wake bout and determined a kynurenine x ZT interaction during both the light phase ($F_{11,209}$ = 3.419, P<0.001) and dark phase ($F_{11,228}$ = 2.339, P<0.01). Each wake bout was shorter at the beginning of the light phase after kynurenine treatment (ZT 1: P<0.0001) and then in the subsequent dark phase, wake bout duration was intermittently reduced (ZT 14, 21 and 22: P<0.05) and finally increased, likely with anticipation of the light switch to occur at ZT 24 (P<0.01; **Supplemental Figure 5E**). Evaluation of wake bout duration by sex in 6-hr bins did not yield any sex-specific findings (**Supplemental Figure 5F**). A shift in home cage activity was found following acute kynurenine treatment (kynurenine x ZT interaction: $F_{23,360}$ = 1.688, P<0.05) wherein on the day of kynurenine exposure, activity levels were notably increased during the first 3 hours (ZT 1: P<0.01, ZT 2: P<0.05, ZT 3: P<0.05), but reduced with the anticipated light change at ZT 12 (P<0.05) and ZT 24 (P<0.05) (**Supplemental Figure 5E**).

KAT II inhibitor PF-04859989 attenuates changes in sleep architecture and spindle dynamics induced by acute kynurenine challenge

Pretreatment with PF-04859989 resulted in reduced *de novo* KYNA synthesis in the brain with systemic kynurenine challenge (**Figure 1**), thus we next explored if PF-04859989 would readily reverse the consequences of kynurenine challenge on sleep architecture. Sleep Study #2 was conducted in a new cohort of adult rats (N = 18), and each subject received the following treatments: i) two vehicle injections, ii) PF-04859989 at ZT 23.5 + vehicle at ZT 0, iii) PF-04859989

at ZT 23.5 + kynurenine at ZT 0. Focusing initially on REM sleep, we found that PF-04859989 pretreatment prevented kynurenine-induced REM deficits, thereby stabilizing REM duration and bout number to vehicle treatment levels. Analyses of data in 1-hr bins, sexes combined, determined that PF-04859989 + vehicle impacted REM duration only during the dark phase (treatment x ZT interaction: $F_{22,374}$ = 1.645, P<0.05) such that REM duration increased at ZT 14 (P<0.001) and decreased at ZT 19 (P<0.01) (**Figure 3A**). Evaluating 6-hr bin data, separated by sex, we determined no differences in REM duration between treatment groups (**Supplemental Figure 6A**). PF-04859989 + vehicle treatment did impact NREM duration (1-hr bins, sexes combined) during the dark phase (treatment x ZT interaction: $F_{22,374}$ = 1.741, P<0.05), with a significant increase at the anticipated light change that occurs after ZT 24 (P<0.001). However, NREM duration during the dark phase was reduced with PF-04859989 treatment alone compared to vehicle (ZT 17: P<0.05, ZT 19: P<0.01) and PF-04859989 + kynurenine treatment (ZT 19: P<0.05) (**Figure 3B**). We determined conspicuous sex-specific differences when data were evaluated in 6-hr bins and separated by sex as enhanced NREM duration with PF-04859989 treatment occurred only in male subjects (main effect of treatment: $F_{2,16}$ = 5.008, P<0.05) from ZT 18-24 (P<0.05) (**Figure 3C**).

While acute kynurenine treatment resulted in fragmentation of sleep bouts, we presently determined that PF-04859989 + vehicle or PF-04859989 + kynurenine treatment stabilized the number of REM bouts (**Supplemental Figures 6B-6C**). PF-04859989 + vehicle decreased the average duration of each REM bout during the dark phase (main effect of treatment: $F_{2,34}$ = 3.311, P<0.05; post hoc, ZT 17: P<0.05, ZT 19: P<0.01) (**Supplemental Figure 6D-6E**). PF-04859989 rescued the NREM bout fragmentation phenotype induced by kynurenine alone (**Supplemental Figures 7A-7B**). No change was observed in the average duration of each NREM bout when comparing treatments (**Supplemental Figures 7C-7D**).

PF-04859989 treatment altered the power spectra during NREM sleep (**Figure 3D**) yet did not impact spectral power during REM sleep (**Supplemental Figure 8**). We found a significant frequency x treatment interaction within the delta range during the light phase ($F_{14,238}$ = 1.792, P<0.05). When given before kynurenine, PF-04859989 enhanced NREM spectral power within the slow wave range of 0.5 Hz (P<0.0001) to 1 Hz (P<0.05) during the light phase, and PF-04859989 + vehicle enhanced only 0.5 Hz oscillations (P<0.05) (**Figure 3D**).

Evaluation of spindle dynamics determined no differences in NREM spindle density between treatments in Sleep Study #2 (**Figure 3E**), indicating that PF-04859989 attenuated the reduction in NREM spindle density elicited by kynurenine (comparison to **Figure 2H**). It is noteworthy that PF-04859989 significantly reduced the peak-to-peak amplitude (main effect of treatment: P<0.01, treatment x sex interaction: P<0.05) in females treated with either vehicle (P<0.001) or kynurenine (P<0.05) (**Figure 3F**). In males, PF-04859989 treatment influenced spectral power within the 10-15 Hz sigma range (treatment x frequency interaction: $F_{20,160}$ = 1.756, P<0.05). With the PF-04859989 + kynurenine, spectral power was increased at 12 Hz (P<0.01) and 12.5 (P<0.05) and with PF-048599889 + vehicle the increase occurred at 13.5 Hz (P<0.05) and 14 Hz (P<0.05) (**Figure 3G**). Average spindle duration was not impacted by PF-04859989 treatment (**Supplemental Figure 7E**).

PF-04859989 prevents kynurenine-induced changes in wake behavior

In Sleep Study #1, we noted increased wakefulness with kynurenine challenge and thereby explored the impact of pretreatment with PF-04859989 on this behavioral phenotype in Sleep Study #2. We determined no significant differences in wake duration between Sleep Study #2 treatment conditions, thereby noting that PF-04859989 prevented kynurenine-induced changes in wake duration (**Supplemental Figure 9A**; 1-hr bins, sexes combined). Analyses of wake duration in 6-hr bins, separated by sex, revealed a treatment x ZT interaction ($F_{2,32}$ = 3.509, P<0.05) and ZT x sex interaction ($F_{1,16}$ = 18.53, P<0.001) during the light phase. In males, PF-

048599889 + vehicle reduced wake duration significantly during the dark phase ($F_{2,16}$ = 5.637, P<0.05; post-hoc, ZT 18: P<0.01) (**Supplemental Figure 9B**). The number of wake bouts, analyzed in 1-hr bins with sexes combined, was not impacted by PF-04859989 (**Supplemental Figure 9C**), however analyses of 6-hr bins, separated by sex, found that in the light phase females had more wake bouts than males across all treatment groups ($F_{1,16}$ = 10.29, P<0.01) (**Supplemental Figure 9D**). PF-04859989 + vehicle treatment significantly reduced the average duration of each wake bout, analyzed in 1-hr bins with sexes combined, during the dark phase (treatment x ZT interaction: $F_{22,374}$ = 1.822, P<0.05; post hoc, ZT 24: P<0.0001). Similarly, PF-04859989 + kynurenine treatment reduced average duration of each wake bout in the dark phase (ZT 16 and 24: P<0.05) (**Supplemental Figures 9E-F**). General home cage activity was impacted by PF-04859989 treatment (treatment x ZT interaction: $F_{46,697}$ = 1.403, P<0.05). Increased activity was noted at ZT 1 (PF-04859989 + vehicle: P<0.01, PF-04859989 + kynurenine: P<0.001). Reduced activity was noted at ZT 4 (PF-04859989 + vehicle: P<0.05; PF-04859989 + kynurenine: P<0.01), ZT 16 (PF-04859989 + vehicle: P<0.05; PF-04859989 + kynurenine: P<0.05), ZT 22 (PF-04859989 + vehicle: P<0.05, PF-04859989 + kynurenine: P<0.01), and ZT 24 (PF-04859989 + vehicle: P<0.01, PF-04859989 + kynurenine: P<0.05) (**Figure 3H**).

Vigilance state transitions impacted by kynurenine challenge and PF-04859989 treatment

To evaluate the impact of kynurenine challenge on the stability of maintaining a wake or sleep episode, the number of transitions into and out of wake, NREM sleep, and REM sleep was quantified by phase. Kynurenine challenge disrupted female sleep transitions (light phase: transition x treatment interaction: $F_{4,44}$ = 3.964, P<0.01; dark phase: transition x treatment interaction: $F_{4,44}$ = 4.740, P<0.01; a main effect of treatment: $F_{1,11}$ = 6.422, P<0.05). Upon kynurenine treatment, females transitioned significantly less from REM to NREM (P<0.05) and NREM to REM (P<0.05) in the light phase, and more from wake to NREM (P<0.0001) and NREM to wake (P<0.01) in the dark phase (**Supplemental Table 2**). PF-04859989 successfully

ameliorated kynurenine-induced changes in sleep transitions in female subjects (**Supplemental Table 3**). No significant differences in transitions were noted in males, regardless of treatment condition.

PF-04859989 treatment attenuates sleep disruptions induced by kynurenine challenge

Sleep spindle density and vigilance state duration data were normalized to a percent change from vehicle treatment to compare results from Sleep Study #1 with Sleep Study #2. Kynurenine challenge resulted in a 12% decrease in spindle density and this impact was significantly attenuated with PF-04859989 + kynurenine treatment (kynurenine compared to PF-04859989 + kynurenine: $P<0.01$). REM duration was reduced by 10% and PF-04859989 substantially rescued this phenotype by increasing REM by 4% (kynurenine compared to PF-04859989 + kynurenine: $P<0.0001$). In summary, we demonstrate the ability of KAT II inhibitor PF-04859989 to reverses kynurenine-induced sleep spindle and REM abnormalities (**Figure 4B**).

Discussion

Altered tryptophan metabolism, including KP dysregulation, impacts sleep and arousal, in normal and pathological conditions.[20,21,45-47] Using telemetric recordings for the acquisition of EEG, EMG, and activity data in freely moving animals, we obtained ethologically relevant polysomnography recordings to evaluate sleep-wake behavior with acute elevation of KYNA (kynurenine challenge) or inhibition of newly produced KYNA in the brain (PF-04859989 + kynurenine). Building upon previous work, which evaluated changes in sleep with a dose-response of kynurenine challenge in male subjects,[20] we presently expanded our understanding of the impact on sleep-wake behaviors by evaluating both sexes along with the translationally relevant studies that reduced *de novo* KYNA formation by inhibiting KAT II with a brain-penetrable and irreversible inhibitor, PF-04859989.

Acute kynurenine challenge notably reduced REM duration in both males and females. We found that females were more resilient to REM architecture changes but had significant disruptions in sleep transitions when KYNA levels were elevated, namely transitioning more frequently between wake and NREM and returning to wake. The enhanced transitioning in females suggests a disruption in maintenance of NREM episodes. With PF-04859989 treatment, male subjects were more vulnerable to somnolence in the subsequent dark phase. Overall, changes in sleep-wake architecture induced by kynurenine challenge were prevented with prophylactic PF-04859989 treatment, and we postulate that the reduced KYNA levels across various brain regions contributed to the attenuated sleep behavior phenotypes. Namely, PF-04859989 effectively reduced KYNA levels in the hypothalamus, basal forebrain, hippocampus, cortex, and prefrontal cortex, suggesting that the majority of KYNA synthesis in these regions is KAT II-dependent. While we saw slight trends towards reduced KYNA levels in the cerebellum and brainstem with PF-04859989 treatment prior to kynurenine challenge, the effects were not significant thereby endorsing evidence of alternative means to regulate KYNA formation within these brain regions.[48] At the molecular level, KAT II mRNA levels encoded by *Aadat* are found prominently in cerebral regions such as the hippocampus, and much less KAT II mRNA has been found within the cerebellum and brainstem.[49] In the case of the cerebellum, it has been suggested that KYNA formation may be more dependent on non-enzymatic formation with a role for reactive oxygen species, thereby peripheral treatment with KAT II inhibitor may be less effective for this particular brain region.[48]

The basal forebrain, a region wherein KYNA was both elevated with kynurenine challenge and reduced with PF-04859989 pre-treatment, is richly implicated in modulation of sleep and arousal behavior.[50-52] A large amount of acetylcholine is released from the basal forebrain during REM sleep,[53-55] and we postulate that KYNA-mediated modulation of cholinergic neurotransmission may impede these dynamics.[56] Within the extracellular milieu, KYNA is

present within low micromolar concentrations and optimally positioned to inhibit receptor targets including α7nACh receptors and NMDA receptors.[57-59] The stark decrease in REM with acute KYNA elevation is presumably related to the inhibition of these targets. The fact that systemic application of PF-04859989 downregulated KYNA production and reversed the consequences of KYNA elevation on sleep behavior provides compelling evidence for the role of KYNA, rather than the alternate arm of the kynurenine pathway that produces the metabolites 3-hydroxykynureine, 3-hydroxyanthranilic acid, and quinolinic acid,[36] in modulating changes in sleep physiology. Ongoing work with mouse models genetically modified to eliminate key enzymes of the kynurenine pathway will provide further support for the implicated role of KYNA in sleep behavior.[60,61]

Several key disruptions of sleep were noted that bring further attention to the concept that KYNA serves as a molecular intersection between sleep and cognitive function.[20,22,23] Reduced REM sleep negatively impacts hippocampal synaptic plasticity and elicits memory deficits.[5,62-64] Reduced amplitude of delta power during NREM and fragmented NREM with elevated KYNA may also influence long-term memory consolidation.[63,65] We evaluated for the first time an impairment in sleep spindles, oscillations produced by the thalamic reticular nucleus that support synaptic plasticity and memory consolidation,[66,67] during NREM sleep when KYNA was elevated. Reduced spindle density is a hallmark endophenotype of sleep dysfunction in patients with psychotic disorders and may serve as a predictor for cognitive and executive dysfunction.[68-73] In addition, several preclinical models relevant to the study of neuropsychiatric illnesses have also noted reduced sleep spindle density in rodents.[43,74,75] Of note is that sleep spindle spectra power during KYNA elevation was initially reduced in the lower sigma range and, perhaps as a consequence of an increased cortical drive to ameliorate reduced spindle density, the power spectra was then elevated in the upper sigma range in male subjects.[76] Yet importantly, prophylactic treatment with PF-04859989 effectively ameliorated kynurenine-induced sleep deficiencies. We find it

particularly noteworthy that PF-04859989 treatment alone enhanced delta power during NREM, suggesting that KAT II inhibition may enhance the quality of slow wave sleep.[77] PF-04859989 treatment also resulted in nuanced sex-specific findings, such as increased spindle spectral power in male subjects and reduced spindle amplitude in female subjects, supporting new reports of sex differences in spindle dynamics.[42]

NREM sleep quality is directly correlated with improved neuropsychological functioning and remains a physiological point of therapeutic interest for cognitive impairment associated with insomnia, neuropsychiatric disorders, and aging.[78] It is plausible that KAT II inhibition modulates NREM sleep by influencing γ-aminobutyric acid (GABA) neurotransmission, as evidenced in microdialysis studies wherein elevated KYNA associates with reduced cortical and hippocampal GABA levels in rats.[79,80] GABA receptor agonists are commonly prescribed for insomnia and NREM sleep disorders,[81,82] yet these drugs, including benzodiazepines or zolpidem, carry a risk of drug dependence and an array of side effects including prolonged sleepiness, delirium, aggression, and complex sleep behaviors such as sleepwalking.[83,84] Our findings place new attention on KAT II inhibitors to modulate NREM sleep quality and improve daytime functioning and arousal. Complementary experiments wherein we consider the use of KAT II inhibitors at different times of day and apply chronobiology to therapeutic approaches may have significant translational value to the treatment of sleep disturbances.

Corresponding to disruptions in sleep parameters, we determined that increased KYNA instigated arousal, but notably during the light phase when rodents acquire homeostatic sleep need.[85] The number of wake bouts during the dark phase increased for all subjects, corresponding with a shift in activity levels and hyperactivity at the light and dark phase transitions, but the effect was notably more profound in females. Fragmented wakefulness may have a negative impact on alertness and cognitive function[86,87] and the ability of PF-04859989 to attenuate this fragmentation backs reports of enhanced cognitive performance with other KAT II inhibitors.[23,39,88]

The finding that PF-04859989 treatment alone elicited sex-specific sedative effects by increasing sleep duration in males, but minimally impacting sleep in females, deserves special consideration. Studies with KAT II inhibition have, however, been conducted in a minimal capacity in both sexes, and effective inhibition of KAT II-dependent KYNA synthesis may present some sex-dependent challenges that warrant further exploration.[21,89] Our biochemical findings did demonstrate inhibition of kynurenine-induced *de novo* KYNA formation in the brain across both sexes with PF-04859989 pretreatment. Yet, we were unable to determine a reduction in basal KYNA with PF-04859989 treatment alone compared to control. Tissue homogenate analyses cannot identify KYNA in the extracellular milieu alone. As such, microdialysis studies with both sexes are warranted with PF-04859989 as it is known that this compound at the dose presently used can readily reduce extracellular KYNA.[27,39]

In summary, our findings indicate an important association between KYNA, the KAT II enzyme, and sleep-wake behavior. Our data suggest that pharmacological agents that inhibit KAT II activity in the brain may attenuate sleep disturbances. KYNA is considered a modulator of mechanisms that mediate both cognition and sleep, thereby KYNA may casually disrupt the relationship between sleep and cognition in pathological conditions. Sleep dysfunction and cognitive impairments are associated with a variety of neurocognitive disorders,[72,90,91] and similarly elevated KYNA levels are found in the brains of patients with these conditions.[28-33,92] As such, regulating KYNA levels with KAT II inhibition should be carefully considered in the development of novel therapeutic strategies to combat sleep and cognitive disturbances, and these strategies may provide value to improving the quality of life in patients afflicted with neurocognitive and associated sleep disruptions.

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Disclosure Statement

There are no conflicts of interest or financial disclosures to report.

Figures

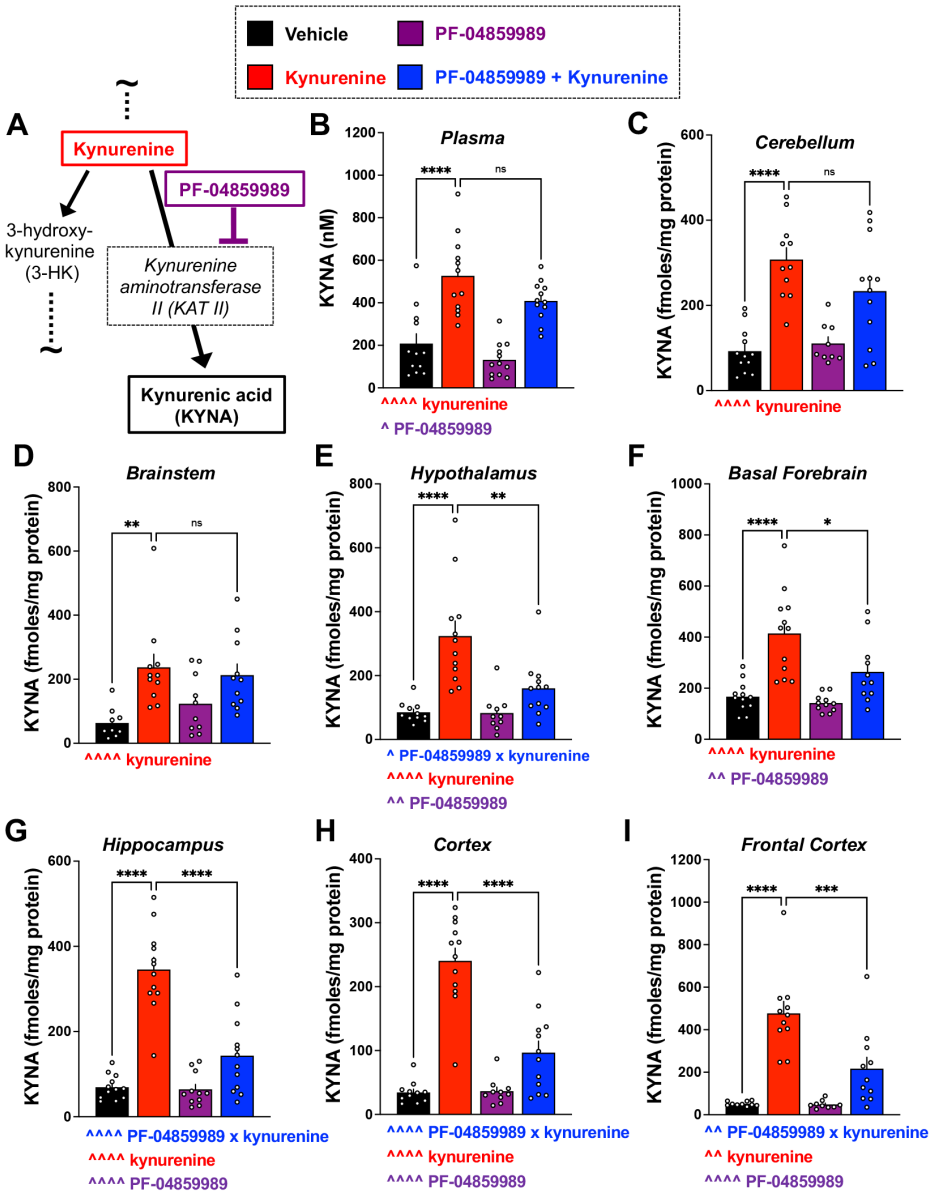

Figure 1. KYNA levels in plasma and across brain regions are increased after the acute kynurenine challenge, and reversed after pretreatment with the KAT II inhibitor, PF-04859989. Adult male and female rats were treated with vehicle, kynurenine (100 mg/kg), PF-04859989 (30 mg/kg), or PF-04859989 + kynurenine at Zeitgeber time (ZT) 0 and tissues harvested at ZT 2. KYNA levels were evaluated in the **(A)** plasma; **(B)** cerebellum; **(C)** brainstem; **(D)** hypothalamus; **(E)** basal forebrain; **(F)** hippocampus; **(G)** cortex; **(H)** frontal cortex KYNA. All data are mean ± SEM. 2-way ANOVA analyses: ^P<0.05, ^^P<0.01, ^^^^P<0.0001; Bonferroni's post hoc test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. N = 9-12 per group.

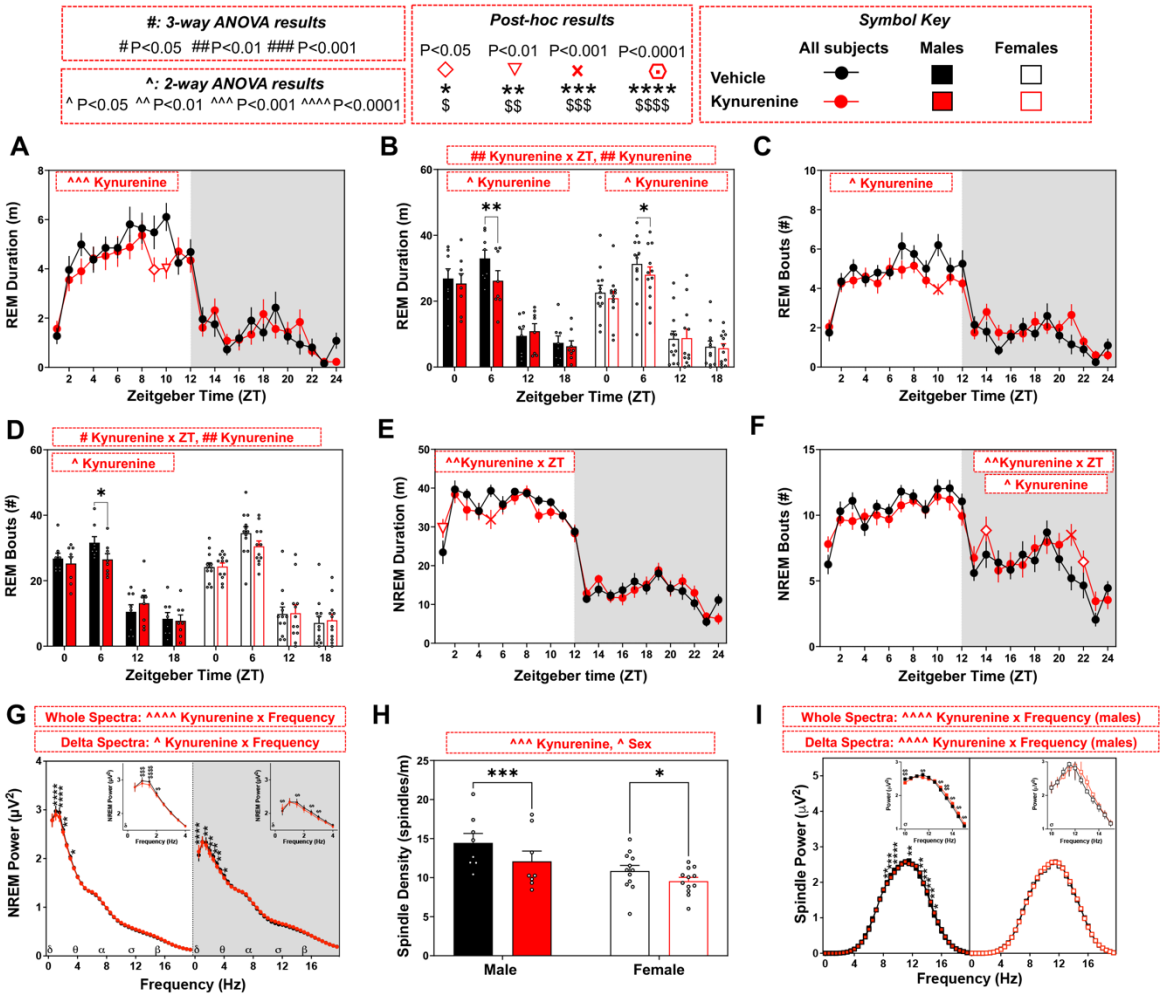

Figure 2. Kynurenine challenge reduces sleep and alters sleep architecture, including reducing spectral power and spindle density. Adult male and female rats were treated with vehicle or kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. **(A)** 1-hr bins of REM duration, sexes combined. **(B)** 6-hr bins of REM duration, analyses separated by sex. **(C)** 1-hr bins of REM bout number, sexes combined. **(D)** 6-hr bins of REM bout number, analyses separated by sex. **(E)** 1-hr bins of NREM duration, sexes combined. **(F)** 1-hr bins of NREM bout number, sexes combined. **(G)** NREM spectral power during the light (ZT 0 – 12; white background) and dark (ZT 12 – 24; grey background) phases, sexes combined. **(H)** NREM spindle density, analyses separated by sex. **(I)** NREM spindle power, analyses separated by sex. All data are mean ± SEM. 3-way RM ANOVA and 2-way RM ANOVA with Fisher's LSD post hoc test. N = 8 males, 12 females.

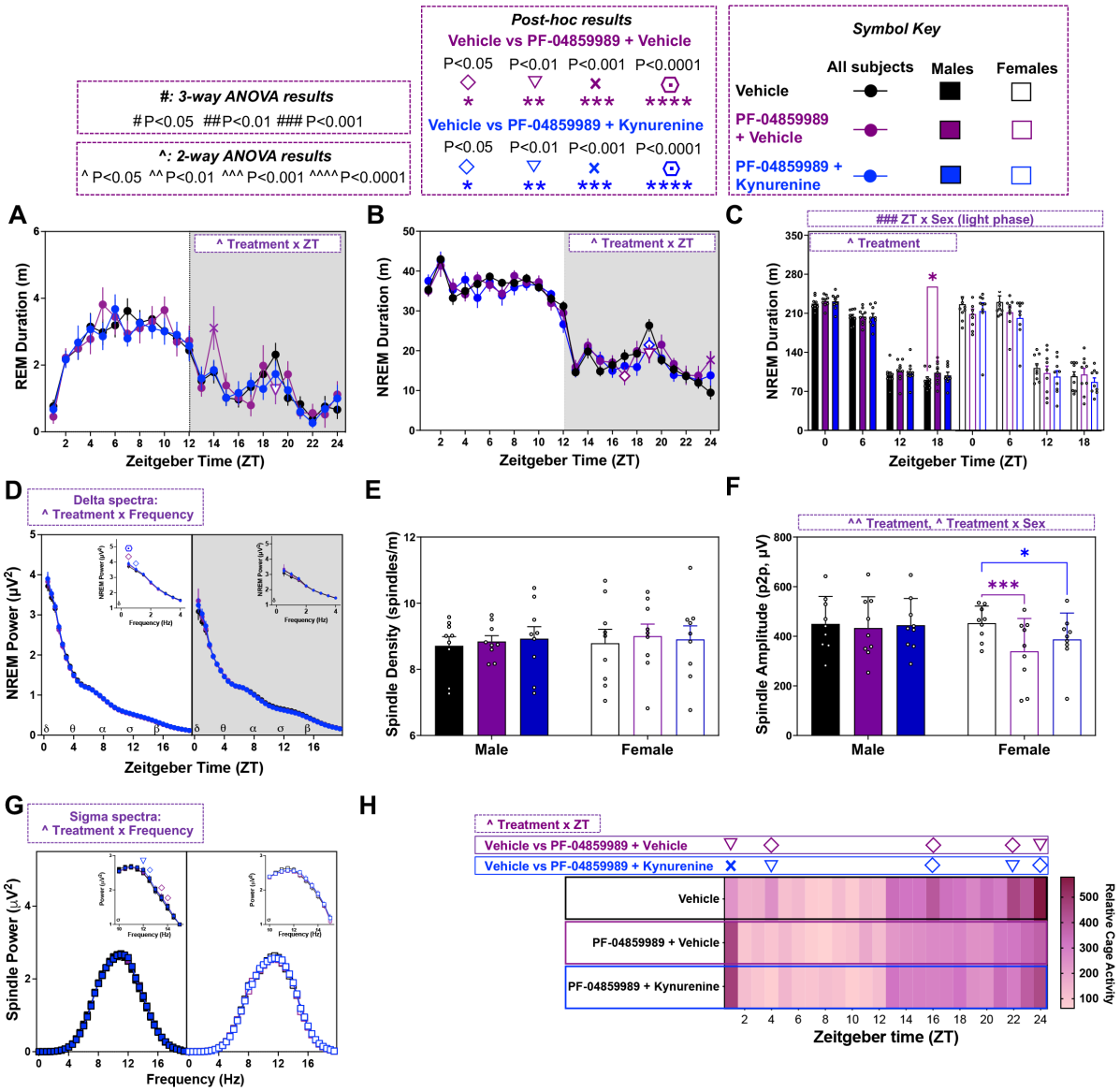

Figure 3. PF-04859989 treatment enhances sleep architecture. Adult male and female rats were treated with vehicle, PF-04859989 (30 mg/kg) + vehicle or PF-04859989 (30 mg/kg) + kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. **(A)** 1-hr bins of REM duration, sexes combined. **(B)** 1-hr bins of NREM duration, sexes combined. **(C)** 6-hr bins of NREM duration, analyses separated by sex. **(D)** NREM spectral power during the light (ZT 0 – 12; white background) and dark (ZT 12 – 24; grey background) phases, sexes combined. **(E)** NREM spindle density, analyses separated by sex. **(F)** NREM spindle amplitude, analysis separated by sex. **(G)** NREM spindle power, analyses separated by sex. **(H)** 1-hr bins of relative home cage activity, sexes combined. All data are mean ± SEM. 3-way RM ANOVA and 2-way RM ANOVA with Fisher's LSD post hoc test. N = 9 males, 9 females.

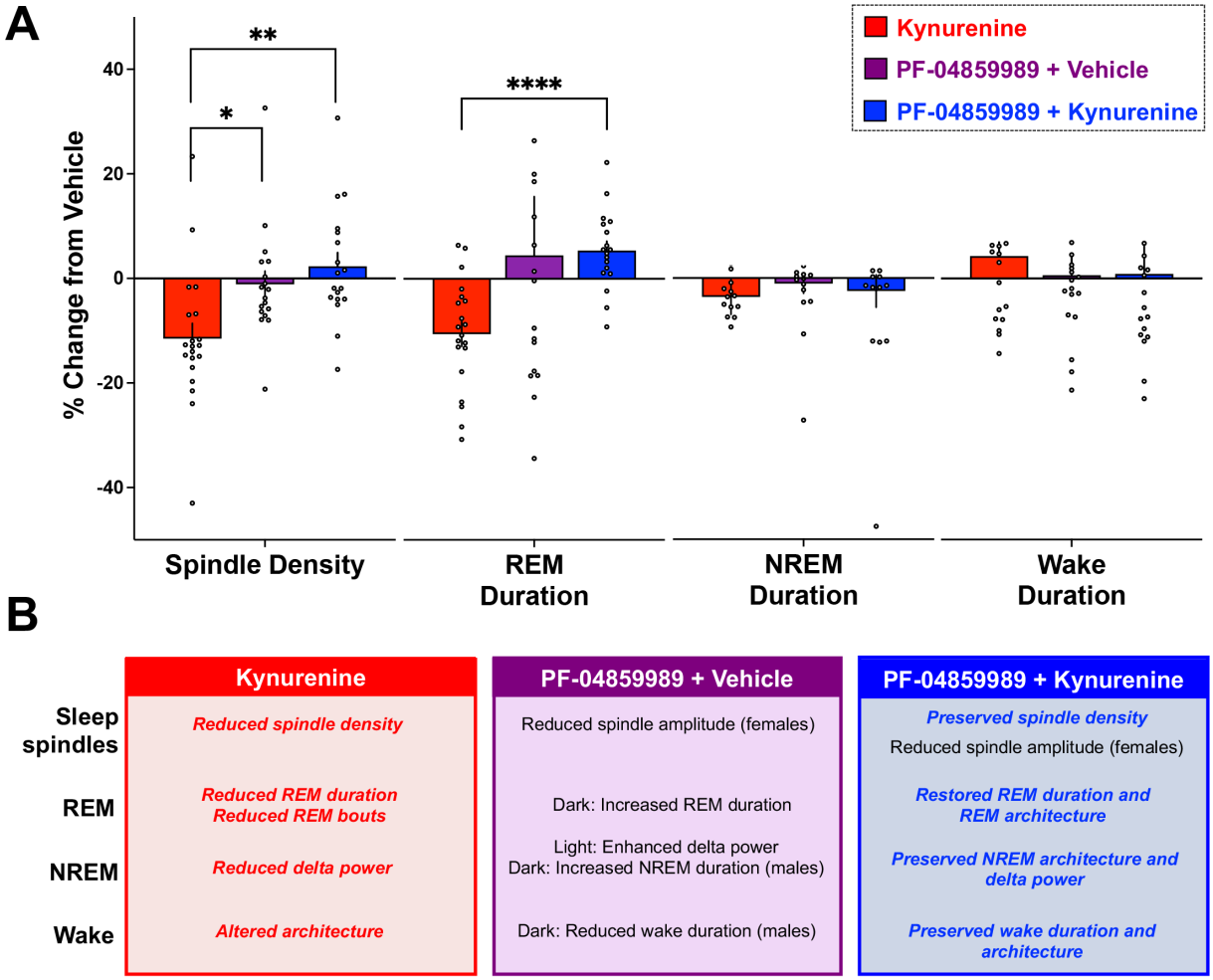

Figure 4. Pretreatment with PF-04859989 attenuates consequences of kynurenine challenge on spindle density and REM duration. Sleep Study #1: Adult male and female rats were treated with vehicle or kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. Sleep Study #2: Adult male and female rats were treated with vehicle, PF-04859989 (30 mg/kg) + vehicle or PF-04859989 (30 mg/kg) + kynurenine (100 mg/kg) at ZT 0. **(A)** To compare results from the two sleep studies, spindle density (ZT 0-4), REM duration (light phase), NREM duration (light phase), and wake duration (light phase) data were evaluated as percent change from vehicle. **(B)** Summarized conclusions. All data are mean ± SEM. Student's T test: *P<0.05, **P<0.01, ****P<0.0001.

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TITLE: **Reducing brain kynurenic acid synthesis precludes kynurenine-induced sleep disturbances**

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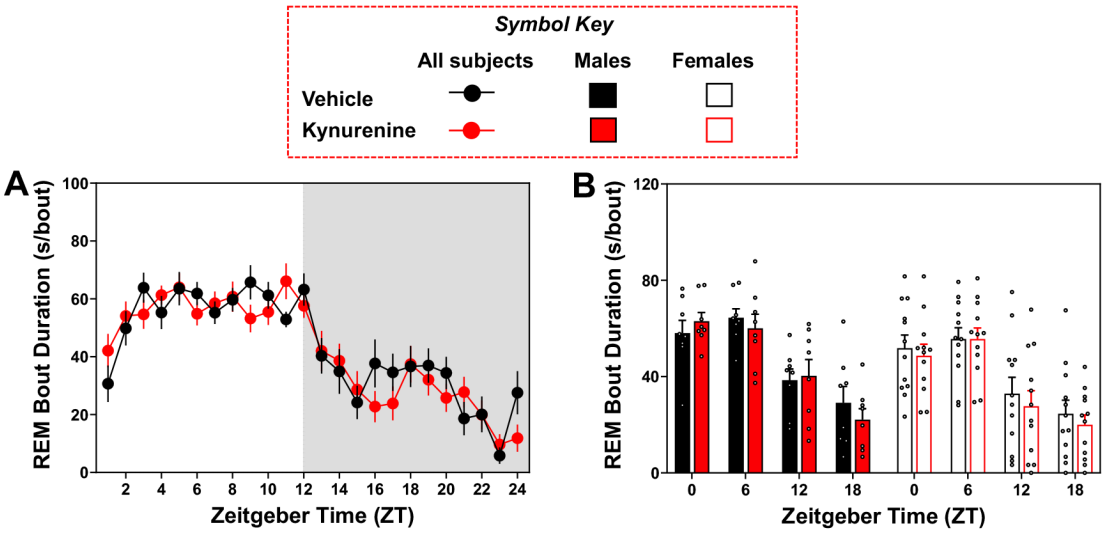
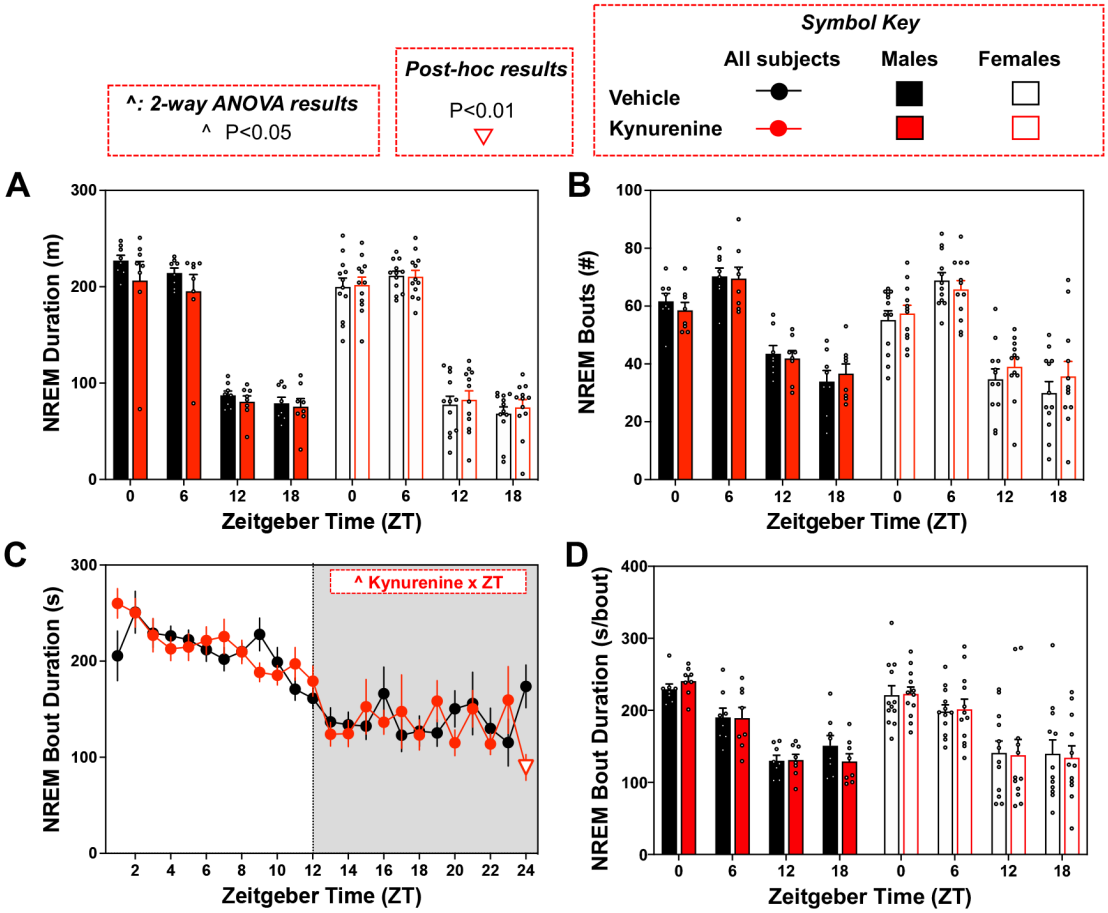
		Vehicle	Kynurenine	PF-04859989	PF-04859989 + Kynurenine	Two-way ANOVA	
						P values	F values
Plasma	Kynurenine (μM)	6.5 ± 0.6	18.3 ± 1.4****	5.3 ± 0.4	16.5 ± 2.0^^^^	P<0.0001	$F_{1,44}$ = 80.86
Cerebellum	Kynurenine (pmoles/ mg protein)	15.2 ± 1.6	94.2 ±18.2****	21.0 ± 3.8	66.8 ± 10.3^	P<0.0001	$F_{1,43}$ = 32.95
Brainstem		21.6 ± 4.8	50.2 ± 4.9**	23.8 ± 3.3	50.1 ± 7.5^^	P<0.0001	$F_{1,41}$ = 24.96
Hypothalamus		18.6 ± 1.7	45.6 ± 2.8****	24.6 ± 2.9	43.4 ± 3.7^^^	P<0.0001	$F_{1,43}$ = 65.02
Basal Forebrain		12.8 ± 0.6	49.9 ± 4.9**	14.0 ± 1.5	46.6 ± 6.1^^^	P<0.0001	$F_{1,32}$ = 34.85
Hippocampus		14.7 ± 3.1	75.8 ± 3.1***	13.7 ± 2.4	70.3 ± 14.2^^^	P<0.0001	$F_{1,37}$ = 45.05
Cortex		15.7 ± 1.0	65.0 ± 5.7****	17.4 ± 1.7	61.4 ± 10.4^^^^	P<0.0001	$F_{1,43}$ = 57.73
Frontal Cortex		26.3 ± 1.6	94.1 ± 5.5***	24.7 ± 2.7	96.5 ± 20.3^^^	P<0.0001	$F_{1,42}$ = 39.76

Kynurenine-induced elevation in kynurenine levels in plasma and across brain regions.
All data are mean ± SEM. Two-way ANOVA, Bonferroni's post hoc analysis:
Vehicle vs. Kynurenine: **P < 0.01, ***P < 0.001, ****P < 0.0001
PF-04859989 vs. PF-04859989 + Kynurenine: ^P < 0.05, ^^P < 0.01, ^^^P < 0.001, ^^^^P < 0.0001.

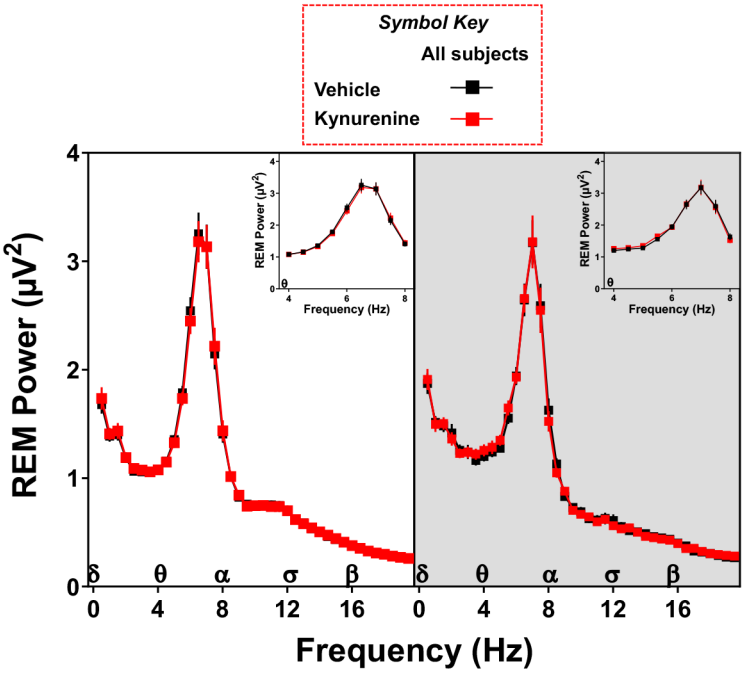
Supplemental Table 1. **Kynurenine challenge significantly increases kynurenine levels in plasma and across brain regions.** Biochemical data for four treatment groups: vehicle, kynurenine (100 mg/kg), PF-04859989 (30 mg/kg), or PF-04859989 + kynurenine. All data are mean ± SEM. N = 4 - 12 per group.



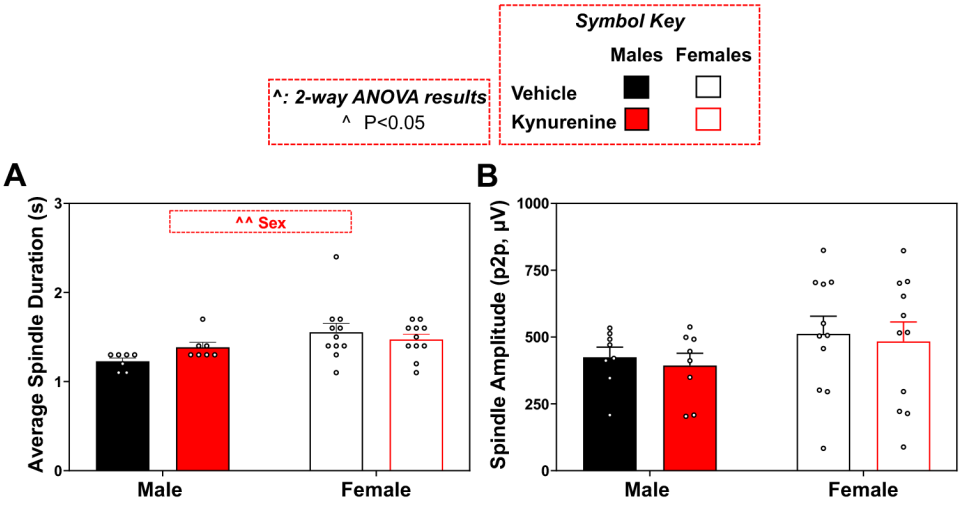
Supplemental Figure 1. Acute kynurenine challenge does not impact REM bout duration. Adult male and female rats were treated with vehicle or kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. **(A)** 1-hr bins of average REM bout duration, sexes combined. **(B)** 6-hr bins of average REM bout duration, analyses separated by sex. All data are mean ± SEM. 3-way RM ANOVA and 2-way RM ANOVA. N = 8 males, 12 females.



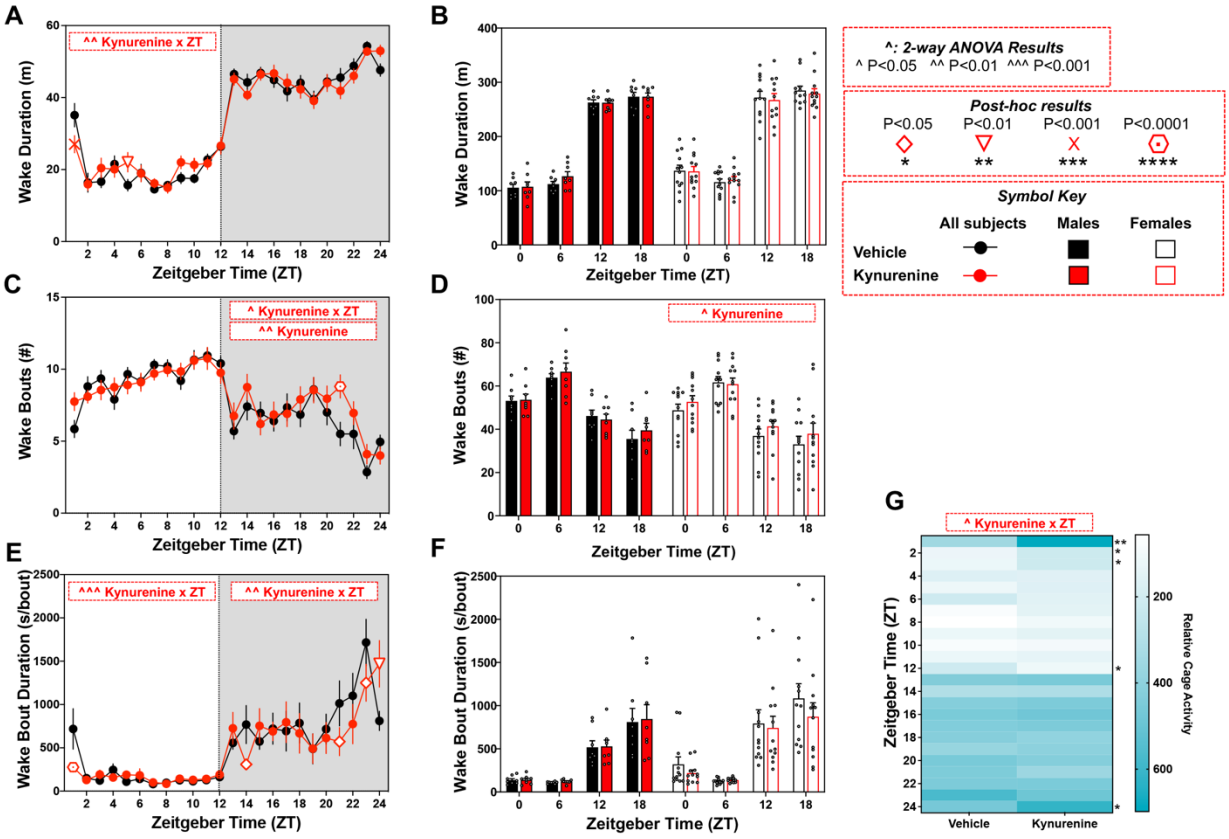
Supplemental Figure 2. Acute kynurenine challenge modestly impacts NREM by decreasing bout duration at the end of the dark phase. Adult male and female rats were treated with vehicle or kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. **(A)** 6-hr bins of NREM duration, analyses separated by sex. **(B)** 6-hr bins of number of NREM bouts, analyses separated by sex. **(C)** 1-hr bins of average NREM bout duration, sexes combined. **(D)** 6-hr bins of average NREM bout duration, analyses separated by sex. All data are mean ± SEM. 3-way RM ANOVA and 2-way RM ANOVA. N = 8 males, 12 females.



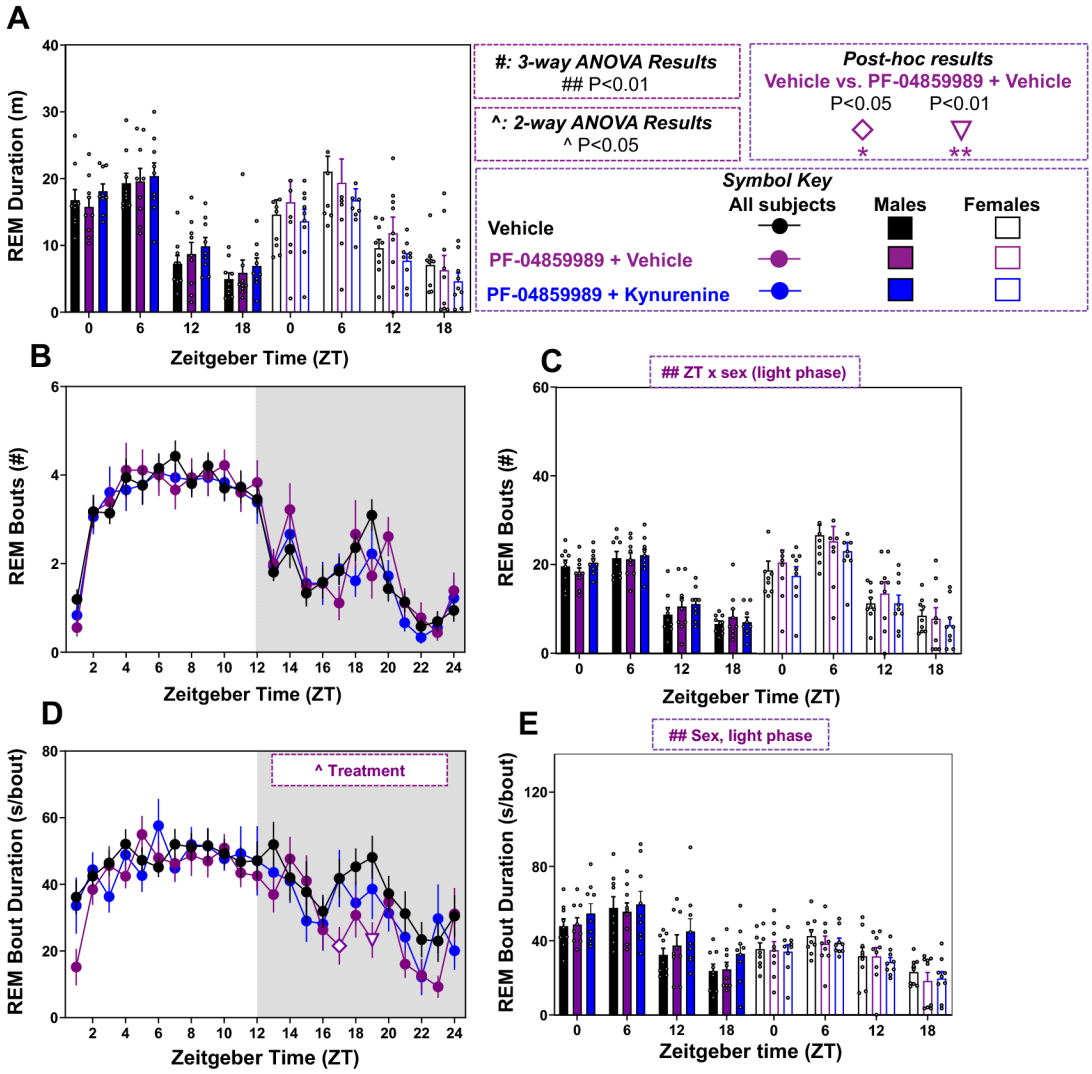
Supplemental Figure 3. Acute kynurenine challenge does not impact REM spectral power. Adult male and female rats were treated with vehicle or kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. REM spectral power during the light (ZT 0 – 12; white background) and dark (ZT 12 – 24; grey background) phases, sexes combined. Inserted graphs shows focused analyses at the theta frequencies (4 - 8 Hz) during each respective phase. All data are mean ± SEM. 2-way RM ANOVA. N = 8 males, 12 females.



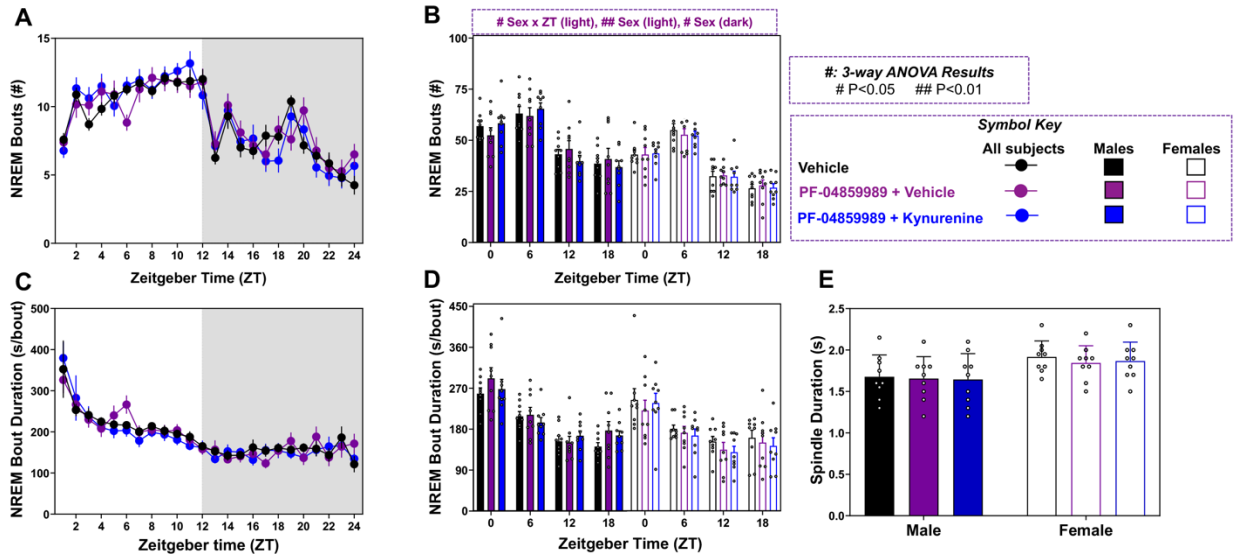
Supplemental Figure 4. Acute kynurenine challenge does not impact average duration of NREM spindles or their peak-to-peak amplitude. Adult male and female rats were treated with vehicle or kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. Analyses of spindles were conducted during the first 4 hr of the light phase, ZT 0 – 4**. (A)** Average spindle duration, sexes separated. **(B)** Peak-to-peak (p2p) amplitude, analyses separated by sex. All data are mean ± SEM. 2-way RM ANOVA. ^P<0.05. N = 8 males, 12 females.



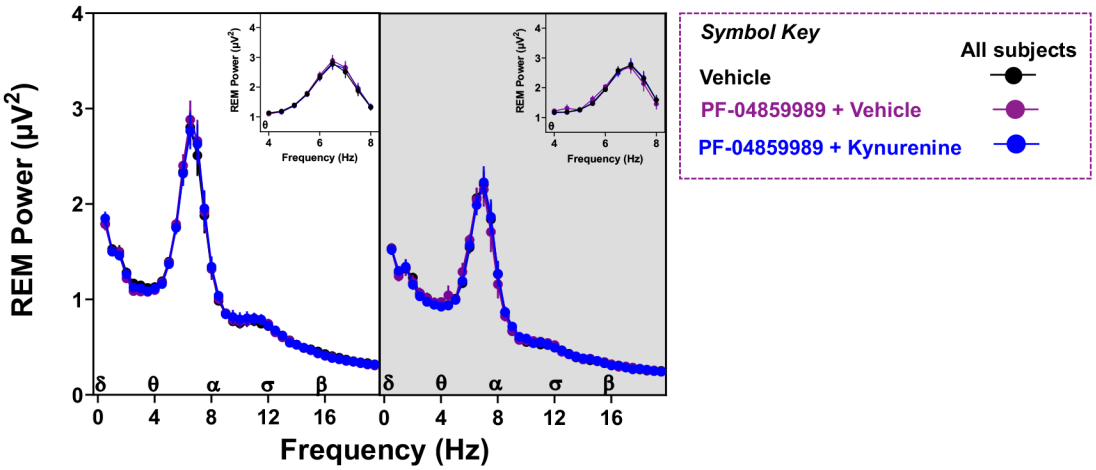
Supplemental Figure 5. Acute kynurenine challenge increases wakefulness during the light phase, altering arousal patterns. Adult male and female rats were treated with vehicle or kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. **(A)** 1-hr bins of wake duration, sexes combined **(B)** 6-hr bins of wake duration, analyses separated by sex **(C)** 1-hr bins of number of wake bouts, sexes combined **(D)** 6-hr bins of number of wake bouts, analyses separated by sex **(E)** 1-hr bins of average wake bout duration, sexes combined **(F)** 6-hr bins of average wake bout duration, analyses separated by sex **(G)** 1-hr bins of relative home cage activity, sexes combined. All data are mean ± SEM. 3-way RM ANOVA and 2-way RM ANOVA with Fisher's LSD post hoc test. N = 8 males, 12 females.



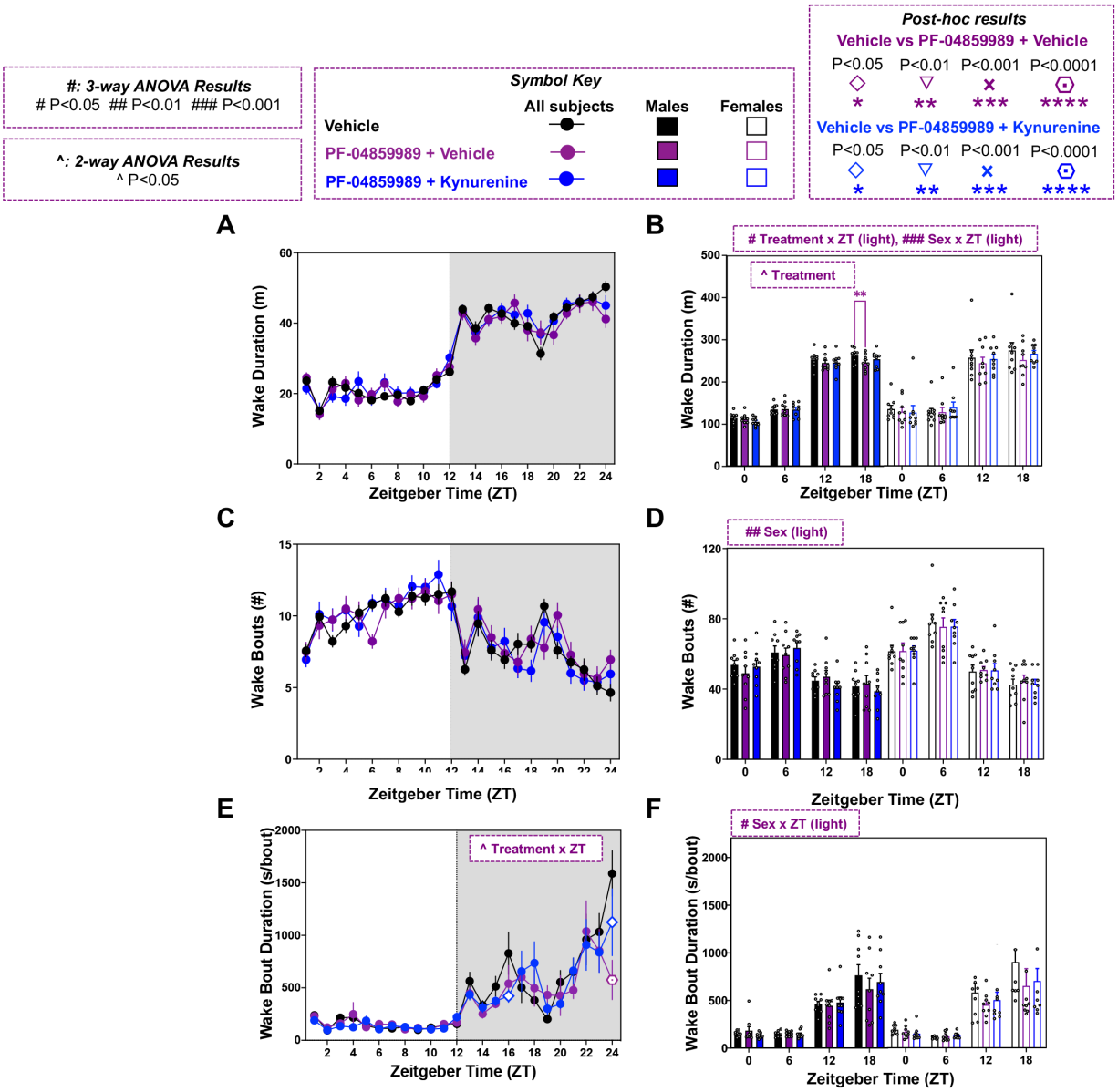
Supplemental Figure 6. PF-04859989 treatment ameliorates the acute kynurenine-induced reduction in REM sleep. Adult male and female rats were treated with vehicle, PF-04859989 (30 mg/kg) + vehicle or PF-04859989 (30 mg/kg) + kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. **(A)** 6-hr bins of REM duration, analyses separated by sex **(B)** 1-hr bins of REM bout number, sexes combined **(C)** 6-hr bins of REM bout number, analyses separated by sex **(D)** 1-hr bins of average REM bout duration, sexes combined **(E)** 6-hr bins of average REM bout duration, analyses separated by sex. All data are mean ± SEM. 3-way RM ANOVA and 2-way RM ANOVA. N = 9 males, 9 females.



Supplemental Figure 7. PF-04859989 ameliorates NREM bout fragmentation elicited by kynurenine and conserves average spindle duration. Adult male and female rats were treated with vehicle, PF-04859989 (30 mg/kg) + vehicle or PF-04859989 (30 mg/kg) + kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. **(A)** 1-hr bins of NREM bout number, sexes combined **(B)** 6-hr bins of NREM bout number, analyses separated by sex **(C)** 1-hr bins of average NREM bout duration, sexes combined **(D)** 6-hr bins of average NREM bout duration, analyses separated by sex **(E)** Analyses of average spindle duration were conducted during the first 4 hr of the light phase, ZT 0 – 4, analyses separated by sex. All data are mean ± SEM. 3-way RM ANOVA and 2-way RM ANOVA. N = 9 males, 9 females.



Supplemental Figure 8. PF-04859989 treatment does not impact REM spectral power. Adult male and female rats were treated with vehicle, PF-04859989 (30 mg/kg) + vehicle or PF-04859989 (30 mg/kg) + kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. REM spectral power during the light (ZT 0 – 12; white background) and dark (ZT 12 – 24; grey background) phases, sexes combined. Inserted graphs show focused analyses at the theta frequencies (4-8 Hz) during each respective phase. All data are mean ± SEM. 2-way RM ANOVA. N = 9 males, 9 females.



Supplemental Figure 9. PF-04859989 prevents kynurenine-induced disruptions in wakefulness, and PF-04859989 treatment alone reduces wakefulness. Adult male and female rats were treated with vehicle, PF-04859989 (30 mg/kg) + vehicle or PF-04859989 (30 mg/kg) + kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. **(A)** 1-hr bins of wake duration, sexes combined **(B)** 6-hr bins of wake duration, analyses separated by sex (C) 1-hr bins of wake bout number, sexes combined (D) 6-hr bins of wake bout number, analyses separated by sex (E) 1-hr bins of average wake bout duration, sexes combined (F) 6-hr bins of average wake bout duration, analyses separated by sex. All data are mean ± SEM. 3-way RM ANOVA and 2-way RM ANOVA with Fisher's LSD post hoc test: N = 9 males, 9 females.

Transition	Vehicle	Kynurenine
MALE		
Wake-NREM	114 ± 3.3	117 ± 5.8
REM-Wake	47 ± 1.8	46 ± 2.7
REM-NREM	10 ± 1.5	6 ± 1.2
NREM-Wake	67 ± 3.3	71 ± 3.5
NREM-REM	57 ± 2.6	52 ± 2.4
Wake-NREM	73 ± 6.6	75 ± 5.4
REM-Wake	18 ± 2.7	20 ± 3.4
REM-NREM	1 ± 0.3	1 ± 0.5
NREM-Wake	55 ± 7.9	55 ± 7.4
NREM-REM	19 ± 2.9	21 ± 3.7
FEMALE		
Wake-NREM	106 ± 4.9	109 ± 5.4
REM-Wake	46 ± 1.9	47 ± 1.5
REM-NREM	11 ± 1.5	6 ± 1.2 *
NREM-Wake	60 ± 3.7	62 ± 4.6
NREM-REM	57 ± 2.5	53 ± 1.7 *
Wake-NREM	59 ± 6.4	77 ± 5.4 ****
REM-Wake	14 ± 2.6	16 ± 3.3
REM-NREM	3 ± 1.7	2 ± 0.7
NREM-Wake	46 ± 6.0	61 ± 7.0 ***
NREM-REM	16 ± 3.9	18 ± 3.7

RM Two-Way ANOVA
Female (Light Phase): ** Transition x Treatment
Female (Dark Phase): ** Transition x Treatment; * Treatment
* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001

Supplemental Table 2. Acute kynurenine challenge disrupts female sleep transitions. Adult male and female rats were treated with vehicle or kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. All data are mean ± SEM. N = 8 - 12 per group

Transition	Vehicle	PF-04859989 + Vehicle	PF-04859989 + Kynurenine
MALE			
Wake-NREM	110 ± 6.1	101 ± 6.6	112 ± 6.2
REM-Wake	37 ± 2.5	36 ± 2.2	37 ± 2.3
REM-NREM	3 ± 1.2	3 ± 1.1	4 ± 1.0
NREM-Wake	73 ± 4.3	68 ± 5.7	74 ± 5.1
NREM-REM	40 ± 2.4	39 ± 2.2	42 ± 2.2
Wake-NREM	77 ± 4.6	82 ± 7.8	72 ± 5.4
REM-Wake	14 ± 2.0	18 ± 3.0	17 ± 2.0
REM-NREM	1 ± 0.2	1 ± 0.4	1 ± 0.4
NREM-Wake	64 ± 4.8	64 ± 8.0	55 ± 5.0
NREM-REM	15 ± 1.9	19 ± 3.2	18 ± 2.0
FEMALE			
Wake-NREM	127 ± 4.2	133 ± 8.7	132 ± 5.9
REM-Wake	37 ± 3.0	42 ± 5.4	36 ± 3.5
REM-NREM	5 ± 1.2	3 ± 0.7	4 ± 1.2
NREM-Wake	89 ± 3.0	91 ± 9.6	96 ± 7.4
NREM-REM	42 ± 3.1	46 ± 5.8	40 ± 3.8
Wake-NREM	77 ± 4.2	85 ± 4.5	85 ± 4.4
REM-Wake	19 ± 1.9	20 ± 4.1	17 ± 2.4
REM-NREM	1 ± 0.3	1 ± 0.3	1 ± 0.3
NREM-Wake	59 ± 3.2	65 ± 6.5	68 ± 3.6
NREM-REM	19 ± 2.0	21 ± 4.3	17 ± 2.7

Supplemental Table 3. PF-04859989 treatment does not alter vigilance state transitions. Adult male and female rats were treated with vehicle, PF-04859989 (30 mg/kg) + vehicle or PF-04859989 (30 mg/kg) + kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. All data are mean ± SEM. N = 9 per group