Reducing brain kynurenic acid synthesis precludes kynurenine-induced sleep disturbances

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ABSTRACT

Patients with neurocognitive disorders often battle sleep disturbances. Kynurenic acid (KYNA) is a tryptophan metabolite of the kynurenine pathway 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. 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. 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 disturbances in REM sleep and sleep spindles, and enhanced NREM sleep. Our findings suggest that reducing KYNA in conditions where the kynurenine pathway is activated may serve as a potential strategy for improving sleep dynamics.

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

Introduction

Healthy sleep promotes synaptic plasticity and is necessary for the maintenance of newly formed memories (Aton et al., 2009). Cortical electroencephalography (EEG) combined with electromyography (EMG) is readily employed to distinguish wake from sleep states. Non-rapid eye movement (NREM) sleep presents as high cortical delta oscillations (0.5-4 Hz) from thalamocortical networks and rapid eye movement (REM) sleep exhibits high frequency theta oscillations (4-8 Hz) from corticolimbic networks. NREM is largely implicated in procedural memory, and REM sleep in considered critical to synaptic pruning, behavioral refinement, and memory consolidation (Abel, Havekes, Saletin, & Walker, 2013; Boyce, Williams, & Adamantidis, 2017). 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 (Lucey et al., 2021; Pocivavsek & Rowland, 2018). 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.

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, supported via the regulatory role of serotonin and melatonin in sleep and arousal (Rapoport & Beisel, 1968). However, only about 5% of tryptophan is degraded through the melatonin pathway. The vast majority of tryptophan is metabolized via the KP , which generates the small neuroactive molecule KYNA that we hypothesize serves a critical role in modulating sleep (Milosavljevic, Smith, Wright, Valafar, & Pocivavsek, 2023; Pocivavsek, Baratta, Mong, & Viechweg, 2017; Rentschler et al., 2021).

Elevations in KYNA disrupt cholinergic and glutamatergic-dependent processes, including learning and memory , and restoring a balance in KYNA promotes cognitive function (Kozak et al., 2014; Pocivavsek et al., 2011). 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 (Linderholm et al., 2012; Miller, Llenos, Dulay, & Weis, 2006; Sathyasaikumar et al., 2011). 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 (Kozak et al., 2014; Pellicciari et al., 2006; Pocivavsek et al., 2011).

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 (Pocivavsek et al., 2017). We selected PF-04859989, a brain penetrable and irreversible KAT II inhibitor (Klausing, Fukuwatari, Bucci, & Schwarcz, 2020; Kozak et al., 2014) and confirmed its effectiveness to reduce *de novo* KYNA formation when administered prior to kynurenine challenge. We thereby hypothesize that KAT II inhibition with PF-04859989 will effectively attenuate dysregulated sleep-wake behavior in response to the acute kynurenine challenge.

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 (Hyderbad, India). PF-04859989 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 100 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 .

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 (Pocivavsek et al., 2017; Rentschler et al., 2021). 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 (Swift et al., 2020).

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-amplitude EMG). 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 duration, 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. Briefly, we applyied a rectified 10 - 15 Hz finite impulse response band pass filter to the EEG signal and computed the cubed root-mean square 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 (Uygun et al., 2019). To ensure scientific rigor, all artifactual spikes with a duration of less than 0.5 s were excluded from spindle analyses criteria. Sleep spindle data were analyzed for the first 4-hr period of the light phase during NREM, acquiring a representative spread of NREM sleep spindles. 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

Normality of data was assessed with Shapiro-Wilk test and data were visually inspected using Q-Q plots to confirm a relative bell-shaped distribution and the absence of outliers. For biochemical analyses, comparisons were made using a 2-way analysis of variance (ANOVA) with kynurenine and/or PF-04859989 treatment as a between-subject factors. Significant main effects were followed by Bonferroni's correction with an adjusted alpha for multiple comparisons. To evaluate sex as a biological variable in sleep architecture data (vigilance state durations, bout number, and average bout duration), we initially employed 3-way repeated measures (RM) ANOVA with treatment and ZT as within-subject factors and sex as a between-subject factor. As no main effects of sex were determined (see **Supplemental Table 1**), the remaining analyses were conducted with sexes combined. Durations were evaluated in 1-hr bins, followed by 6-hr bins, while bout number and average bout duration were evaluated in 6-hr bins employing 2-way RM ANOVA with treatment and ZT as within-subject factors. REM theta power spectra and NREM

delta power spectra were evaluated during the light phase by 2-way RM ANOVA with frequency and treatment as within-subject factors. Spindle density, average spindle duration, and spindle amplitude were evaluated by paired Student's t-test. Power spectra during spindles were evaluated with 2-way RM ANOVA with frequency and treatment as within-subject factors. Vigilance state transitions were evaluated by paired Student's t-test. Relative cage activity was analyzed by 3-way RM ANOVA with treatment and ZT as within-subject factors, and sex as a between-subject factor. Post hoc analysis was conducted with Bonferroni's correction in Sleep Study #1 and Dunnett test in Sleep Study #2, to compare drug treatments to vehicle treatment condition. Percent of vehicle calculations (REM duration, NREM slow-wave spectral power and spindle density) allowed comparisons between Sleep Study #1 and Sleep Study #2, thereby comparing PF-04859989 + kynurenine to kynurenine treatment alone with unpaired Student's t-test. All statistical analyses were performed using Prism 9.0 (GraphPad Software, La Jolla, CA, USA) and significance was defined as $P < 0.05$.

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 (**Supplemental Table 2**). Kynurenine-treated rats exhibited a 2.8-fold increase in plasma kynurenine levels compared with vehicle-treated rats ($P<0.0001$). As kynurenine promptly enters the brain from the blood, we confirmed significant elevation in brain kynurenine levels in the cerebellum (6.2-fold; $P<0.0001$), brainstem (2.3-fold; $P<0.0001$), hypothalamus (2.5-fold; $P<0.0001$), basal forebrain (3.9-fold; $P<0.0001$), hippocampus (5.2-fold; $P<0.0001$), cortex (4.1-fold; $P<0.0001$), and frontal cortex

(3.6-fold; P<0.0001) compared to vehicle. Levels of kynurenine in plasma and brain remained unchanged after pretreatment with PF-04859989.

Upon kynurenine treatment at ZT 0, plasma KYNA increased 2.5-fold compared to 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<0.0001), hypothalamus (3.8-fold; $F_{1,41}$ = 27.20, P<0.001), 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). PF-04859989 significantly impacted KYNA levels noted as a main effect of treatment 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<0.0001). 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 frontal cortex ($F_{1,37}$ = 9.248, P<0.01). Post-hoc analysis noted a significant reduction in KYNA 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<0.01). PF-04859989 pretreatment did not significantly reduce KYNA formation in the plasma, 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 sleep and alters architecture, spectral power, and spindle dynamics

Sleep Study #1 was conducted in a cohort of rats used exclusively for sleep monitoring experiments. No main effects of sex were determined when sleep data were evaluated separately by sex (**Supplemental Table 1)** and vigilance state durations in 1-hr bins are shown separately for males and females in **Supplemental Figure 1**. For the remaining visualizations of vigilance state data, we evaluated with male and female subjects combined. REM duration decreased significantly, per 1-hr bin analysis, when kynurenine treatment was compared to vehicle treatment (ZT x treatment: $F_{23,437}$ = 1.635, P<0.05; treatment: $F_{1,19}$ = 15.14, P<0.01). The starkest REM duration decrease occurred in the later portion of the light phase at ZT 10 (P<0.01(**Figure 2A**). Analyses in 6-hr time bins supported the notion that REM was disrupted with kynurenine challenge, particularly in the latter half of the light phase (ZT 6-12). REM duration (treatment: $F_{1,19}$ = 15.15, P<0.001)(**Figure 2B**) and REM bouts (treatment: $F_{1,19}$ = 8.510, P<0.01)(**Figure 2C**) were significantly reduced with kynurenine challenge. Parameters that were not altered with kynurenine treatment related to REM included the average duration of each REM bout (**Supplemental Figure 2A**) and spectra power in the theta range during the light phase (**Supplemental Figure 2B**).

Kynurenine treatment induced changes to NREM duration that varied across 1-hr bin analysis (treatment x ZT interaction: $F_{23,437}$= 2.032, P<0.01). NREM duration decreased significantly at ZT 5 (P<0.01) with kynurenine treatment compared to vehicle (**Figure 2D**). Analyses in 6-hr time bins revealed no significant changes in NREM duration (**Figure 2E**), NREM bout number (**Supplemental Figure 2C**), and average duration of NREM bouts (**Supplemental Figure 2D**). Delta (0.5 – 4 Hz) spectral power was highest compared with theta, alpha, sigma, and beta during NREM sleep (light phase: $F_{38,722}$ = 414.2, P<0.0001; data not shown). Thus, we subsequently evaluated spectral power during NREM within the delta range. Kynurenine treatment significantly reduced NREM delta spectral power (treatment x frequency interaction: $F_{7,133}$ = 2.165, P<0.05). Lower NREM spectral power was found at 1 Hz (P<0.001) and 1.5 Hz (P<0.0001) during the light phase (**Figure 2F**).

During 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 (P<0.001)(**Figure 3A**), yet the average duration of each spindle (**Figure 3B**), peak to peak amplitude (**Figure 3C**) and the frequency distribution of spindle wavelets (**Figure 3D**) remained unchanged when comparing kynurenine and vehicle treatments.

Kynurenine challenge alters wake architecture and home cage activity

To understand how the observed changes in REM and NREM impacted arousal, wake state architecture and general home cage activity were evaluated. Analysis of wake durations in 1-hr bins revealed a kynurenine x ZT interaction ($F_{123,437}$ = 2.078, P<0.01) such that kynurenine initially reduced wake duration within the first hour (P<0.01)**(Supplemental Figure 3A)**. When wake parameters were evaluated in 6-hr bins, we did not find any significant changes in wake duration (**Supplemental Figure 3B**), number of wake bouts (**Supplemental Figure 3C**), or average duration of each wake bout (**Supplemental Figure 3D**). Of note, sex significantly impacted home cage activity (3-way ANOVA, $F_{1,347}$ = 5.118, P<0.05), along with time of day ($F_{22,396}$ = 25.40, P<0.0001), yet kynurenine treatment did not change home cage activity across time (**Supplemental Figure 3E**).

Vigilance state transitions are impacted by kynurenine challenge

The stability of maintaining a wake or sleep episode upon kynurenine challenge was assessed by examining the number of transitions into and out of wake, NREM sleep, and REM sleep by light or dark phase (**Supplemental Table 3**). Kynurenine challenge reduced REM to NREM (P < 0.0001) and NREM to REM (P < 0.01) transitions during the light phase. In the subsequent dark phase, kynurenine treatment resulted in enhanced wake to NREM (P < 0.05) and REM to wake (P <0.05) transitions.

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 (ZT 23.5 s.c., ZT 0 i.p.), ii) PF-04859989 at ZT 23.5 + vehicle at ZT 0, iii) PF-04859989 at ZT 23.5 + kynurenine at ZT 0. Our analysis focused on vigilance state parameters that were altered with kynurenine challenge, as reported above. Thus, focusing initially on REM sleep, we found that PF-04859989 pretreatment prevented kynurenine-induced REM deficits, thereby stabilizing REM duration (1-hr bins: **Figure 4A**; 6-hr bins: **Figure 4B**), REM bout number (**Figure 4C**), average duration of each REM bout (**Supplemental Figure 4A**), and theta spectral power during REM sleep (**Supplemental Figure 4B**) to vehicle treatment levels. PF-04859989 pretreatment did no influence NREM duration (1-hr bins: **Figure 4D**; 6-hr bins: **Figure 4E**), NREM bout number (**Supplemental Figure 4C**), and average duration of each NREM bout (**Supplemental Figure Figure 4D**). Of note, PF-04859989 treatment, with or without kynurenine, altered the power spectra during NREM sleep (2-way RM ANOVA, $F_{14,238}$ = 1.792, P < 0.05) by enhancing delta power within the slow wave range of 0.5 Hz compared to vehicle treatment alone (PF-04859989 + vehicle: P<0.05; PF-04859989 + kynurenine: P < 0.001)(**Figure 4F**).

Evaluation of spindle dynamics determined no differences in NREM spindle density between treatments in Sleep Study #2 (**Figure 5A**), indicating that PF-04859989 attenuated the reduction in NREM spindle density elicited by kynurenine (comparison to **Figure 3A**). Average spindle duration was not impacted by PF-04859989 treatment (**Figure 5B**). However, it is noteworthy that PF-04859989 alone significantly reduced the peak-to-peak amplitude (RM ANOVA, $F_{2,34}$= 5.133,

P < 0.05; PF-04859989 + vehicle: P<0.01)(**Figure 5C**). Spectral power within the 10-15 Hz sigma range was also not impacted by PF-04859989 treatment (**Figure 5D**).

PF-04859989 prevents kynurenine-induced changes in wake behavior

We determined no significant differences in wake duration between Sleep Study #2 treatment conditions when evaluating data in 1-hr bins (**Supplemental Figure 5A**) or 6-hr bins (**Supplemental Figure 5B**). Wake bout number (**Supplemental Figure 5C**) and average duration of each wake bout (**Supplemental Figure 5D**) were also not altered by PF-04859989 treatment. As in Sleep Study #1 home cage activity data, we similarly evaluated activity separately by sex in Sleep Study #2. Home cage activity was significantly impacted by an interaction between ZT and treatment in males, noting increased activity at ZT 1 (PF-04859989 + vehicle: P<0.001, PF-04859989 + kynurenine: P<0.001), and reduced activity at ZT 4 (PF-04859989 + kynurenine: P<0.05) and ZT 24 (PF-04859989 + vehicle: P<0.001, PF-04859989 + kynurenine: P<0.01) (**Supplemental Figure 5E**). PF-04859989 treatment with or without kynurenine did not change home cage activity in female subjects.

Vigilance state transitions are not impacted by PF-04859989 treatment

PF-04859989 successfully ameliorated kynurenine-induced changes in sleep transitions (**Supplemental Table 4**), as in Sleep Study #2 no significant differences in transitions were noted between treatment conditions.

PF-04859989 treatment attenuates sleep disruptions induced by kynurenine challenge

Vigilance state durations, sleep spindle density, and NREM slow wave spectral power 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 11% decrease in REM duration and this impact was significantly attenuated with PF-04859989 + kynurenine treatment (P<0.0001)(**Figure 6A**). PF-04859989 given prior to kynurenine challenge significantly attenuated the reduction in

slow wave (0.5 – 2 Hz) spectra power during NREM with kynurenine challenge alone (**Figure 6B)**. Lastly, spindle density was reduced by 12% with kynurenine challenge and PF-04859989 + kynurenine treatment substantially rescued this phenotype ($P<0.01$)(**Figure 6C)**. Taken together, we demonstrate the ability of KAT II inhibitor PF-04859989 to reverse kynurenine-induced sleep spindle and REM sleep abnormalities (**Figure 6**).

CONCLUSION

Altered tryptophan metabolism, including KP dysregulation, impacts sleep and arousal in normal and pathological conditions (Milosavljevic et al., 2023; Mukherjee et al., 2018; Pocivavsek et al., 2017; Rentschler et al., 2021; Yamashita, 2020). 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 (Pocivavsek et al., 2017), we presently expanded our understanding of the impact on sleep-wake behaviors by inhibiting KAT II, thereby reducing de novo KYNA formation, with a brain-penetrable and irreversible compound, PF-04859989 in male and female subjects.

Acute kynurenine challenge notably reduced the total duration of REM sleep and the architecture of sleep, denoted as reduced REM bouts and transitions from NREM to REM and vice versa. 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 frontal 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 (Blanco Ayala et al., 2021). At the transcriptome level, KAT II mRNA 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 (Song et al., 2018). 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 (Blanco Ayala et al., 2021).

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 (Lelkes, Abdurakhmanova, & Porkka-Heiskanen, 2018; Szymusiak, Alam, & McGinty, 2000). A large amount of acetylcholine is released from the basal forebrain during REM sleep (Jing et al., 2020), and we postulate that KYNA-mediated modulation of cholinergic neurotransmission may impede these dynamics (Zmarowski et al., 2009). Within the extracellular milieu, KYNA is present within low micromolar concentrations and optimally positioned to inhibit receptor targets including α7nACh receptors and NMDA receptors (Albuquerque & Schwarcz, 2013; Konradsson-Geuken et al., 2010). 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 (Schwarcz, Bruno, Muchowski, & Wu, 2012), 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 (Erhardt et al., 2017).

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 (Pocivavsek et al., 2017; Pocivavsek et al., 2011). Reduced REM sleep negatively impacts hippocampal synaptic plasticity and elicits memory deficits (Boyce, Glasgow, Williams, & Adamantidis, 2016; Tartar et al., 2006). Reduced amplitude of delta power during NREM and disrupted transitions between NREM and REM with elevated KYNA may also influence long-term memory consolidation (Tartar et al., 2006). 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 (Fernandez & Luthi, 2020), 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 (D'Agostino et al., 2018; Ferrarelli & Tononi, 2017; Manoach et al., 2014). Preclinical models relevant to the study of neuropsychiatric illnesses have also noted reduced sleep spindle density in rodents (Aguilar, Strecker, Basheer, & McNally, 2020; Czekus et al., 2022; Thankachan et al., 2019). We show presently for the first time that inhibition of KAT II and reduction of brain-derived KYNA, achieved with prophylactic PF-04859989 treatment, effectively ameliorated kynurenine-induced spindle density deficiencies.

We also find it particularly noteworthy that PF-04859989 treatment alone enhanced NREM delta power, suggesting that KAT II inhibition may enhance the quality of slow wave sleep (Lundahl, Deacon, Maurice, & Staner, 2012). 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 (Wilckens, Ferrarelli, Walker, & Buysse, 2018). 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 (Beggiato et al., 2014; Wright et al., 2021). GABA receptor agonists are commonly prescribed for insomnia and NREM sleep disorders (Lankford et al., 2008; Walsh et al., 2011), 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 (Earl & Van Tyle, 2020). 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 (Milosavljevic et al., 2023). Despite a number of disruptions in sleep parameters, we did not find major changes in arousal phenotypes with increased KYNA. However, enhanced home cage activity with PF-04859989 immediately after treatment warrants further investigation into its short term efficacy in attentional tasks, as KAT II inhibitors have been shown to acutely enhance cognition (Klausing et al., 2020; Pocivavsek et al., 2011).

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 causally disrupt the relationship between sleep and cognition in pathological conditions. Sleep dysfunction and cognitive impairments are associated with a variety of neurocognitive disorders (Brzecka et al., 2018; Manoach et al., 2014), and similarly elevated KYNA levels are found in the brains of patients with these conditions (Linderholm et al., 2012; Miller et al., 2006; Sathyasaikumar et al., 2011). 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 disorders and associated sleep disruptions.

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TABLES AND TABLE LEGENDS

	Plasma	Cerebellum	Brainstem	Hypothalamus	Basal Forebrain	Hippocampus	Cortex	Frontal Cortex
	% Increase from vehicle							
Kynurenine	201±30****	232±30****	273±65**	280±55****	148±29****	399±41****	599±58****	824±110****
	% Reduction from kynurenine challenge							
PF-04859989 + Kynurenine	34±8	34±17	14±20	69±11**	61±15*	73±10****	70±9****	61±13***

Relative change in KYNA levels after kynurenine challenge and following PF-04859989 pretreatment.
All data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. N = 11-12 per group.

Table 1. Relative change in KYNA levels after kynurenine challenge and following PF-04859989 pretreatment. Adult rats were peripherally injected at Zeitgeber time (ZT) 0 with kynurenine (100 mg/kg) to induce *de novo* kynurenic acid (KYNA) formation. PF-04859989 (30 mg/kg), systemically active KAT II inhibitor, was given 30 minutes prior at ZT 23.5. Tissues were harvested at ZT 2. All data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. N = 11-12 per group.

FIGURE TITLES AND LEGENDS

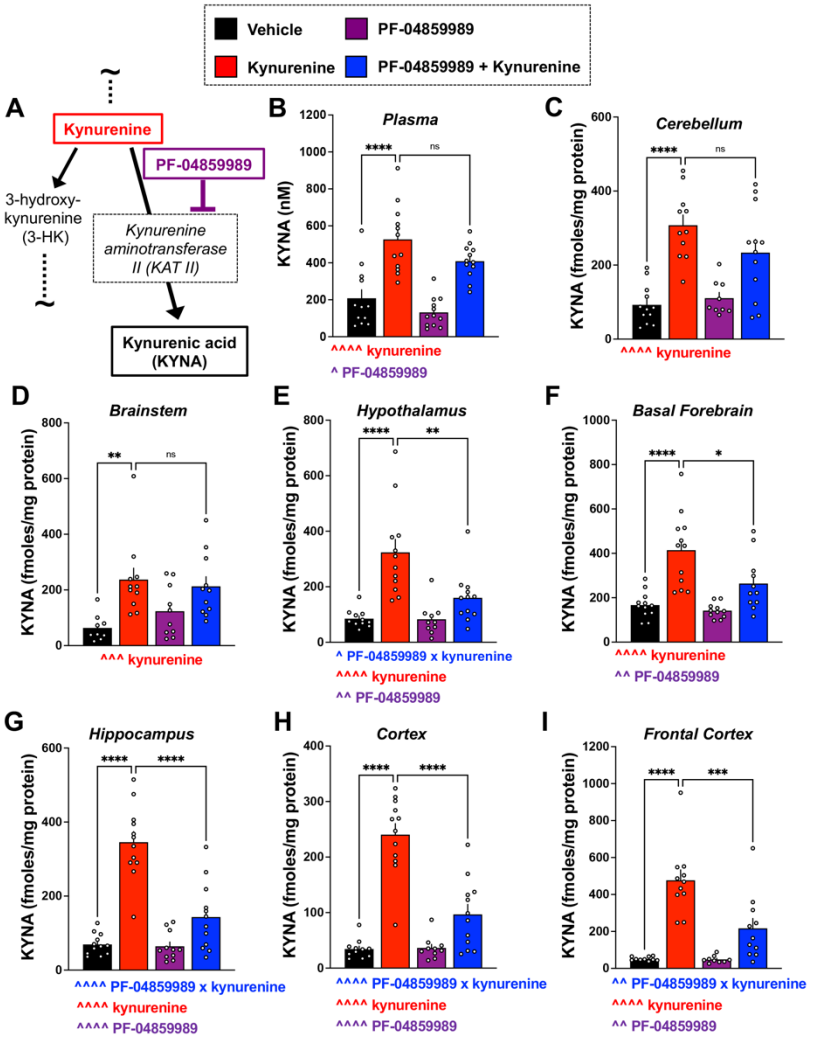

Figure 1. KYNA levels in plasma and across brain regions are increased after the acute kynurenine challenge, and reversed after pretreatment with the KAT II inhibitor, PF-04859989. (A) Schematic representation of truncated kynurenine pathway demonstrating key metabolites and pharmacological tools used in the present study. Adult rats were peripherally injected at Zeitgeber time (ZT) 0 with kynurenine (100 mg/kg) to induce *de novo* kynurenic acid (KYNA) formation. PF-04859989 (30 mg/kg), systemically active KAT II inhibitor, was given 30 minutes prior at ZT 23.5. Tissues were harvested at ZT 2. KYNA levels were evaluated in the **(B)** plasma; **(C)** cerebellum; **(D)** brainstem; **(E)** hypothalamus; **(F)** basal forebrain; **(G)** hippocampus; **(H)** cortex; **(I)** frontal cortex. All data are mean ± SEM. Two-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.

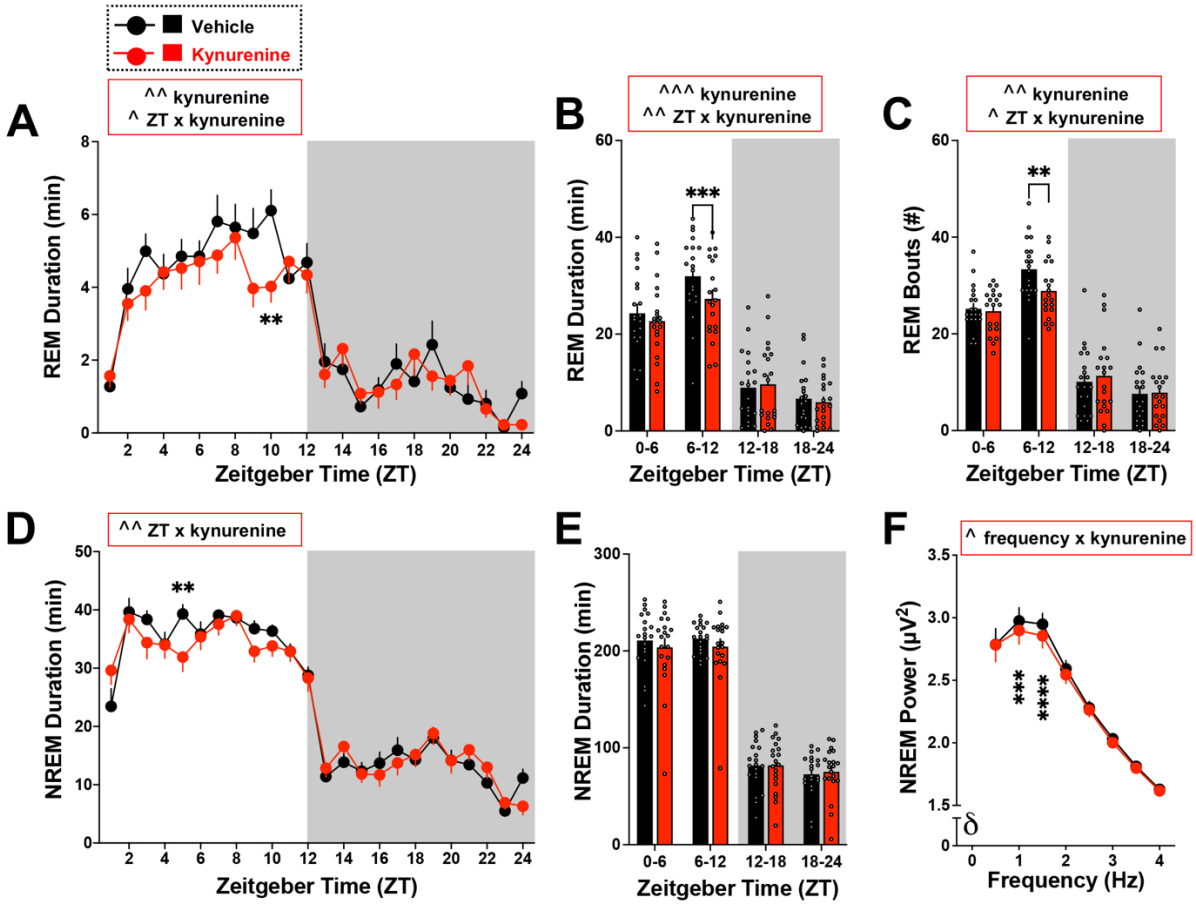

Figure 2. Kynurenine challenge reduces sleep and alters sleep architecture. Adult rats were treated with vehicle or kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. Data are mean ± SEM, analyzed by two-way RM ANOVA, with significance shown in graphs as ^P<0.05, ^^P<0.01, ^^^P<0.001 and Bonferroni's post hoc test **P<0.01, ***P<0.001, ****P<0.0001. **(A)** 1-hr bins of REM duration. ZT x treatment: $F_{23,437}$ = 1.635, P<0.05; treatment: $F_{1,19}$ = 15.14, P<0.01; ZT: $F_{23,437}$ = 30.82, P<0.0001. **(B)** 6-hr bins of REM duration. ZT x treatment: $F_{3,57}$ = 4.162, P<0.01; treatment: $F_{1,19}$ = 15.15, P<0.001; ZT: $F_{3,57}$ = 99.50, P<0.0001. **(C)** 6-hr bins of REM bout number. ZT x treatment: $F_{3,57}$ = 3.74, P<0.05; treatment: $F_{1,19}$ = 8.510, P<0.01; ZT: $F_{3,57}$ = 112.9, P<0.0001. **(D)** 1-hr bins of NREM duration. ZT x treatment: $F_{23,437}$ = 2.032, P<0.01; ZT: $F_{23,437}$ = 69.02, P<0.0001 **(E)** 6-hr bins of NREM duration. ZT: $F_{3,57}$= 247.3, P<0.0001 **(F)** NREM spectral power in the delta frequency range during the light phase. Frequency x treatment: $F_{7,133}$ = 2.165, P<0.05; Frequency: $F_{7,133}$ = 82.34, P<0.0001. N = 20 per group (8 males, 12 females).

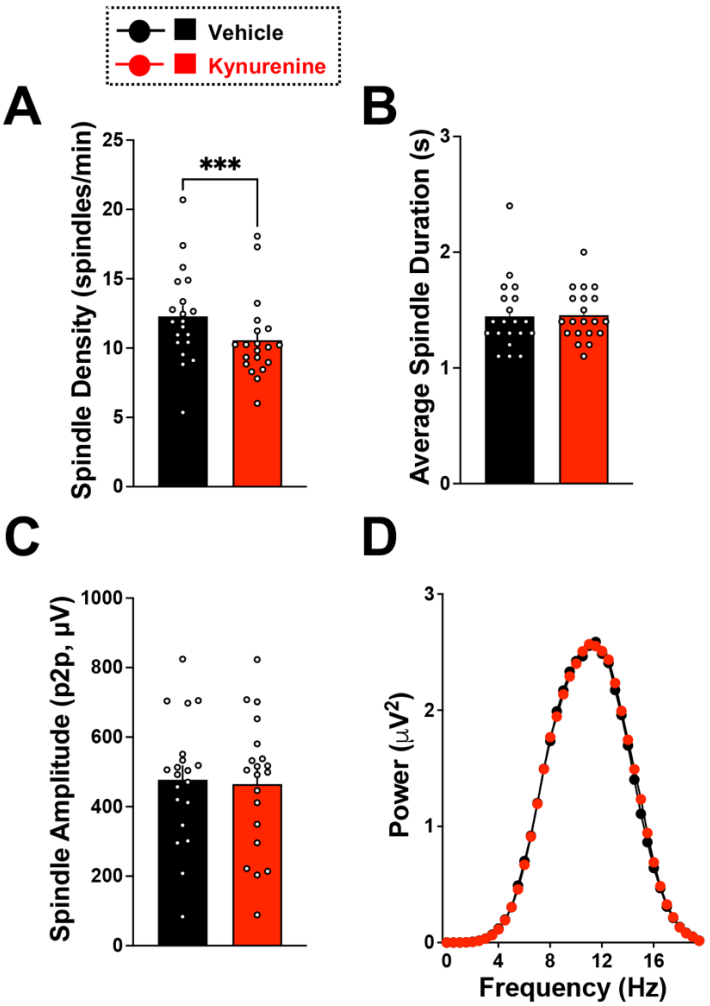

Figure 3. Kynurenine challenge reduces NREM sleep spindle density. Adult rats were treated with vehicle or kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. Sleep spindles were evaluated from ZT 0 – ZT 4. Data are mean ± SEM, analyzed by paired Student's t-test (A-C) or two-way RM ANOVA (D). **(A)** Spindle density. $t_{19} = 4.571$, ***P<0.001. **(B)** Average spindle duration. $t_{19} = 0.1327$, P=0.8958. **(C)** Spindle peak to peak (p2p) amplitude. $t_{19} = 0.9167$, P=0.3708. **(D)** NREM spindle power. Frequency: $F_{39,741} = 185.0$, P<0.0001. N = 20 per group (8 males, 12 females).

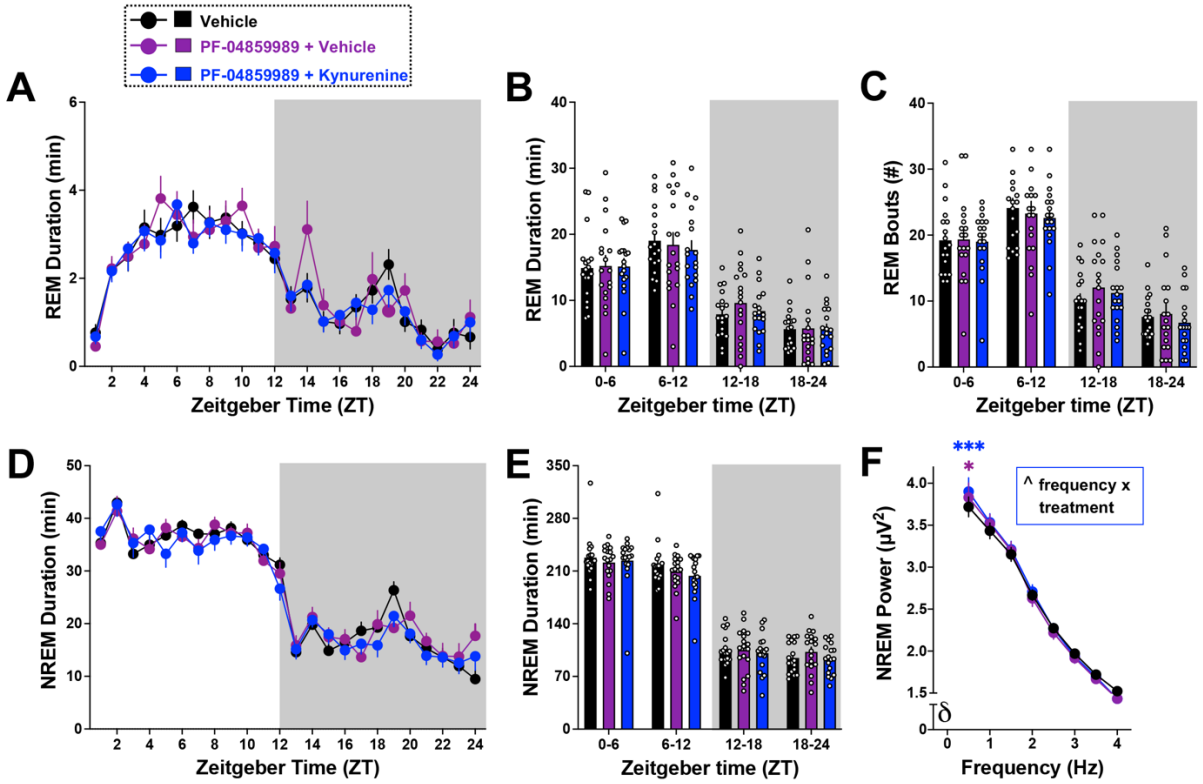

Figure 4. PF-04859989 treatment enhances NREM sleep architecture. Adult rats were treated with vehicle, PF-04859989 (30 mg/kg), or PF-04859989 + kynurenine (100 mg/kg) at the start of the light phase. Data are mean ± SEM, analyzed by two-way RM ANOVA, with significance shown in graphs as ^P<0.05, and Dunnett's post hoc test *P<0.05 (PF-04859989 + vehicle vs. vehicle), ***P<0.001 (PF-04859989 + kynurenine vs. vehicle). **(A)** 1-hr bins of REM duration. ZT: $F_{23,391}$= 23.98, P<0.0001. **(B)** 6-hr bins of REM duration. ZT: $F_{3,51}$ = 62.44, P<0.0001. **(C)** 6-hr bins of REM bout number. ZT: $F_{3,51}$ = 76.02, P<0.0001. **(D)** 1-hr bins of NREM duration. ZT: $F_{23,391}$= 86.51, P<0.0001 **(E)** 6-hr bins of NREM duration. ZT: $F_{3,51}$= 304.4, P<0.0001 **(F)** NREM spectral power in the delta frequency range during the light phase. Frequency x treatment: $F_{14,238}$ = 1.792, P<0.05; Frequency: $F_{7,119}$ = 161.9, P<0.0001. N = 18 per group (9 males, 9 females).

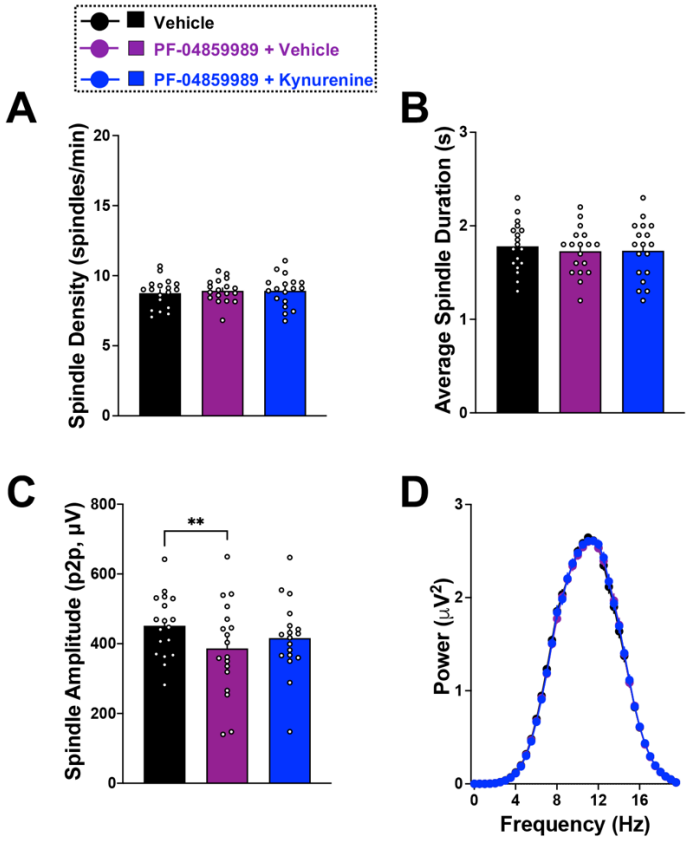

Figure 5. Pretreatment with PF-04859989 prior to kynurenine challenge restores NREM spindle dynamics. Adult rats were treated with vehicle, PF-04859989 (30 mg/kg), or PF-04859989 + kynurenine (100 mg/kg) at the start of the light phase. Sleep spindles were evaluated from ZT 0 – ZT 4. Data are mean ± SEM, analyzed by RM ANOVA. **(A)** Spindle density. $F_{2,34}$ = 0.4918, P=0.6158. **(B)** Average spindle duration. $F_{2,34}$ = 1.653, P=0.2065. **(C)** Spindle peak to peak (p2p) amplitude. $F_{2,34}$ = 5.133, **P<0.01. **(D)** NREM spindle power. Frequency: $F_{39,663}$ = 840.3, P<0.0001. N = 18 per group (9 males, 9 females).

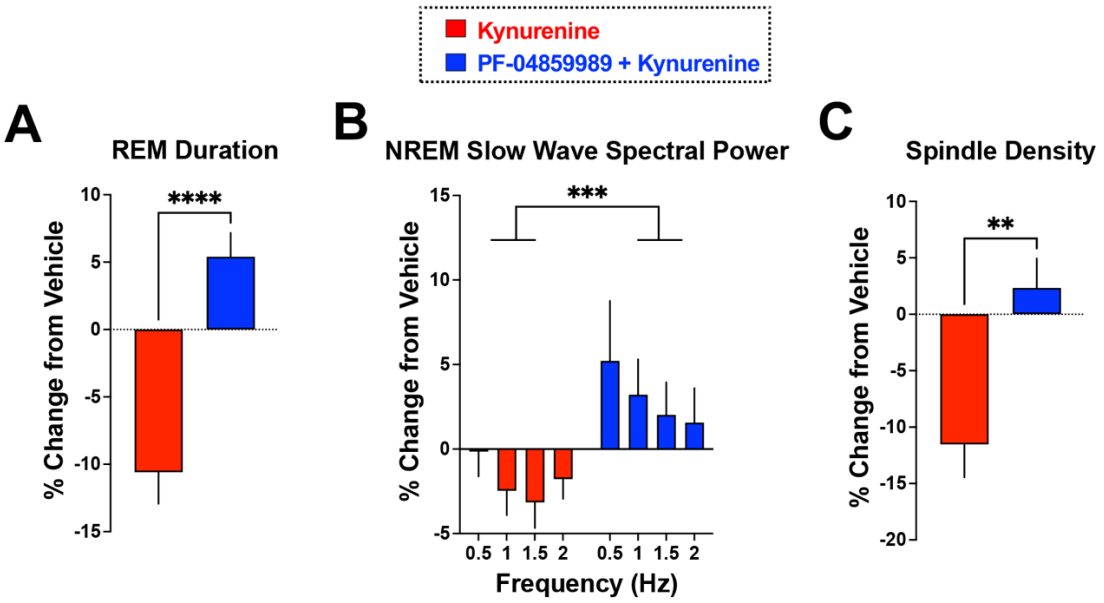

Figure 6. Pretreatment with PF-04859989 attenuates consequences of kynurenine challenge on REM and NREM sleep parameters. In Sleep Study #1, adult rats were treated with vehicle or kynurenine (100 mg/kg) at Zeitgeber time (ZT) 0. In Sleep Study #2, adult rats were treated with vehicle (ZT 23. 5 and ZT 0), PF-04859989 (30 mg/kg; ZT 23.5) + vehicle (ZT 0) or PF-04859989 (30 mg/kg; ZT 23.5) + kynurenine (100 mg/kg; ZT 0). To compare results from the two sleep studies, data were evaluated as percent change from vehicle. **(A)** REM duration (ZT 0-12); Student's T test, $t_{36} = 5.344$, ****P<0.0001 **(B)** NREM slow wave spectral power (ZT 0-12), two-way ANOVA, $F_{1,144} = 12.31$, ***P<0.001 **(C)** spindle density (ZT 0-4); Student's T test, $t_{36} = 3.495$, **P<0.01. All data are mean ± SEM. Kynurenine: N = 20; PF-04859989 + Kynurenine: N = 18.

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