SLEEP, 2022, 1–14

https://doi.org/10.1093/sleep/zsac083
Advance Access Publication Date: 8 April 2022
Original Article

ORIGINAL ARTICLE

Tonic endocannabinoid signaling supports sleep through development in both sexes

Shenée C. Martin[1,†], Sean M. Gay[1,†], Michael L. Armstrong[2], Nila M. Pazhayam[1], Nichole Reisdorph[2] and Graham H. Diering[1,3,*]

[1]Department of Cell Biology and Physiology and the UNC Neuroscience Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA, [2]Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, Aurora, CO, USA and [3]Carolina Institute for Developmental Disabilities, Carrboro, NC, USA

[†]These authors contributed equally to this work.

[*]Corresponding author. Graham H. Diering, 111 Mason Farm Road, 5200 Medical and Biomolecular Research Building, Chapel Hill, NC 27599-7545, USA. Email: graham_diering@med.unc.edu.

Abstract

Sleep is an essential behavior that supports brain function and cognition throughout life, in part by acting on neuronal synapses. The synaptic signaling pathways that mediate the restorative benefits of sleep are not fully understood, particularly in the context of development. Endocannabinoids (eCBs) including 2-arachidonyl glycerol (2-AG) and anandamide (AEA), are bioactive lipids that activate cannabinoid receptor, CB1, to regulate synaptic transmission and mediate cognitive functions and many behaviors, including sleep. We used targeted mass spectrometry to measure changes in forebrain synaptic eCBs during the sleep/wake cycle in juvenile and adolescent mice of both sexes. We find that eCBs lack a daily rhythm in juvenile mice, while in adolescents AEA and related oleoyl ethanolamide are increased during the sleep phase in a circadian manner. Next, we manipulated the eCB system using selective pharmacology and measured the effects on sleep behavior in developing and adult mice of both sexes using a noninvasive piezoelectric home-cage recording apparatus. Enhancement of eCB signaling through inhibition of 2-AG or AEA degradation, increased dark-phase sleep amount and bout length in developing and adult males, but not in females. Inhibition of CB1 by injection of the antagonist AM251 reduced sleep time and caused sleep fragmentation in developing and adult males and females. Our data suggest that males are more sensitive to the sleep-promoting effects of enhanced eCBs but that tonic eCB signaling supports sleep behavior through multiple stages of development in both sexes. This work informs the further development of cannabinoid-based therapeutics for sleep disruption.

Statement of Significance

Sleep is an essential behavior seen through all stages of life. However, the molecular mechanisms that mediate the restorative benefits of sleep are incompletely understood, particularly in the context of development. The endocannabinoid (eCB) system has been shown to promote sleep behavior. There is a considerable interest in the development of therapeutics based on the eCB system. However, the majority of research on this topic using rodent model systems has been conducted using only adult males. Here, we have systematically examined the effects of pharmacological manipulations of the eCB system using male and female mice, at developmental time points and in adults. We show that eCB signaling is required to maintain sleep in developing and adult mice of both sexes.

Key words: endocannabinoid signaling; cannabinoid receptor; sleep regulation; brain development; sexual dimorphism

Submitted: 13 May, 2021; **Revised:** 7 March, 2022

Introduction

Sleep is an essential conserved behavior seen throughout life. Chronic sleep disruption is associated with many diseases ranging from neurodevelopmental disorders in earlier life to neurodegenerative diseases later in life. Indeed, sleep disruption is a major comorbidity in autism spectrum disorder, reported in up to 80% of affected individuals [1, 2]. Sleep disruption is also believed to increase the risk of developing Alzheimer's disease (AD), and likely plays a role in the progression of AD pathology and cognitive decline [3]. Therefore, sleep is an important therapeutic target through all stages of life. Multiple recent studies using transcriptomics, proteomics, phosphoproteomics, or electron microscopy, together with a large body of previous literature show that synapses of the mouse forebrain undergo widespread and profound remodeling during sleep, or in response to sleep deprivation [4–8], strongly supporting synapses as a major locus for sleep's restorative effects [9–11]. Sleep and synapses are highly dynamic during development [12–14]. In humans and other mammals, sleep amount peaks at birth and declines sharply through childhood and adolescence. Sleep composition is also dynamic: rapid-eye-movement (REM) sleep dominates early life while non-REM (NREM), also called slow-wave sleep, becomes more prominent during adolescence [12, 14–18]. These findings suggest that the regulation and function of sleep may be very different at different stages of development. Therefore, treatment of sleep disruption throughout the lifespan will require further molecular insights into the synaptic signaling process activated during sleep at each life stage.

The endocannabinoid (eCB) system forms a prominent signaling pathway that regulates synaptic transmission throughout much of the brain [19] and is known to play a role in the regulation of sleep, cognition, and other behaviors [20, 21]. eCBs are bioactive lipids synthesized in postsynaptic compartments that signal in a retrograde manner by acting on presynaptic CB1 receptors to regulate neurotransmitter release [22]. The two most well-studied eCBs, and known endogenous agonists for presynaptic CB1 receptors are 2-arachidonyl glycerol (2-AG) and anandamide (also called arachidonoyl ethanolamide, AEA) [22–25]. Importantly, these two eCB metabolites have completely nonoverlapping synthetic and degradative pathways, strongly suggesting that they are independently regulated and serve distinct functions. 2-AG is synthesized by the rate limiting enzyme diacylglycerol lipase alpha (DAGLα) and degraded by monoacylglycerol lipase (MAGL). AEA is part of a larger family of *N*-acylethanolamides (NAEs), that are synthesized by *N*-acyl phosphatidylethanolamine-specific phospholipase D, or through multiple secondary pathways [22]. NAEs are degraded by a single lipase, fatty acid amide hydrolase (FAAH) [22]. The pharmaceutical industry and basic research have devoted considerable effort to the development of drugs that can act as selective agonists or antagonists for CB1 receptors as well as selectively upregulating 2-AG or NAEs through inhibition of MAGL or FAAH, respectively [22, 26, 27].

Previous studies have implicated eCBs in both NREM and REM sleep [20]. NREM sleep involves highly coordinated neuronal activity throughout much of the brain, particularly the cortex. Cortical slow waves (0.5–4 Hz) form the most prominent features in electroencephalogram (EEG) recordings during NREM sleep. Expression of eCB system components is prominent in the forebrain, and eCB signaling has been shown to support the cortical up-state [28], a prominent mode of microcircuit activity within the cortex that underlies the generation of cortical slow waves [29]. Multiple studies have used pharmacology or genetics to target the eCB system in rodents and have shown that enhancement of eCB signaling promotes NREM stability, whereas inhibition of eCB signaling causes NREM fragmentation [20, 28, 30–34]. Thus, eCB signaling is well positioned to support the highly coordinated neuronal activity that likely promotes the restorative actions of NREM sleep. Direct infusion of eCBs into the cerebral ventricles, hippocampus, or lateral hypothalamus have been shown to promote REM sleep [35–38]. Thus, it is likely that eCB signaling affects sleep behavior and physiology through cortical and subcortical brain regions, including the suprachiasmatic nucleus [20, 39, 40]. However, it is not known if eCB signaling promotes sleep in the context of development in both sexes.

In the current study, we combine quantitative mass spectrometry (MS) and behavioral pharmacology in mice to examine the role of eCB signaling in promoting sleep during development and in adulthood in both sexes. Our data show that the expression of synaptic eCBs undergo a clear maturation where AEA and related NAEs are decreased during sleep deprivation in juveniles, transitioning to expression of AEA and related metabolites by circadian mechanisms in adolescents. We confirm previous findings that enhancement of eCB signaling promotes sleep stability and suppression of eCB signaling drives sleep fragmentation in adult males [30, 32], and we extend these findings to include developmental ages in both sexes. eCB signaling is important to sustain sleep in both males and females. However, we find that developing and adult males are far more sensitive to the sleep-promoting effects of eCB boosting drugs than females. Thus, our findings further support a role for the eCB system in promoting sleep and uncover differences in this system during development vs. adulthood and between sexes.

Methods

Mice

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina (UNC) at Chapel Hill and were performed in accordance with the guidelines of the U.S. National Institutes of Health. All experiments were performed using C57Bl/6J mice of both sexes purchased from Jackson Labs, or bred in house. Experiments were performed using juvenile (postnatal day P21–P35), adolescent (P42–P56), or adult (>P90) mice. For experiments using adult mice, experimental animals were purchased from Jackson Labs and were allowed to acclimate to the housing at UNC for at least 2 weeks prior to the start of the experiment. For experiments using juvenile or adolescent mice, experimental animals were bred in house to provide the best control over the target ages, using adult breeders purchased from Jackson Labs. Breeders for our colony were replaced every 3 months with mice supplied from Jackson Labs.

Drugs and treatments

PF3845, JZL195, MJN110, and AM251 were purchased from Cayman Chemicals. PF3845 inhibits the enzyme FAAH and is expected to drive a selective increase in the levels of AEA and related NAEs [26]. MJN110 inhibits the enzyme MAGL and is expected to drive a selective increase in 2-AG [27] (see Figure 2, A).

AM251 is an inverse agonist of CB1. JZL195 is a dual inhibitor of both MAGL and FAAH [41] and is used to preserve the integrity of eCB metabolites during sample preparation (see below). Drugs were dissolved in DMSO and then prepared into vehicle solution (5% dimethyl sulfoxide [DMSO], 5% Kolliphor, 90% of a 1% NaCl solution) at the doses indicated. For metabolomics experiments, mice were treated with vehicle or the drugs indicated by intraperitoneal injection (IP) at Zeitgeber time 0 (ZT0) and then sacrificed 4 h later at ZT4, followed by isolation of the forebrain and synaptosome preparation. For sleep behavior experiments, mice were injected immediately after the onset of the light phase (ZT0) or immediately preceding the onset of the dark phase (ZT12). Each mouse received an injection of vehicle or drug followed by a second injection 72 h later of vehicle or drug with a cross over design. Sleep behavior was examined for 24 h following each injection. The effects of the drug were determined by comparing 24 h following drug injection to the 24 h following vehicle injection for each mouse.

Synaptosome preparation

Male and female juvenile mice postnatal day 21 (P21), or adolescents P56, were sacrificed during the sleep phase (ZT4), wake phase (ZT16), or following 4 h of sleep deprivation (SD4) from ZT0 to ZT4. Sleep deprivation was achieved using gentle handling, a low stress method that involves tapping on the mouse cage and disturbing the bedding material [42]. Mouse forebrains, consisting of whole cortex and hippocampus, were dissected in ice-cold phosphate buffered saline, and then frozen on dry ice, and kept at –80°C until further processing. Frozen mouse forebrains were homogenized using 12 strokes from a glass homogenizer in ice-cold homogenization solution (320 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM 2,2',2",2"'-(Ethane-1,2-diyldinitrilo) tetraacetic acid [EDTA], 5 mM Na pyrophosphate, 1 mM Na_3VO_4, 200 nM okadaic acid, 50 nM JZL195, protease inhibitor cocktail [Roche]). Brain homogenate was then centrifuged at 1000 × *g* for 10 min at 4°C to obtain the P1 (nuclear) and S1 (postnuclear) fractions. The S1 fraction was then layered on top of a discontinuous sucrose density gradient (0.8, 1.0, or 1.2 M sucrose in 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid [HEPES], pH 7.4, 1 mM EDTA, 5 mM Na pyrophosphate, 1 mM Na_3VO_4, 200 nM okadaic acid, 50 nM JZL195, protease inhibitor cocktail [Roche]) and then subjected to ultra-centrifugation at 82 500 × *g* for 2 h at 4°C. Material accumulated at the interface of 1.0 and 1.2 M sucrose (synaptosomes) was collected. Synaptosomes were diluted using 10 mM HEPES, pH 7.4 (containing protease, phosphatase, and lipase inhibitors) to restore the sucrose concentration back to 320 mM. The diluted synaptosomes were then pelleted by centrifugation at 100 000 × *g* for 30 min at 4°C. The synaptosome pellet was then resuspended in homogenization buffer (containing inhibitors). The protein concentration was determined using Bradford assay and material was frozen at –80°C prior to targeted MS. Note the addition of dual MAGL/FAAH lipase inhibitor JZL195 50 nM was added to all steps of sample preparation to maintain the integrity of eCB metabolites during sample preparation.

Targeted MS for eCB quantification

Frozen forebrain synaptosome fractions were prepared for eCB analysis as follows. Briefly, synaptosomes in homogenization buffer were removed from –80°C freezer and thawed on ice. The samples were then immediately placed into a microcentrifuge at 14 000 RPM for 10 min at 4°C. The supernatant was removed and then 440 µl of methanol, 50 µl of internal standard containing 200 ng/mL each of arachidonyl ethanolamide-d4 and oleoyl ethanolamide-d4 and 2000 ng/mL of 2-arachidonlyl glycerol-d5, and 10 µl of 5 mg/mL butylated hydroxytoluene (BHT) in ethanol was added. The synaptosome pellet was resuspended and then vortexed for 5 s. The sample was then centrifuged at 14 000 RPM for 10 min at 4°C. The supernatant was removed and then placed into a capped autosampler vial for analysis. LC/MS/MS analysis of eCBs was performed as previously described [43], with some modifications. Briefly, mass spectrometric analysis was performed on an Agilent 6490 triple quadrupole mass spectrometer in positive ionization mode. Calibration standards were analyzed over a range of concentrations from 0.2 to 40 pg on column for all of the ethanolamides. The following lipids were quantified: 2-AG, AEA, docosahexaenoyl ethanolamide (DHEa), docosatetraenoyl ethanolamide (DEA), linoleoyl ethanolamide (LEA), oleoyl ethanolamide (OEA), palmitoleoyl ethanolamide (POEA), palmitoyl ethanolamide (PEA), and stearoyl ethanolamide (SEA). Quantitation of eCBs was performed using Agilent Masshunter Quantitative Analysis software. All results were normalized to protein concentration.

For presentation and analysis of our synaptic eCB data, each sample in the juvenile and adolescent ZT16, ZT4, and SD4 groups were normalized to the mean concentration of each metabolite from the ZT16 condition within each cohort. Note: the juvenile dataset includes two independent cohorts (set1 and set2) that were analyzed several months apart, each cohort included male and female mice from all three conditions. The two juvenile sample sets were first normalized to their respective mean ZT16 condition prior to combining the results of set1 and set2. The absolute quantification of eCB metabolites from juvenile set1 and set2, and adolescent mice is presented in Supplementary Table 1. Although absolute quantification is comparable between set1 and set2, variations in sample preparation and preparation of internal standards used for targeted quantification between set1 and set2 result in small but systematic differences in absolute quantification between sets, meaning that only the ZT16-normalized results can be meaningfully combined. In order to compare results obtained between sexes, male and female samples in the juvenile and adolescent ZT16, ZT4, and SD4 groups were normalized to the mean concentration of each metabolite from the male ZT16 condition within each cohort, data separated by sex are presented in Supplementary Figures 1 and 2.

Sleep phenotyping and behavior analysis

C57/BL6J mice were moved to our wake/sleep behavior satellite facility on a 12 h:12 h light:dark cycle (lights on 7 am to 7 pm). Mice were individually housed in 15.5 cm^2 cages with bedding, food, and water. Mice were given at least two full dark cycles to acclimate to the recording cages before the beginning of data collection. No other animals were housed in the room during these experiments. Sleep and wake behavior were recorded using a noninvasive home-cage monitoring system, PiezoSleep 2.0 (Signal Solutions, Lexington, KY). The system uses a piezoelectric mat underneath the cage to detect vibrational movement of the mouse including breathing. Customized software (SleepStats, Signal Solutions, Lexington, KY) uses an algorithm

to process the signal and discern sleeping respiratory patterns from waking respiratory patterns. Sleep was characterized primarily by periodic (2–3 Hz) and regular amplitude signals, which is typical of respiration from a sleeping mouse. In contrast, signals characteristic of wake were both the absence of characteristic sleep signals and higher amplitude, and irregular signals were associated with volitional movements, even subtle head movements during quiet wake. The piezoelectric signals in 2-s epochs were classified by a linear discriminant classifier algorithm based on multiple signal variables to assign a binary label of "sleep" or "wake." Data collected from the cage system were binned over specified time periods: 1 h bins to generate a daily sleep trace, 12 h bins for average light- or dark-phase percent sleep or sleep bout lengths. To eliminate the impact of short and ambiguous arousals on the sleep bout length statistics, a sleep bout length count is initiated when a 30-s interval contains greater than 50% sleep and terminates when a 30-s interval has less than 50% sleep. This algorithm has been validated in adult mice by using electroencephalography, electromyography, and visual evaluation [44–48]. It is assumed here that the respiration patterns used to distinguish sleep from wake are similar in adult and developing mice.

Statistical analysis

Microsoft Excel or Prism 8.0 (GraphPad Software Inc.) was used to analyze data from sleep behavior experiments outlined above. Paired two-tailed Student's *t*-test analyses were used to compare 24 h sleep behavior, separated into 12 h light and dark periods, between vehicle injections and drug injections in the same animals. Comparisons of eCB metabolites between wake, sleep, and sleep deprivation conditions, or comparing vehicle treated groups to drug treatment was conducted using one-way ANOVA with post hoc unpaired two-tailed Student's *t*-test with Bonferroni correction for multiple comparisons. All data are presented as mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Results

Sleep and circadian regulation of synaptic eCBs

Daily fluctuations in eCBs have been reported in blood and cerebral spinal fluid, from animal models and humans [49–51]. However, it is not clear how these daily rhythms reflect the activity of eCBs at synapses, where eCBs are generated in postsynaptic neurons in response to neuronal activity [19]. Moreover, it is not clear how daily expression of eCBs may differ during development compared to adulthood. Therefore, we developed a targeted MS assay to quantify 2-AG, AEA, and related NAEs, from mouse forebrain synaptosome fractions. In preliminary assessment of this assay, we quantified eCBs from whole forebrain homogenate, the S2 fraction (cytosol and extracellular fluid), and synaptosomes. We found that proportionally greater levels of eCBs were detected in synaptosomes compared to whole forebrain homogenate, and eCBs were essentially absent from the soluble S2 fraction (not shown), suggesting that brain eCBs are primarily localized to synaptic fractions and are retained during sample preparation. All steps of synaptosome isolation included the dual MAGL/FAAH inhibitor JZL195 [41] to preserve the integrity of eCBs during sample preparation.

We then went on to examine the sleep-dependent regulation of synaptic eCBs in juvenile and adolescent mice. These experiments include both sexes, but were not specifically designed or powered to examine sex differences in eCB expression. Juvenile (P21) or adolescent (P56) mice of both sexes were sacrificed 4 h into the light (sleep) or dark (wake) phase, ZT4 or ZT16, respectively, or following 4 h of near total sleep deprivation (SD4) using gentle handling [42] starting from light onset (ZT0–ZT4) (Figure 1, A). Wakefulness during SD is assessed by continual observation. Juvenile mice (preweening) were exposed to SD in the presence of their dam, in order to mitigate the stress inherently associated with SD [42]. Forebrains (hippocampus and cortex) were dissected followed by isolation of synaptosomes and targeted MS quantification. Figure 1 presents the summarized results obtained from both sexes and is normalized to the wake (ZT16) condition. eCB quantification separated by sex for juveniles and adolescents is presented in Supplementary Figures 1 and 2, respectively, and is shown normalized to the male wake (ZT16) condition. Absolute quantification is shown in Supplementary Table 1.

In juveniles we found that there was no difference in any of the eCBs compounds between wake and sleep phases, although AEA showed a trend to increase during the sleep phase compared to the wake phase. Compared to the sleep phase, SD4 caused a significant decrease in the levels of AEA and most of the NAE species measured (Figure 1, B). These findings show that in juveniles, synaptic NAEs lack a daily rhythm but do show a clear response to sleep deprivation. It is possible that the SD induced decrease in NAEs may result from the stress associated with SD [42]. In clear contrast, in adolescents we found that AEA and OEA were significantly increased during the sleep phase or SD4 (both collected at ZT4) compared to the wake phase (Figure 1, C). All of the NAE species are FAAH substrates and showed a clear trend toward higher expression during the sleep phase, suggesting that NAE species may have a coordinated expression mechanism. Consistent with this observation we recently showed using proteomics that in mice aged P56–P70, synapse associated FAAH protein in the forebrain is 42% higher during the wake phase compared to the sleep phase [4], suggesting that daily fluctuations in NAEs in P56 mice may be regulated by synaptic levels of FAAH protein. 2-AG showed no changes between the three conditions at either age, suggesting that the expression of synaptic 2-AG in the forebrain may be independent of the sleep/wake cycle. Together these data show that as the mice mature toward adulthood, components of the eCB system transition to regulation by the circadian rhythm, where synaptic AEA/OEA are increased during the sleep phase.

Although a systematic comparison of synaptic eCBs between sexes was not the goal of this experiment, it is of interest to note whether compelling trends toward sex differences exist in these datasets. In the juvenile dataset we did not observe any significant differences or compelling trends between males and females for any of the eCB metabolites analyzed (Supplementary Figure 1). Similarly, in the adolescent dataset we did not observe any significant differences or compelling trends between sexes for the major CB1 agonists, 2-AG or AEA (Supplementary Figure 2). Interestingly, when separating the quantification of the remaining NAE species between sexes, the increased expression of NAEs during the sleep phase (ZT4) seems to be driven primarily by the males, and for two metabolites, OEA and POEA, we did observe a significant increase in males compared to females at ZT4 (Supplementary Figure 2). While these comparisons are

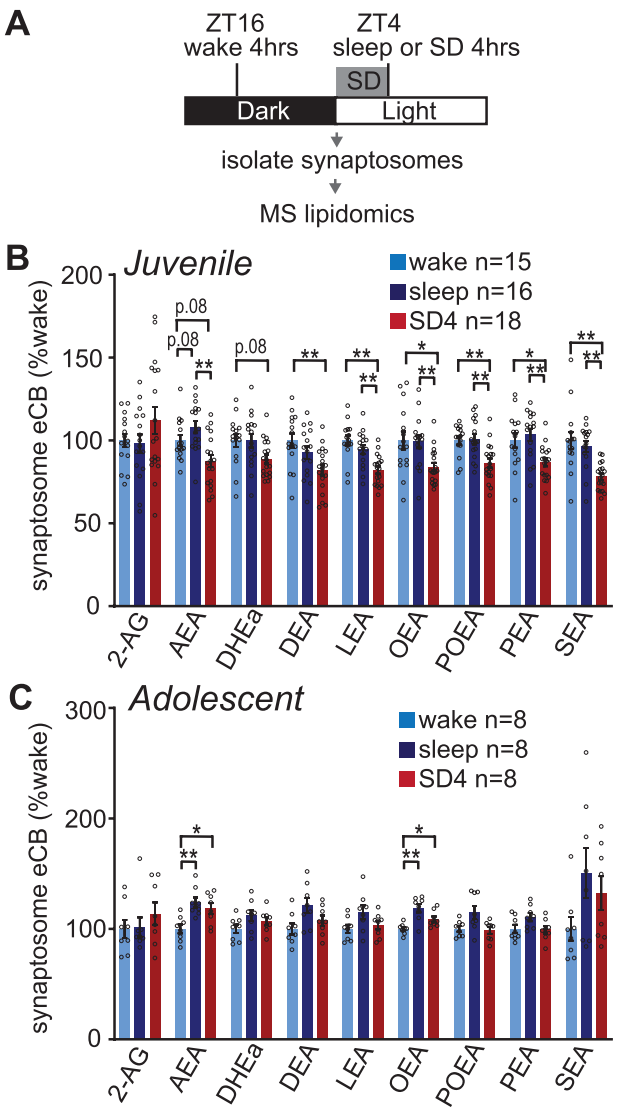

Figure 1. Regulation of synaptic eCBs during the sleep–wake cycle in juvenile (P21) and adolescent mice (P56). (A) Experimental design: mice were maintained on 12:12 h light/dark schedule. Mouse tissue was collected 4 h into the wake period (ZT16) or sleep period (ZT4), or after 4 h of gentle handling sleep deprivation (SD4) during the light period (ZT0–ZT4). The forebrain was dissected, the synaptosome fraction isolated, and quantified with MS. (B) Quantification of synaptic eCBs in P21 juveniles. Data are normalized to the wake condition. $n = 15–18$ per condition, includes males and females. (C) Quantification of synaptic eCBs in P56 adolescents. Data are normalized to the wake condition. $n = 8$ per condition, includes males and females. Lipids quantified: 2-AG, AEA, DHEa, DEA, LEA, OEA, POEA, PEA, and SEA. $^{*}p < 0.05$, $^{**}p < 0.01$ (one-way ANOVA with post hoc unpaired two-tailed Student's *t*-test with Bonferroni correction). Error bars indicate ± SEM. See also Supplementary Table 1, and Supplementary Figures 1 and 2.

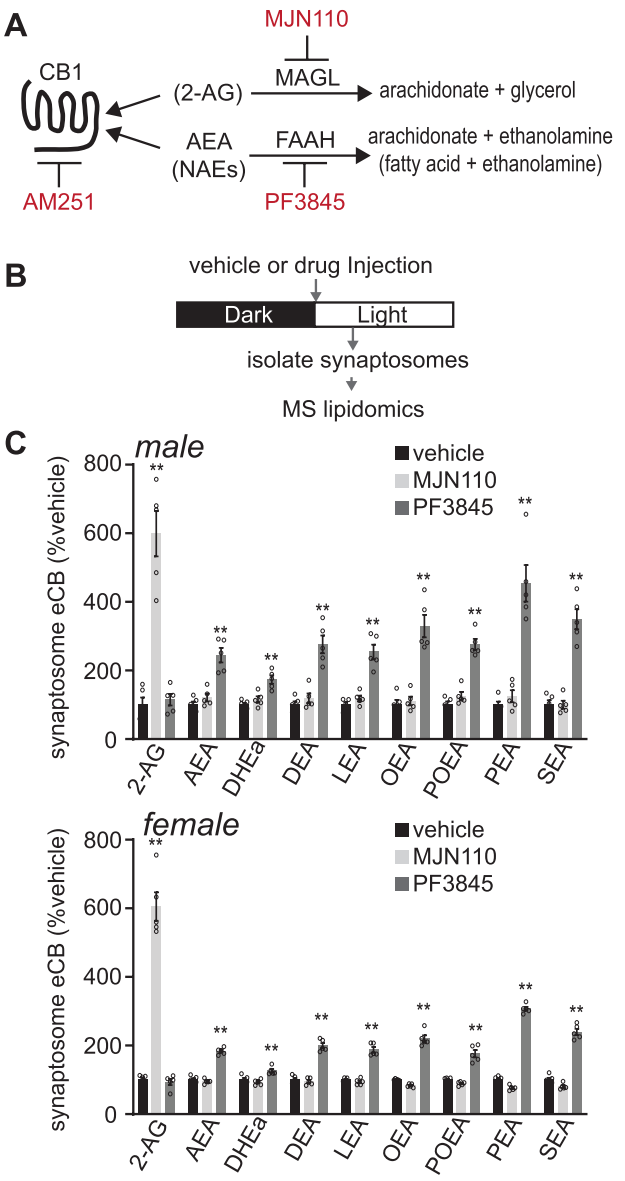

Figure 2. Pharmacological manipulations of synaptic eCB metabolites. (A) Schematic demonstrates the expected effects of the drugs used in this study. (B) Experimental design: mice were treated with vehicle or drugs by IP at ZT0. Mice were sacrificed at ZT4, forebrain dissected, synaptosomes isolated and quantified with targeted MS analysis. (C) Quantification of synaptic eCBs in vehicle, MJN110 (5 mg/kg), or PF3845 (10 mg/kg) treated adult male and female mice. Data are normalized to the vehicle treatment condition. $n = 5$ per sex/condition. $^{**}p < 0.01$ indicates significant difference between drug treatment and vehicle treatment control (one-way ANOVA with post hoc unpaired two-tailed Student's *t*-test with Bonferroni correction). Error bars indicate ± SEM. See also Supplementary Table 2.

suggestive that sex differences may exist in the sleep-dependent regulation of synaptic NAE species other than AEA, additional studies specifically designed for sex comparisons will be needed to support this conclusion.

Pharmacological inhibition of eCB degradation

Previous studies have shown that eCB boosting drugs promote sleep behavior in adult male mice [20,30]. Based on the distinct daily patterns of synaptic eCBs and different effects of SD in juvenile and adolescent mice (Figure 1), we hypothesized that pharmacological manipulation of eCBs using inhibitors of MAGL or FAAH would have differential sleep-promoting effects in developing mice vs. adults. Selective inhibitors MJN110 (5 mg/kg) or PF3845 (10 mg/kg) were used to block MAGL or FAAH, respectively [26,27] (Figure 2, A). To confirm the action of these compounds, and to show that these treatments are able to manipulate synaptic eCBs in both sexes, we treated adult (P90) male and female mice at ZT0 by IP injection, followed by sacrifice at ZT4 and targeted quantification of eCBs in forebrain synaptosomes (Figure 2, B).

As expected, compared to vehicle treated controls, MAGL inhibitor MJN110 caused a sixfold increase in 2-AG, and no change in AEA and the related NAEs (Figure 2, C). FAAH inhibitor PF3845 caused no change in 2-AG and a two- to fourfold increase in AEA and NAE species (Figure 2, C). Near identical effects were seen in males and females (Figure 2, C). Absolute quantification of eCB metabolites in vehicle and drug treated males and females are shown in Supplementary Table 2. These data show that MJN110 and PF3845 achieve similar levels of target engagement in both sexes.

Sleep-promoting effects of 2-AG or NAEs in developing and adult males, but not females

We next went on to examine how pharmacological manipulations of eCB signaling affect sleep behavior in the context of development and between sexes. We measured sleep behavior in juvenile (P21), adolescent (P42), or adult (P90) male and female mice using a noninvasive piezoelectric home-cage monitoring system, PiezoSleep, that uses highly sensitive piezoelectric polymers to measure mouse motion and breathing. This mechanical signal is then analyzed using custom software to score wake and sleep behavior. The PiezoSleep system has been previously validated using simultaneous EEG and video recordings [44–48], that showed an overall excellent match between EEG/electromyography and piezo scoring, but also found that PiezoSleep reports a small but significant increase in light phase sleep compared to EEG [44]. Note the ages indicated represent the age at the beginning of the experiment. Mice were given 2–3 days to acclimate to the recording cages prior to data collection. In the following experiments, male and female mice of indicated ages were treated by 2 consecutive IP injections of either vehicle or drug, separated by 3 days, using a random crossover design. Injections occurred either at ZT0 (lights on) or immediately prior to ZT12 (lights off) and sleep behavior was examined for 24 h following injection (separated into 12 h blocks of light and dark). The effects of the drug were compared directly to the effects of vehicle control for each mouse. Quantification of total sleep time and bout lengths following vehicle or drug treatments are summarized in Supplementary Table 3.

Multiple previous studies have shown that increased eCB signaling by direct agonist treatment, or inhibition of MAGL or FAAH, promotes sleep in adult male mice or male rats [30, 35]. Whether similar effects are also seen in developing mice or females has not been tested. Quantification of synaptic eCBs (Figure 1) showed that juvenile mice lacked a daily rhythm of synaptic eCBs whereas adolescents showed an increase in AEA/OEA during the sleep phase. These findings led us to hypothesize that AEA/OEA may promote sleep only after mice had matured through the juvenile period. Accordingly, our expectation was that pharmacologically increasing the levels of eCBs in males or females would have a more profound effect on sleep behavior in adults than in juveniles. We began by treating mice with MJN110 or PF3845 immediately prior to dark onset (ZT12), the beginning of the active phase, where sleep-promoting drugs are expected to show a more profound effect. In males, MJN110 treatment compared to vehicle control caused a significant increase in dark-phase total sleep time and sleep bout length (Figure 3, A–C). Contrary to our expectations, qualitatively similar results were observed in male juveniles, adolescents, and adults. PF3845 treatment also significantly increased dark-phase total sleep time and bout length in male juveniles, adolescents, and adults (Figure 3, D–F). The effects of either MJN110 or PF3845 were largely seen within the first 12 h of treatment and had minimal effects on subsequent light-phase sleep, with the exception of modest reduction in total sleep in some ages (Figure 3, B and E). In contrast, light-phase injection of MJN110 or PF3845 in males had no significant effect on sleep amount or bout length at any age (Supplementary Figure 3). These findings show that in males, increased activity of 2-AG or NAEs in the dark phase can promote sleep and increase sleep bout length independent of developmental age. These data also confirm the dark-phase sleep-promoting effects of similar compounds tested in a previous study examining adult males [30].

Identical dark-phase MJN110 or PF3845 treatments caused minimal effects in female mice, in striking contrast to males (Figure 4). MJN110 treatment did not have any effect on dark-phase total sleep in females at any age, but caused a significant increase in dark-phase sleep bout length only in juvenile females. MJN110 also caused a modest decrease in subsequent light-phase total sleep time in female adolescents (Figure 4, A–C). PF3845 showed no sleep-promoting effects during the dark phase in females at any age and caused a decrease in subsequent light-phase sleep bout length in female juveniles (Figure 4, D–F). Light-phase injection of MJN110 caused a modest increase in light-phase total sleep in female juveniles, but at the expense of reduced sleep bout length (Supplementary Figure 4). Light-phase injection of PF3845 in females had no measurable effect on sleep at any age (Supplementary Figure 4).

We had expected to see differential sleep-promoting effects of MJN110 or PF3845 with respect to developmental age, but not sex. Instead, we found that MJN110 or PF3845 treatments clearly promote sleep in males, independent of age, while females show minimal to no effects. Consistent with a previous report, the sleep-promoting effects of MAGL and FAAH inhibitor drugs in males are only observed in the dark phase [30], perhaps due to a ceiling effect where light-phase sleep is already at high levels that cannot be increased further by increased cannabinergic signaling.

CB1 signaling sustains sleep in developing and adult males and females

Pharmacological enhancement of 2-AG or NAEs, showed clear sleep promotion in developing and adult males, but not females. We hypothesize that an eCB tone supports sleep in both sexes, but this tone may be saturating in females, limiting the effects of MJN110 or PF3845, whereas in males the eCB tone may be unsaturated in the dark phase, allowing for the sleep-promoting effects of MJN110 and PF3845. To test whether an eCB tone supports sleep in males and females, we examined the effects of treatment by a CB1 receptor inverse agonist, AM251 (10 mg/kg) on sleep behavior. Previous studies (using males only) have shown mixed results of this, or similar treatments, in mice and rats. In some studies, CB1 inhibitors have no or modest effect [52–54], while in others these treatments suppress sleep behavior and cause sleep fragmentation [30, 32]. We suspected that these discrepancies may result from the different doses used. Therefore, we used AM251 at 10 mg/kg, the higher end of the dose ranges reported in the literature. We first examined the effect of AM251 in the sleep (light) phase, when sleep disrupting compounds may be expected to show a more profound effect. Compared

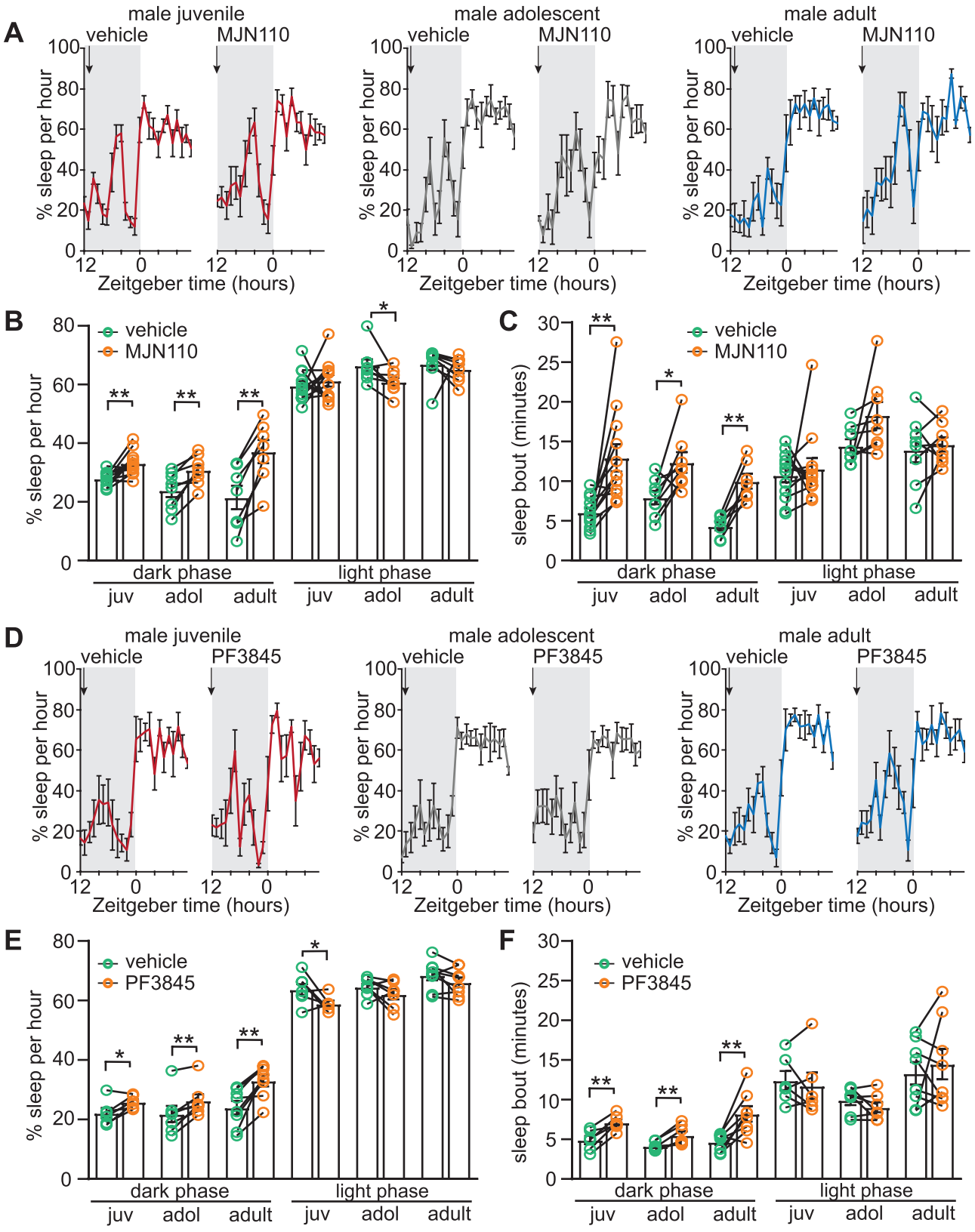

Figure 3. Increased 2-AG or AEA promotes dark-phase sleep behavior in developing and adult males. (A) Twenty-four hours trace of average hourly sleep of male juveniles (juv, left), adolescents (adol, middle), and adults (right) treated with vehicle or MJN110 (5 mg/kg) at the onset of the dark phase (ZT12), injection indicated by arrow. Gray bars in sleep traces indicate dark phase. Quantification of average hourly sleep (B) and sleep bout length in minutes (C) for 24 h following vehicle or MJN110 injection. Data separated into 12 h of dark and light phases. $N = 12$ juveniles, 8 adolescents, 7 adults. (D) Twenty-four hours trace of average hourly sleep of male juveniles (left), adolescents (middle), and adults (right) treated with PF3845 (10 mg/kg) at the onset of the dark phase (ZT12), injection indicated by arrow. Quantification of average hourly sleep (E) and sleep bout length in minutes (F) for 24 h following vehicle or PF3845 injection. Data separated into 12 h of dark and light phases. $N = 6$ juveniles, 7 adolescents, 8 adults. $^{*}p < 0.05$, $^{**}p < 0.001$ (paired two-tailed Student's *t*-test). Error bars indicate ± SEM. See also Supplementary Figure 3.

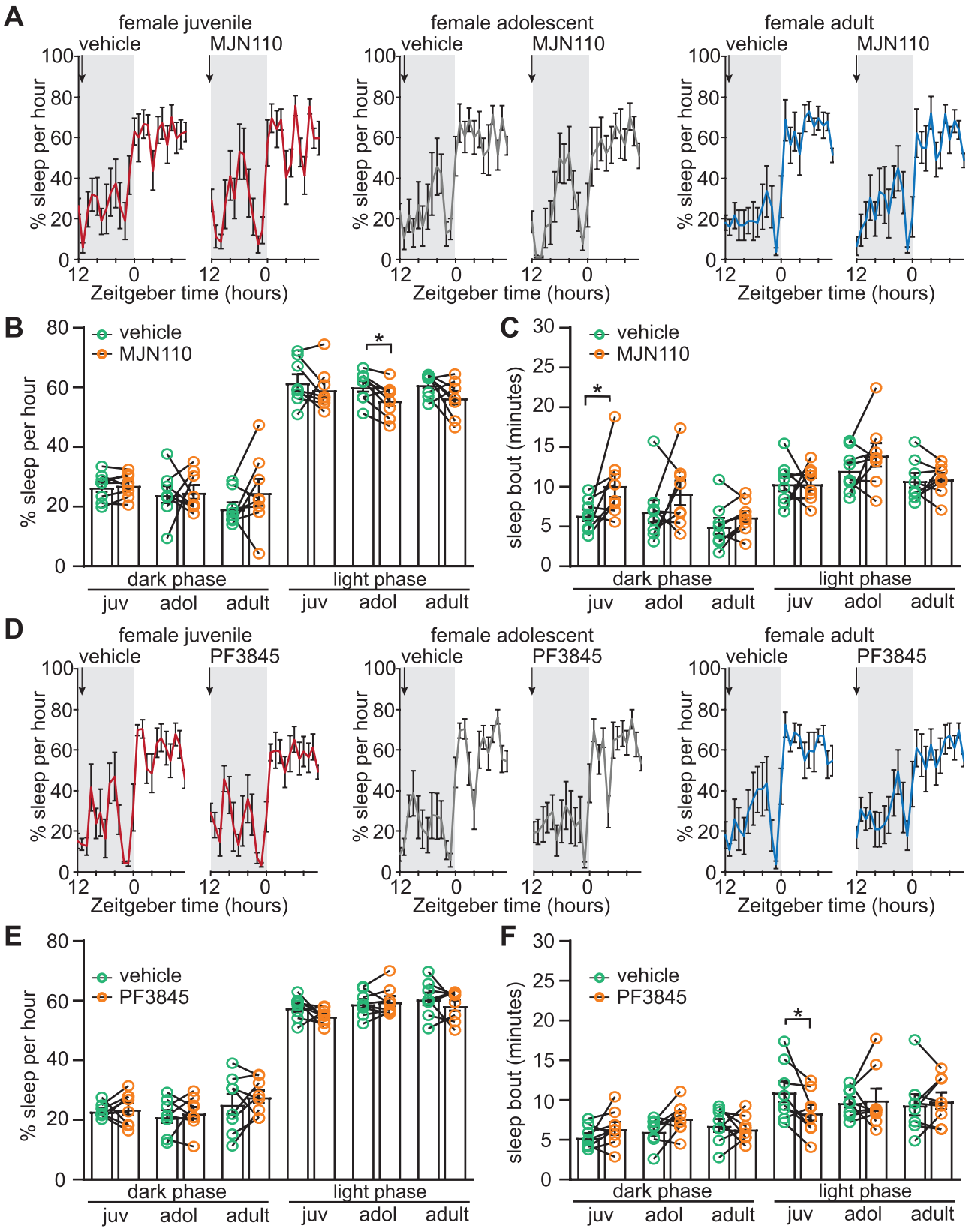

Figure 4. Increased 2-AG or AEA has minimal effects on dark-phase sleep behavior in developing and adult females. (A) Twenty-four hours trace of average hourly sleep of female juveniles (left), adolescents (middle), and adults (right) treated with vehicle or MJN110 (5 mg/kg) at the onset of the dark phase (ZT12), injection indicated by arrow. Gray bars in sleep traces indicate dark phase. Quantification of average hourly sleep (B) and sleep bout length in minutes (C) for 24 h following vehicle or MJN110 injection. Data separated into 12 h of dark and light phases. $N = 8$ juveniles, 8 adolescents, 8 adults. (D) Twenty-four hours trace of average hourly sleep of female juveniles (left), adolescents (middle), and adults (right) treated with PF3845 (10 mg/kg) at the onset of the dark phase (ZT12), injection indicated by arrow. Quantification of average hourly sleep (E) and sleep bout length in minutes (F) for 24 h following vehicle or PF3845 injection. Data separated into 12 h of dark and light phases. $N = 8$ juveniles, 8 adolescents, 8 adults. *$p < 0.05$ (paired two-tailed Student's t-test). Error bars indicate ± SEM. See also Supplementary Figure 4.

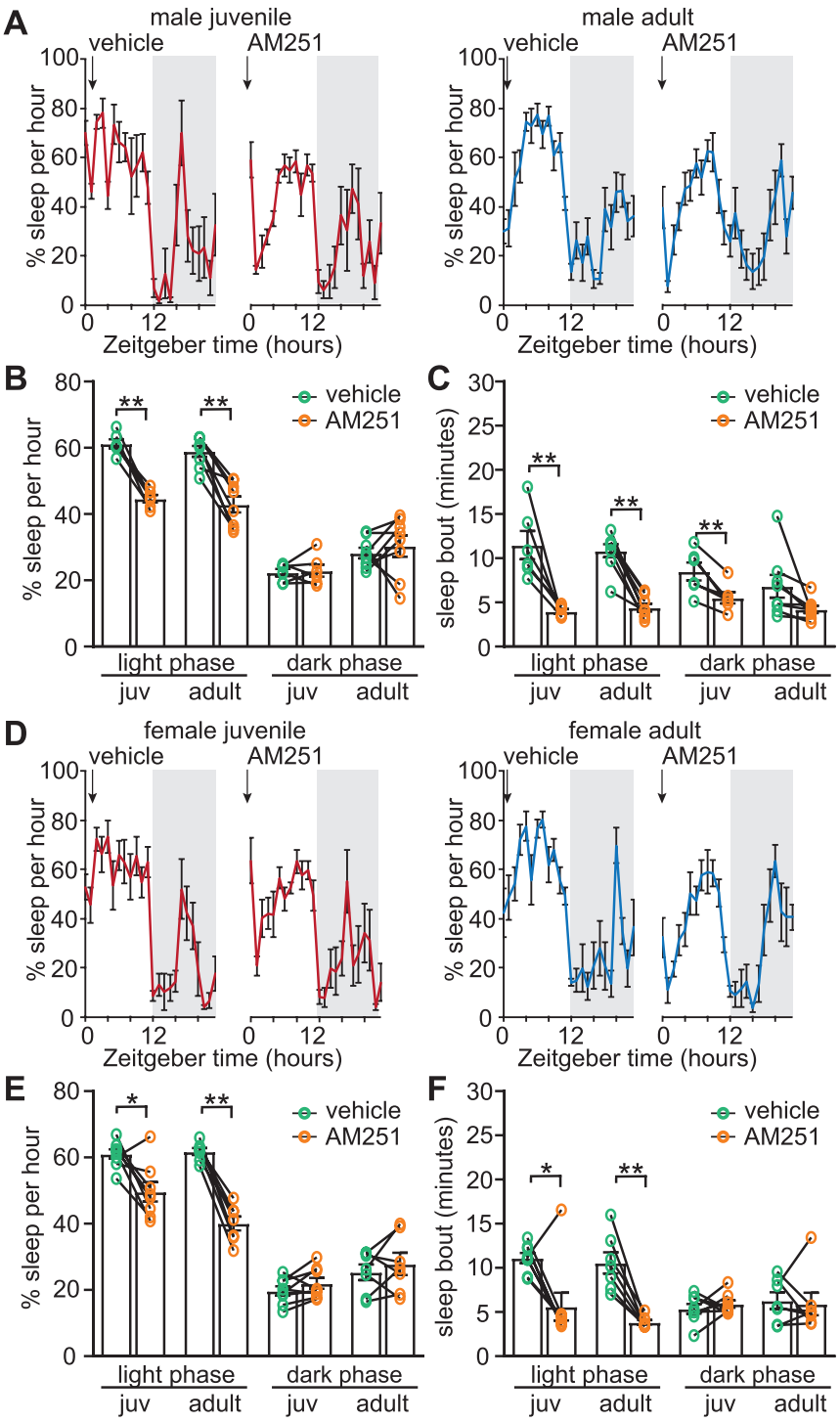

Figure 5. Tonic CB1 signaling sustains light-phase sleep behavior in developing and adult males and females. (A) Twenty-four hours trace of average hourly sleep of male juveniles (left), and adults (right) treated with vehicle or AM251 (10 mg/kg) at the onset of the light phase (ZT0), injection indicated by arrow. Gray bars in sleep traces indicate dark phase. Quantification of average hourly sleep (B) and sleep bout length in minutes (C) for 24 h following vehicle or AM251 injection. Data separated into 12 h of light and dark phases. $N = 6$ juveniles, 8 adults. (D) Twenty-four hours trace of average hourly sleep of female juveniles (left), and adults (right) treated with vehicle or AM251 (10 mg/kg) at the onset of the light phase (ZT0). Quantification of average hourly sleep (E) and sleep bout length in minutes (F) for 24 h following vehicle or AM251 injection. Data separated into 12 h of light and dark phases. $N = 8$ juveniles, 7 adults. $^{*}p < 0.05$, $^{**}p < 0.001$ (paired two-tailed Student's *t*-test). Error bars indicate ± SEM. See also Supplementary Figure 5.

to vehicle control, light-phase AM251 treatment caused a significant decrease in light-phase total sleep time and sleep bout length, in juveniles and adults, both males and females (Figure 5). The reduction in sleep bout length was particularly striking (Figure 5, C and F), suggesting that in the absence of CB1 signaling, sleep becomes highly fragmented, consistent with previous reports where CB1 was inhibited by pharmacology or genetic knockout [28, 30, 32, 34]. In the accompanying sleep traces

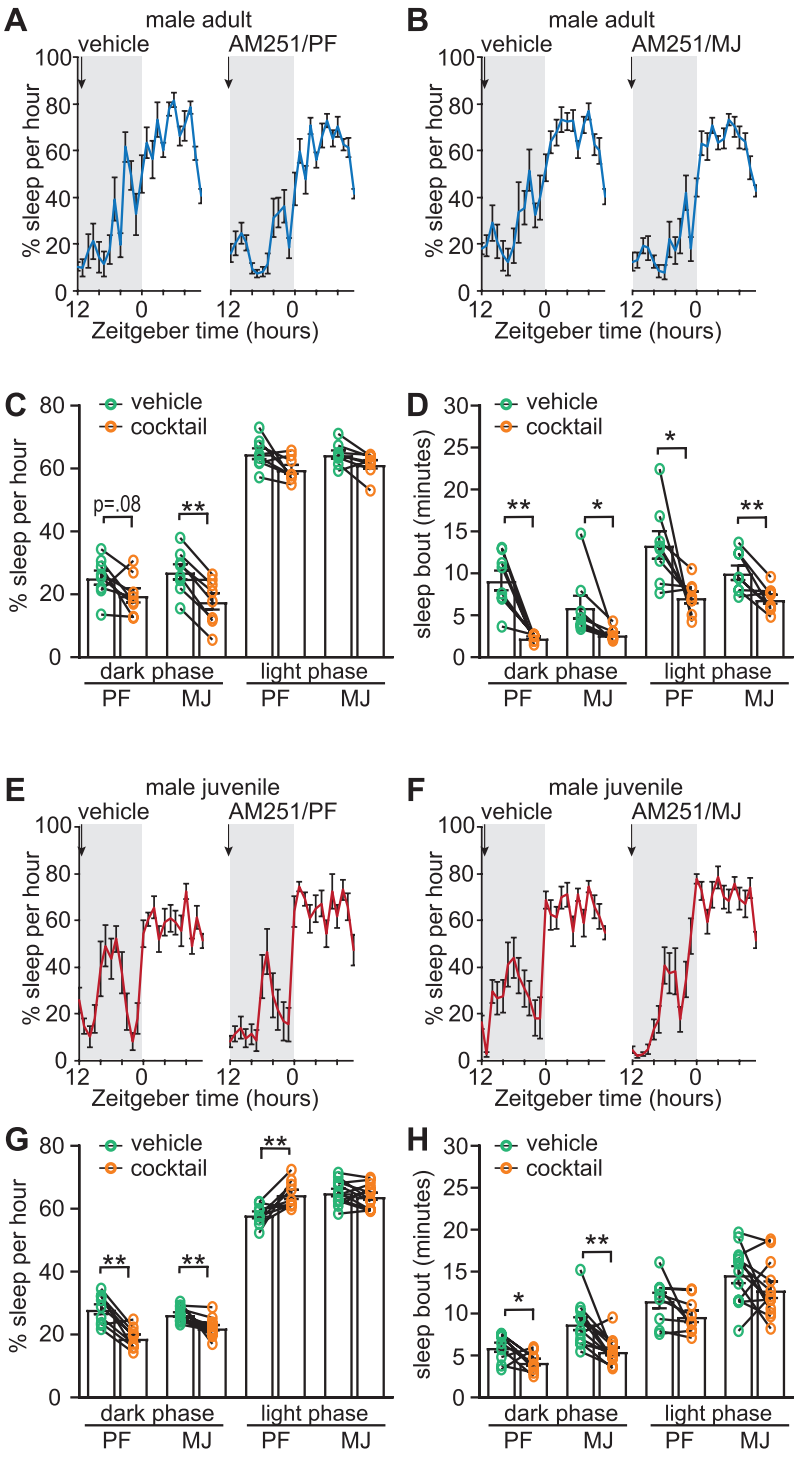

Figure 6. Sleep-promoting effects of enhanced 2-AG or AEA in developing and adult males requires CB1. Twenty-four hours trace of average hourly sleep of adult males treated with vehicle or combined AM251 (10 mg/kg)/MJN110 (5 mg/kg) (A), or AM251 (10 mg/kg)/PF3845 (10 mg/kg) (B). Injections, indicated by arrow, at the onset of the dark phase (ZT12). Gray bars in sleep traces indicate dark phase. Quantification of average hourly sleep (C) and sleep bout length in minutes (D) for 24 h following vehicle or combined drug injection. Data separated into 12 h of dark and light phases. N = 8 AM251/MJN110, 8 AM251/PF3845. Twenty-four hours trace of average hourly sleep of juvenile males treated with vehicle or combined AM251 (10 mg/kg)/MJN110 (5 mg/kg) (E) or AM251 (10 mg/kg)/PF3845 (10 mg/kg) (F). Quantification of average hourly sleep (G) and sleep bout length in minutes (H) for 24 h following vehicle or combined drug injection. Data separated into 12 h of dark and light phases. N = 12 AM251/MJN110, 9 AM251/PF3845. *$p < 0.05$, **$p < 0.001$ (paired two-tailed Student's t-test). Error bars indicate ± SEM.

(Figure 5, A and D), the sleep suppressing effects of AM251 are particularly notable in the first hours of the light phase, probably reflecting the pharmacokinetics of this compound. The effects of AM251 were largely restricted to the first 12 h (light phase), but sleep fragmentation was observed in the subsequent dark phase only in juvenile males. Adolescents were not tested in this experiment but our expectation based on these findings is that this age would respond similarly to juveniles and adults.

Dark-phase injection of AM251 in juvenile and adult males and females also caused a significant suppression of total sleep and sleep fragmentation within the first 12 h (Supplementary Figure 5). Together these findings support our idea that an eCB tone supports sleep in both males and females, during development and adulthood, and that this eCB tone supports sleep in the light and dark phases.

Sleep promotion by MJN110 or PF3845 require CB1

We wanted to confirm that increases in 2-AG or NAEs, via inhibition of MAGL or FAAH, respectively, promote sleep in males by acting on CB1 receptors. This is particularly important for PF3845, because inhibition of FAAH increases synaptic levels all NAE metabolites (Figure 2, C) [22], many of which are known to be bioactive. For example, FAAH substrates OEA and PEA are known to act as endogenous agonists for peroxisome proliferator-activated receptors (PPARs) nuclear transcription factors [22]. Adult or juvenile male mice were injected prior to the dark phase with vehicle control or a cocktail containing AM251 (10 mg/kg) together with either MJN110 (5 mg/kg) or PF3845 (10 mg/kg). Unlike MJN110 or PF3845 alone, which in males caused significant increases in total dark-phase sleep and bout length, cocktail injections in adult males caused significant reductions, or a strong trend, in total sleep and bout length (Figure 6, A–D). In juvenile males, combined treatment with AM251 prevented the sleep-promoting effects of MJN110 or PF3845 (Figure 6, E–H). These results strongly suggest that the male-specific sleep-promoting effects of MJN110 or PF3845 require CB1 as expected.

Discussion

Previous studies have shown that the eCB system can promote sleep in adult males, primarily through a stabilization of the sleep state, independent of the homeostatic sleep drive [20, 28, 30]. It is unknown whether eCBs also play a role in sleep behavior during development or in females. Further, how synaptic eCBs are regulated by the sleep/wake cycle in the context of development has not been examined. In the current study, we used quantitative MS to show that the expression of forebrain synaptic AEA and related NAEs lack a daily rhythm in juvenile mice (P21) of both sexes, but transition to regulation by the circadian rhythm during maturation, where expression of forebrain synaptic AEA and related OEA are higher during the sleep phase. Based on this clear difference in the sleep-dependent expression of AEA during development, we hypothesized that the previously described sleep-promoting effects of eCBs would emerge during maturation toward adulthood. We confirmed previous reports that enhancing the levels of 2-AG or AEA increased sleep amount and bout length in adult males [20, 30]. However, counter to our hypothesis, we show similar sleep-promoting effects in juvenile and adolescent males. Identical treatments in developing and adult females had minimal effects. These findings suggest that, although components of the eCB system are undergoing maturation between P21 and P56, the neural circuits underlying the male-specific sleep-promoting effects of MAGL and FAAH inhibitors are already sufficiently matured even prior to sexual maturity. Inhibition of CB1 with a direct antagonist, AM251, caused a very clear suppression of sleep amount and sleep fragmentation at all ages tested, in both males and females. The latter finding suggests that an eCB tone is important to sustain sleep in both sexes and throughout development, consistent with previous conclusions that eCBs promote sleep stability [20]. Our findings highlight two areas of interest for future studies. First, what mechanisms regulate the expression of synaptic eCBs in developing mice, and what are the physiological implications of the transition toward circadian control of forebrain AEA and OEA? Second, what is the cellular and circuit basis for the male-specific sleep-promoting effects of MAGL or FAAH inhibition?

Circadian expression of synaptic AEA and sleep ontogenesis

Sleep behavior is broadly believed to be under the control of two major influences, the circadian clock that promotes sleep behavior at the ecologically appropriate time of day, and the homeostatic sleep drive that promotes sleep in proportion to time spent awake [55]. Previous studies using pharmacological or genetic manipulations of CB1 conclude that eCB signaling supports sleep behavior, primarily through stabilization of sleep episodes, independent of the homeostatic sleep drive [20, 28, 30, 34]. In our adolescent mice, we observed higher expression of AEA and OEA at ZT4 compared to ZT16, under both undisturbed and sleep deprived conditions, leading us to conclude that the expression of forebrain AEA/OEA metabolites is likely controlled by circadian mechanisms. This conclusion is in general agreement with previous studies that have shown a circadian expression of AEA in human plasma and in multiple regions of the rodent brain [50, 56]. Studies on the ontogenesis of sleep in rodents have shown that the circadian rhythm begins to have a strong influence on sleep behavior during the third or fourth week following birth [57–59]. Our data suggest that a circadian expression of AEA emerges after P21. In future studies, it will be of interest to determine with greater resolution when the circadian expression of synaptic AEA emerges. EEG studies in developing mice have shown that sleep behavior is highly fragmented at P10–P14, with very short sleep bouts and frequent state transitions. Sleep bout lengths undergo a profound consolidation from P14 to P21 [17], and this process continues at a more gradual pace from P21 to P60 [18]. It is possible that the emergence of a circadian control of synaptic AEA may be mechanistically linked with the emergent role of the circadian rhythm on sleep behavior and consolidation of sleep bouts during this period of maturation.

Sex-specific effects of eCB boosting drugs

We began our study be asking whether forebrain synaptic eCB metabolites are regulated in a sleep- and development-dependent manner, independent of sex. While the study design included both sexes, it was not specifically designed or powered for a clear sex comparison and we limit our main conclusions to the role of developmental age. This design is in accordance with recent recommendations that experiments should include both sexes, even if not powered for sex comparisons [60]. Nonetheless, in comparing the expression levels of synaptic eCB metabolites between the sexes in the current dataset we see no significant

differences, or compelling trends, in any of the eCB species examined at the juvenile age or in the major CB1 agonists 2-AG and AEA at the adolescent age (Supplementary Figures 1 and 2). Interestingly, our data are suggestive that several NAE species, show a higher expression level during the sleep phase in adolescent males only (Supplementary Figure 2). OEA and PEA are a well-described agonists for the nuclear transcription factor peroxisome proliferator-activated receptor alpha (PPARα) which has been implicated in sleep behavior [61]. Whether OEA and related NAEs indeed show a male-specific increase during sleep will require additional dedicated studies. Moreover, the expression of eCB metabolites in females has been shown to have a bidirectional interaction with gonadal hormones during the estrous cycle [62, 63], an important variable that was not accounted for in the current dataset. Future studies designed to provide a comprehensive understanding of the sleep-dependent expression of eCBs in females may need to take into account the regular fluctuations in gonadal hormones.

Based on the data contained in this study we suggest that the male-specific sleep-promoting effects of MAGL or FAAH inhibitors is likely due to sex-specific differences in the expression of CB1, or downstream signaling mechanisms, within specific brain regions, and not related to sex differences in eCB metabolites, at least in the forebrain. First, the male-specific sleep-promoting effects of eCB boosting drugs is apparent even in juvenile mice before the onset of puberty, an age where we see no evidence of sex differences in synaptic eCB expression. Second, we also have confirmed that our MAGL and FAAH inhibitor compounds selectively increase the levels of 2-AG and AEA/NAEs, respectively, and that near identical responses are seen in both sexes (Figure 2), thus ruling out differences in target engagement of these drugs between sexes. Finally, 2-AG and AEA each act as CB1 agonists, but diverge in their other targets [22], and increasing the level of either 2-AG or AEA promoted sleep in males, suggesting that signaling events downstream of CB1 activation are relevant for male-specific sleep promotion. In support of this we show that the sleep-promoting effects of MAGL/FAAH inhibitors in males are blocked by CB1 antagonist AM251 (Figure 6).

Previous literature shows clear sex differences in behavioral responses to cannabinoids or eCB targeted pharmacology [64, 65]. Sex differences in the expression of CB1 mRNA or protein, or in the density of CB1 receptor binding using radio-labeled ligands, have been described in mice and rats but are highly region selective [66–68]. It is also plausible that sex differences may exist in the signaling machinery downstream of CB1 activation. Sex-specific behavioral responses to eCB/CB1 targeted pharmacology are likely due to sexual dimorphism in specific cell populations and neural circuits. The neural circuits that underlie the sleep-promoting effects of eCBs, particularly in males are not known, although the eCB system is most prominently expressed in the mouse forebrain, and CB1 mRNA is expressed at higher density in many regions of the male cortex [68]. We hypothesize that CB1 expression is higher in males in brain regions underlying the sleep-promoting effects of MJN110 or PF3845 treatment, allowing for the larger behavioral response to these drugs. Thus, while the sexually divergent response to MJN110 and PF3845 we report here is in general agreement with the greater literature [64, 65], the underlying molecular, cellular, or circuit basis for this difference between sexes is not clear and will require further research.

AEA vs. 2-AG

AEA and 2-AG are well-characterized endogenous agonists for the CB1 receptor. Does it matter that there are two distinct eCB classes when either one can activate CB1 and promote sleep? 2-AG and AEA have completely nonoverlapping enzymes that control their synthesis and degradation [22], suggesting that the two CB1 agonists undergo independent regulation and may serve distinct physiological functions. Accordingly, our targeted quantification of synaptic eCBs show that AEA and related NAEs are suppressed by sleep deprivation in juveniles, transitioning to circadian regulation of AEA/OEA in adolescents. Whereas, synaptic 2-AG appears to be expressed independently of the sleep–wake cycle at both ages. Unlike other neurotransmitters that are stored in vesicles for regulated release, eCBs are synthesized from postsynaptic membranes in response to neuronal activity, commonly described as synthesized "on demand." Prominent forms of eCB-dependent short-term plasticity including depolarization induced suppression of excitation or inhibition (DSE and DSI) have been shown to result from rapid and transient synthesis of 2-AG, but not AEA [69, 70]. In other settings, eCB signaling is better described as being "tonic," where an eCB tone likely due to AEA and not 2-AG, is constitutively acting on CB1 receptors over longer time scales [28, 71, 72]. 2-AG has been characterized as a "full-agonist" for CB1 receptors, but sustained increases in 2-AG through high doses of MAGL inhibitor drives the desensitization of CB1, leading to drug tolerance and secondary effects consistent with suppression of CB1 signaling [21, 22, 30, 73–75]. In contrast, AEA is a "partial agonist" but does not drive desensitization, allowing for sustained CB1 signaling [73, 76]. Thus, we speculate that the two major eCBs may play distinct roles with respect to the sleep/wake cycle: AEA serves a tonic signaling function that stabilizes the sleep state, whereas 2-AG acts in a phasic manner to regulate short-term synaptic plasticity independent of the sleep/wake cycle. During maturation of the cortex, the circadian rhythm emerges as the main influence of AEA expression, perhaps to promote sleep activity in adolescent and adult mice. In support of this, mice with deletion of FAAH have been shown to have sustained elevations in AEA and other NAEs, and to have increased total amount and bout lengths of NREM sleep, and higher levels of NREM delta power compared to wild-type mice [33].

Considerable interest is growing around the potential of the eCB system as a therapeutic target in a number of conditions. Our data support a role for the eCB system in promoting sleep, and we further extend this work to examine developmental time points. Our results also strongly emphasize that sex as a biological variable will require considerable attention in the ongoing development of eCB- and cannabinoid-based medicines.

Supplementary Material

Supplementary material is available at *SLEEP* online.

Funding

This work was supported by grants from the National Institutes of Health to GHD (RF1AG068063) and to SCM (F31-AG074649-01), and from the National Science Foundation to SMG (DGE-2040435).

Acknowledgments

The authors thank Julia Lord and Michael Ye for technical assistance and critical feedback with this work.

Disclosure Statement

None declared.

References

1. Mazzone L, *et al*. The relationship between sleep problems, neurobiological alterations, core symptoms of autism spectrum disorder, and psychiatric comorbidities. *J Clin Med*. 2018;**17**. doi:10.3390/jcm7050102
2. Missig G, *et al*. Sleep as a translationally-relevant endpoint in studies of autism spectrum disorder (ASD). *Neuropsychopharmacology*. 2020;**45**(1):90–103.
3. Wang C, *et al*. Bidirectional relationship between sleep and Alzheimer's disease: role of amyloid, tau, and other factors. *Neuropsychopharmacology*. 2020;**45**(1):104–120. doi:10.1038/s41386-019-0478-5
4. Diering GH, *et al*. Homer1a drives homeostatic scaling-down of excitatory synapses during sleep. *Science*. 2017;**355**(6324):511–515.
5. Wang Z, *et al*. Quantitative phosphoproteomic analysis of the molecular substrates of sleep need. *Nature*. 2018;**558**(7710):435–439.
6. de Vivo L, *et al*. Ultrastructural evidence for synaptic scaling across the wake/sleep cycle. *Science*. 2017;**355**(6324):507–510.
7. Noya SB, *et al*. The forebrain synaptic transcriptome is organized by clocks but its proteome is driven by sleep. *Science*. 2019;**366**. doi:10.1126/science.aav2642
8. Bruning F, *et al*. Sleep-wake cycles drive daily dynamics of synaptic phosphorylation. *Science*. 2019;**366**. doi:10.1126/science.aav3617
9. Tononi G, *et al*. Sleep and the price of plasticity: from synaptic and cellular homeostasis to memory consolidation and integration. *Neuron*. 2014;**81**(1):12–34.
10. Diekelmann S, *et al*. The memory function of sleep. *Nat Rev Neurosci*. 2010;**11**(2):114–126.
11. Frank MG, *et al*. The function(s) of sleep. *Handb Exp Pharmacol*. 2019;**253**:3–34.
12. Roffwarg HP, *et al*. Ontogenetic development of the human sleep-dream cycle. *Science*. 1966;**152**(3722):604–619.
13. Penzes P, *et al*. Dendritic spine pathology in neuropsychiatric disorders. *Nat Neurosci*. 2011;**14**(3):285–293.
14. Frank MG. Sleep and synaptic plasticity in the developing and adult brain. *Curr Top Behav Neurosci*. 2015;**25**:123–149.
15. Kurth S, *et al*. Mapping of cortical activity in the first two decades of life: a high-density sleep electroencephalogram study. *J Neurosci*. 2010;**30**(40):13211–13219.
16. Wintler T, *et al*. Sleep, brain development, and autism spectrum disorders: insights from animal models. *J Neurosci Res*. 2020;**98**(6):1137–1149.
17. Rensing N, *et al*. Longitudinal analysis of developmental changes in electroencephalography patterns and sleep-wake states of the neonatal mouse. *PLoS One*. 2018;**13**(11):e0207031.
18. Nelson AB, *et al*. Sleep patterns and homeostatic mechanisms in adolescent mice. *Brain Sci*. 2013;**3**(1):318–343.
19. Castillo PE, *et al*. Endocannabinoid signaling and synaptic function. *Neuron*. 2012;**76**(1):70–81.
20. Kesner AJ, *et al*. Cannabinoids, endocannabinoids and sleep. *Front Mol Neurosci*. 2020;**13**:125.
21. Busquets-Garcia A, *et al*. Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses. *Biol Psychiatry*. 2011;**70**(5):479–486.
22. Di Marzo V. New approaches and challenges to targeting the endocannabinoid system. *Nat Rev Drug Discov*. 2018;**17**(9):623–639.
23. Devane WA, *et al*. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science*. 1992;**258**(5090):1946–1949.
24. Sugiura T, *et al*. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun*. 1995;**215**(1):89–97.
25. Mechoulam R, *et al*. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol*. 1995;**50**(1):83–90.
26. Ahn K, *et al*. Discovery and characterization of a highly selective FAAH inhibitor that reduces inflammatory pain. *Chem Biol*. 2009;**16**(4):411–420.
27. Niphakis MJ, *et al*. Evaluation of NHS carbamates as a potent and selective class of endocannabinoid hydrolase inhibitors. *ACS Chem Neurosci*. 2013;**4**(9):1322–1332.
28. Pava MJ, *et al*. Endocannabinoid modulation of cortical up-states and NREM sleep. *PLoS One*. 2014;**9**(2):e88672.
29. Steriade M, *et al*. Intracellular analysis of relations between the slow (<1 Hz) neocortical oscillation and other sleep rhythms of the electroencephalogram. *J Neurosci*. 1993;**13**(8):3266–3283.
30. Pava MJ, *et al*. Endocannabinoid signaling regulates sleep stability. *PLoS One*. 2016;**11**(3):e0152473.
31. Santucci V, *et al*. Arousal-enhancing properties of the CB1 cannabinoid receptor antagonist SR 141716A in rats as assessed by electroencephalographic spectral and sleep-waking cycle analysis. *Life Sci*. 1996;**58**(6):PL103–PL110.
32. Bogáthy E, *et al*. AM-251, a cannabinoid antagonist, modifies the dynamics of sleep-wake cycles in rats. *Front Pharmacol*. 2019;**10**:831.
33. Huitron-Resendiz S, *et al*. Characterization of the sleep-wake patterns in mice lacking fatty acid amide hydrolase. *Sleep*. 2004;**27**(5):857–865. doi:10.1093/sleep/27.5.857
34. Silvani A, *et al*. Cardiorespiratory anomalies in mice lacking CB1 cannabinoid receptors. *PLoS One*. 2014;**9**(6):e100536.
35. Murillo-Rodríguez E, *et al*. Anandamide modulates sleep and memory in rats. *Brain Res*. 1998;**812**(1–2):270–274.
36. Pérez-Morales M, *et al*. 2-AG into the lateral hypothalamus increases REM sleep and cFos expression in melanin concentrating hormone neurons in rats. *Pharmacol Biochem Behav*. 2013;**108**:1–7.
37. Rueda-Orozco PE, *et al*. Intrahippocampal administration of anandamide increases REM sleep. *Neurosci Lett*. 2010;**473**(2):158–162.
38. Herrera-Solís A, *et al*. Acute and subchronic administration of anandamide or oleamide increases REM sleep in rats. *Pharmacol Biochem Behav*. 2010;**95**(1):106–112.
39. Acuna-Goycolea C, *et al*. Cannabinoids excite circadian clock neurons. *J Neurosci*. 2010;**30**(30):10061–10066.
40. Hablitz LM, *et al*. Cannabinoid signaling recruits astrocytes to modulate presynaptic function in the suprachiasmatic nucleus. *eNeuro*. 2020;**7**. doi:10.1523/ENEURO.0081-19.2020
41. Long JZ, *et al*. Dual blockade of FAAH and MAGL identifies behavioral processes regulated by endocannabinoid crosstalk in vivo. *Proc Natl Acad Sci U S A*. 2009;**106**(48):20270–20275.

42. Suzuki A, *et al*. Behavioral and biochemical dissociation of arousal and homeostatic sleep need influenced by prior wakeful experience in mice. *Proc Natl Acad Sci U S A*. 2013;**110**(25):10288–10293.
43. Gouveia-Figueira S, *et al*. Validation of a tandem mass spectrometry method using combined extraction of 37 oxylipins and 14 endocannabinoid-related compounds including prostamides from biological matrices. *Prostaglandins Other Lipid Mediat*. 2015;**121**(Pt A):110–121.
44. Mang GM, *et al*. Evaluation of a piezoelectric system as an alternative to electroencephalogram/electromyogram recordings in mouse sleep studies. *Sleep*. 2014;**37**(8):1383–1392. doi:10.5665/sleep.3936
45. Donohue KD, *et al*. Assessment of a non-invasive high-throughput classifier for behaviours associated with sleep and wake in mice. *Biomed Eng Online*. 2008;**7**:14.
46. Flores AE, *et al*. Pattern recognition of sleep in rodents using piezoelectric signals generated by gross body movements. *IEEE Trans Biomed Eng*. 2007;**54**(2):225–233.
47. Wang C, *et al*. A comparative study of sleep and diurnal patterns in house mouse (*Mus musculus*) and Spiny mouse (*Acomys cahirinus*). *Sci Rep*. 2020;**10**:10944.
48. Yaghouby F, *et al*. Unsupervised estimation of mouse sleep scores and dynamics using a graphical model of electrophysiological measurements. *Int J Neural Syst*. 2016;**26**(4):1650017.
49. Valenti M, *et al*. Differential diurnal variations of anandamide and 2-arachidonoyl-glycerol levels in rat brain. *Cell Mol Life Sci*. 2004;**61**(7–8):945–950.
50. Murillo-Rodriguez E, *et al*. Diurnal variation of arachidonoylethanolamine, palmitoylethanolamide and oleoylethanolamide in the brain of the rat. *Life Sci*. 2006;**79**(1):30–37.
51. Hillard CJ. Circulating endocannabinoids: from whence do they come and where are they going? *Neuropsychopharmacology*. 2018;**43**(1):155–172.
52. Goonawardena AV, *et al*. A pilot study into the effects of the CB1 cannabinoid receptor agonist WIN55,212-2 or the antagonist/inverse agonist AM251 on sleep in rats. *Sleep Disord*. 2011;**2011**:178469.
53. Calik MW, *et al*. Effects of cannabinoid agonists and antagonists on sleep and breathing in Sprague-Dawley rats. *Sleep*. 2017;**40**(9). doi:10.1093/sleep/zsx112
54. Goonawardena AV, *et al*. Modulation of food consumption and sleep-wake cycle in mice by the neutral CB1 antagonist ABD459. *Behav Pharmacol*. 2015;**26**(3):289–303.
55. Borbély AA. A two process model of sleep regulation. *Hum Neurobiol*. 1982;**1**(3):195–204.
56. Vaughn LK, *et al*. Endocannabinoid signalling: has it got rhythm? *Br J Pharmacol*. 2010;**160**(3):530–543.
57. Frank MG, *et al*. Development of circadian sleep regulation in the rat: a longitudinal study under constant conditions. *Sleep*. 2017;**40**(3). doi:10.1093/sleep/zsw077
58. Frank MG. The ontogenesis of mammalian sleep: form and function. *Curr Sleep Med Rep*. 2020;**6**(4):267–279.
59. Ibuka N. Ontogenesis of circadian sleep-wakefulness rhythms and developmental changes of sleep in the altricial rat and in the precocial guinea pig. *Behav Brain Res*. 1984;**11**(3):185–196.
60. Shansky RM. Are hormones a "female problem" for animal research? *Science*. 2019;**364**(6443):825–826.
61. Murillo-Rodriguez E. The role of nuclear receptor PPARα in the sleep-wake cycle modulation. A tentative approach for treatment of sleep disorders. *Curr Drug Deliv*. 2017;**14**(4):473–482.
62. Santoro A, *et al*. The complex interplay between endocannabinoid system and the estrogen system in central nervous system and periphery. *Int J Mol Sci*. 2021;**22**. doi:10.3390/ijms22020972
63. Gorzalka BB, *et al*. Minireview: endocannabinoids and gonadal hormones: bidirectional interactions in physiology and behavior. *Endocrinology*. 2012;**153**(3):1016–1024.
64. Craft RM, *et al*. Sex differences in cannabinoid pharmacology: a reflection of differences in the endocannabinoid system? *Life Sci*. 2013;**92**(8–9):476–481.
65. Cooper ZD, *et al*. Sex-dependent effects of cannabis and cannabinoids: a translational perspective. *Neuropsychopharmacology*. 2018;**43**(1):34–51.
66. Reich CG, *et al*. Differential effects of chronic unpredictable stress on hippocampal CB1 receptors in male and female rats. *Behav Brain Res*. 2009;**203**(2):264–269.
67. Riebe CJ, *et al*. Estrogenic regulation of limbic cannabinoid receptor binding. *Psychoneuroendocrinology*. 2010;**35**(8):1265–1269.
68. Liu X, *et al*. Sexual dimorphic distribution of cannabinoid 1 receptor mRNA in adult C57BL/6J mice. *J Comp Neurol*. 2020;**528**(12):1986–1999.
69. Yoshino H, *et al*. Postsynaptic diacylglycerol lipase mediates retrograde endocannabinoid suppression of inhibition in mouse prefrontal cortex. *J Physiol*. 2011;**589**(Pt 20):4857–4884.
70. Tanimura A, *et al*. The endocannabinoid 2-arachidonoylglycerol produced by diacylglycerol lipase alpha mediates retrograde suppression of synaptic transmission. *Neuron*. 2010;**65**(3):320–327.
71. Gonzalez-Islas C, *et al*. Tonic and transient endocannabinoid regulation of AMPAergic miniature postsynaptic currents and homeostatic plasticity in embryonic motor networks. *J Neurosci*. 2012;**32**(39):13597–13607.
72. Kim J, *et al*. Reduction in endocannabinoid tone is a homeostatic mechanism for specific inhibitory synapses. *Nat Neurosci*. 2010;**13**(5):592–600.
73. Schlosburg JE, *et al*. Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat Neurosci*. 2010;**13**(9):1113–1119.
74. Kinsey SG, *et al*. Repeated low-dose administration of the monoacylglycerol lipase inhibitor JZL184 retains cannabinoid receptor type 1-mediated antinociceptive and gastroprotective effects. *J Pharmacol Exp Ther*. 2013;**345**(3):492–501.
75. Sugiura T, *et al*. 2-Arachidonoylglycerol, a putative endogenous cannabinoid receptor ligand, induces rapid, transient elevation of intracellular free Ca^{2+} in neuroblastoma × glioma hybrid NG108-15 cells. *Biochem Biophys Res Commun*. 1996;**229**(1):58–64.
76. Burkey TH, *et al*. Relative efficacies of cannabinoid CB1 receptor agonists in the mouse brain. *Eur J Pharmacol*. 1997;**336**(2–3):295–298.