Published in final edited form as:

Eur J Neurosci. 2012 June ; 35(11): 1789–1798. doi:10.1111/j.1460-9568.2012.08112.x.

5'-ectonucleotidase knockout mice lack non-REM sleep responses to sleep deprivation

Mark R. Zielinski, Ping Taishi, James M. Clinton, and James M. Krueger

Sleep and Performance Research Center, Programs in Neuroscience, and WWAMI Medical Education Program, Washington State University, Spokane, WA, USA 99210-1495

Abstract

Adenosine and extracellular adenosine triphosphate (ATP) have multiple physiological central nervous system (CNS) actions including regulation of cerebral blood flow, inflammation, and sleep. However, their exact sleep regulatory mechanisms remain unknown. Extracellular ATP and adenosine diphosphate (ADP) are converted to adenosine monophosphate (AMP) by the enzyme ectonucleoside triphosphate diphosphohydrolase 1, also known as CD39, and extracellular AMP is in turn converted to adenosine by the 5'-ectonucleotidase enzyme CD73. We investigated the role of CD73 in sleep regulation. Duration of spontaneous non-rapid eye movement sleep (NREMS) was greater in CD73 knockout (KO) mice compared to C57BL/6 controls whether determined in our laboratory or by others. After sleep deprivation (SD), NREMS was enhanced in controls but not CD73 KO mice. Interleukin-1 beta (IL1 β) enhanced NREMS in both strains indicating that the CD73 KO mice were capable of sleep responses. Electroencephalographic (EEG) power spectra during NREMS in the 1.0–2.5 Hz frequency range was significantly enhanced after SD in both CD73 KO and WT mice; the increases were significantly greater in the WT mice vs. CD73 KO mice. Rapid eye movement sleep did not differ between strains in any of the experimental conditions. With the exception of CD73 mRNA, the effects of SD on various adenosine-related mRNAs were small and similar in both strains. These data suggest that sleep is regulated, in part, by extracellular adenosine derived from the actions of CD73.

Keywords

Purine receptor; cytokine; adenosine; EEG delta power; 5' ectonucleotidases

Introduction

Adenosine has multiple central nervous system (CNS) functions including the regulation of cerebral blood flow, inflammation, and sleep (Porkka-Heiskanen & Kalinchuk, 2010). Intracellular adenosine levels are regulated by enzymes such as adenosine kinase (ADK) and adenosine deaminase (ADA). Adenosine nucleotide/adenosine ratios are about 10,000:1 (Masino *et al.*, 2009); several enzymes, including all ATPases, are involved in the conversion of adenosine nucleotides to adenosine. Extracellular adenosine is derived, in part, from extracellular ATP (Kumar & Sharma, 2009). Extracellular adenosine is also derived from intracellular adenosine via nucleoside transporters such as equilibrative transporter-1 (ENT1) and concentrative transporter-2 (CNT2). Extracellular ATP and ADP are converted to AMP by the enzyme CD39 (also called 5'-ectonucleoside triphosphate

diphosphohydrolase-1). Extracellular AMP is, in turn, converted to adenosine by the enzyme CD73 (also known as 5'-ectonucleotidase). How this litany of anabolic and catabolic enzymes and transporters are choreographed to regulate sleep remains unknown, although there is evidence that the flux of extracellular adenosine is enhanced in the cortex and basal forebrain during extended wakefulness (Porkka-Heiskanen *et al.*, 2000).

Extracellular ATP has a role in sleep regulation (Krueger *et al.*, 2008). Recently, we showed that an ATP agonist enhances sleep and purine type 2 (P2) receptor antagonists inhibit sleep, and that these actions are mediated, in part, via P2 X7 receptors (P2X7R) (Krueger *et al.*, 2010). ATP is released into the extracellular space during neuro- and glia-transmission (Fields & Burnstock, 2006; Verderio & Matteoli, 2011). Further, in glia and immunocytes, extracellular ATP enhances the release of sleep-regulatory cytokines (Krueger, 2008) including interleukin-1 beta (IL1 β), tumor necrosis factor-alpha (TNF α), and brain-derived neurotrophic factor (BDNF) (Su *et al.*, 2010; Suzuki *et al.*, 2004; Takasaki *et al.*, 2008). Thus, it is plausible that CD73 could alter sleep.

Herein we report that non-rapid eye movement sleep (NREMS) responses to sleep deprivation (SD) were absent in mice lacking CD73 [CD73 knockout (KO) mice], although their NREMS responses to IL1 β remained intact. Rapid eye movement sleep (REMS) was enhanced in CD73 KO and control mice after SD but not after IL1 β administration. The results provided here are consistent with the hypothesis that extracellular adenosine and extracellular ATP are involved in NREMS regulation.

Materials and Methods

Animals and Surgery

CD73 knockout (KO) mice with a C57BL/6J genetic background were generated at the University of Oklahoma (Oklahoma City, OK) as previously described (Thompson *et al.*, 2004). These mice were bred for 2–3 generations at Washington State University. C57BL/6J wild-type (WT) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and served as controls for the CD73 KO mice. All mice were genotyped from tail snips by Transnetyx (Cordova, TN). In Experiments #1, #2, and #3, the same two groups of mice [WT ($n = 9$) and CD73KO ($n = 9$); 10–12 weeks of age] were used concurrently for polysomnographic studies. In Experiment #4, different groups of mice [WT ($n = 18–20$) and CD73KO ($n = 18–20$); 10 weeks of age] were used for somatosensory cortex and hypothalamus mRNA analyses of tissue obtained after SD.

Mice used for polysomnographic analyses were anesthetized with an intraperitoneal (ip) injection of ketamine-xylazine (87 and 13 mg/kg, respectively) for surgical implantation of electroencephalographic (EEG) electrodes over the right and left parietal cortices and over the cerebellum, which acted as a ground as previously described (Krueger *et al.*, 2010). In addition, a stainless steel electromyogram (EMG) electrode was placed in the dorsal neck muscles. The electrodes were formed into a pedestal and fixed on the head with dental cement. Mice were allowed at least 7 days to recover from the surgical procedure and to acclimate to the tethered polysomnographic cables. Mice were housed individually in standard caging and maintained at a thermoneutral ambient temperature (29 ± 1 °C) in sound-attenuated environmental chambers on a 12:12 h light:dark cycle (light onset, time = 0). Food and water were available *ad libitum*. All experimental protocols were approved by the Washington State University Animal Care and Use Committee and were in compliance with the National Institutes of Health guidelines.

Polysomnographic Recording and Analyses

Polysomnographic recording cables were connected to commutators that were attached to amplifiers. The analog EEG and EMG signals from the amplifiers were converted to digital signals (128 Hz sampling rate) and recorded. EEG signals were filtered below 0.1 Hz and above 40 Hz. Polysomnographic analyses were conducted using Sleep Sign Software (Kissei Comtec Co., Ltd, Japan). Vigilance states including wakefulness, NREMS, and rapid-eye movement sleep (REMS) were manually determined off-line in 10 second epochs as described previously (Krueger *et al.*, 2010). Briefly, NREMS was characterized by high-amplitude EEG signals and low EMG activity. REMS was identified by regular low-amplitude EEG and minimal EMG activity. Wakefulness was determined when the signal exhibited low amplitude fast EEG and high EMG activity. Time spent in the vigilance states and the durations of vigilance state episodes were calculated in 2 h and 12 h time blocks, respectively. EEG signals (μV^2) were analyzed by fast Fourier transformation for two types of analyses. NREMS EEG slow wave activity (SWA) (0.5–4 Hz) values were determined within each epoch. The means of the 24 h EEG SWA control values for each mouse were used as a reference value to normalize the data for both the control and experimental treatment days as previously described (Krueger *et al.*, 2010). EEG SWA values for each animal were determined in 2 h time bins and presented as a percentage of the reference value. Power spectrum analysis was also performed. EEG power spectra (μV^2) were calculated in 0.5 Hz bins in the frequency range of 0.5 to 20 Hz for 24 h periods. For these calculations, the sums of the total power from each of the 40, 0.5 Hz wide, frequency bands was used to normalize the values in individual frequency bins. Thus, the numbers within each frequency bin are the fraction of total power occurring during the time period analyzed. For values within the delta range, EEG power spectra values are close to EEG SWA values, but can differ because the normalization is within the time period analyzed, whereas for EEG SWA values the 24 h values for the 0.5–4.0 Hz frequency band are used to normalize. The time blocks used to assess cortical EEG power spectra were; a) the 12 h light and dark periods for baseline values; b) the first 2 h post-SD (12–13 h) (a time when EEG SWA during NREMS epochs is enhanced in mice)(Krueger *et al.*, 2010) and; c) 9–12 h post-SD (20–0 h)(a time when a negative rebound in EEG SWA during NREMS epochs typically occurs in mice); and d) 12 h (12–0 h) post-IL1 β administration (times when EEG SWA during NREMS epochs are attenuated) (Huber *et al.*, 2000; Jhaveri *et al.*, 2007; Krueger *et al.*, 2010).

Experimental Protocols

Experiment #1: Spontaneous sleep in CD73 KO and control mice

After the seven-day acclimation period, spontaneous sleep was determined from 24 h recordings that began at dark onset for both strains of mice. These values also served as a baseline for post-SD recovery sleep.

Experiment #2: Responses to SD

The day immediately after spontaneous sleep recordings were taken, mice were deprived of sleep by the gentle handling method for 6 h prior to dark onset (Szentimai *et al.*, 2007). Immediately thereafter, recovery sleep after the deprivation was determined from 24 h recordings that began at dark onset.

Experiment #3: Responses to IL1 β

Two days after SD recovery, saline (0.2 ml) was injected into the peritoneal cavity between 15–30 min before the beginning of the dark period to serve as the baseline for IL1 β administration. Sleep after the saline injection was determined from 24 h recordings that began immediately after the injection at dark onset. After the baseline recording, mice were

given 400 ng of IL1 β in 0.2 ml of saline ip and sleep was determined from 24 h recordings that began at dark onset.

Experiment #4: mRNA expressions in hypothalamic and somatosensory cortex in response to SD

CD73KO and WT mice were sleep deprived by the gentle-handling method for 6 h prior to dark onset or allowed to sleep *ad libitum*. Mice were then sacrificed at dark onset ($n = 9-10$ per group). Mice were decapitated, their brains were removed, and dissected as follows. The hypothalamus was dissected from the ventral surface of the brain posterior to the optic chiasm and anterior to the mammillary bodies (Bregma -0.34 mm to -2.70 mm) (Paxinos & Franklin, 2001). Bilateral somatosensory cortices were dissected (Bregma 1.98 mm to -2.46 mm) ± 2.0 mm in the dorsal-ventral directions with respect to the parietotemporal ridge. These brain regions were then immediately snap frozen in liquid nitrogen and stored at -80°C . Real-time polymerase chain reaction (RT-PCR) was used to analyze mRNA levels of CD73, CD39, CNT2, ADK, ADA, ENT1, adenosine receptor A1 (AdorA1), adenosine receptor A2a (AdorA2a), P2X7R, purine type Y1 receptor (P2Y1R), IL1 β , and TNF α . The primers used are shown in Table 1. The tissue was homogenized, RNA was extracted using Trizol reagent, and cDNA was prepared and analyzed for the genes of interest. Cyclophilin A mRNA was used to compare expression of mRNAs as previously described (Krueger et al., 2010). These data were analyzed by the delta threshold cycle (C_t) value method (Taishi et al., 2004).

Statistical Analysis

The analysis of variance (ANOVA) statistical analyses used for the sleep data are outlined in Table 2. Two mice were excluded from EEG power analysis because of artifacts in their EEG. In addition to the ANOVAs, post-hoc comparisons were made with independent or paired t-tests when appropriate for the sleep data. RT-PCR data were analyzed with independent t-tests for strain (i.e., *ad libitum* sleep) or for each strain's response to SD (*ad libitum* sleep vs. SD). $P < 0.05$ was considered to be significant.

Results

Experiment #1

Spontaneous sleep architecture—Spontaneous NREMS had a diurnal rhythm in both CD73 KO and WT mice [time: $F_{(1, 16)} = 7.011$, $p = 0.018$] with a greater duration of NREMS, expressed as a percentage of total recording time, occurring during the light period compared to the dark period (Table 3). The amount of NREMS during the dark period in CD73 KO mice was significantly greater than that occurring in WT mice [$t_{(1, 16)} = 2.505$, $p = 0.023$](Table 3). The amount of NREMS exhibited during the dark period in CD73 KO mice was also greater than other C57BL/6 strains (i.e., C57BL/6N and C57BL/6NTAC) assessed in our laboratory and those of C57BL/6 strains reported in the literature (Olivadoti & Opp, 2008)(Table 3). CD73 KO mice had lower individual NREMS episode durations during spontaneous sleep compared to WT mice [strain: $F_{(1, 16)} = 6.112$, $p = 0.025$; CD73 KO: light (1.21 ± 0.12 min), dark (1.08 ± 0.07 min) vs. WT: light (1.37 ± 0.16 min), dark (1.56 ± 0.09 min)]. NREMS episode frequency was greater in the light period than the dark period for both strains [light vs. dark: $F_{(1, 16)} = 6.303$, $p = 0.023$; CD73 KO: light ($324.44 \pm 0.20.93$), dark (296.00 ± 20.11) vs. WT: light (236.22 ± 18.87), dark (194.33 ± 17.63)]. CD73 KO mice had a greater frequency of NREMS episodes than WT mice resulting in enhanced NREMS durations [$F_{(1, 16)} = 15.992$, $p = 0.001$].

Duration of spontaneous REMS in both strains had the typical diurnal rhythm characteristic of mouse sleep [time: $F_{(1, 16)} = 4.645$, $p = 0.047$](Table 3). There were no significant

differences between CD73 KO and WT mice for spontaneous REMS values. The frequency [CD73 KO: light (68.44 ± 9.45), dark (44.78 ± 6.52) vs. WT: light (68.00 ± 9.81), dark (34.89 ± 3.77)] and duration [CD73 KO: light (0.92 ± 0.05 min), dark (0.90 ± 0.06 min) vs. WT: light (0.85 ± 0.07 min), dark (0.74 ± 0.08 min)] of individual REMS episodes also were similar in both strains. Further, significant enhanced REMS episode frequencies occurred during the light periods compared to dark periods [light vs. dark: $F_{(1, 16)} = 32.522$, $p < 0.001$].

EEG SWA during NREMS displayed a diurnal rhythm across the 24 h recording period with higher values occurring during the dark period than during the light [time: $F_{(1, 14)} = 5.574$, $p = 0.033$]. The diurnal rhythm of EEG SWA during NREMS was similar to that previously described for mice (Kapas *et al.*, 2008; Krueger *et al.*, 2010). NREMS EEG SWA (0.5–4.0 Hz) in CD73 KO mice did not significantly differ from that in WT mice during either the dark or light cycles (data not shown), although there were strain differences in EEG power spectra (1.0–2.5 Hz) (Figure 1).

Compared to WT mice, CD73 KO mice had lower NREMS EEG power within the delta power frequency range, while greater NREMS EEG power in the higher frequency ranges (~10–20 Hz) during light and dark periods [strain $\times$ frequency: $F_{(39, 560)} = 1.703$, $p = 0.006$] (Figure 1A). No significant differences in REMS EEG power were found between strains in the lower frequency ranges (~0–9 Hz) during light and dark periods, but CD73 KO mice had significantly lower REMS EEG power than WT mice in the higher frequency ranges (~10–20 Hz) (Figure 1B).

Experiment #2

Sleep responses after sleep deprivation—NREMS duration increased in WT mice relative to baseline during the first 12 h post-SD [treatment: $F_{(1, 8)} = 67.530$, $p < 0.001$]; this effect was most evident in the first 6 h after SD (Figure 2A). In contrast, in CD73 KO mice, NREMS duration after SD was not significantly different from their baseline NREMS values. However, during the first 12 h dark period after SD, WT and CD73 KO mice spent similar amounts of time in NREMS (340.1 ± 14.11 min vs. 340.3 ± 16.5 min, respectively). SD induced a smaller enhancement in NREMS episode durations in CD73 KO mice than WT mice [$F_{(1, 16)} = 5.084$, $p = 0.039$]. SD reduced NREMS episode frequency during both dark and light periods [$F_{(1, 16)} = 22.707$, $p < 0.001$; CD73 KO: light (-95.44 ± 22.05), dark (-72.22 ± 17.95) vs. WT: light (-33.22 ± 14.58), dark (-5.11 ± 18.21)]. CD73 KO mice had reduced NREMS episode frequencies during both dark and light periods compared to baseline values [strain: $F_{(1, 16)} = 11.608$, $p = 0.004$]. This did not occur in WT mice.

REMS was enhanced after SD in both CD73 KO and WT mice over the 24 h post-treatment recording [$F_{(1, 16)} = 4.859$, $p = 0.042$] (Figure 2B). Duration of REMS in both strains of mice was significantly enhanced during the immediate 12 h following SD. After SD during the first 12 h dark period WT mice spent 50.46 ± 5.23 min in REMS whereas CD73 KO mice spent 54.15 ± 6.42 min in REMS. SD significantly enhanced REMS episode duration in both WT and CD73 KO strains [treatment: $F_{(1, 16)} = 16.650$, $p = 0.001$; CD73 KO: light ($+0.11 \pm 0.06$ min), dark ($+0.18 \pm 0.08$ min) vs. WT: light ($+0.11 \pm 0.08$ min), dark ($+0.31 \pm 0.08$ min)]. REMS episode frequencies were greater during the light period vs. dark period in both strains [$F_{(1, 16)} = 44.572$, $p < 0.001$]. A significant interaction was found where SD enhanced the frequency of REMS episodes during the dark period while reducing REMS episode frequencies during the light period [light vs. dark $\times$ treatment: $F_{(1, 16)} = 7.221$, $p = 0.016$; CD73 KO: light (-5.89 ± 10.90), dark ($+4.56 \pm 6.64$) vs. WT: light (-10.89 ± 9.01), dark ($+14.22 \pm 7.39$)].

NREMS EEG SWA was significantly enhanced during the first 2 h post-SD in WT mice [$t_{(1, 7)} = 2.708$, $p = 0.030$] but not CD73 KO mice. NREMS EEG SWA was reduced during 9–12 h post-SD (i.e., 20–0 h) [$F_{(1, 14)} = 7.312$, $p = 0.017$]. This reduction in NREMS EEG SWA after SD was only found in WT mice [$F_{(1, 7)} = 12.293$, $p = 0.010$]. Between strains or within each strain, no significant differences between spontaneous sleep and post-SD were found for NREMS EEG SWA during waking episodes (data not shown).

In WT mice, the SD-enhanced NREMS EEG spectral power during the first 2 h post-SD period occurred in the 1.0–2.5 Hz range [treatment $\times$ frequency: $F_{(39, 560)} = 4.281$, $p < 0.001$]. In contrast, the parallel responses in CD73 KO mice in the 1.0–2.5 Hz range to SD were significantly diminished compared to WT mice [strain: $F_{(1, 39)} = 6.804$, $p = 0.009$], although smaller enhancements were found in higher frequencies for CD73 KO mice [treatment $\times$ strain: $F_{(1, 560)} = 9.892$, $p = 0.002$; treatment $\times$ frequency $\times$ strain: $F_{(39, 560)} = 1.962$, $p = 0.001$]. WT mice exhibited a significant negative rebound in EEG NREMS power (0.5–20 Hz) during 7–12 h post-SD compared to spontaneous sleep (h 20–0; Figure 3C). CD73 KO mice did not display this effect [treatment $\times$ strain effect: $F_{(1, 560)} = 38.734$, $p < 0.001$]. After SD, no significant differences in waking EEG spectral power were found between strains or for each strain (data not shown).

Experiment #3

Sleep responses to saline—In both strains duration of sleep after saline injections was similar to that occurring during spontaneous sleep (Table 3). The duration of NREMS exhibited a diurnal rhythm after saline injections in both CD73 KO and WT mice with greater durations of NREMS occurred during the light period than during the dark period [time: $F_{(1, 16)} = 5.042$, $p = 0.039$] (Table 3). Duration of NREMS was greater in the CD73 KO mice than in the WT mice after saline injections [$F_{(1, 16)} = 5.471$, $p = 0.033$]. WT and CD73 KO mice had greater NREMS episode durations during the light vs. dark period after saline injections [$F_{(1, 16)} = 6.743$, $p = 0.019$; CD73 KO: light (1.89 ± 0.13 min), dark (1.58 ± 0.15 min) vs. WT: light (1.95 ± 0.22 min), dark (1.63 ± 0.15 min)]. Compared to WT mice, CD73 KO mice had greater frequencies of NREMS episodes over the 24 h period after saline administration [strain: $F_{(1, 16)} = 5.471$, $p = 0.033$; CD73 KO: light (212.38 ± 14.48), dark (200.00 ± 14.38) vs. WT: light (195.56 ± 19.65), dark (181.44 ± 21.58)]. No other differences in the frequency of NREMS episodes were found after saline injections for either mouse strain.

REMS duration also exhibited a diurnal rhythm after saline injections in both strains with greater amounts of REMS occurring during the light vs. dark period [time: $F_{(1, 16)} = 6.902$, $p = 0.018$] (Table 3). No significant differences in REMS episode durations after saline injections were found between strains [CD73 KO: light (1.01 ± 0.05 min), dark (0.88 ± 0.05 min) vs. WT: light (0.89 ± 0.05 min), dark (0.88 ± 0.06 min)]. REMS episodes were more frequent during the light compared to dark period in both strains after saline injections [light vs. dark: $F_{(1, 16)} = 15.304$, $p = 0.001$; CD73 KO: light (65.00 ± 4.69), dark (36.89 ± 5.17) vs. WT: light (58.44 ± 10.30), dark (38.33 ± 5.90)]. This effect was similar to that found during spontaneous sleep.

NREMS EEG SWA varied over the 24 h period with higher values occurring during the dark hours [time: $F_{(1, 14)} = 8.652$, $p = 0.011$] (Figure 5A). There were no significant differences observed between strains in NREMS EEG SWA after saline injections. Further, there were no significant differences in waking EEG SWA between strains after saline injections (data not shown).

Sleep responses to IL1 β —A main effect of IL1 β enhanced NREMS duration was found in both CD73 KO and WT mice during the 24 h post injection period compared to saline

injections [treatment: $F_{(1, 16)} = 15.283$, $p = 0.001$] (Figure 4A). This effect was greatest during the first 4 h post-IL1 β . WT and CD73 KO mice had similar enhanced NREMS duration responses to IL1 β administration above their respective saline control values during this 4 h period (65.5 ± 15.2 and 56.44 ± 16.2 min of excess NREMS, respectively). Compared to saline injections, no significant differences in NREMS episode duration or the frequency of these episodes were found after IL1 β treatment in dark or light periods, [CD73 KO: light (-2.11 ± 5.47 min), dark ($+6.78 \pm 10.73$ min) vs. WT: light (-7.22 ± 10.84 min), dark ($+11.33 \pm 14.47$ min); CD73 KO: light (-5.11 ± 5.31), dark ($+33.44 \pm 31.54$) vs. WT: light (-1.33 ± 4.91), dark ($+14.22 \pm 31.80$), respectively]. Further, no significant differences between strains were found for NREMS episode duration responses to IL1 β .

Compared to saline control, no significant differences in REMS duration were found after IL1 β administration (Figure 4B). REMS episode durations after IL1 treatments were similar to saline treatments for both strains [CD73 KO: light ($+0.08 \pm 0.06$ min), dark ($+0.00 \pm 0.07$ min) vs. WT: light (-0.04 ± 0.03 min), dark (-0.02 ± 0.07 min)]. REMS episode frequencies were greater during the light period compared to the dark period in both strains [$F_{(1, 16)} = 14.972$, $p = 0.001$; [CD73 KO: light (-5.11 ± 5.31), dark (-3.00 ± 3.88 min) vs. WT: light (-1.33 ± 4.91), dark (-5.22 ± 6.92)]. No other significant differences were found for REMS episode frequencies.

Compared to the saline control, a reduction in NREMS EEG SWA occurred for 12 h after the IL1 injection [treatment: $F_{(1, 14)} = 5.067$, $p = 0.041$] (Figure 5A). Specifically, IL1 β reduced NREMS EEG SWA in CD73 KO mice but not in WT mice [$F_{(1, 7)} = 5.996$, $p = 0.044$]. No significant differences in waking EEG SWA were found between strains after saline or IL1 β treatments (data not shown).

NREMS EEG power in the 0.5–20 Hz band was reduced in the first 12 h post-IL1 β administration (12–0 h) compared to saline administration [treatment: $F_{(1, 560)} = 48.314$, $p < 0.001$] (Figure 5B). The reduction in NREMS EEG power was largely contained within the delta frequency range [treatment $\times$ frequency: $F_{(39, 560)} = 2.167$, $p < 0.001$], and these EEG power reductions occurred in CD73 KO but not WT mice [treatment $\times$ strain: $F_{(1, 560)} = 9.590$, $p = 0.002$]. IL1 β did not significantly alter waking EEG spectral power in either strain (data not shown).

Experiment #4

In CD73 KO mice sacrificed at dark onset after *ad libitum* sleep conditions, CD73 mRNA was not detectable, although it was present in the WT control mice in all brain areas examined (data not shown). In contrast, there were few differences in mRNA levels between strains in these control conditions regardless of the brain area examined for the RNA species examined with the exception of ADA. ADA mRNA levels were significantly higher in the somatosensory cortex of CD73KO mice compared to WT mice (data not shown).

Several mRNA species were up-regulated after SD, although these differences were relatively small and seldom exceeded a 2-fold increase (Table 4). Similar to baseline results, after SD, CD73 mRNA was not detectable in the CD73 KO mice. In the WT mice, CD73 mRNA was significantly reduced in the hypothalamus but not in the somatosensory cortex. A few mRNA species changed as a consequence of SD in both strains of mice. CD39 mRNA significantly increased in both strains after SD but only in the somatosensory cortex. In both strains, somatosensory cortex and hypothalamic ADA and CNT2 mRNAs significantly increased and decreased after SD, respectively. SD significantly increased IL1 β mRNA in the somatosensory cortex of WT mice but not in CD73 KO mice. Conversely, hypothalamic IL1 β mRNA was significantly reduced in CD73 KO mice after SD but not in WT mice. TNF α mRNA levels significantly increased in the somatosensory cortex after SD

in both strains. SD significantly reduced hypothalamic TNF α mRNA, although this finding was only significant in WT mice. AdorA1 and AdorA2a mRNAs were not significantly altered by SD in the somatosensory cortex or hypothalamus of WT mice. In contrast in the CD73 KO mice, AdorA1 mRNA was significantly increased after SD in the somatosensory cortex but not the hypothalamus. AdorA2a mRNA was not changed after SD for CD73 KO mice in either brain region. In the WT mice, P2X7R mRNA was significantly reduced in the hypothalamus after SD but not in the somatosensory cortex. Hypothalamic and somatosensory cortex P2X7R mRNA was not significantly altered by SD in the CD73 KO mice. P2Y1R mRNA was not changed by SD in the hypothalamus for either strain. In the somatosensory cortex, CD73 KO mice had significantly increased P2Y1R after SD, while the WT mice exhibited a similar but non-significant trend.

Discussion

Current results implicate CD73 in sleep regulation to the extent that mice lacking CD73 have more spontaneous NREMS than WT controls and do not exhibit the typical sleep rebound after acute sleep loss exhibited in WT mice (Hu *et al.*, 2011; Krueger *et al.*, 2010; Szentirmai *et al.*, 2007). Nevertheless, the capacity of CD73 KO mice to respond to somnogens is evidenced by their robust sleep responses to IL1 β . Differences between strains in gene expression and the pattern of genes up-regulated by SD are not notable except for CD73 mRNA suggesting that sleep differences are due to the lack of CD73. Collectively, these data are consistent with the hypothesis that adenosinergic mechanisms are involved in sleep regulation, although they likely extend beyond adenosine to include ATP catabolic and signaling mechanisms.

Studies involving kidney tissue and epithelial cells derived from CD73 KO mice suggest that CD73 KO mice have a reduced capacity to enhance extracellular adenosine levels than WT mice (Colgan *et al.*, 2006). Seemingly, brain extracellular adenosine in CD73 KO mice may be lower than in WT mice after SD. However, the measurement of extracellular adenosine *in vivo* is controversial (Clapp-Lilly *et al.*, 1999; Grabb *et al.*, 1998; Latini & Pedata, 2001). Thus, we did not pursue the measurement of brain extracellular adenosine. Further, sleep affects many variables that change adenosine metabolism including responses to tissue damage inflicted by the dialysis probe, cerebral blood flow, brain temperature, cell activity, and stressors. These issues render adequate controls very difficult because one is unable to isolate sleep/sleep loss as the independent variable. Nevertheless, using microdialysis, sleep loss is associated with an enhancement of the flux of extracellular adenosine (Porkka-Heiskanen *et al.*, 1997; 2000). In those experiments, inadequate recovery time from probe-induced injury and local inflammatory responses was allowed and adenosine molar values were not provided. Regardless, those measurements were interpreted as demonstrating a correlation of enhanced extracellular adenosine with increased sleep pressure. Further, there is a large pharmacological literature suggesting that adenosine receptor agonists promote sleep and EEG SWA while adenosine receptor antagonists inhibit sleep (Benington *et al.*, 1995; Porkka-Heiskanen & Kalinchuk, 2010).

The enhanced spontaneous NREMS occurring in CD73 KO mice compared to C57BL/6 mice, albeit small, seems to be real. We compared nighttime values of duration of NREMS occurring in the CD73 KO mice to additional C57BL/6 strains recorded under the same conditions in other experiments in our laboratory and to those reported in other studies (Olivadoti & Opp, 2008)(Table 3). All the WT C57BL/6 strains slept less than the CD73 KO mice during the night. Spontaneous NREMS was fragmented in CD73 KO mice, with shorter more frequent episodes compared with WT mice. However, after SD, both strains increased the duration of NREMS episodes although the frequency of NREMS episodes was reduced in CD73 KO mice. This NREMS consolidation, along with the small increase in

NREMS EEG delta power during the subsequent first two hours of recovery that occurred in the CD73 KO mice, indicates that some mechanism of sleep homeostasis, e.g. extracellular adenosine derived from intracellular adenosine, is still functioning in CD73 KO mice, albeit at a reduced effectiveness. Regardless, current results cannot exclude the possibility that the enhanced spontaneous NREMS in the CD73 KO mice might be attributable to other abnormalities in these mice that manifest during the nighttime hours.

Cell activity is associated with ATP release into the extracellular space (Krueger, 2008). During SD neuron activity is enhanced (Vyazovskiy *et al.*, 2009), suggesting an even greater transient increase in extracellular ATP levels during prolonged wakefulness. Extracellular ATP is catabolized to adenosine via CD39 and CD73 and this process would be disrupted in the CD73 KO mice. Indeed, we found that CD39 mRNA expression is enhanced after SD in CD73 KO mice suggesting mechanisms upstream of adenosine are involved in sleep regulation. Extracellular ATP also acts directly on P2 receptors to enhance the processing and release of sleep regulatory substances from glia such as IL1 β (Bianco *et al.*, 2005; Ferrari *et al.*, 2006), TNF α (Hide *et al.*, 2000; Suzuki *et al.*, 2004), or BDNF (Trang *et al.*, 2009; Verderio *et al.*, 2006). These substances, including ATP agonists, in turn promote sleep (Krueger *et al.*, 2010). It seems likely, although speculative, that the lack of CD73 would be associated with enhanced extracellular AMP/ATP levels that would in turn enhance sleep via P2 receptors and the associated release of sleep regulatory substances; such an interpretation is consistent with the enhanced spontaneous NREMS occurring during the dark period in CD73 KO mice compared to C57BL/6 mice. However, it is inconsistent with the absence of sleep NREMS rebound in the CD73 KO mice after SD. Nevertheless, unknown developmental compensation mechanisms in the CD73 KO mice limit the interpretation of these results.

Adenosine, acts on purine type 1 (P1) receptors. Which P1 receptors that are involved in sleep regulation is debated (Benington & Heller, 1995; Blanco-Centurion *et al.*, 2006; Huang *et al.*, 2005; Methippara *et al.*, 2005; Stenberg *et al.*, 2003). Although AdoraA1 and AdoraA2a agonists are somnogenic (Benington & Heller, 1995; Coleman *et al.*, 2006; Methippara *et al.*, 2005; Scammell *et al.*, 2001), the duration of spontaneous NREMS and SD-associated NREMS rebound in AdoraA1 KO or AdoraA2a KO mice are similar to WT mice (Huang *et al.*, 2005; Stenberg *et al.*, 2003). Further, though caffeine is believed to inhibit sleep by acting on P1 receptors, caffeine inhibits sleep in *Drosophila* by attenuating cAMP phosphodiesterase activity independent of P1 receptors (Wu *et al.*, 2009). As mentioned, extracellular ATP acts through P2 receptors to activate IL1 β and TNF α (Monif *et al.*, 2010). IL1 β and TNF α , in turn, activate nuclear factor-kappa B to enhance AdoraA2a transcription (Morello *et al.*, 2006). Thus, the current CD73 KO mice experiments emphasize the ambiguities surrounding the interactions of adenosine, purine receptors, and cytokines in sleep regulation.

That IL1 β -induced sleep responses were greater than those induced by SD, suggests that the lack of sleep responses to SD in CD73 KO mice was not due to ceiling effects. Regardless, the similar IL1 β -induced sleep responses in CD73 KO and WT mice suggest that extracellular adenosine derived from extracellular ATP is not part of the sleep response to IL1 β . However, adenosine derived from intracellular stores could be involved in sleep responses to IL1 β (Luk *et al.*, 1999). Further, it is possible that the mechanisms responsible for SD-induced sleep responses are independent of those mechanisms responsible for enhanced sleep following systemic administration of IL1. Although sleep rebound is attenuated in mice lacking the IL1 Type I receptor and the tumor necrosis factor 55 kD receptor (Baracchi and Opp 2008) and anti-IL1 antibodies attenuate sleep rebound in rats and rabbits (reviewed Krueger *et al.*, 2008), in the current study, exogenous IL1 given systemically to CD73 KO mice decreased EEG delta power during NREMS whereas SD

enhanced it, suggesting independent mechanisms are at play. Thus we cannot exclude a ceiling effect as an explanation for the absence of sleep rebound in the CD73 KO mice.

Since rebounds in REMS after SD and spontaneous duration of REMS were not different between CD73 KO mice and WT controls, CD73 might not be involved in the regulation of REMS. AdorA1 KO and AdorA2a KO mice both exhibit similar spontaneous REMS and REMS responses to sleep loss as their WT controls further suggesting that adenosine has little role in regulating REMS (Huang *et al.*, 2005; Stenberg *et al.*, 2003). Nevertheless, AdorA1 agonists can suppress or enhance REMS (Benington *et al.*, 1995; Alam *et al.*, 2009), while AdorA1 antagonists suppress REMS suggesting adenosine receptors have an influence on REMS (Thakkar *et al.*, 2008; Alam *et al.*, 2009). Further, AdorA2a agonists and AdorA2a antagonists have the capacity to alter REMS (Coleman *et al.*, 2006; Satoh *et al.*, 1996, 2006).

EEG SWA is often used as a measure for sleep intensity (Borbely, 1982), e.g., it increases after SD (Pappenheimer *et al.*, 1975). CD73 KO mice exhibited a small increase in NREMS EEG delta power during the first two hours post-SD, although this effect was much smaller than that occurring in WT mice (Hu *et al.*, 2011; Krueger *et al.*, 2010; Szentirmai *et al.*, 2007). In WT mice, the EEG SWA increases were followed by negative rebounds in NREMS EEG SWA thereby confirming prior results (Jhaveri *et al.*, 2007; Krueger *et al.*, 2010; Olivadoti & Opp, 2008). However, CD73 KO mice lacked the subsequent negative NREMS EEG SWA rebound. We do not have a parsimonious explanation for this, perhaps it is related to adenosine-modulated cerebral blood flow (Kusano *et al.*, 2010). Blood flow affects EEG power and the direction of these effects is brain region-dependent (Holfe *et al.*, 1997). In contrast to the current results, mice lacking the P2X7 receptor had much attenuated NREMS EEG SWA during the first 2 h post-SD period but the subsequent negative EEG power phase remained intact (Krueger *et al.*, 2010). Regardless, current results are consistent with prior conclusions that sleep duration is regulated independently from NREMS EEG SWA (Davis *et al.*, 2011).

SD-induced reductions in CNT2 mRNA and enhanced ADA mRNA occurred in both strains; these responses confirm previous reports (Guillen-Gomez *et al.*, 2004; Mackiewicz *et al.*, 2000). Further, ENT1 and ADK mRNA levels were not altered by SD in either strain (data not shown); our data are consistent with the lack of differences in spontaneous sleep or responses to SD found in heterozygous mice with a disrupted ADK gene compared to WT controls (Palchykova *et al.*, 2010). Basheer and colleagues reported increased basal forebrain AdorA1 mRNA levels after SD in rats (Basheer *et al.*, 2001, 2007). That we found increased somatosensory cortex AdorA1a mRNAs in CD73 KO but not WT mice after SD suggests that AdorA1a involvement in sleep regulation varies by brain region. Our finding that SD increases cortical IL1 β mRNA and TNF α mRNA in WT mice is consistent with previous reports (Mackiewicz *et al.*, 1996; Taishi *et al.*, 1998, 1999). The lack of enhanced cortical IL1 β mRNA may have contributed to the lack of sleep rebound after SD in CD73 mice. However, the SD-induced reduction in hypothalamic IL1 β and TNF α mRNAs in both strains of mice is opposite that which occurs in rats where SD enhances these hypothalamic mRNAs (Taishi *et al.*, 1998, 1999). Opposing findings in the expression of cortical mRNAs vs. hypothalamic mRNAs have been previously reported (Mackiewicz *et al.*, 2000).

Differences in cortical and hypothalamic gene expression may be a consequence of activity-dependency. There are parts of the hypothalamus, e.g. the ventrolateral preoptic area, that show increased neuronal activity during sleep (Sherin *et al.*, 1996). Expressions of cortical IL1 β and TNF α are activity-dependent (Churchill *et al.*, 2008; Hallett *et al.*, 2010). For instance, rat whisker stimulation enhances TNF α expression in corresponding whisker barrel cortices (Churchill *et al.*, 2008). Such stimulation or direct application of TNF α to a

cortical column enhances the probability of the cortical column, receiving the enhanced afferent input or TNF α , being in a sleep-like state (Churchill *et al.*, 2008; Rector *et al.*, 2005). These data suggest that sleep is initiated within local neuronal/glia networks and that it is activity-dependent. Glio- and neuro-transmissions are associated with ATP release; this is fundamentally a local use-dependent process. The resulting transient increases in extracellular ATP via P2 receptors enhance sleep regulatory substance levels, e.g., IL1 β and TNF α , and thereby translate glial and neuronal activity into a longer-lived sleep regulatory signal (reviewed Krueger *et al.*, 2008). That CD73 plays a critical role in this process, albeit complex is evident from current results.

In conclusion, CD73 is involved in sleep regulation. The attenuated sleep responses to sleep loss in CD73 KO mice compared further indicates the role of adenosine and mechanisms upstream of adenosine such as extracellular ATP and AMP in sleep regulation.

Acknowledgments

We thank Dr. Linda F. Thompson, Oklahoma Medical Research Foundation, Oklahoma City, for providing CD73 knockout mice and for reviewing a draft of this manuscript. We also thank Dr. Chris Davis for help in harvesting and dissecting brain samples for RNA analyses. This work was supported in part by the National Institutes of Health, grant numbers (NS025378, NS031453).

References

- Alam, MdN; Kumar, S.; Rai, S.; Methippara, M.; Szymusiak, R.; McGinty, D. Role of adenosine A1 receptor in the perifornical-lateral hypothalamic area in sleep-wake regulation in rats. *Brain Research*. 2009; 1304:96–104. [PubMed: 19781535]
- Basheer R, Halldner L, Alanko L, McCarley RW, Fredholm BB, Porkka-Heiskanen T. Opposite changes in adenosine A1 and A2A receptor mRNA in the rat following sleep deprivation. *Neuroreport*. 2001; 12:1577–1580. [PubMed: 11409719]
- Basheer R, Bauer A, Elmenhorst D, Ramesh V, McCarley RW. Sleep deprivation upregulates A1 adenosine receptors in the rat basal forebrain. *Neuroreport*. 2007; 18:1895–1899. [PubMed: 18007182]
- Benington JH, Heller HC. Restoration of brain energy metabolism as the function of sleep. *Prog Neurobiol*. 1995; 45:347–360. [PubMed: 7624482]
- Benington JH, Kodali SK, Heller HC. Stimulation of A1 adenosine receptors mimics the electroencephalographic effects of sleep deprivation. *Brain Research*. 1995; 692:79–85. [PubMed: 8548323]
- Bianco F, Pravettoni E, Colombo A, Schenk U, Moller T, Matteoli M, Verderio C. Astrocyte-derived ATP induces vesicle shedding and IL-1 beta release from microglia. *J Immunol*. 2005; 174:7268–7677. [PubMed: 15905573]
- Blanco-Centrurion C, Xu M, Murillo-Rodriguez E, Gerashchenko D, Shiromani AM, Salin-Pascual RJ, Hof PR, Shiromani PJ. Adenosine and sleep homeostasis in the basal forebrain. *J Neurosci*. 2006; 26:8092–8100. [PubMed: 16885223]
- Borbely AA. A two process model of sleep regulation. *Hum Neurobiol*. 1982; 1:195–204. [PubMed: 7185792]
- Baracchi F, Opp MR. Sleep-wake behavior and responses to sleep deprivation of mice lacking both interleukin-1 beta receptor 1 and tumor necrosis factor-alpha receptor 1. *Brain Behav. Immun*. 2008; 22:982–993. [PubMed: 18329246]
- Churchill L, Rector DM, Yasuda K, Fix C, Rojas MJ, Yasuda T, Krueger JM. Tumor necrosis factor alpha: activity dependent expression and promotion of cortical column sleep in rats. *Neuroscience*. 2008; 156:71–80. [PubMed: 18694809]
- Clapp-Lilly KL, Roberts RC, Duffy LK, Irons KP, Yong H, Drew KL. An ultrastructural analysis of tissue surrounding a microdialysis probe. *J Neurosci Methods*. 1999; 90:129–142. [PubMed: 10513596]

- Coleman CG, Baghdoyan HA, Lydic R. Dialysis delivery of an adenosine A2A agonist into the pontine reticular formation of C57BL/6J mouse increases pontine acetylcholine release and sleep. *J Neurochem.* 2006; 96:1750–1759. [PubMed: 16539690]
- Colgan SP, Eltzschig HK, Eckle T, Thompson LF. Physiological roles for ecto-5'-nucleotidase (CD73). *Purinergic Signal.* 2006; 2:351–360. [PubMed: 18404475]
- Davis CJ, Clinton JM, Jewett KA, Zielinski MR, Krueger JM. Delta wave power: an independent sleep phenotype or epiphenomenon? *J Clin Sleep Med.* 2011; 7:S16–S18. [PubMed: 22003323]
- Ferrari D, Pizzirani C, Adinolfi E, Lemoli R, Curti A, Idzko M, Panther E, Di Virgilio F. The P2X7 receptor: A key player in IL-1 processing and release. *J Immunol.* 2006; 176:3877–3883. [PubMed: 16547218]
- Fields RD, Burnstock G. Purinergic signaling in neuron-glia interactions. *Nat Rev Neurosci.* 2006; 7:423–436. [PubMed: 16715052]
- Grabb MC, Sciotti VM, Gidday JM, Cohen SA, Van Wylen DGL. Neurochemical and morphological responses to acutely and chronically implanted brain microdialysis probes. *J Neurosci Methods.* 1998; 82:25–34. [PubMed: 10223512]
- Guillen-Gomez E, Calbet M, Casado J, de Lecea L, Soriano E, Pastor-Anglada M, Burgaya F. Distribution of CNT2 and ENT1 transcripts in rat brain: selective decrease of CNT2 mRNA in the cerebral cortex of sleep-deprived rats. *J Neurochem.* 2004; 90:883–893. [PubMed: 15287894]
- Hallett H, Churchill L, Taishi P, De A, Krueger JM. Whisker stimulation increases expression of nerve growth factor- and interleukin-1 beta-immunoreactivity in the rat somatosensory cortex. *Brain Res.* 2010; 1333:48–56. [PubMed: 20338152]
- Hide I, Tanaka M, Inoue A, Nakajima K, Kohsaka S, Inoue K, Nakata Y. Extracellular ATP triggers tumor necrosis factor- α release from rat microglia. *J Neurochem.* 2000; 75:965–972. [PubMed: 10936177]
- Holfe N, Paus T, Reutens D, Fiset P, Gotman J, Evans AC, Jones BE. Regional cerebral blood flow changes as a function of delta and spindle activity during slow wave sleep in humans. *J Neurosci.* 1997; 17:4800–4808. [PubMed: 9169538]
- Hu WP, Li JD, Colwell CS, Zhou QY. Decreased REM sleep and altered circadian sleep regulation in mice lacking vasoactive intestinal polypeptide. *Sleep.* 2011; 34:49–56. [PubMed: 21203371]
- Huang ZL, Qu WM, Eguchi N, Chen JF, Schwarzschild MA, Fredholm BB, Urade Y, Hayaishi O. Adenosine A(2A), but not A(1), receptors mediate the arousal effect of caffeine. *Nat Neurosci.* 2005; 8:858–859. [PubMed: 15965471]
- Huber R, Deboer T, Tobler I. Topography of EEG dynamics after sleep deprivation in mice. *J Neurophysiol.* 2000; 84:1888–1893. [PubMed: 11024081]
- Jhaveri KA, Trammell RA, Toth LA. Effect of environmental temperature on sleep, locomotor activity, core body temperature and immune responses of C57BL/6J mice. *Brain Behav Immunity.* 2007; 21:975–987.
- Kapas L, Bohnet S, Traynor T, Majde JA, Szentirmai E, Magrath P, Taishi P, Krueger JM. Spontaneous and influenza virus-induced sleep are altered in TNF double receptor deficient mice. *J. Appl. Physiol.* 2008; 105:1187–1198. [PubMed: 18687977]
- Krueger JM. The role of cytokines in sleep regulation. *Curr Pharm Des.* 2008; 14:3408–3416. [PubMed: 19075717]
- Krueger JM, Rector DM, Roy S, Van Dongen HPA, Belenky G, Panksepp J. Sleep as a fundamental property of neuronal assemblies. *Nature Revs Neurosci.* 2008; 9:910–919. [PubMed: 18985047]
- Krueger JM, Taishi P, De A, Davis CJ, Winters BD, Clinton J, Szentirmai E, Zielinski MR. ATP and the purine type 2 X7 **receptor affect sleep. *J Appl Physiol.* 2010; 109:1318–1327. [PubMed: 20829501]
- Kumar V, Sharma A. Adenosine: an endogenous modulator of innate immune system with therapeutic potential. *Eur J Pharmacol.* 2009; 616:7–15. [PubMed: 19464286]
- Kusano Y, Echeverry G, Miekisiak G, Kulik TB, Aronhime SN, Chen JF, Winn HR. Role of adenosine A2 receptors in regulation of cerebral blood flow during induced hypotension. *J Cereb Blood Flow Metab.* 2010; 30:808–815. [PubMed: 19920844]
- Latini S, Pedata F. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J Neurochem.* 2001; 79:463–484. [PubMed: 11701750]

- Luk WP, Zhang Y, White TD, Lue FA, Wu C, Jiangt C, Zhang L, Moldofsky H. Adenosine: a mediator of interleukin-1 β -induced hippocampal synaptic inhibition. *J Neuroscience*. 1999; 19:4238–4244.
- Mackiewicz M, Nikonova EV, Bell CC, Galante RJ, Zhang L, Geiger JD, Pack AI. Activity of adenosine deaminase in the sleep regulatory areas of the rat CNS. *Brain Res Mol Brain Res*. 2000; 80:252–255. [PubMed: 11038259]
- Mackiewicz M, Shockley KR, Romer MA, Galante RJ, Zimmerman JE, Naidoo N, Baldwin DA, Jensen ST, Churchill GA, Pack AI. Macromolecule biosynthesis: a key function of sleep. *Physiol Genomics*. 2007; 31:441–457. [PubMed: 17698924]
- Mackiewicz M, Sollars PJ, Ogilvie MD, Pack AI. Modulation of IL-1 β gene expression in the rat CNS during sleep deprivation. *Neuroreport*. 1996; 7:529–533. [PubMed: 8730822]
- Masino SA, Kawamura M, Wasser CA, Pomeroy LT, Ruskin DN. Adenosine, ketogenic diet and epilepsy: the emerging therapeutic relationship between metabolism and brain activity. *Curr Neuropharmacol*. 2009; 7:257–268. [PubMed: 20190967]
- Methippara MM, Kumar S, Alam MN, Szymusiak R, McGinty D. Effects on sleep of microdialysis of adenosine A1 and A2a receptor analogs into the lateral preoptic area of rats. *Am J Physiol Regul Integr Comp Physiol*. 2005; 289:R1715–R1723. [PubMed: 16109808]
- Monif M, Burnstock G, Williams DA. Microglia: proliferation and activation driven by the P2X7 receptor**. *Int J Biochem Cell Biol*. 2010; 42:1753–1756. [PubMed: 20599520]
- Morello S, Ito K, Yamamura S, Lee KY, Jazrawi E, Desouza P, Barnes P, Cicala C, Adcock IM. IL-1 β and TNF- α regulation of the adenosine receptor (A2A) expression: differential requirement of NF- κ B binding to the proximal promoter. *J Immunol*. 2006; 177:7173–7183. [PubMed: 17082635]
- Olivadoti MD, Opp MR. Effects of I.C.V. administration of interleukin-1 on sleep and body temperature of interleukin-6-deficient mice. *Neuroscience*. 2008; 153:338–348. [PubMed: 18367337]
- Palchikova S, Winsky-Sommerer R, Shen HY, Boison D, Gerling A, Tobler I. Manipulation of adenosine kinase affects sleep regulation in mice. *J Neurosci*. 2010; 30:13157–13165. [PubMed: 20881134]
- Pappenheimer JR, Koski G, Fencel V, Karnovsky ML, Krueger J. Extraction of sleep-promoting factor S from cerebrospinal fluid and from brains of sleep-deprived animals. *J Neurophysiol*. 1975; 38:1299–1311. [PubMed: 1221075]
- Paxinos, G.; Franklin, KBJ. *Mouse brain in stereotaxic coordinates* (deluxe edition). second edition. Academic Press; 2001.
- Porkka-Heiskanen T, Kalinchuk AV. Adenosine, energy metabolism and sleep homeostasis. *Sleep Med Rev*. 2010; 15:123–135. [PubMed: 20970361]
- Porkka-Heiskanen T, Strecker RE, Thakkar M, Bjorkum AA, Greene RW, McCarley RW. Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. *Science*. 1997; 276:1265–1268. [PubMed: 9157887]
- Porkka-Heiskanen T, Strecker RE, McCarley RW. Brain site specificity of extracellular concentration changes during sleep deprivation and spontaneous sleep: an in vivo microdialysis study. *Neuroscience*. 2000; 99:507–517. [PubMed: 11029542]
- Rector DM, Topchiy IA, Carter KM, Rojas MJ. Local functional state differences between rat cortical columns. *Brain Res*. 2005; 1047:45–55. [PubMed: 15882842]
- Satoh S, Matsumura H, Suzuki F, Hayaishi O. Promotion of sleep mediated by the A2a-adenosine receptor and possible involvement of this receptor in the sleep induced by prostaglandin D2 in rats. *Proc Natl Acad Sci USA*. 1996; 93:5980–5984. [PubMed: 8650205]
- Satoh S, Matsumura H, Kanbayashi T, Yoshida Y, Urakami T, Nakajima T, Kimura N, Nishino S, Yoneda H. Expression pattern of FOS in orexin neurons during sleep induced by an adenosine A2A receptor agonist. *Behav Brain Res*. 2006; 170:277–286. [PubMed: 16621044]
- Scammell TE, Gerashchenko DY, Mochizuki T, McCarthy MT, Estabrooke IV, Sears CA, Saper CB, Urade Y, Hayaishi O. An adenosine A2a agonist increases sleep and induces Fos in ventrolateral preoptic neurons. *Neuroscience*. 2001; 107:653–663. [PubMed: 11720788]

- Sherin JE, Shiromani PJ, McCarley RW, Saper CB. Activation of ventrolateral preoptic neurons during sleep. *Science*. 1996; 271:216–219. [PubMed: 8539624]
- Stenberg D, Litnius E, Halldner L, Johansson B, Fredholm BB, Porkka-Heiskanen T. Sleep and its homeostatic regulation in mice lacking the adenosine A1 receptor. *J Sleep Res*. 2003; 12:283–290. [PubMed: 14633239]
- Su D, Gu Y, Wang Z, Wang X. Lidocaine attenuates proinflammatory cytokine production induced by extracellular adenosine triphosphate in cultured rat microglia. *Anesth Analg*. 2010; 111:768–774. [PubMed: 20686009]
- Suzuki T, Hide I, Ido K, Kohsaka S, Inoue K, Nakata Y. Production and release of neuroprotective tumor necrosis factor by P2X7 receptor-activated microglia. *J Neurosci*. 2004; 24:14715–14719. [PubMed: 14715932]
- Szentirmai E, Kapas L, Sun Y, Smith RG, Krueger JM. Spontaneous sleep and homeostatic sleep regulation in ghrelin knockout mice. *Am J Physiol Regul Integr Comp Physiol*. 2007; 293:R510–R517. [PubMed: 17409264]
- Taishi P, Gardi J, Chen Z, Fang J, Krueger JM. Sleep deprivation increases the expression of TNF alpha mRNA and TNF 55kD receptor mRNA in rat brain. *The Physiologist*. 1999; 42:A4.
- Taishi P, Chen Z, Obal F Jr, Hansen MK, Zhang J, Fang J, Krueger JM. Sleep-associated changes in interleukin-1 β mRNA in the brain. *J Interferon Cytokine Res*. 1998; 18:793–798. [PubMed: 9781819]
- Taishi P, De A, Alt J, Gardi J, Obal F Jr, Krueger JM. Interleukin-1 β stimulates growth hormone-releasing hormone receptor mRNA expression in the rat hypothalamus in vitro and in vivo. *J Neuroendocrinol*. 2004; 16:113–118. [PubMed: 14763997]
- Takasaki I, Takarada S, Tatsumi S, Azegami A, Yasuda M, Fukuchi M, Tabuchi A, Kondo T, Tabuchi Y, Tsuda M. Extracellular adenosine 5'-triphosphate elicits the expression of brain-derived neurotrophic factor exon IV mRNA in rat astrocytes. *Glia*. 2008; 56:1369–1379. [PubMed: 18649393]
- Thakkar MM, Engemann SC, Walsh KM, Sahota PK. Adenosine and the homeostatic control of sleep: effects of A1 receptor blockade in the perifornical lateral hypothalamus on sleep-wakefulness. *Neuroscience*. 2008; 153:875–880. [PubMed: 18440150]
- Thompson LF, Eltzschig HK, Ibla JC, Van De Wiele CJ, Resta R, Morote-Carcia JC, Colgan SP. Crucial role for ecto-5'-nucleotidase (CD73) in vascular leakage during hypoxia. *J Exp Med*. 2004; 200:1395–1405. [PubMed: 15583013]
- Trang T, Beggs S, Wan X, Salter MW. P2X4-receptor-mediated synthesis and release of brain-derived neurotrophic factor in microglia is dependent on calcium and p38-mitogen-activated protein kinase activation. *J Neurosci*. 2009; 29:3518–3528. [PubMed: 19295157]
- Verderio C, Bianco F, Blanchard MP, Bergami M, Canossa M, Scarfone E, Matteoli M. Cross talk between vestibular neurons and Schwann cells mediates BDNF release and neuronal regeneration. *Brain Cell Biol*. 2006; 35:187–201. [PubMed: 17957483]
- Verderio C, Matteoli M. ATP in neuron-glia bidirectional signaling. *Brain Res Rev*. 2011; 66:106–114. [PubMed: 20451555]
- Vyazovskiy VV, Olcese U, Lazimy YM, Faraguna U, Esser SK, Williams JC, Cirelli C, Tononi G. Cortical firing and sleep homeostasis. *Neuron*. 2009; 63:865–878. [PubMed: 19778514]
- Wu MN, Ho K, Crocker A, Yue Z, Koh K, Sehgal A. The effects of caffeine on sleep in *Drosophila* require PKA activity, but not the adenosine receptor. *J Neurosci*. 2009; 29:11029–11037. [PubMed: 19726661]

Figure 1. NREMS and REMS EEG power spectra (0.5–20 Hz frequency range) in WT (○) and CD73 KO (●) mice during dark and light periods. CD73 KO mice had lower NREMS EEG power within the delta range and greater NREMS EEG power within the higher frequencies (~10–20 Hz range) during both dark and light periods than WT mice. CD73 KO mice had lower REMS EEG power in the higher frequencies (~10–20 Hz range) than WT mice. Bars and (*) indicate significant differences between strains at $p < 0.05$.

Figure 2.

A: NREMS responses to SD were greater in WT mice (○) compared to that of CD73 KO mice (●). CD73 KO mice did not demonstrate significant alterations in NREMS after SD compared to spontaneous sleep values. B: Both WT and CD73 KO mice had similar enhanced REMS durations after SD. Bar and (*) indicate significant difference in sleep parameter changes from baseline and after SD between strains. Significance was set at $p < 0.05$.

Figure 3.

A: WT and CD73 KO mice had enhanced NREMS EEG SWA after SD (●) compared to spontaneous sleep (○). In contrast, CD73 KO mice did not exhibit the negative rebound in NREMS EEG SWA that was found in WT mice. B: NREMS EEG power was significantly enhanced predominantly in the delta frequency range during the first 2 h post-SD in both strains. C: CD73 KO mice did not exhibit the typical negative rebound in NREMS EEG power during 9–12 h post-SD (i.e., 20–0 h) after SD. Bars and (*) indicate significant differences between spontaneous sleep and responses to SD for each strain. Significance was set at $p < 0.05$.

Figure 4.

A: WT (○) and CD73 KO mice (●) had similar enhanced NREMS responses to IL1 (400 ng, i.p.) compared to saline responses. B: REMS responses to IL1 were also similar between strains. (*) indicate significant difference in sleep parameter changes from saline and after IL1 between strains. Significance was set at $p < 0.05$.

Figure 5.

CD73 KO mice exhibited significant reductions in NREM EEG SWA after IL1 injections (●) compared to saline (○), which were not evident in WT mice. Compared to saline, IL1 significantly reduced NREMS EEG power in CD73 KO mice but not WT mice. Bars and (*) indicate significant differences between responses to saline and IL1 for each strain set at $p < 0.05$.

Table 1

Primers used for RT-PCR

Gene	5'-3'	Primer	Size (bp)
CD73	F	CTGACCCAGGAAGACTACCT	133
	R	GCTGAGGAAGACATGGCTCT	
CD39	F	CATCCAAGCATCACCAGACT	154
	R	CATCCAAGCATCACCAGACT	
ADK	F	CTATGCTGCCGAGAACAACA	226
	R	TGCCTCTTCGAGTTCACCTT	
ADA	F	AAGCATTTGGCATCAAGGTC	116
	R	CATAGCCACCACGGTCTTCT	
CNT2	F	CAAACCCCGTGGTCTCTTAC	112
	R	TCCCCTCACATCCACTCCTA	
ENT1	F	TGCCTCTTCGAGTTCACCTT	91
	R	CCGTGAAGATGAAGCAGACA	
Adora1	F	ATCCCTCTCCGTACAAGACAGT	120
	R	ACTCAGGTTGTTCCAGCCAAAC	
Ador2a	F	CCGAATTCCACTCCGGTACA	131
	R	CAGTTGTTCCAGCCCAGCAT	
IL1	F	CAACCAACACGTGATATTCTCCATG	152
	R	GATCCACACTCTCCAGCTGCA	
TNF	F	GGGACAGTGACCTGGACTGT	77
	R	GCTCCAGTGAATTCGAAAG	
P2X7R	F	GCCAACTATGAACGGCTCTT	133
	R	TACCCATGATTCTCCCTGA	
P2Y1	F	GGCAGGCTCAAGAAGAAGAA	220
	R	AGATCAGCACCAAAGGGATG	

Table 2

ANOVAs used for sleep data analyses

Comparisons	ANOVA Used	Independent Factor	Repeated Factor
NREMS, REMS, and EEG SWA during spontaneous sleep or after saline administration	2-way ANOVA across 24 h on 2 h blocks	Strain	Time
NREMS and REMS episode durations and frequencies during spontaneous sleep or after saline administration	2-way ANOVA	Strain	Light vs. dark
Spontaneous NREMS and REMS EEG power spectrum	3-way ANOVA	Strain, frequency	Light vs. dark
NREMS, REMS, and EEG SWA during spontaneous sleep or after saline administration	3-way ANOVA across 24 h on 2 h blocks	Strain	Time, treatment
NREMS and REMS episode durations and frequencies	3-way ANOVA	Strain	Light vs. dark, treatment
NREMS and waking EEG power spectrum treatments (i.e., spontaneous sleep vs. SD or saline vs. IL1)	3-way ANOVA	Strain, frequency	Treatment

Spontaneous sleep in C57BL/6 mice compared to CD73 KO mice for 12 h light and dark periods.

Table 3

		% NREMS (Current Study)	% NREMS (Literature)	Range +	% REMS (Current Study)	% REMS (Literature)
		Spontaneous	Saline	Range +	Spontaneous	Saline
WT	Dark	34.7 ± 2.1 *	36.3 ± 3.0	22–37	3.5 ± 0.4	4.0 ± 0.7
	Light	49.9 ± 2.1	52.2 ± 3.5	49–57	7.9 ± 1.2	4.7 ± 0.8
CD73 KO	Dark	43.8 ± 3.0 **++	42.8 ± 3.8 **++		5.7 ± 1.0	7.6 ± 1.1
	Light	52.7 ± 3.0	54.1 ± 1.7		8.8 ± 1.4	9.2 ± 0.9

⁺ includes 3 in house studies using C57BL/6 strains of mice.
* indicates numerical values in % of time in state for the 12 h block.
⁺⁺ indicates means ± SEMs are outside the literature range means and means ± SEMs of current study controls.
^{**} indicates significant differences from current study controls ($p < 0.05$).

Table 4WT and CD73 KO *ad libitum* sleep and SD CD73 and related brain region mRNA levels.

	WT	WT SD	CD73 KO	CD73 KO SD
Sctx				
CD73	1.01 ± 0.05	0.98 ± 0.11	Not detectable	Not detectable
CD39	1.02 ± 0.07	1.28 ± 0.05 *	1.02 ± 0.06	1.19 ± 0.06 *
CNT2	1.03 ± 0.08	0.62 ± 0.06 *	1.04 ± 0.10	0.49 ± 0.06 *
Ada	1.02 ± 0.08	1.97 ± 0.14 *	1.01 ± 0.04	1.70 ± 0.14 *
AdorA1	0.97 ± 0.01	1.00 ± 0.05	1.00 ± 0.02	1.50 ± 0.06 *
AdorA2a	1.00 ± 0.04	1.09 ± 0.10	1.06 ± 0.13	0.81 ± 0.07
P2X7R	1.06 ± 0.13	0.87 ± 0.09	1.06 ± 0.12	1.36 ± 0.11
P2Y1R	1.04 ± 0.09	1.31 ± 0.13	1.02 ± 0.07	1.31 ± 0.10 *
IL1	1.02 ± 0.06	1.32 ± 0.10 *	0.97 ± 0.06	1.12 ± 0.10
TNF	1.07 ± 0.14	1.51 ± 0.15 *	1.05 ± 0.12	2.38 ± 0.32 *
Hypothalamus				
CD73	1.02 ± 0.05	0.81 ± 0.06 *	Not detectable	Not detectable
CD39	1.01 ± 0.04	1.06 ± 0.07	1.02 ± 0.06	1.02 ± 0.16
CNT2	1.02 ± 0.08	0.55 ± 0.07 *	1.11 ± 0.16	0.51 ± 0.06 *
Ada	1.00 ± 0.07	1.23 ± 0.08 *	1.03 ± 0.08	1.35 ± 0.10 *
AdorA1	1.02 ± 0.06	0.98 ± 0.06	1.03 ± 0.08	1.39 ± 0.36
AdorA2a	1.00 ± 0.02	0.95 ± 0.04	1.03 ± 0.09	1.09 ± 0.07
P2X R	1.04 ± 0.04	0.77 ± 0.07 *	1.05 ± 0.11	1.11 ± 0.12
P2Y1R	1.00 ± 0.05	0.94 ± 0.08	1.06 ± 0.11	1.22 ± 0.15
IL1	1.04 ± 0.10	0.80 ± 0.10	1.01 ± 0.14	0.62 ± 0.10 *
TNF	1.01 ± 0.09	0.63 ± 0.06 *	1.05 ± 0.12	0.79 ± 0.12

Data (Mean ± SEM) are expressed as fold change in sleep deprived mice relative to *ad libitum* sleep for both WT and CD73 KO strains.* Significant differences in mRNA levels after SD were set at $p < 0.05$.