Title: ATP and the purine type 2 X7 receptor affect sleep

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Running head: ATP via P2 receptors regulates sleep

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Abstract

Sleep is dependent upon prior brain activities, e.g. after prolonged wakefulness sleep rebound occurs. These effects are mediated, in part, by humoral sleep regulatory substances such as cytokines. However, the property of wakefulness activity that initiates production and release of such substances and thereby provides a signal for indexing prior waking activity is unknown. We propose that extracellular ATP, released during neuro- and glio-transmission and acting via purine type 2 (P2) receptors, is such a signal. ATP induces cytokine release from glia. Cytokines in turn affect sleep. We show here that a P2 receptor agonist, BzATP, increased non-rapid eye movement sleep (NREMS) and electroencephalographic (EEG) delta power while two different P2 receptor antagonists, acting by different inhibitory mechanisms, reduced spontaneous NREMS in rats. Rat P2X7 receptor protein varied in the somatosensory cortex with time of day and P2X7 mRNA was altered by interleukin-1 treatment, sleep deprivation and with the time of day in the hypothalamus and somatosensory cortex. Mice lacking functional P2X7 receptors had attenuated NREMS and EEG delta power responses to sleep deprivation but not to interleukin-1 treatment compared to wild type mice. Data are consistent with the hypothesis that extracellular ATP, released as a consequence of cell activity and acting via P2 receptors to release cytokines and other sleep regulatory substances, provides a mechanism by which the brain could monitor prior activity and translates it into sleep.

Keywords

Cytokine, purine receptor, interleukin-1, sleep deprivation
Introduction

It has been known for 100 years that waking activity induces the accumulation of sleep regulatory substances (SRSs) in the brain (23, 31). Such substances enhance or inhibit sleepiness and sleep. Several SRSs, including brain cytokines such as interleukin-1 beta (IL1) and tumor necrosis factor alpha (TNF), are well-characterized for their sleep regulatory roles in health and disease (reviewed in 29, 27). For instance, injection of IL1 or TNF into humans (thereby mimicking their endogenous accumulation during sleep loss) induces symptoms associated with sleep loss such as, sleepiness, fatigue, excess sleep, cognitive dysfunction, and enhanced sensitivity to kindling and pain stimuli (reviewed in 29). Although SRSs provide a mechanism by which the brain can keep track of prior activity (reviewed in 36), the literature has failed to characterize what it is about wakefulness that causes enhanced SRS activity. We posit that it is neuro- and glio-transmission-associated release of ATP (29).

ATP is co-released with neurotransmitters including sites within multiple areas of brain (reviewed in 6, 7). ATP is also released from glia cells into the extracellular space (reviewed in 15). Independent studies demonstrated the ATP-enhanced release of cytokines from immunocytes via purine P2 receptors (reviewed in 14). In the brain, ATP induces IL1 (3, 50), TNF (19) and brain-derived neurotrophic factor (BDNF) (53) release from glia via purine P2 receptors. Extracellular ATP, released from synapses, glia or immunocytes is important for IL1 processing and release (14, 41). ATP stimulation of microglia via P2X7 receptors, a purine P2 receptor, enhances TNF production and the neuro-protective effects of TNF in neuron-glia co-cultures exposed
to glutamate (44). Since these cytokine gliotransmitters are also putative SRSs we posit that the brain keeps track of prior sleep/wake activity via ATP interaction with purine P2 receptors and the subsequent induction of cytokine release. Conversely, it seems likely that sleep would affect P2 receptor expression and that cytokines may provide a feedback signal to alter P2 receptors. In this report, we show that an ATP agonist promotes non-rapid eye movement sleep (NREMS) while ATP antagonists inhibit NREMS in rats. We also provide evidence that P2X7 receptor expression and protein levels vary in brain with the sleep-wake cycle and are affected by injections of exogenous IL1. Finally, we show that mice lacking the P2X7 receptor have attenuated sleep responses to sleep deprivation but not to IL1. Results are consistent with the hypothesis that release of ATP during neuro- and glio-transmission initiates a series of steps that ultimately provide a mechanism by which the brain tracks its prior state history.

**Materials and Methods:**

**Agents**

The P2 receptor agonist 2′(3′)-O-(4-Benzoylbenzoyl) adenosine 5′-triphosphate triethylammonium (BzATP) and the P2 receptor antagonist periodate oxidized ATP (OxATP) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). The P2 receptor antagonists, A438079 and AZ10606120 were purchased from Tocris Bioscience (Ellisville, MO). Murine and human IL1 were purchased from R&D Systems (Minneapolis, MN).
Animals and Surgery

Experimental procedures were approved by the Washington State University Institutional Animal Care and Use Committee and were in compliance with the National Institutes of Health guidelines.

Rats:

Male Sprague-Dawley rats (250–300 g) from Taconic Farms (Germantown, NY) were kept on a 12:12-hr light/dark cycle (lights on at 00:00 h) at 22 ± 2°C ambient temperature. Surgeries were performed using ketamine/xylazine (87 and 13 mg/kg, respectively) anesthesia. Stainless-steel screws for electroencephalographic (EEG) recording were implanted over the left frontal (5 mm anterior and 2 mm lateral from bregma), and the right parietal lobes (5 mm posterior and 6 mm lateral from bregma) and a ground electrode was over the left occipital cortex (11 mm posterior and 4 mm lateral from bregma). The EEG signal used for analyses was that with the least amount of signal artifact. An electromyogram (EMG) electrode was implanted into the nuchal muscles. The leads from the EEG and EMG electrodes were routed to a plastic pedestal (Plastics One, Roanoke, VA). The EEG leads were attached to the skull with dental cement (Patterson Dental Supply, Saint Paul, MN). An intracerebroventricular (icv) guide cannula was implanted (0.8mm posterior, 1.5mm lateral from bregma). The patency of the icv guide cannulae was verified by the icv injection of 40 ng of angiotensin II (Sigma-Aldrich) in 4 µl isotonic NaCl (30) 3-4 days after the surgery. This treatment evokes an immediate drinking response. The rats with positive drinking
responses were allowed at least 1 week to recover from the surgery before experiments commenced.

**Mice:**

Male P2X7 receptor homozygous knockout (P2X7 KO) and wild type control (WT) mice were purchased from Jackson Laboratories (Bar Harbor, ME, strains B6.129P2-P2rx7<tm1Gab>/J and C57BL/6J respectively). Animals were maintained in sound attenuated environmental chambers at an ambient temperature of 29° C ± 2° C (mouse thermo-neutral temperature), a 12:12 h light-dark cycle (lights on 00:00, lights out 12:00), and free access to food and water. Mice were housed in individual cages with cedar shaving bedding provided. Mouse genotypes were confirmed by Transnetyx Inc. (Memphis, TN) from tail-tip samples. Mice were 10 to 19 weeks old at the time of experiments.

Mice were anesthetized using intraperitoneal (ip) injections of ketamine and xylazine (87 and 13 mg/kg respectively). The animals were provided a stainless steel wire EMG electrode in the nuchal muscle and 3 gold-plated wire EEG electrodes (Plastics One) over the parietal and frontal cortices as previously described (45, 46). As with rats, the EEG signal with the least amount of movement artifact was used. All electrode leads were routed to a plastic pedestal socket (Plastics One) and fixed to the skull with dental cement. Animals were allowed to recover after surgery for at least 7 days before experimental recordings. During the recovery period, animals were connected to the recording cables and handled daily to habituate them to the experimental conditions.
Recording and Analyses

The EEG and EMG were recorded as previously described (46, 47). Recording cables were connected to the amplifiers via commutators (Plastics One). EEG and EMG data were amplified using polygraph amplifiers (Grass-Telefactor, Inc., West Warwick, RI) then digitized at 128 Hz. EEG signals were filtered below 0.1 Hz and above 40 Hz. EMG signals were filtered below 100 Hz and above 4000 Hz. Sleep-wake activity was recorded and analyzed for 24 h after each treatment except in Experiment 1 where only 22 h were recorded. Vigilance states were determined visually in 10 s epochs using Sleep Sign for Animal software (Kissei Comtec, Nagano, Japan) and employing previously described criteria (26). Briefly, NREMS was characterized by high amplitude EEG slow waves in the 0.5 to 4 Hz range and REMS was characterized by very regular low amplitude waves with a pronounced theta component (6 to 9 Hz) and a lack of EMG activity; whereas waking was characterized by less regular low amplitude EEG waves in a variety of frequencies and EMG activity. Sleep data are presented as minutes spent in NREMS or REMS in 2 h or 12 h time bins.

In addition, the EEG waveforms were analyzed by fast Fourier transformation using two types of analyses. First, delta power (0.5-4 Hz) during NREMS was determined. Within each NREMS epoch, delta wave (0.5-4 Hz) [slow-wave amplitude (SWA)] power was determined. EEG SWA values obtained during control recording periods were averaged for each animal and used as a reference value to normalize data across the 24 h data collection period for both the baseline day and experimental day as previously described (26). Values (µV^2) obtained during NREMS for each animal for
each 2 h time bin during control or experimental recordings were expressed as a percentage of the reference value. The second type of analysis was power spectra analysis. On the baseline day, EEG power spectra were calculated in 1.0 Hz bins in the 0.5-20 Hz frequency range for the entire 24-h period. EEG power on the baseline and experimental day was expressed as percentage of total baseline power in 0.1 Hz bins. The time blocks analyzed for power spectra varied between experiments; in most cases, the 0-6 h post-injection period was analyzed. In some cases, the time blocks selected were those within which maximum sleep duration effects occurred.

Experimental Protocols

**Experiment 1: Effects of BzATP on Sleep in Rats.**

*Dark onset injections:*

Three groups of rats (n=8 for each group) received 4 µl of pyrogen free sterile saline and on a separate experimental day one dose of BzATP (0.04, 0.4 or 4.0 nmol; one dose per group) icv during the last 30 min of the light period. EEG and EMG were recorded for 22 h. One rat from the 0.04 nmol BzATP dose was excluded from the EEG SWA and EEG power analysis due to EEG artifacts. *Light onset injections:*

Another three groups of rats were used in this experiment and were subjected to the same protocol as that used at dark onset except saline or BzATP [0.04 (n=9), 0.4 (n=7) or 4.0 nmol (n=8)] in 4 µl saline; one dose per group) was injected icv within 30 min of light onset and EEG and EMG were recorded for the next 22 h. One rat from the 0.4 nmol BzATP dose was excluded from the EEG SWA and EEG power analysis due to EEG artifacts. Both light and dark onset injections were done because baseline sleep
is substantially lower during the dark period compared to that occurring during the light period.

**Experiment 2: Effects of P2 receptor antagonists on sleep in rats.**

*A438079:* Three groups of rats (n=8 for each group) received icv injection of saline on the control day and a competitive P2X7 receptor antagonist A438079 (1.0, 10.0 or 1000.0 nmol in 4 µl) on the following day 30 min prior to light onset. EEG was recorded for 24 h after both injections. One rat from the 1.0 nmol group was eliminated from data analyses due to technical problems with its headpiece.

*AZ10606120:* Two groups of rats (n=8 for each group) were treated as in the previous experiment except that 24 h after the saline injection an allosteric P2 receptor antagonist, AZ10606120 (1.0, or 10.0 nmol in 4 µl saline) was administered.

*OxATP:* The experimental protocols for this experiment were identical as those for Experiment 1 except 2 doses of the non-competitive inhibitor OxATP (0.4 or 4.0 nmol, n=8 for each dose), were injected icv at light onset.

**Experiment 3: Diurnal variations of P2X7 receptor mRNA and protein levels and their changes after sleep deprivation and IL1 treatment in rats.**

Male Sprague-Dawley rats (280 – 350 g, from Taconic Farms) were kept on a 12:12 h light:dark cycle at 23°C ambient temperature. Three experiments were performed. Experiment 3a: The diurnal variation of P2X7 receptor mRNA and protein levels were determined at 3 h (mRNA) or 6 h (protein) intervals (n= 6 for each group). Experiment 3b: P2X7 receptor mRNA levels in somatosensory cortex and hypothalamus were
determined after 6 h of sleep deprivation by gentle handing starting at light onset (n=10 for both control and experimental groups). Control animals were allowed to sleep ad
libitum. Samples from control and sleep deprived rats were collected immediately after the end of sleep deprivation (0600h). Experiment 3c: P2X7 receptor mRNA levels were determined 2 h and 5 h after icv injection of IL1 (n=8 for each group). Rats in this experiment were provided an icv guide cannula as described for the sleep experiments. Only rats exhibiting positive drinking response to angiotensin II were used. IL1 injections were performed 6 h after light onset 4 days after the surgery. Four groups of rats were used. Two groups received 4 µl physiological saline and another two groups were injected with a somnogenic dose (2.5 ng) of human (H) recombinant IL-1 (R&D Systems). Somatosensory cortex and hypothalamic samples were harvested 2 h and 5 h after control and IL1 injections then frozen in liquid nitrogen.

Real-time RT-PCR: RNA was extracted using Trizol reagent, cDNA prepared and analyzed for IL1, P2X7 receptor and cyclophilin mRNA levels using semi-quantitative real-time RT-PCR as previously described (49). Data analysis of mean values (2-ΔΔct) and one-way ANOVA followed by a post hoc analysis (Tukey test) was used. For P2X7 receptor primers were: 5’-CTGCAAGATGTCAAAGGTCAAGAC-3’, 5’-
TGGACCTAGGAACCGCTTCTATC-3’(152 bp) and IL1 and cycA primers were those as previously reported (49).

Western blots: Protein was isolated from somatosensory cortical samples taken from the opposite side of the brain as the samples analyzed for mRNA. Four groups sampled every 6 h at 03:00, 09:00, 15:00 and 21:00 h were used. The tissues were
homogenized in RIPA lysis buffer with protease inhibitor mixture and 1% phosphatase inhibitor Cocktail I and II (both from Sigma-Aldrich). After centrifugation the supernatant (20 µg) were subjected to 4-20% SDS-PAGE gel (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes then incubated overnight with a rabbit polyclonal antibody against rat P2X7 receptor (1:15,000, Alomone Labs, Jerusalem, Israel) and 1:40,000 dilution of monoclonal mouse antibody β-actin (Sigma-Aldrich). Immunostaining was performed using ECL blot detection (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The signals from the protein bands were scanned and analyzed using Image J quantification software. The protein levels were normalized with their corresponding β-actin signal and the results were expressed as percent of P2X7 receptor protein / β-actin ratio.

**Experiment 4: Sleep in P2X7 receptor KO mice.**

Spontaneous sleep-wake activity of P2X7 KO and WT mice (n = 8 for each genotype) was recorded over a 24-hour period beginning from dark onset. On the next day, mice were sleep deprived for the 6 h preceding dark onset (06:00-12:00h) by the gentle handling method. When mice were visually determined to be resting, for example by exhibiting nesting behavior, stillness, or eye closure, they were gently prohibited from sleeping using a soft artists paint brush usually by rearranging the bedding material or if necessary softly touching the animal. Starting immediately after deprivation EEG and EMG were recorded for 24 h.

**IL-1 treatment:** The same groups of KO and WT mice used in the sleep deprivation experiment were given 0.1 ml sterile saline ip on the control day and 400 ng of carrier-
free recombinant mouse IL-1 (R&D Systems) in 0.1 ml sterile saline 5 min before dark onset (12:00). EEG and EMG were recorded for 24 h after both injections.

Statistics:

Two- or three-way repeated measures ANOVA was performed on sleep and power spectra data (factors: time, treatment and genotype). Where ANOVA indicated a significant effect, paired, or independent when appropriate, Student’s t-tests were used for post hoc analyses. In Experiments 1, 2 and 4, time spent in sleep (NREMS and REMS) and EEG SWA data were analyzed in 2-h time blocks comparing the baseline day and the experimental days in each group. For the P2X7 receptor antagonist experiments, 12 h blocks of time spent in REMS or NREMS were analyzed using Students paired t-tests. Animals in the first 6 h after the BzATP injections exhibited significant increases in NREMS; therefore, power spectrum for NREMS was analyzed for the first 6-h time block following the injections. Spectral data from the same time block was analyzed after P2-antagonist administration. In addition after OxATP, animals exhibited significant decreases in NREMS during h 3-4 post-injection; therefore, the power spectrum for NREMS was analyzed for this time block and for a time block when NREMS was not affected (22-24 h). Two way ANOVA (repeated factor: treatment, independent factor frequency) was used to analyze the power spectrum data during NREMS. $P < 0.05$ was considered to be significant.
**Results:**

**Experiment 1:**

Dark onset administration of all three doses of BzATP increased duration of NREMS during the first 6-8 hours post-injection (Figure 1 and Table 1). After the 0.04 nmol dose, the rats had about 51 min of extra NREMS during the first 6 hours following injections. During the daylight hours, 12-22 hours post-injection (00:00-10:00 h), the two lower doses of BzATP tested failed to alter duration of NREMS compared to control values. The highest dose, 4.0 nmol BzATP, slightly, but significantly, increased duration of NREMS during this period (243.9±13.4 min on the control day vs 274.4±9.3 min on the experimental day). The lower two doses of BzATP had no effect on REMS during the first 12 hours post-injection but significantly decreased duration of REMS in post-injection hours 12-22 (Figure 1; 00:00-10:00 h). The 4 nmol dose of BzATP increased duration of REMS by about 10 min for the first 4 hours post-injection (12:00-16:00 h) but had no significant effects on duration of REMS during the rest of the recording period.

BzATP induced an increase in EEG SWA during NREMS after all 3 doses although the effects were statistically significant only after the 0.4 nmol dose (Table 1). This dose also decreased EEG SWA during NREMS during the light period 14-16 hours post-injection (02:00-04:00 h). The BzATP-enhanced EEG delta power was evident in the power spectra after the 0.04 nmol dose (Figure 1). Power in higher frequencies after the 0.04 nmol dose was not affected by BzATP nor was power in any frequency significantly altered after the higher two doses of BzATP during the first 6 post-injection
Light onset administration of BzATP increased duration of NREMS (Figure 2). After about a 2 hour delay, NREMS increased after all 3 doses tested (Table 1). The effects were largest after the 0.4 nmol dose, NREMS increased by 62.0 ± 10.6 min after BzATP in the light period (Figure 2). Increased NREMS persisted into the first 4 hours of the dark period (12:00-16:00 h). The other two doses tested 0.04 and 4 nmol, significantly increased duration of NREMS only during post-injection hours 2-4 (Figure 2). The lower dose of BzATP injected during the daytime had no effect on duration of REMS. In contrast, the higher two doses inhibited REMS during the first 12 hours post-injection then promoted duration of REMS during post-injection hours 12-22 although these effects were only significant during post-injection hours 14-16 after the 0.4 nmol dose (Figure 2).

Light onset administration of BzATP increased EEG SWA during NREMS (Table 1). These effects were evident after all 3 doses and were statistically significant after the higher two doses (Figure 2). EEG power spectra analyses indicated that delta power was also significantly increased after all 3 doses during post-injection hours 1-6 (Figure 2). EEG power in other frequency bands was also increased although the effects were much smaller (Figure 2).
Experiment 2:

Light onset administration of the high dose, 1000 nmol, of A438079 (the competitive inhibitor of P2X7 receptors) suppressed the duration of NREMS (Figure 3, top right). A slight but non-significant decrease in NREMS was observed during the light period while the decrease was statistically significant during the subsequent dark period. In the first 24 hours after the A438079 injection, the rats were awake for an additional 60 min as compared to the saline injection day. The two lower doses did not alter NREMS duration or any other sleep parameter (data not shown). Light onset injections of the high dose of A438079 had little effect on EEG power during the first 6 h post-injection. However, it inhibited EEG power during NREMS from 5-8 Hz (theta range), during 7 and 8 hours following injection (data not shown). Conversely, A438079 treatment elicited a robust increase in the EEG power spectrum during the latter part of the dark period (22:00-23:00 h) in the 1-6 Hz range. REMS duration decreased with A438079 administration, but the effect was not significant.

Light onset administration of 10 nmol of the allosteric P2X7 receptor inhibitor, AZ10606120, appeared to suppress NREMS and REMS duration in the dark period (data not shown) but these effects were not statistically significant (p=0.086). Moreover, there were no AZ10606120-induced differences in the power spectra at any frequency across the 24 hour post-injection times.

Light onset administration of the irreversible P2X7 receptor antagonist OxATP inhibited duration of NREMS during the light period and decreased duration of NREMS in the
subsequent dark period although the latter effect was not significant (Figure 3 left top).

For the 24 h post-injection period the rats were awake about 50 minutes more than controls. Light onset injections of OxATP inhibited EEG SWA during NREMS during the first 12 h post-injection period (Figure 3, 00:00-12:00 h). During the first 6 h post-injection EEG power was greater in the 1-5 Hz frequency range after OxATP treatment (Figure 3, lower left).

**Experiment 3:**

There was a diurnal variation in the levels of the P2X7 receptor mRNA in the hypothalamus and the somatosensory cortex (Figure 4). In the somatosensory cortex, higher levels of both the P2X7 receptor mRNA and protein occurred during daylight hours and lower levels at night (Figure 4). These changes paralleled changes in IL1 mRNA although the changes in P2X7 receptor levels were more pronounced.

In the somatosensory cortex after 6 hours of sleep deprivation, P2X7 receptor mRNA levels were reduced to about half of those occurring spontaneously at the time samples were harvested (03:00 h) (Figure 5 left). In contrast, in the hypothalamus sleep deprivation induced a slight but significant increase in P2X7 receptor mRNA.

Similarly, the effects of IL1 on P2X7 receptor expression were opposite in the somatosensory cortex and hypothalamus. Icv injection of a somnogenic IL1 dose enhanced levels of P2X7 receptor mRNA 2 and 5 hours after injection in the
somatosensory cortex (Figure 5 left). In contrast, in the hypothalamus, this dose of IL1 decreased P2X7 receptor mRNA levels at both time points.

Experiment 4:

Spontaneous NREMS, REMS and EEG SWA in P2X7 receptor KO mice did not differ from that observed in WT mice (compare baseline data in Figure 7). Both strains of mice showed the typical diurnal variations in sleep parameters with more NREMS and REMS during the light period and less during the dark. Six hours of sleep deprivation induced different sleep responses in WT and KO mice. The WT mice showed significantly larger increases in NREMS duration after sleep deprivation compared to the P2X7 receptor KO mice; during the first 2 hours after sleep deprivation the WT mice had 30 min of additional NREMS while the P2X7 receptor KO mice only had 11 min of additional NREMS above that observed during baseline recordings (Figure 6 insert; p<0.02, t-test). Although both strains increased duration of REMS in response to sleep deprivation, the REMS duration was not significantly higher in either mouse strain. Both strains responded to sleep deprivation with changes in EEG. The initial increase in EEG SWA values during NREMS were greater in the WT compared to the P2X7 receptor KO mice during the first 2 h post-sleep deprivation (p<0.03, t-test). These initial sleep deprivation-induced increases were followed by decreases in EEG SWA below baseline values that extended for the next 10 hours of recording in both strains of mice as was previously described by Jhaveri et al (24) (Figure 6).
Changes in sleep-wake activity after IL1 injections were similar in the two genotypes (Figure 7). In both strains, NREMS duration significantly increased roughly to the same extent compared to ip saline injections (2-way ANOVA, treatment effect first 12 hours WT: F(1,14)=30.17, p=0.001; KO: F(1,14)=7.13, p=0.018). The duration of REMS was not altered by IL1. IL1 depressed EEG SWA during NREMS in both genotypes, consistent with the previously reported effect of IL1 on EEG SWA in mice (37). There was a reduction in EEG power across the spectrum during the first 2 hours post-injection (Figure 7, 2-way ANOVA treatment effect WT: F(1,14)=6.37, p=0.024; KO: F(1,14)=35.69, p=0.0001). In the subsequent daytime hours, 12 hours after injection, EEG SWA during NREMS and the EEG power spectra returned to control values in both strains.

**Discussion**

The results implicate extracellular ATP and P2 receptors in sleep regulation. We demonstrate that an ATP agonist increases sleep, ATP antagonists inhibit spontaneous sleep, and expression of the P2X7 receptor varies with sleep propensity and, if removed, reduces sleep deprivation-induced sleep responses. Previously extracellular ATP had been posited to be involved in sleep regulation (29) and our preliminary report supported this hypothesis (28). Further, the expression patterns of P2X receptors in Zebra fish and ectonucleotidases in zebra fish and rats suggested a role for extracellular ATP in sleep regulation (1, 2). Collectively, current and prior reports suggest an involvement of extracellular ATP-P2 receptors in sleep regulation.
There is substantial data, primarily pharmacological, in support of a role for adenosine in sleep regulation (reviewed in 4) suggesting the possibility that the somnogenic actions of BzATP result from its release of adenosine. Thus, icv administration of adenosine is reported to enhance duration of NREMS in rats within a limited dose range (39). However, when we administered the most effective dose, 100 nmol adenosine icv, to rats in preliminary studies, there was no effect on sleep. These results suggest that the sleep-promoting actions of BzATP do not result from its catabolism to adenosine. It seems likely that extracellular adenosine’s involvement in sleep regulation is dependent upon its continuous production, some of which would come from extracellular ATP.

The inhibition of NREMS by the P2 antagonists provides evidence that extracellular ATP via P2 receptors is involved in spontaneous sleep. That the P2X7 KO mice had normal spontaneous sleep is likely explained by either developmental compensation or that the multiple P2X and P2Y receptors have redundancy of action. Regardless, P2 agonists and antagonists lack specificity for individual P2 receptors (6, 7); thus the contribution of antagonist inhibition of P2 receptors, in addition to P2X7 receptors, to their sleep inhibitory actions cannot be ruled out.

Halassa et al. (16) described a role for astrocytes in sleep regulation; ATP is released from glia as a consequence of Ca^{2+} activation. Halassa and colleagues (16) interpreted their results as being the consequence of reduced extracellular adenosine derived from extracellular ATP. Their data are consistent with the attenuated EEG
delta power responses to sleep deprivation observed in conditional KO mice lacking
the purine type I A1a receptor. However, the typical enhancements in duration of
NREMS after sleep loss are not affected by either the conditional removal of the A1a
receptor (5) or inhibition of glio-transmission (16). Further, in A1a KO mice (43), or in
drosophila lacking adenosine receptors (55), sleep responses to sleep loss are normal.
These results stand in contrast to current data where the P2X7 KO mice had deficits in
both duration of NREMS and EEG delta power during recovery sleep following sleep
deprivation. Further, if the P2X7 KO mice are given IL1, which is downstream from the
ATP-P2 receptor interaction, they have responses indistinguishable from WT controls.

The conversion of extracellular ATP associated with neuro- and glio-transmission to
adenosine is regulated enzymatically in the brain (9) (Figure 8). Ectonucleoside
triphosphate diphosphohydrolase1 (also called CD39) catalyzes ATP and ADP to
5’AMP (40). Ecto-5’-nucleotidase (also called CD73) catalyzes 5’adenosine
monophosphate to adenosine (9). If extracellular adenosine is involved in sleep, it
most likely is derived from extracellular, rather than intracellular, ATP. For instance,
mice lacking the ability to convert extracellular ATP to adenosine (CD73 KO) have
more spontaneous sleep than WT controls (Zielinski et al. unpublished observations).
Cell metabolism, whether neuronal or glia, does not seem a likely source for
extracellular adenosine because for every degree increase in temperature there is
about a 13% increase metabolism (22). However, fever per se does not increase sleep
(e.g. see 25, 26, 51). Such considerations are consistent with the premise that
extracellular ATP-modulated $K^+$ channels (P2X receptors) mediate cortical network response state (12).

Some of the released extracellular ATP acts on glia P2 receptors and causes the release of IL1, TNF and brain-derived neurotrophic factor (BDNF) as well as additional ATP [see Figure 8 and (29)]. The IL1 precursor, pro-IL1, is processed by caspase-1 and this is triggered by ATP-activation of P2X7 receptors (14, 41). TNF and IL1 released from glia act on adjacent neurons leading to the activation of nuclear factor kappa B (NFkB). NFkB promotes transcription of receptor mRNAs such as the adenosine A1a receptor and the glutamate AMPA receptor-gluR1 mRNAs (29).

Translation of those mRNAs into their respective proteins and the subsequent receptor membrane expression would change the sensitivity of the postsynaptic neuron. This is a proto-typical scaling effect since the expression of postsynaptic receptors is modulated by the activity of the presynaptic neuron (i.e. the amount of released ATP). Thus, the sensitivity of the postsynaptic neuron is scaled to the prior use of the synapse. Indeed, both TNF and BDNF are the two molecules most firmly linked to synaptic scaling (52). We view these ATP-P2-cytokine interactions as key sleep regulatory events (29). Mechanistically, the transient increase in extracellular ATP consequent to neuro- and glio-transmission, by interacting with nearby P2 receptors (10), causes the release of IL1, TNF and BDNF. Such a mechanism offers the possibility for the translation of prior cellular activity into local levels of SRSs (29). SRSs in turn, by a slow process (gene transcription/translation), alter electrical properties of nearby neurons by altering their own production and that of receptor
populations. The SRSs by a fast process (diffusion over short distances) also directly interact with receptors on neurons to alter electrical properties (e.g. 13, 42). Further, some ATP is converted to extracellular adenosine that in turn acts on P1 receptors to influence sleep on a more rapid time scale. These events are posited to happen within local networks (10) and the collective electrical changes result in a shift in input-output relationships within the networks that originally exhibited the enhanced activity, i.e., a state shift. Current results are consistent with this hypothesis.

Sleep is dependent upon the nature of activity during wakefulness as illustrated in the sleep and learning, sleep and exercise, and sleep and infectious disease literatures. Regardless, the specific aspects of our hypothesis for the translation of activity into sleep are thus far tested only indirectly. For instance, we showed that IL1 and TNF expression in neurons is dependent upon prior neuronal activity. From that work we are aware that different patterns of waking stimulation resulted in slightly different patterns of cytokine expression in the cortex (8). Such results suggest that SRS expression/release and subsequent SRS-induced sleep are stimulation-pattern dependent. Indeed, ATP release is disproportionally greater during high frequency stimulation (11, 54). IL1 and TNF are also up-regulated in brain after excessive stimulation as occurs during kindling (38, 56), during sleep deprivation (18, 21, 33, 48), after whisker stimulation in the somatosensory cortex (17), or during brain inflammation (32). The current demonstration of sleep- and IL1-dependent expressions of P2X7 receptor mRNA suggests that sleep mechanisms feedback to influence brain expression of P2X7 receptors. Our results are consistent with the prior report that IL1
transiently enhances P2X7 receptor mRNA and protein in astrocytes (35). Such findings are also consistent with diurnal variations in P2X7 receptor mRNA and protein reported herein to the extent that sleep propensity co-varies with expression. How the differential P2R expressions observed in the somatosensory cortex vs the hypothalamus are integrated into sleep regulation remains unknown.

The well-known wake-promoting actions of caffeine are often thought to result from the antagonist actions of caffeine on P1 receptors. However, caffeine inhibits sleep in drosophila by inhibiting cAMP phosphodiesterase activity independently of adenosine receptors (55). Caffeine also inhibits TNF production via activation of the cAMP/protein kinase A pathway (20); TNF is a well-characterized SRS (29). Finally, ATP-induced currents in PC12 neurons in vitro are also dependent on cAMP-dependent protein kinase (34). Such findings reinforce the posited role for extracellular ATP in sleep regulation and suggest that the paradigm that caffeine promotes wakefulness via adenosine antagonism is incomplete.

In conclusion, the work reported here suggests that extracellular ATP and P2 receptors have a role in sleep regulation and link neuro- and glio-transmission to sleep. Because such actions are also linked to molecules known to be involved in central nervous system connectivity they suggest that sleep mechanisms cannot be separated from neuronal connectivity.
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Bibliography:


Figure Legends:

**Figure 1:** Nighttime (shaded area) injection of BzATP enhanced non-rapid eye movement sleep (NREMS). Rats received one of 3 doses of BzATP as indicated at dark onset. All three doses enhanced duration of NREMS during the initial 6-8 hours post-injection. The highest dose also enhanced duration of NREMS in the subsequent light period. BzATP inhibited duration of rapid eye movement sleep (REMS) during the daylight period occurring 12 hours after the injection after the lower two doses. After the highest dose, REMS was enhanced during the first 4 hours post-injection. BzATP induced enhancement of electroencephalographic slow-wave activity (EEG SWA). BzATP at the lowest dose enhanced EEG delta power; this effect was absent after the higher doses. EEG power values were obtained during the first 6 hours post-injection. Open circles represent control values while closed circles were obtained after BzATP injections. Asterisks indicate significant differences between control and test days (p < 0.05, paired t test).

**Figure 2:** Daytime (light areas) injection of BzATP enhanced duration of rat NREMS EEG SWA during NREMS and EEG spectral power. In contrast, duration of REMS
was decreased during daylight hours but enhanced in the subsequent dark period.

EEG power values were obtained during hours 1-6 post-injections. Open circles indicate values from control recordings while closed circles represent values obtained after BzATP injections. Asterisks indicate significant differences between control and test days (p < 0.05, paired t test).

Figure 3: Both P2X7 receptor antagonists, injected at light onset (black bars or solid circles), suppressed NREMS as compared with saline injections (light bars or open circles). OxATP attenuated SWA during the first 12 hours at the 1-4 Hz frequencies. Although EEG SWA was not significantly affected by A438079 during the first 6 hours, using select 2 hour time bins A438079 induced a biphasic effect initially suppressing but then augmenting the EEG power spectra at different frequencies (see text for details, data not shown). Two smaller doses of A438079 were also tested, 1 nmol and 10 nmol; neither dose affected any of the parameters measured (asterisks indicate p<0.05 between control and test days).

Figure 4: Diurnal variation in the relative amount of interleukin-1 beta (IL1) mRNA and P2X7 receptor mRNA and protein in the hypothalamus and somatosensory cortex. A) Western blot analyses of somatosensory cortex samples showed variation of P2X7 receptor protein levels at 15:00 (3 hours after light onset) or 3:00 h (3 hours after dark onset). No differences in time of day were observed in the standard β-actin. B) Time course of P2X7 receptor mRNA (open triangle), protein (closed triangle and IL1 mRNA (closed circle). The levels of P2X7 receptor protein (somatosensory cortex) and mRNA
(hypothalamus and somatosensory cortex) were greater during the light period than during the dark period (black horizontal bar indicates dark period). Expression of protein levels were normalized to β-actin levels. Statistical significance (p< 0.05) from the values obtained at 21:00 hour is noted by the asterisks; significant differences from dark onset indicated by plus signs; significant differences from values obtained 12 hours prior, represented by pound symbols.

Figure 5: Expression of P2X7 receptor mRNA in the somatosensory cortex and hypothalamus after 6 h of sleep deprivation (SD) or 2 hours and 5 hours after intracerebroventricular injection of 2.5 ng IL1. Left: SD significantly decreased the levels of P2X7 receptor mRNA in the somatosensory cortex and IL1 enhanced P2X7 mRNA levels both 2 and 5 hours after the injection. Right: In the hypothalamus SD enhanced P2X7 mRNA levels and IL1 significantly decreased P2X7 receptor mRNA. Asterisks indicate significant difference from corresponding control (C).

Figure 6: Sleep responses induced by sleep deprivation were attenuated in the P2X7 receptor knockout (KO) mice (open bars) compared to wild type (WT) controls (shaded bars). Mice were deprived of sleep from 06:00-12:00 hours then allowed to sleep ad libitum. WT mice exhibited typical NREMS rebound (right, insert) and EEG SWA enhancements (left) during the first two hours post-deprivation as previously reported (24). In contrast, the P2X7 receptor KO mice had attenuated NREMS and EEG SWA responses during the same period. Both strains had reduced EEG SWA for post-
deprivation hours 6-22 (18:00-10:00 h) as previously reported (24). Asterisks indicate p<0.05 difference between WT and KO mice for NREMS and EEG SWA.

**Figure 7:** IL1 enhances duration of NREMS and inhibits EEG SWA during NREMS after intraperitoneal injections in P2X7 receptor KO and WT mice. Closed circles represent data from IL1-treated mice while the open circles represent values from saline treated control mice. Unlike intracerebroventricular or intravenous injections in rats, intraperitoneal injections of IL1-reduced EEG power as previously reported (37). The responses to IL1 were similar in both genotypes (WT left and KO right) suggesting that IL1’s influence on sleep results from a step downstream from the ATP-P2 receptor interaction. Asterisks indicate p<0.05 difference between baseline and test days.

**Figure 8:** Extracellular ATP involvement in sleep regulation. ATP is released into the extracellular space as a consequence of cell activity during neuro- or glio-transmission. Extracellular ATP then activates P2-receptors, e.g. P2X7, that in turn are involved in interleukin-1 (IL1) processing and release as well as release of other sleep regulatory substances such as tumor necrosis factor and brain-derived neurotrophic factor (not shown). IL1 in turn activates nuclear factor kappa B (NFkB) leading to changes in receptor trafficking. This changes the cell’s long-term sensitivity to neurotransmitters such as glutamate (glu) and to neuromodulators such as adenosine. Extracellular ATP is also catabolized to adenosine via the actions of ectonucleotidases such as CD39 and CD73; this action is faster than the ATP-P2-induces changes in transcription and translation. Both actions of ATP are likely involved in sleep regulation.
Figure 1
Figure 2.
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Figure 4
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Table 1. Statistical results for changes in non-rapid-eye movement sleep (NREMS), rapid-eye movement sleep (REMS), electroencephalographic slow-wav activity (EEG SWA) and EEG power during NREMS in the post-injection hours 1-6 after three doses of BzATP injected dark or light onset. p < 0.05: * = significant difference between baseline and treatment condition.

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