ATP and the purine type 2 X7 receptor affect sleep

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Krueger JM, Taishi P, De A, Davis CJ, Winters BD, Clinton J, Szentirmai É, Zielinski MR. ATP and the purine type 2 X7 receptor affect sleep. J Appl Physiol 109: 1318–1327, 2010. First published September 9, 2010; doi:10.1152/japplphysiol.00586.2010.—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 gliotransmission 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, 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate (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, by sleep deprivation, and with 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 with 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 translate it into sleep.

ATP is coreleased with neurotransmitters including sites within multiple areas of the brain (reviewed in Refs. 6, 7). ATP is also released from glia cells into the extracellular space (reviewed in Ref. 15). Independent studies demonstrated the ATP-enhanced release of cytokines from immunocytes via purine type 2 (P2) receptors (reviewed in Ref. 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 (purine P2 receptors) enhances TNF production and the neuroprotective effects of TNF in neuron-glia cocultures 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 gliotransmission 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 (St. Louis, MO). The P2 receptor antagonists A438079 and AZT066120 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 National Institutes of Health guidelines. Rats. Male Sprague-Dawley rats (250–300 g) from Taconic Farms (Germantown, NY) were kept on a 12:12-h light-dark cycle (lights on at 0000) at 22 ± 2°C ambient temperature. Surgeries were performed with 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 right parietal (5 mm posterior and 6 mm lateral from bregma) lobes, 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, St. Paul, MN). An intracerebroventricular (ICV) guide cannula was implanted (0.8 mm posterior, 1.5 mm lateral from bregma). The patency of the ICV guide cannulas was verified by the ICV injection of 40 ng of angiotensin II (Sigma-Aldrich) in 4 μl of 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 wk 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 with an ambient temperature of 29 ± 2°C (mouse thermoneutral temperature), a 12:12-h light-dark cycle (lights on 0000, lights out 1200), and free access to food and water. Mice were housed in individual cages with cedar shaving bedding provided. Mouse genotypes were confirmed by Transnetx (Memphis, TN) from tail tip samples. Mice were 10–19 wk old at the time of experiments.

Mice were anesthetized with 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 three 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...
Experiment 1: effects of BzATP on sleep in rats. DARK-ONSET INJECTIONS. Three groups of rats \( (n = 8 \text{ for each group}) \) received 4 \( \mu \text{g} \) of pyrogen-free sterile saline and on a separate experimental day one dose of BzATP (0.04, 0.4, or 4.0 \( \text{nml} \); 1 dose/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 because of 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 that saline or BzATP [0.04 \((n = 9)\), 0.4 \((n = 7)\), or 4.0 \((n = 8)\) \( \text{nmol} \)] in 4 \( \mu \text{mol} \) of saline was injected at dark onset by 10.220.33.5 on April 6, 2017 http://jap.physiology.org/ Downloaded from

Table 1. Changes in NREMS, REMS, EEG slow-wave activity, and EEG power during NREMS in postinjection hours 1–6 after BzATP injection

<table>
<thead>
<tr>
<th>BzATP</th>
<th>0.04 nmol</th>
<th>0.4 nmol</th>
<th>4 nmol</th>
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<tr>
<td>Dark-onset administration</td>
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<tr>
<td>NREMS Treatment</td>
<td>( F_{(1,7)} = 1.63 ) &amp; ( F_{(1,7)} = 34.3^* ) &amp; ( F_{(1,7)} = 35.7^* ) &amp; ( F_{(1,7)} = 35.7^* )</td>
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<tr>
<td>Interaction</td>
<td>( F_{(10,70)} = 5.0^* ) &amp; ( F_{(10,70)} = 4.0^* ) &amp; ( F_{(10,70)} = 5.2^* ) &amp; ( F_{(10,70)} = 5.2^* )</td>
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<tr>
<td>REMS Treatment</td>
<td>( F_{(1,7)} = 34.3^* ) &amp; ( F_{(1,7)} = 5.5^* ) &amp; ( F_{(1,7)} = 0.0 ) &amp; ( F_{(1,7)} = 0.0 )</td>
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<tr>
<td>Interaction</td>
<td>( F_{(10,70)} = 4.0^* ) &amp; ( F_{(10,70)} = 4.0^* ) &amp; ( F_{(10,70)} = 4.1^* ) &amp; ( F_{(10,70)} = 4.1^* )</td>
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<tr>
<td>EEG SWA Treatment</td>
<td>( F_{(1,7)} = 5.3 ) &amp; ( F_{(1,6)} = 0.2 ) &amp; ( F_{(1,7)} = 0.1 ) &amp; ( F_{(1,7)} = 0.1 )</td>
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<td>Interaction</td>
<td>( F_{(10,60)} = 3.8^* ) &amp; ( F_{(10,60)} = 3.8^* ) &amp; ( F_{(10,60)} = 3.2 ) &amp; ( F_{(10,60)} = 3.2 )</td>
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<tr>
<td>EEG power hours 1-6 Treatment</td>
<td>( F_{(1,140)} = 2.6 ) &amp; ( F_{(1,120)} = 4.2 ) &amp; ( F_{(1,140)} = 0.6 ) &amp; ( F_{(1,140)} = 0.6 )</td>
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<tr>
<td>Interaction</td>
<td>( F_{(19,140)} = 5.5^* ) &amp; ( F_{(19,120)} = 3.3^* ) &amp; ( F_{(19,140)} = 2.5^* ) &amp; ( F_{(19,140)} = 2.5^* )</td>
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<tr>
<td>Light-onset administration</td>
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<tr>
<td>NREMS Treatment</td>
<td>( F_{(1,8)} = 6.4^* ) &amp; ( F_{(1,6)} = 29.9^* ) &amp; ( F_{(1,7)} = 0.2 ) &amp; ( F_{(1,7)} = 0.2 )</td>
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<tr>
<td>Interaction</td>
<td>( F_{(10,80)} = 2.4^* ) &amp; ( F_{(10,60)} = 2.2^* ) &amp; ( F_{(10,70)} = 2.0^* ) &amp; ( F_{(10,70)} = 2.0^* )</td>
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<td>REMS Treatment</td>
<td>( F_{(1,8)} = 2.1 ) &amp; ( F_{(1,6)} = 0.3 ) &amp; ( F_{(1,7)} = 8.6^* ) &amp; ( F_{(1,7)} = 8.6^* )</td>
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<tr>
<td>Interaction</td>
<td>( F_{(10,80)} = 0.9 ) &amp; ( F_{(10,60)} = 4.4^* ) &amp; ( F_{(10,70)} = 3.4^* ) &amp; ( F_{(10,70)} = 3.4^* )</td>
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<td>EEG SWA Treatment</td>
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<tr>
<td>Interaction</td>
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<td>EEG power hours 1-6 Treatment</td>
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<tr>
<td>Interaction</td>
<td>( F_{(19,152)} = 3.0^* ) &amp; ( F_{(19,144)} = 13.2^* ) &amp; ( F_{(19,133)} = 13.2^* ) &amp; ( F_{(19,133)} = 13.2^* )</td>
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Values are statistical results for changes in non-rapid eye movement sleep (NREMS), rapid eye movement sleep (REMS), electroencephalographic (EEG) slow-wave activity (EEG SWA), and EEG power during NREMS in postinjection hours 1–6 after 3 doses of 2’(3’)-O-(4-benzoylbenzoyl)adenosine 5’-triphosphate (BzATP) injected at dark or light onset. *Significant difference between baseline and treatment condition \( (P < 0.05) \).
IL-1 (R&D Systems). Somatosensory cortex and hypothalamic samples were harvested 2 h and 5 h after control and IL-1 injections and then frozen in liquid nitrogen.

REAL-TIME RT-PCR. RNA was extracted with TRIzol reagent, and cDNA was prepared and analyzed for IL-1, P2X7 receptor, and cyclophilin mRNA levels by semiquantitative real-time RT-PCR as previously described (49). Data analysis of mean values (ΔCt, where Ct is threshold cycle) and one-way ANOVA followed by a post hoc analysis (Tukey test) were used. For P2X7 receptor primers were 5'-CTGCAAGATGTCAAAGGTCAAGAC-3' and 5'-TGGACCTAG-GAACCGCTTCTATC-3' (152 bp), and IL-1 and cyclophilin A primers were those 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 0300, 0900, 1500, and 2100 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) was subjected to 4–20% SDS-PAGE gel (Bio-Rad, Hercules, CA), transferred to nitrocellulose membranes, and 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 with ECL blot detection (GE Healthcare Bio-Sciences, Piscataway, NJ). The signals from the protein bands were scanned and analyzed with Image J quantification software. The protein levels were normalized with their corresponding β-actin signal, and the results were expressed as percentage of P2X7 receptor protein-to-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-h period beginning from dark onset. On the next day, mice were sleep deprived for the 6 h preceding dark onset (0600–1200) 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 with a soft artist’s paintbrush, 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-1β TREATMENT. The same groups of KO and WT mice used in the sleep deprivation experiment were given 0.1 ml of sterile saline IP on the control day and 400 ng of carrier-free recombinant mouse IL-1 (R&D Systems) in 0.1 ml of sterile saline 5 min before dark onset (1200). 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

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**Fig. 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 postinjection hours 1–6. ○, Values from control recordings; ●, values obtained after BzATP injections. *Significant differences between control and test days (P < 0.05, paired t-test).
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 with Student’s 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 after the injections. Spectral data from the same time block were analyzed after P2 antagonist administration. In addition, after OxATP animals exhibited significant decreases in NREMS during postinjection hours 3–4; 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 h after injection (Fig. 1 and Table 1). After the 0.04-nmol dose, the rats had ~51 min of extra NREMS during the first 6 h following injections. During the daylight hours, 12–22 h after injection (0000–1000), the two lower doses of BzATP tested failed to alter duration of NREMS compared with 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 control day vs. 274.4 ± 9.3 min on experimental day). The lower two doses of BzATP had no effect on REMS during the first 12 h after injection but significantly decreased duration of REMS in postinjection hours 12–22 (Fig. 1; 0000–1000). The 4-nmol dose of BzATP increased duration of REMS by ~10 min for the first 4 h after injection (1200–1600) 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 three 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 h after injection (0200–0400). The BzATP-enhanced EEG delta power was evident in the power spectra after the 0.04-nmol dose (Fig. 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 postinjection hours (1200–1800), although after the higher dose there were small, but significant, increases in the 11–15 Hz band.

Light-onset administration of BzATP increased duration of NREMS (Fig. 2). After an ~2-h delay, NREMS increased after all three 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 (Fig. 2). Increased NREMS persisted into the first 4 h of the dark period (1200–1600). The other two doses tested, 0.04 and 4 nmol, significantly increased duration of NREMS only during postinjection hours 2–4 (Fig. 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 h after injection and then promoted duration of REMS during postinjection hours 12–22, although these effects were only significant during postinjection hours 14–16 after the 0.4-nmol dose (Fig. 2).

Light-onset administration of BzATP increased EEG SWA during NREMS (Table 1). These effects were evident after all three doses and were statistically significant after the higher two doses (Fig. 2). EEG power spectrum analyses indicated that delta power was also significantly increased after all three doses during postinjection hours 1–6 (Fig. 2). EEG power in other frequency bands was also increased, although the effects were much smaller (Fig. 2).

Experiment 2

Light-onset administration of the high dose, 1,000 nmol, of A438079 (the competitive inhibitor of P2X7 receptors) sup-

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Fig. 3. Both P2X7 receptor antagonists, injected at light onset (filled bars, •), suppressed NREMS compared with saline injections (open bars, ○). Oxidized ATP (OxATP) attenuated SWA during the first 12 h at the 1–4 Hz frequencies. Although EEG SWA was not significantly affected by A438079 during the first 6 h. With select 2-h 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 (*P < 0.05 between control and test days).
pressed the duration of NREMS (Fig. 3, top right). A slight but nonsignificant decrease in NREMS was observed during the light period, while the decrease was statistically significant during the subsequent dark period. In the first 24 h after the A438079 injection, the rats were awake for an additional 60 min compared with the saline injection day. The two lower doses did not alter NREMS duration or any other sleep parameter (data not shown). Light-onset injection of the high dose of A438079 had little effect on EEG power during the first 6 h after injection. However, it inhibited EEG power during the subsequent dark period, although the latter effect was not significant (Fig. 3, top left). For the 24-h postinjection period these rats were awake ~50 min more than control rats. Light-onset injections of OxATP inhibited EEG SWA during NREMS during the first 12-h postinjection period (Fig. 3, 0000–1200). During the first 6 h after injection EEG power was greater in the 1–5 Hz frequency range after OxATP treatment (Fig. 3, bottom left).

**Experiment 3**

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

In the somatosensory cortex after 6 h of sleep deprivation, P2X7 receptor mRNA levels were reduced to about half of those occurring spontaneously at the time samples were harvested (0300) (Fig. 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 h after injection in the somatosensory cortex (Fig. 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 those observed in WT mice (compare baseline data in Fig. 7). Both strains of mice

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**Fig. 4.** Diurnal variation in the relative amount of interleukin-1β (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 1500 (3 h after light onset) or 0300 (3 h after dark onset). No differences in time of day were observed in the standard, β-actin. B: time course of P2X7 receptor mRNA and protein (▲) and IL1 mRNA (■). 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 was normalized to β-actin levels. *Statistical significance (P < 0.05) from values obtained at 2100; +significant differences from dark onset; #significant differences from values obtained 12 h before.

**Fig. 5.** Expression of P2X7 receptor mRNA in the somatosensory cortex and hypothalamus after 6 h of sleep deprivation (SD) or 2 h and 5 h after intracerebroventricular injection of 2.5 ng of 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 h after the injection. Right: in the hypothalamus SD enhanced P2X7 mRNA levels, and IL1 significantly decreased P2X7 receptor mRNA. *Significant difference from corresponding control (C).
showed the typical diurnal variations in sleep parameters, with more NREMS and REMS during the light period and less during the dark period. Six hours of sleep deprivation induced different sleep responses in WT and KO mice. WT mice showed significantly larger increases in NREMS duration after sleep deprivation compared with P2X7 receptor KO mice; during the first 2 h after sleep deprivation WT mice had 30 min of additional NREMS while P2X7 receptor KO mice only had 11 min of additional NREMS above that observed during baseline recordings (Fig. 6, inset; P < 0.02, t-test). Although both strains increased duration of REMS in response to sleep deprivation, 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 was greater in WT compared with P2X7 receptor KO mice during the first 2 h after 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 h of recording in both strains of mice, as previously described by Jhaveri et al. (24) (Fig. 6).

Changes in sleep-wake activity after IL1 injections were similar in the two genotypes (Fig. 7). In both strains, NREMS duration significantly increased to roughly the same extent compared with IP saline injections [2-way ANOVA, treatment effect first 12 h 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 IL-1 on EEG SWA in mice (37). There was a reduction in EEG power across the spectrum during the first 2 h after injection [Fig. 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 h 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 (28) supported this hypothesis. Furthermore, the expression patterns of P2X receptors in zebrafish and ectonucleotidases in zebrafish and rats suggested a role for extracellular ATP in sleep regulation (1, 2). Collectively, the present and prior reports suggest an involvement of extracellular ATP-P2 receptors in sleep regulation.

There are substantial data, primarily pharmacological, in support of a role for adenosine in sleep regulation (reviewed in...
Ref. 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 Ca2+ 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 gliotransmission (16). Furthermore, 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 the present data, where the P2X7 KO mice had deficits in both duration of NREMS and EEG delta power during recovery sleep following sleep deprivation. Furthermore, if P2X7 KO mice are given IL1, which is downstream from the ATP-P2 receptor interaction, they have responses indistinguishable from WT control mice.

The conversion of extracellular ATP associated with neuro- and gliotransmission to adenosine is regulated enzymatically in the brain (9) (Fig. 8). Ectonucleoside triphosphate diphosphohydrolase 1 (also called CD39) catalyzes ATP and ADP to 5’-adenosine monophosphate (5’-AMP) (40). Ecto-5’-nucleotidase (also called CD73) catalyzes 5’-AMP 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 control mice (Zielinski et al., unpublished observations). Cell metabolism, whether neuronal or glial, does not seem a likely source for extracellular adenosine because for every degree of increase in temperature there is an ~13% increase metabolism (22). However, fever per se does not increase sleep (e.g., see Refs. 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 BDNF as well as additional ATP (see Fig. 8 and Ref. 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-κB (NF-κB). NF-κB 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 prototypical scaling effect since the expression of postsynaptic protein increases or decreases by 13% increase. ATP is released into the extracellular space as a consequence of cell activity during neuro- or gliotransmission. Extracellular ATP then activates P2 receptors (R), e.g., P2X7, that in turn are involved in 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-κB (NF-κB), leading to changes in receptor trafficking. This changes the cell’s long-term sensitivity to neurotransmitters such as glutamate (glu) and to neuropeptides 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-induced changes in transcription and translation. Both actions of ATP are likely involved in sleep regulation. AMPA, amino-3-hydroxy-5-methyl-4-isoxazole propionic acid.
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, 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 gliotransmission, 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 (see, e.g., Refs. 13, 42). Furthermore, some ATP is converted to extracellular adenosine that in turn acts on P1 receptors to influence sleep on a more rapid timescale. 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. The present 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. Nevertheless, 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 on 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 upregulated 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 present demonstration of sleep- and IL1-dependent expressions of P2X7 receptor mRNA suggests that sleep mechanisms feed back 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 here to the extent that sleep propensity covaries with expression. How the differential P2 receptor expression observed in the somatosensory cortex versus 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 gliotransmission 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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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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