Adenosine inhibits glutamatergic input to basal forebrain cholinergic neurons

J. M. Hawryluk, L. L. Ferrari, S. A. Keating, and E. Arrigoni
Department of Neurology, Beth Israel Deaconess Medical Center, Boston, Massachusetts

Submitted 10 June 2011; accepted in final form 15 February 2012

Hawryluk JM, Ferrari LL, Keating SA, Arrigoni E. Adenosine inhibits glutamatergic input to basal forebrain cholinergic neurons. J Neurophysiol 107: 2769–2781, 2012. First published February 22, 2012; doi:10.1152/jn.00528.2011.—Adenosine has been proposed as an endogenous homeostatic sleep factor that accumulates during waking and inhibits wake-active neurons to promote sleep. It has been specifically hypothesized that adenosine decreases wakefulness and promotes sleep recovery by directly inhibiting wake-active neurons of the basal forebrain (BF), particularly BF cholinergic neurons. We previously showed that adenosine directly inhibits BF cholinergic neurons. Here, we investigated 1) how adenosine modulates glutamatergic input to BF cholinergic neurons and 2) how adenosine uptake and metabolism are involved in regulating extracellular levels of adenosine. Our experiments were conducted using whole cell patch-clamp recordings in mouse brain slices. We found that in BF cholinergic neurons, adenosine reduced the amplitude of AMPA-mediated evoked glutamatergic excitatory postsynaptic currents (EPSCs) and decreased the frequency of spontaneous and miniature EPSCs through presynaptic A1 receptors. Thus we have demonstrated that in addition to directly inhibiting BF cholinergic neurons, adenosine depresses excitatory inputs to these neurons. It is therefore possible that both direct and indirect inhibition may synergistically contribute to the sleep-promoting effects of adenosine in the BF. We also found that blocking the influx of adenosine through the equilibrative nucleoside transporters or inhibiting adenosine kinase and adenosine deaminase increased endogenous adenosine inhibitory tone, suggesting a possible mechanism through which adenosine extracellular levels in the basal forebrain are regulated.

IN THE LAST EIGHTY YEARS, a large number of studies have been directed toward identifying the endogenous sleep factors that could build during the waking period to drive the need for sleep and then be dissipated by sleep itself (Achermann and Borbely 2003; Borbely and Tobler 1985). Among several endogenous sleep factors, one of the best candidates to serve as a homeostatic sleep-promoting signal is adenosine (Basheer et al. 2004; Stenberg 2007). Adenosine is a purine nucleoside; it is a by-product of ATP hydrolysis and might represent a cellular signal for energy demand in the brain (Benington and Heller 1995; Chagoya de Sanchez et al. 1993). Adenosine has been shown to promote slow-wave sleep and rapid eye movement (REM) sleep in cats and rats (Basheer et al. 2004; Porkka-Heiskanen et al. 2002; Radulovacki 1995; Radulovacki et al. 1984) and more recently in mice (Coleman et al. 2006; Oishi et al. 2008; Urade et al. 2003; Van Dort et al. 2009). In the early 1990s it was hypothesized that adenosine accumulates in the extracellular space during waking where, on reaching sufficiently high extracellular levels, it could produce sleep through inhibition of wake-active neurons, in particular those of the basal forebrain (BF) (Rainnie et al. 1994). Since then, several studies have shown that extracellular adenosine levels rise in the BF during prolonged wakefulness and decline during recovery sleep and that adenosine can inhibit BF wake-active neurons through A1 receptors (Alam et al. 1999; Arrigoni et al. 2006; Kalinchuk et al. 2003; Porkka-Heiskanen et al. 1997; Thakkar et al. 2003a). In addition, accumulation of adenosine in the BF is correlated with both increased electroencephalographic (EEG) slow-wave activity and sleep amount and has been shown to contribute to homeostatic sleep drive following sleep deprivation (McCarley 2007; Porkka-Heiskanen et al. 1997; Portas et al. 1997; Stenberg 2007; Streekar et al. 2000; Thakkar et al. 2003b).

The BF contains a heterogeneous population of neurons including corticopetal cholinergic, GABAergic, glutamatergic, and peptidergic neurons as well as interneurons (Gritti et al. 1993, 2006; Hur and Zaborszky 2005; Schwaber et al. 1987; Zaborszky et al. 1999, 2005; Zaborszky and Duque 2000, 2003). BF cholinergic neurons provide the major cholinergic innervation of the cerebral cortex (Gritti et al. 1997; Manns et al. 2001; Saper 1984; Woolf 1991); they fire in association with cortical activation during wakefulness and REM sleep, which is when cortical acetylcholine release is maximal, and their firing rate positively correlates with gamma EEG activity across the sleep-wake cycle (Hassani et al. 2009; Jaser and Tessier 1971; Jones 2004; Lee et al. 2005; Marrosu et al. 1995). More importantly, activation of BF cholinergic neurons is sufficient to promote cortical activation and increases wakefulness and REM sleep (Cape et al. 2000).

BF cholinergic neurons also receive robust glutamatergic inputs (Hur et al. 2009). These projections appear to arise from multiple sources and include the lateral hypothalamus, the amygdala, the intralaminar and midline nuclei of the thalamus, and the brain stem (Carnes et al. 1990; Espana et al. 2005; Fadel et al. 2005; Jolkkonen et al. 2002; Materi et al. 2000; Rasmussen et al. 1994; Wu et al. 2004; Zaborszky et al. 1984, 1991). As such, glutamatergic inputs from these predominately wake-promoting structures could promote arousal through activation of BF cholinergic neurons. We have previously reported that adenosine directly inhibits BF cholinergic neurons through A1 receptors and the subsequent activation of an inwardly rectifying potassium conductance (Arrigoni et al. 2006). It is also possible, however, that adenosine may inhibit BF cholinergic neurons indirectly by reducing glutamatergic input to the BF. We therefore examined in the present study 1) how adenosine modulates glutamatergic input to BF cholinergic neurons and 2) which enzymes control the level of extracellular adenosine in the BF.
MATERIAL AND METHODS

Animals. In this study we used male and female (5–20 days old) C57BL/6 mice and an additional cohort of six mature (6 wk old) C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME). Mice were housed in a pathogen-free barrier animal research facility and maintained on a 12:12-h light-dark cycle (lights on at 7:00 AM) at 22°C ambient temperature and with ad libitum access to food and water. Care of the mice met the National Institutes of Health standards, as set forth in the Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Beth Israel Deaconess Medical Center and Harvard Medical School Institutional Animal Care and Use Committees.

Prelabeling of BF cholinergic neurons. Under isoflurane anesthesia, mice were injected into the lateral cerebroventricle [intracerebroventricular (icv) injections] with indocarbocyanine (Cy3)-coupled antibodies raised against mouse p75 neurotrophin receptors (Cy3-p75NTR-IgG; Advanced Targeting Systems, San Diego, CA). Cy3-p75NTR-IgGs (100–150 nl, 0.4 mg/ml) were slowly (over 5 min) air pressure-injected using a silane-coated glass pipette (25–30-μm tip diameter) (Scammell et al. 1998). Coordinates for the left lateral cerebroventricle for the mice < 21 days old were anterioposterior (AP) = –0.1 mm, dorsoventral (DV) = –0.8 mm, and mediolateral (ML) = –1.4 mm, and those for the 6-wk-old mice were AP = –0.2 mm, DV = –2 mm, and ML = –1 mm (Franklin and Paxinos 1997). One to 3 days after the surgery, brain slices were prepared from mice for in vitro recordings. Labeled neurons were found ipsi- and contralateral to the injection (Hartig et al. 1998), and recordings were made from both hemispheres.

Slice preparation and whole cell patch-clamp recordings. Brain slices from immature mice (<21 days old) were prepared as follows. The animals were anesthetized (isoflurane inhalation) to the point of respiratory arrest and then decapitated. Coronal brain slices (300-μm thickness) were cut with a vibrating microtome (VT1000; Leica, Bannockburn, IL) in ice-cold artificial cerebrospinal fluid (ACSF; HEPES-buffered solution) oxygenated with 100% O2. Brain slices from the cohort of 6-wk-old mice were prepared as follows (Peca et al. 2011; Zhao et al. 2011). The animals were deeply anesthetized by intraperitoneal injection of a ketamine-xylazine mixture (150 mg/kg i.p.). The animals were then transcardially perfused with ice-cold artificial cerebrospinal fluid (ACSF) [N-methyl-D-glucamine (NMDG)-based solution, carbogemated with 95% O2 and 5% CO2]. Mice were then decapitated, and coronal brain slices (300-μm thickness) were cut in ice-cold cutting ACSF (NMDG-based). Slices containing the BF were immersed in ACSF (HEPES-buffered solution) until transferred to the recording chamber.

NC33S were recorded at room temperature, submerged, and perfused (2 ml/min) with oxygenated ACSF (HEPES-buffered solution), and recordings were made from Cy3-p75NTR-IgG-labeled neurons of the magnocellular preoptic nucleus and substantia innominata (MCPO/SI). Whole cell recordings were guided by combined fluorescence and infrared differential interference contrast (IR-DIC) video microscopy using a fixed-stage upright microscope (Axioskop 2FS; Carl Zeiss MicroImaging, Thornwood, NY) equipped with a Normarki water-immersion lens (×40, 0.8 NA). IR-DIC and fluorescence images were detected with an IR-sensitive charge-coupled device camera (ORCA-ER; Hamamatsu, Bridgewater, NJ) and displayed on a computer screen in real time using AxioVision software (Carl Zeiss MicroImaging). Whole cell recordings were made using a Multiclamp 700B amplifier, a Digidata 1322A interface, and Clampex 9 software (Molecular Devices, Foster City, CA).

Evoked excitatory postsynaptic currents (eEPSCs) were induced by local stimulation with a bipolar electrode (Frederick Haer, Bowdoinham, ME) placed within a 200-μm radius of the recorded cell. Current pulses (0.1- to 0.6-ms duration, repeated every 20 s) were delivered with a constant-current source (Stimulus Isolator A360; World Precision Instruments, Sarasota, FL), triggered by Clampex software (Molecular Devices), and the stimulus strength was adjusted (0.1–0.6 mA) to give maximum evEPSC amplitude. Evoked EPSCs were displayed as an average of 5–10 consecutive traces, and trace baselines were superimposed. Recordings in which the evEPSCs displayed multiple peaks or were unstable over time, or in which the stimulations evoked antidromic or orthodromic action potentials, were discarded. The paired-pulse facilitation (PPF) test was delivered using two identical electrical stimulating pulses (40-ms interval, repeated every 20 s). Paired-pulse ratio (PPR) was calculated as the average of six consecutive traces by dividing the evEPSC peak amplitude of the second pulse by the evEPSC amplitude of the first pulse. The effects of adenosine on the PPF were represented by scaling the traces during adenosine application so that the evEPSC (1st pulse) matches the control evEPSC (1st pulse) (see Fig. 3B).

Reagents and solutions. Composition of the ACSF used for recording and for preparation of the brain slices of immature mice was (in mM) 140 NaCl, 3 KCl, 1.3 MgSO4, 2.4 CaCl2, 1.4 NaH2PO4, 11 glucose, and 5 HEPES (pH 7.2 with NaOH, 315–320 mosmol/l). Composition of the cutting ACSF (NMDG-based solution) used for the preparation of brain slices from 6-wk-old mice was (in mM) 119 NaCl, 2.5 KCl, 1.25 NaH2PO4, 26 NaHCO3, 25 glucose, 2 thioracil, 5 t-ascorbic acid, 3 Na-pyruvate, 0.5 CaCl2, and 10 MgSO4 (pH 7.3 with HCl when carbogemated with 95% O2 and 5% CO2). Composition of the pipette solution was (in mM) 120 K-gluconate, 10 KCl, 3 MgCl2, 10 HEPES, 2.5 K-ATP, 0.5 Na-GTP, and Lucifer yellow CH-ammonium salt (0.1%) (pH 7.2 adjusted with KOH, 280 mosmol/l). Miniature EPSCs (mEPSCs) were recorded in tetrodotoxin (TTX; 1 μM), bicuculline methiodide (10 μM), and a pipette solution containing (in mM) 110 d-gluceric acid, 10 CsCl, 10 HEPES, 3 MgCl2, 1 CaCl2, 11 EDTA, 20 d-sorbitol, 2 MgATP, 0.3 NaGTP, and Lucifer yellow CH-ammonium salt (0.1%) (pH 7.2 adjusted with CsOH; 280 mosmol/l). Bicuculline methiodide, 6,7-dinitroquinoxalin-2,3-dione (DNQX) disodium salt, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzof[quinoxaline-7-sulfonamide (NBQX) disodium salt, 4-aminopyridine (4-AP), TTX, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), N'-cyclopentyladenosine (CPA), CGS-21680, 2-Cl-IB-MECA, 2-phenylaminoadenosine (CV-1808), dipyradimole (DIPY), 5-iodotubercidin (5-IT), and etorphyro-9-(2-hydroxy-3-nonyl)adenine hydrochlorid (EHNA) were purchased from Tocris Bioscience (Elvissville, MO). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of 4-AP, bicuculline methiodide, DNQX, NBQX, and EHNA were prepared in H2O. Stock solutions of adenosine, DPCPX, CPA, CGS-21680, CV-1808, 2-Cl-IB-MECA, 5-(4-nitrobenzyl)-6-thioinosine (NBTI), DIPY, and 5-IT were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the ACSF was <0.1%.

Data analysis and statistics. Data were analyzed using Clampfit 9 (Molecular Devices), IGOR Pro 6 (WaveMetrics, Lake Oswego, OR), Mini Analysis 6 (Synaptosoft, Leonia, NJ), Origin (Microcal, Northampton, MA), and StatView software (SAS Institute, Cary, NC). The A-current was isolated using a standard two-step voltage protocol [holding potential (Vh) = –90 and –40 mV] (Burdakov et al. 2004). The peak A-type conductance (gA) was calculated using Eq. 1:

\[ g_A = \frac{V_{m} \cdot (V_{m} \cdot 1 + V_{m} + V_{S_0} \cdot k)}{I_{V_{m}} \cdot (V_{m} \cdot 1 + V_{m} + V_{S_0} \cdot k)} \]

where \( V_{S_0} \) is the test pulse potential and \( V_{m} \) is the test pulse potential that gives half-maximal activation. Curve fitting was performed using Origin (Microcal). Evoked EPSC peak amplitudes were normalized by dividing the values of control and treatment samples by the mean of the control sample. This normalization conserves the distribution and the relative variance of the samples, allowing the subsequent use of a t-test (Valcu and Valcu 2011). Data were compared using paired t-tests. Spontaneous EPSCs (sEPSCs) and mEPSCs were semiautomatically analyzed off-line using Mini Analysis software. Traces (5...
PBS, and then cut into 40-10% formalin, equilibrated in 20% sucrose and 0.02% sodium azide in 20 ml of 10% formalin. Brains were removed, postfixed overnight in isoflurane and perfused transcardially with 20 ml of PBS followed by IgG-injected mice for ChAT double immunolabeling studies. One day

Table 1. Cell counting in BF nuclei of neurons labeled by Cy3-p75NTR-IgG and positive for ChAT immunoreactivity

<table>
<thead>
<tr>
<th></th>
<th>%ChAT(+) Cells Labeled by Cy3-p75NTR-IgG</th>
<th>%Cy3-p75NTR-IgG(+) Cells</th>
<th>No. of ChAT(+) Cells</th>
<th>No. of Cy3-p75NTR-IgG(+) Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS ipsilateral</td>
<td>57.7 ± 4.5</td>
<td>5.5 ± 0.9</td>
<td>982</td>
<td>639</td>
</tr>
<tr>
<td>MS contralateral</td>
<td>58.8 ± 4.6</td>
<td>4.5 ± 1.0</td>
<td>871</td>
<td>575</td>
</tr>
<tr>
<td>hDB ipsilateral</td>
<td>53.6 ± 5.9</td>
<td>4.7 ± 1.8</td>
<td>1422</td>
<td>866</td>
</tr>
<tr>
<td>hDB contralateral</td>
<td>58.3 ± 5.0</td>
<td>3.8 ± 0.8</td>
<td>1848</td>
<td>1172</td>
</tr>
<tr>
<td>MCPO ipsilateral</td>
<td>50.2 ± 6.8</td>
<td>3.3 ± 1.5</td>
<td>1357</td>
<td>774</td>
</tr>
<tr>
<td>MCPO contralateral</td>
<td>51.7 ± 6.9</td>
<td>3.4 ± 1.3</td>
<td>1605</td>
<td>882</td>
</tr>
<tr>
<td>SI ipsilateral</td>
<td>44.6 ± 4.8</td>
<td>4.5 ± 1.6</td>
<td>485</td>
<td>237</td>
</tr>
<tr>
<td>SI contralateral</td>
<td>43.1 ± 5.1</td>
<td>4.0 ± 1.9</td>
<td>640</td>
<td>279</td>
</tr>
</tbody>
</table>

Percentage values are means ± SE from 7 mice, indicating the percentage of choline acetyltransferase-positive [ChAT(+)] neurons labeled with indocarbocyanine-coupled antibodies raised against mouse p75 neurotrophin receptors (Cy3-p75NTR-IgG) and the percentage of neurons labeled with Cy3-p75NTR-IgG that are ChAT(-). Cell counts were performed in the medial septum (MS), horizontal limb of the diagonal band (hDB), magnocellular preoptic nucleus (MCPO), and substantia innominata (SI) ipsilateral and contralateral to the Cy3-p75NTR-IgG intracerebroventricular injections.

RESULTS

In the BF, the neurotrophin receptor p75 (p75NTR) is expressed almost exclusively on cholinergic neurons across species, including rats and mice (Rossner et al. 2000; Springer et al. 1987; Tremere et al. 2000). In recent years, a number of studies have used fluorescent conjugated anti-rat p75NTR antibodies (192IgG) to label in vivo BF cholinergic neurons in rats (Arrigoni et al. 2006; Hartig et al. 1998; Wu et al. 2000). More recently, a new polyclonal fluorescent antibody against murine p75NTR (Cy3-p75NTR IgG; Advanced Targeting Systems) has become available. To determine whether this antibody specifically labels the cholinergic population within the BF and can therefore be used for in vitro electrophysiological recordings in mice, we injected seven mice in the lateral cerebroventricle with anti-murine Cy3-p75NTR-IgG and used them for a ChAT-immunohistochemical double-label study. From the MS to the SI region, we found that ~50% of ChAT-positive neurons were labeled with Cy3-p75NTR-IgG (Table 1 and Fig. 1). More importantly we found that Cy3-p75NTR-IgGs were internalized almost exclusively by ChAT-positive neurons. Only 3–5% of the Cy3-p75NTR-IgG-labeled neurons in BF were not ChAT positive (Table 1), indicating that similar to what has been reported for the fluorescent 192IgG in rats (Hartig et al. 1998), the fluorescent antibody against murine p75NTR is a useful tool to label BF cholinergic neurons for in vitro electrophysiological recordings in mice.

In this study, we identified MCPO/SI cholinergic neurons based on the presence of internalized Cy3-p75NTR-IgG and/or by post hoc immunoreactivity for ChAT (Fig. 2). We found that mouse MCPO/SI cholinergic neurons were mostly silent.
Fig. 1. Cy3-coupled antibodies raised against mouse p75 neurotrophin receptors (Cy3-p75NTR-IgGs) specifically label cholinergic neurons in the basal forebrain (BF). Photographs show neurons of the horizontal limb of the diagonal band (hDB), magnocellular preoptic nucleus (MCPO), and substantia innominata (SI) nuclei. p75NTR-IgG label is shown in red and choline acetyltransferase (ChAT) immunoreactivity in green. A: ChAT immunostaining in the hDB shown at low magnification (left; scale bar = 100 μm). ChAT-positive neurons and p75NTR-IgG-positive neurons (boxed area) are shown at higher magnification at middle and right, respectively (scale bars = 25 μm). B: ChAT immunostaining in the MCPO and SI at the level of the crossing fibers of the anterior commissure is shown at low magnification (top row, left; scale bar = 200 μm). ChAT-positive neurons and p75NTR-IgG-positive neurons of the MCPO (bottom boxed area) and of the SI (top boxed area) are displayed at higher magnification at top row, middle and right, for MCPO and at bottom row, left and middle, for SI (scale bars = 25 μm).

Fig. 2. A: in vivo labeling of MCPO/SI cholinergic neurons using fluorescent anti-murine p75NTR-IgGs. Two MCPO neurons were labeled with Cy3-p75NTR-IgG (left) and visualized under infrared differential interference contrast (right), and the lower cell was filled with Lucifer yellow from the recording pipette (right). B: the recorded/Lucifer yellow-filled neuron (in green) is positive for ChAT immunoreactivity (in red). C: coronal diagrams (modified from Franklin and Paxinos 1997) represent the distribution of 17 recorded cholinergic neurons. hDB, MCPO, and SI nuclei are highlighted in gray. The number at the bottom of each diagram indicates mm anterior to bregma. D and E: firing properties of MCPO/SI neurons during depolarizing (+40 pA, from −83 mV; top traces) and hyperpolarizing current pulses (−20 pA, from −50 mV; bottom traces) showing no I mediated depolarizing sag, a voltage-dependent rectification compatible with the presence of an inwardly rectifying K+ current, and delayed rebound firing on recovery from hyperpolarizing pulses due to activation of an A-type current (arrowheads) that is abolished by 5 mM 4-aminopyridine (4-AP). F: voltage-clamp recordings (1 μM TTX) of the A-type current in MCPO/SI cholinergic neurons shown as a transient outward current that is abolished by 4-AP. G: activation curve for A-type conductance (gA; see MATERIALS AND METHODS) expressed as a fraction of maximal conductance (gAmax) and plotted against the test pulse potential (holding potential Vh = −90 mV; n = 9). The curve is the best fit of a sigmoidal function (V1/2 = −28.3 mV and k = 9.59 mV; χ2 < 0.001). Con, control.
current pulses with a voltage-dependent rectification compatible with the presence of an inwardly rectifying potassium conductance. Collectively, these data indicate that MCPO/SI cholinergic neurons of mice have firing patterns very similar to those of the BF cholinergic neurons previously described in rats and guinea pigs (Arrigoni et al. 2006; Khateb et al. 1995; Wu et al. 2000).

Adenosine inhibits the evoked glutamatergic input to MCPO/SI cholinergic neurons through presynaptic adenosine A1 receptors. To investigate the effects of adenosine on the excitatory input to the MCPO/SI cholinergic neurons, we placed a bipolar stimulating electrode in the MCPO/SI region within a 200-μm radius of the recorded cell. Focal electrical stimulation of 100- to 600-μs duration produced glutamatergic AMPA receptor-mediated evEPSCs ($V_h = -70$ mV, in 10 μM bicuculline) that were completely blocked by the AMPA receptor antagonists DNQX (20 μM; $n = 4$) and NBQX (10 μM; $n = 3$). Adenosine (100 μM) reduced the amplitude of glutamatergic evEPSCs to 47.6 ± 6.0% ($n = 30$; $P < 0.001$, paired $t$-test), and this effect was reversible by washout of adenosine (Fig. 3A). Additional recordings were conducted on a cohort of 6-wk-old mice. Similar to the effect of adenosine in the immature mice, adenosine reduced the amplitude of glutamatergic evEPSCs (to 52.9 ± 4.1%; $n = 5$; $P = 0.001$, paired $t$-test) in the 6-wk-old mice.

To determine whether adenosine acted presynaptically, we used a paired-pulse test in which two evEPSCs were evoked in rapid sequence (40-ms interstimulus time) by two identical stimulating pulses. The ratio of the evEPSC amplitude in response to the second pulse to that of the first pulse, the so-called PPR, depends on the probability of vesicular release at the synapse, and changes in PPR have been used as a measure of changes in the release probability (Debanne et al. 1996; Dobrunz and Stevens 1997). We found that adenosine (100 μM) increased PPR from 1.26 ± 0.07 (in control) to 1.53 ± 0.11 (in adenosine; $n = 7$; $P = 0.007$, paired $t$-test), and PPR returned to 1.38 ± 0.11 with adenosine washout, indicating that adenosine inhibited the glutamatergic input to the MCPO/SI neurons by reducing the probability of glutamate release at the synaptic terminals (Fig. 3B).

We next asked which adenosine receptor subtypes were responsible for this effect. We found that the A1 receptor antagonist DPCPX (100 nM) abolished the effect of adenosine (Fig. 3C). Additional recordings were conducted on a cohort of 6-wk-old mice. Similar to the effect of adenosine in the immature mice, adenosine reduced the amplitude of glutamatergic evEPSCs (to 52.9 ± 4.1%; $n = 5$; $P = 0.001$, paired $t$-test) in the 6-wk-old mice.

### Figure 3

A: adenosine (100 μM) inhibits glutamatergic AMPA-receptor-mediated evoked excitatory postsynaptic currents (evEPSCs); 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) was used at 10 μM. AD, adenosine. B: adenosine increases paired-pulse facilitation (PPF; paired-pulses test using 40-ms interstimulus interval). At right, scaled traces to match evEPSC (1st pulses) are represented to show the increased PPF by adenosine. C and D: adenosine inhibition of evEPSCs is blocked by the A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 100 nM) and is mimicked by the A1 agonist N6-cyclopentyladenosine (CPA; 100 nM). E–G: evoked glutamatergic EPSCs are unaffected by the A2A receptor agonist CGS-216800 (0.1–3 μM), the A2 receptor agonist CV-1808 (250 nM), or the A3 receptor agonist CI-IB-MECA (100 nM). H and I: DPCPX (100 nM) increases the amplitude of evEPSCs by blocking endogenous adenosine. J: summary graph showing the averaged effects of adenosine, adenosine in the presence of DPCPX, CPA, CPA in the presence of DPCPX, CGS-216800 (0.1 and 3 μM), CV-1808, CI-IB-MECA, and DPCPX on evEPSC amplitude. *$P < 0.05$; **$P < 0.01$, paired $t$-test, comparing evEPSC amplitude in control artificial cerebrospinal fluid (ACSF) and during drug applications. All evEPSCs were recorded at $V_h = -70$ mV.
on the evEPSC amplitude (DPCPX + adenosine: 108.9 ± 22.5%; n = 7; P = 0.19, paired t-test, compared with evEPSC amplitude in DPCPX), whereas the A1 agonist CPA (100 nM) mimicked the adenosine effects (CPA: 63.6 ± 16.4%; n = 7; P = 0.0013; CPA + DPCPX: 107.6 ± 12.3%; n = 6; P = 0.875, paired t-test, compared with evEPSC amplitude in control and DPCPX), indicating that adenosine inhibited the glutamatergic transmission to the MCPO/SI cholinergic neurons through A1 receptors (Fig. 3, C and D). Similar effects were also found in the 6-wk-old mice in which the effect of adenosine was completely blocked by DPCPX (DPCPX; P = 0.51, paired t-test, compared with evEPSC amplitude in DPCPX).

We found no effects of the A2A receptor agonist CGS-21680 (0.1–3 μM), the dual A2A and A3b Receptor agonist CV-1808 (250 nM), and the A3 receptor agonist CI-IB-MECA (100 nM) on the evEPSC amplitude [CGS-2168 (100 nM): 97.2 ± 17.1%; n = 6; P = 0.51; CGS-2168 (3 μM): 96.5 ± 13.6%; n = 9; P = 0.58; CV-1808: 99.8 ± 9.8%; n = 4; P = 0.76; and IB-MECA: 99.4 ± 13.4%; n = 6; P = 0.95, paired t-test, compared with evEPSC amplitude in control], which suggests that there is no involvement of the A2 and A3 receptors in the inhibition of glutamatergic input to MCPO/SI cholinergic neurons (Fig. 3, E–G). In addition, we found that DPCPX alone increased the evEPSC amplitude to 147.1 ± 24.5% (n = 6; P = 0.034, paired t-test, compared with evEPSC in control), indicating that the glutamatergic input was under an inhibitory tone by endogenous adenosine (Fig. 3, H and I).

Adenosine inhibits spontaneous glutamatergic input to MCPO/SI cholinergic neurons through presynaptic adenosine A1 receptors. We next examined the effects of adenosine on spontaneous glutamatergic input to MCPO/SI cholinergic neurons. Glutamatergic sEPSCs were recorded at a holding potential of −70 mV in bicuculline (10 μM) and were completely blocked by DNQX (20 μM; n = 4). We found that adenosine (100 μM) reduced the frequency of the glutamatergic sEPSCs and that this effect was reversed after 20 min in washout (Fig. 4). Adenosine reduced the sEPSC frequency to 53.9 ± 11.9% (control: 3.58 ± 0.67 Hz; adenosine: 1.93 ± 0.43 Hz; n = 12; P < 0.001, paired t-test; washout: 3.58 ± 0.86 Hz) but had no effects on the sEPSC amplitude (control: 12.71 ± 1.27 pA; adenosine: 11.98 ± 1.87 pA; n = 12; P = 0.41, paired t-test; washout: 11.72 ± 1.64 pA). Application of DPCPX (100 nM) blocked the effects of adenosine on sEPSC frequency (DPCPX: 4.91 ± 1.40 Hz; DPCPX + adenosine: 4.97 ± 1.50 Hz; n = 9; P = 0.88, paired t-test), indicating that the inhibition of the glutamatergic input was mediated by adenosine A1 receptors (Fig. 4).

To determine whether adenosine acted on the presynaptic glutamatergic terminals, we tested the effects of adenosine on glutamatergic mEPSCs recorded in the presence of TTX (1 μM; Vh = −70 mV). We found that adenosine decreased the frequency of glutamatergic mEPSCs to 64.6 ± 14.5% (control: 2.20 ± 0.37 Hz; adenosine: 1.42 ± 0.32 Hz; n = 7; P = 0.002, paired t-test; washout: 2.72 ± 0.52 Hz) without affecting the mEPSC amplitude (control: 16.26 ± 1.54 pA, adenosine: 17.26 ± 2.09 pA; P = 0.32, paired t-test; washout: 17.95 ± 1.41 pA), suggesting a presynaptic effect. Application of DPCPX (100 nM) abolished adenosine effects on mEPSC frequency (DPCPX: 1.72 ± 0.31 Hz; DPCPX + adenosine: 1.61 ± 0.32 Hz; n = 7; P = 0.42, paired t-test), indicating that the presynaptic adenosine receptors were A1 receptors (Fig. 4).

Endogenous extracellular adenosine. Extracellular adenosine is cleared through two main mechanisms. Adenosine is

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Fig. 4. Adenosine inhibits spontaneous glutamatergic input through presynaptic A1 receptors. A: effects of adenosine (100 μM) on the glutamatergic spontaneous EPSC (sEPSC). B: cumulative distribution plots of the sEPSC interevent intervals and amplitude (number of sEPSCs: control = 2,118; AD = 1,180; and wash = 1,830) of the cell represented in A. C: bar graphs showing the mean effects of adenosine applied alone and in the presence of DPCPX on sEPSC frequency and amplitude. In control ACSF: adenosine effects on sEPSC frequency [F(2,11) = 6.662, P = 0.011, 1-way ANOVA (**P < 0.01, Fisher’s LSD)] and on sEPSC amplitude [F(2,11) = 1.103, P = 0.363, 1-way ANOVA]. In the presence of DPCPX: adenosine effects on sEPSC frequency [F(2,11) = 0.222, P = 0.886, 1-way ANOVA] and on sEPSC amplitude [F(2,11) = 0.346, P = 0.573, 1-way ANOVA]. D: effects of adenosine on glutamatergic miniature EPSCs (mEPSCs) recorded in TTX (1 μM). E: cumulative distribution plots of mEPSC interevent intervals and amplitude (number of mEPSCs: control = 531; AD = 245; and wash = 432) of the cell represented in D. F: bar graphs showing the mean effects of adenosine applied alone and in the presence of DPCPX on mEPSC frequency and amplitude. In control ACSF: adenosine effects on mEPSC frequency [F(2,11) = 11.157, P = 0.003, 1-way ANOVA (**P < 0.01, Fisher’s LSD)] and on mEPSC amplitude [F(2,11) = 0.901, P = 0.437, 1-way ANOVA]. In the presence of DPCPX: adenosine effects on mEPSC frequency [F(2,11) = 0.022, P = 0.886, 1-way ANOVA] and on mEPSC amplitude [F(2,11) = 0.744, P = 0.421, 1-way ANOVA]. sEPSCs and mEPSCs were recorded at Vh = −70 mV.

J Neurophysiol • doi:10.1152/jn.00528.2011 • www.jn.org
first taken up by neurons and astrocytes through the equilibrative nucleoside transporters and concentrative transporters, and it is then either phosphorylated to AMP by adenosine kinase or converted to inosine by adenosine deaminase. In addition, adenosine can be metabolized by adenosine deaminase in the extracellular matrix (Baldwin et al. 2004; Dunwiddie and Masino 2001; Fredholm et al. 2005; Noji et al. 2004). To determine the involvement of adenosine uptake (by the equilibrative nucleoside transporters: ENT1 and ENT2), adenosine phosphorylation (by adenosine kinase), and adenosine metabolism (by adenosine deaminase) in the regulation of extracellular adenosine in BF, we tested the effects of specific blockers for these enzymes on the amplitude of the evEPSC in MCPO/SI cholinergic neurons. We found that blocking the equilibrative nucleoside transporters ENT1 and ENT2 with NBTI (5 μM) + DIPY (10 μM) (Dunwiddie and Diao 1994; Frenguelli et al. 2007; Wall et al. 2007) reduced the amplitude of the evEPSCs to 79.7 ± 8.4% (n = 9; P = 0.003, paired t-test, compared with evEPSC amplitude in control). The effect of NBTI + DIPY was completely blocked by 500 nM DPCPX (DPCPX + NBTI + DIPY: 98.7 ± 9.5%; n = 5; P = 0.902, paired t-test, compared with evEPSC amplitude in DPCPX), indicating the presence of an influx of adenosine through the equilibrative nucleoside transporters (Fig. 5A).

We next tested the role of adenosine kinase and adenosine deaminase in the regulation of extracellular adenosine. We found that both the adenosine kinase inhibitor 5-IT and the adenosine deaminase inhibitor EHNA reduced the evEPSC amplitude, and their effects were blocked by DPCPX (Fig. 5, B and C). Application of 5-IT (7 μM) reduced the evEPSC amplitude to 74.3 ± 10.1% (n = 9; P = 0.0014, paired t-test, compared with evEPSC amplitude in control), and the effect of 5-IT was blocked by DPCPX (DPCPX + 5-IT: 99.1 ± 4.6%; n = 7; P = 0.892, paired t-test, compared with evEPSC amplitude in DPCPX). Application of EHNA (10 μM) reduced the evEPSC amplitude to 68.2 ± 6.3% (n = 10; P = 0.001, paired t-test, compared with evEPSC amplitude in control), and this effect was blocked by DPCPX (DPCPX + EHNA: 96.0 ± 8.4%; n = 8; P = 0.499, paired t-test, compared with evEPSC amplitude in DPCPX). These results indicate that adenosine kinase and adenosine deaminase are both important in controlling extracellular adenosine levels and hence in the regulation of the inhibitory tone within the BF by adenosine.

**DISCUSSION**

In the present study we found that adenosine inhibits glutamatergic inputs to BF cholinergic neurons through the activation of presynaptic A1 receptors and that this glutamatergic input is under tonic inhibition by endogenous adenosine. We also found that extracellular adenosine levels are regulated by the combined activity of the equilibrative nucleoside transporters adenosine kinase and adenosine deaminase.

**Technical considerations on the in vivo labeling of BF cholinergic neurons.** In the BF, the p75NTR is exclusively expressed on cholinergic neurons, and this is true in a number of mammalian species (Hefti et al. 1986; Maclean et al. 1997; Rossner et al. 2000; Springer et al. 1987; Tremere et al. 1998). The function of this receptor in the BF is still not fully understood, although there is increasing evidence that it might...
regulate axonal outgrowth and retraction (Coulson et al. 2009). Antibodies against the p75NTR conjugated with either the ribosome-inactivating protein saporin or a fluorescent dye have been used previously to both lesion and label the BF cholinergic neurons, respectively (Arrigoni et al. 2006; Fuller et al. 2011; Hartig et al. 1998; Kalinchuk et al. 2008; Kaur et al. 2008; Wu et al. 2000). An anti-murine p75NTR antibody (Advanced Targeting Systems) that recognizes the extracellular domain of murine p75NTR (Huber and Chao 1995; Rao and Anderson 1997) is now commercially available in the saporin- and Cy3-conjugated forms (Berger-Sweeney et al. 2001; Moreau et al. 2008). The aim of the double-labeling work in the present study was to determine whether this anti-murine Cy3-p75NTR-IgG could specifically label BF cholinergic neurons and whether it could be used for in vitro electrophysiological recordings in mouse brain slices. Our results show that the anti-murine Cy3-p75NTR-IgG is highly selective for BF cholinergic neurons and that about 50% of the BF cholinergic neurons, ipsi- and contralateral to the injection site, internalized the Cy3-p75NTR-IgG. There was a slightly higher percentage of cholinergic neurons labeled for Cy3-p75NTR-IgG in the rostromedial region of BF, consistent with the report that the cholinergic neurons that project to the amygdala, which are located in the caudolateral region, are devoid of p75NTR (Heckers et al. 1994). We also found a lower percentage of cholinergic neurons labeled with Cy3-p75NTR-IgG compared with previous reports (Hartig et al. 1998; Kordower et al. 1988; Rossner et al. 2000). This discrepancy may, however, be due to the detection limits of our methods. In most of the previous studies, the p75NTR-IgG-positive cells were determined by p75NTR immunoreactivity, whereas in our study we quantified the double-labeled cholinergic neurons based on the native fluorescence of the internalized Cy3-p75NTR-IgG.

It is also important to note that, as a labeling tool, this method has two distinct disadvantages: 1) it requires stereotaxic icv injections; and 2) the label is not permanent. The highest number of labeled cells was found between 24 and 48 h post-icv injection, and by days 4 and 5 the number of labeled cells and the intensity of the labeling were both significantly reduced. On the other hand, this method does offer the advantage that it can be used in wild-type and knockout mice, whereas a transgenic approach would require the crossing of two or more mouse lines (Rosi et al. 2011). Taken together, our results demonstrate that the anti-murine Cy3-p75NTR-IgG is highly selective and labels a large number, if not all, of the BF cholinergic neurons, and it is therefore a suitable marker for the in vivo labeling of BF cholinergic neurons in mice. This becomes particularly useful when recording from the SI region, where the cholinergic neurons are less densely distributed.

### Adenosine inhibits the excitatory input to BF cholinergic neurons by presynaptic A1 receptors

Previous electrophysiological studies in BF have investigated spontaneous and evoked glutamatergic EPSCs in cholinergic neurons (Momiyama et al. 1996; Sim and Griffith 1996; Zhang and Trussell 1994). The glutamate release at these synapses is controlled by multiple types of presynaptic Ca2+ channels (P/Q- and N-types), and these channels undergo postnatal developmental changes in which the contribution of the P/Q-type increases with age at the expense of the N-type Ca2+ channels (Momiyama 2010; Momiyama and Fukazawa 2007; Sim and Griffith 1996). Furthermore, the glutamatergic transmission to the BF cholinergic neurons has been shown to be inhibited by muscarinic M2, dopamine D1, and somatostatin SST1/4 presynaptic receptors, but unlike dopamine and somatostatin, which only act presynaptically, acetylcholine and adenosine, as shown in the present study, have both pre- and postsynaptic effects (Arrigoni et al. 2006; Momiyama 2010; Momiyama and Fukazawa 2007; Momiyama et al. 1996; Momiyama and Zaborszky 2006; Sim and Griffith 1996).

Previous studies have shown that adenosine, through its action on A1 receptors, inhibits the discharge of BF wake-active neurons, reduces acetylcholine release in the cortex, and directly inhibits cholinergic MCPO/SI neurons (Alam et al. 1999; Arrigoni et al. 2006; Materi et al. 2000; Thakkar et al. 2003a; Van Dort et al. 2009). In this study, we demonstrate that adenosine reduces the excitatory drive to BF cholinergic neurons, a finding that provides an additional putative inhibitory mechanism for adenosine on BF cholinergic neurons. Adenosine induces membrane hyperpolarization and inhibits glutamatergic transmission in several other brain regions, and as we have found in BF cholinergic neurons, often the pre- and postsynaptic effects of adenosine are synergistic (Arrigoni et al. 2001; Greene and Haas 1985; Kuwahata 2004; Liu and Gao 2007; Proctor and Dunwiddie 1987; Rainnie et al. 1994; Wu and Saggau 1994).

We also found that adenosine inhibits both evoked and spontaneous glutamatergic transmission to BF cholinergic neurons through a presynaptic effect. Adenosine increases evEPSC PPF, indicating that adenosine decreases glutamate release probability (Debanne et al. 1996; Dobrunz and Stevens 1997), and in the presence of TTX, adenosine decreases the frequency of the mEPSCs without affecting their amplitude, indicating that adenosine acts at the synaptic terminals (Redman 1990). This finding is consistent with our previous data showing that adenosine inhibits mEPSC frequency but not mEPSC amplitude in neurons of the brain stem cholinergic nuclei (Arrigoni et al. 2001). It remains unknown how adenosine affects glutamate release at the synaptic terminals. Adenosine could reduce the presynaptic Ca2+ influx through either the inhibition of the presynaptic voltage-dependent Ca2+ channels and/or the activation of an inwardly rectifying K+ current, which leads to the hyperpolarization of the synaptic terminal (Bean 1989; Dolphin et al. 1986; Greene and Haas 1991; Wu and Saggau 1994). In addition, adenosine could also inhibit glutamate release downstream of Ca2+ influx (Brambilla et al. 2005; Scanziani et al. 1992; Scholz and Miller 1992). In BF, for instance, dopamine inhibits the GABA release to cholinergic neurons by a presynaptic Ca2+-independent mechanism (Momiyama and Sim 1996).

Adenosine activates four subtypes of G protein-coupled adenosine receptors (A1, A2A, A2B, and A3) (Fredholm et al. 2001). MCPO/SI nuclei express A1 receptors, whereas A2A/A2B receptors and A3 receptor mRNAs and proteins are undetectable in the BF (Allen-Brain-Atlas 2007; Basheer et al. 2001). Consistent with this receptor distribution, MCPO/SI neurons respond to A1 but not to A2 receptor agonists (Alam et al. 1999; Arrigoni et al. 2006; Thakkar et al. 2003a). However, since glutamatergic inputs to BF cholinergic neurons can originate from many sources, it remains possible that the effects of adenosine on these afferents could be mediated by any of the four adenosine receptor subtypes. Taken from tests on a series of specific adenosine receptor agonists and antagonists, our
results are consistent with adenosine signaling being mediated solely through \( A_1 \) receptors, suggesting that the neuronal sources of glutamatergic input to BF cholinergic neurons express only \( A_1 \) receptors or that their terminals contacting BF cholinergic neurons only express \( A_1 \) receptors.

**Possible sources of the glutamatergic input to BF cholinergic neurons.** BF cholinergic neurons respond to glutamate, and they are innervated by a robust glutamatergic input that represents \( >50\% \) of their afference (Hur et al. 2009; Khatib et al. 1995; Momiyama and Fukazawa 2007; Sim and Griffith 1996). This input appears to arise from multiple sources, including the lateral hypothalami, the amygdala, the intralaminar and midline nuclei of the thalamus, and the brain stem, but not the cortex, which sends glutamatergic connections back to the BF, but only to noncholinergic neurons (Carnes et al. 1990; Fadel et al. 2005; Jolkkonen et al. 2002; Zaborszky et al. 1984, 1991, 1997). It remains unclear, however, which of these regions serves as the source of the excitatory glutamatergic input to BF cholinergic neurons. Orexin neurons of the lateral hypothalamus, which also colocalize glutamate, are in direct contact with BF cholinergic neurons and also express adenosine \( A_1 \) receptors, and so are a possible source of glutamatergic input to BF cholinergic neurons that would also be depressed by adenosine (Abrahamson et al. 2001; Liu and Guo 2007; Thakkar et al. 2002; Wu et al. 2004). Moreover, because orexin and glutamate localize at the same terminals, it is possible that adenosine could inhibit the release of both orexin and glutamate to BF cholinergic neurons (Torrealba et al. 2003). It is also the case that the pedunculopontine tegmentum (PPT) region could provide a wake-promoting input via the BF and that this input could be glutamatergic (Materi et al. 2000; Rasmussen et al. 1994). The cholinceptive neurons of the medial pontine reticular formation (mPRF), which are implicated in the generation of REM sleep and wakefulness, could be another possible source of glutamatergic input to the BF (Jones 1995; Semba et al. 1988a; Steriade and McCarley 2005). These neurons are under the control of adenosine, although indirectly, through an \( A_2 \)- and \( A_1 \)-mediated modulation of the acetylcholine release (Coleman et al. 2006; Marks et al. 2003; Tanase et al. 2003). Whether mPRF neurons or their terminals respond directly to adenosine is a possibility of considerable interest. In addition, the brain stem parabrachial (PB) nucleus contains glutamatergic neurons that project to the BF (Fuller et al. 2011; Hur and Zaborszky 2005). Lesions of the PB significantly reduce wakefulness, suggesting that the PB might provide an ascending excitatory drive that activates BF cholinergic neurons to promote EEG and behavioral arousal (Fuller et al. 2011). Finally, glutamatergic BF neurons themselves could be a source of the glutamatergic input to the BF cholinergic neurons that are depressed by adenosine. These neurons in fact constitute more than half of the BF neuronal population, and about 60\% of glutamatergic BF neurons fire in correlation with cortical EEG activation (Gritti et al. 2006; Hassani et al. 2009). Although the primary activating effects of the glutamatergic BF neurons on the cortex are likely mediated by direct projections to the cortex, it is also possible that these activating effects may be in part attributed to collaterals to local BF cholinergic neurons (Hajszan et al. 2004; Henny and Jones 2008; Hur et al. 2009; Hur and Zaborszky 2005). Indeed, corticopetal neurons in the BF have local axon collaterals displaying numerous boutons (Semba et al. 1987), and further-
facilitate the movement of adenosine across the cell membrane along its concentration gradient, and therefore blocking the transporters can inhibit either adenosine release or adenosine uptake depending on the direction of the adenosine gradient across the membrane (Gu et al. 1995; Latini and Pedata 2001). In several regions of the central nervous system, including the BF, blockade of the adenosine transporters increases extracellular adenosine levels (Ackley et al. 2003; Dunwiddie and Diao 1994; Porkka-Heiskanen et al. 1997; Safiulina et al. 2005; Wall et al. 2007). In our study we found that blocking adenosine equilibrative transporters with NBTI and DIPY depresses glutamatergic input and that this effect was blocked by prior application of an adenosine antagonist, indicating an influx of adenosine and a buildup of extracellular adenosine following the inhibition of its uptake.

We next investigated the involvement of adenosine kinase and adenosine deaminase in the clearance of adenosine. Adenosine kinase is thought to play a primary role in adenosine metabolism, whereas adenosine deaminase may be more important under pathophysiological conditions when adenosine levels are higher. This assumption is based mainly on the higher affinity of adenosine kinase for adenosine (2–3 orders of magnitudes higher than adenosine deaminase) and on reports that inhibitors for adenosine kinase are more effective than adenosine deaminase inhibitors at increasing extracellular levels (Latini and Pedata 2001). Interestingly, we found that the adenosine kinase inhibitor 5-IT and the adenosine deaminase inhibitor EHNA were equally effective at depressing glutamatergic input to BF cholinergic neurons, suggesting that basal extracellular levels of adenosine in the BF are likely regulated by the activity of both enzymes. The role of adenosine kinase and adenosine deaminase in controlling adenosine basal levels might vary among brain regions or might depend on the postnatal developmental age. In contrast with our results in the BF, in the hippocampus or cerebellum adenosine kinase appears to have a greater relevance than adenosine deaminase (Lloyd and Fredholm 1995; Wall et al. 2007), but whereas these studies were conducted in postweaning age animals, our work was done in immature mice. Postnatal developmental changes in the enzymes involved in the metabolism of adenosine metabolism cannot be excluded and should be addressed by future studies.

Evidence supporting a role for adenosine deaminase in sleep regulation is provided by a recent study showing that genetic variability of the adenosine deaminase gene is linked to the duration and intensity of sleep in humans (Retey et al. 2005). Furthermore, adenosine deaminase is highly expressed in the leptomeninges, where infusion of its inhibitors increases extracellular adenosine levels and promotes slow-wave sleep (Okada et al. 2003). In the brain, its highest levels are found in posterior hypothalamus and in particular in the neurons of the tuberomammillary nucleus (TMN) (Geiger and Nagy 1986; Wada et al. 1991). Because these neurons are important for the maintenance of behavioral vigilance (Lin 2000; Staines et al. 1986; Takahashi et al. 2006), the TMN is expected to be particularly sensitive to the sleep-promoting effects of adenosine deaminase inhibitors, but future studies are needed to address this possibility. Furthermore, a moderate number of adenosine deaminase-immunoreactive neurons and levels of enzymatic activity have been found in BF and other sleep regulatory nuclei, supporting the concept that its role in sleep regulation might extend beyond the TMN and the leptomeninges (Geiger and Nagy 1986; Mackiewicz et al. 2000, 2003; Staines et al. 1988). Of particular note is the finding that the ectoform of adenosine deaminase is functionally associated with the A1 receptors, and therefore it is strategically placed where the extracellular adenosine is sensed (Ciruela et al. 1996; Torvien et al. 2002).

This could mean that if coupled to the presynaptic A1 receptors in BF, adenosine deaminase could be effective in gauging the excitatory drive onto BF cholinergic neurons.

In conclusion, our findings demonstrate that adenosine inhibits glutamatergic input to BF cholinergic neurons through activation of presynaptic A1 receptors. This effect provides an additional putative mechanism by which the adenosine that accumulates during prolonged periods of wakefulness can progressively reduce the excitability of BF wake-active neurons and thereby promote sleep. The clearance of extracellular adenosine is mediated mainly by the combined effects of uptake and metabolism. Both adenosine kinase and adenosine deaminase appear to be important for maintaining the extracellular adenosine inhibitory tone within the BF.

ACKNOWLEDGMENTS

We thank Sofia Z. Iqbal for helping with the animal surgeries, Dr. Patrick M. Fuller for helpful comments on this work, and Dr. Clifford B. Saper for support and advice.

GRANTS

This study was supported by National Institutes of Health (NIH) Grant 1R01 NS061863 and NIH Administrative Supplement Utilizing Recovery Act Funds 3R01 NS061863-01A1S3 and 1PO1 HL095491-01A1.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: J.M.H., L.L.F., S.A.K., and E.A. performed experiments; J.M.H., L.L.F., and E.A. analyzed data; J.M.H., L.L.F., and E.A. interpreted results of experiments; J.M.H., L.L.F., and E.A. prepared figures; J.M.H., L.L.F., S.A.K., and E.A. approved final version of manuscript; E.A. conception and design of research; E.A. drafted manuscript; E.A. edited and revised manuscript.

REFERENCES


The role of the p75
low-voltage-release by orexin A.
discharge properties of identified neurons in the basal forebrain.
central nervous system.
responses.
function of sleep.
sleep-wake regulation.
GABAergic in comparison to cholinergic and putative glutamatergic basal
fluorescence labelling of cholinergic neurons containing p75(NTR) in the rat
novel glutamatergic local circuit cells.
munoreactive input to septohippocampal parvalbumin-containing neurons:
hippocampal slices.
neurons containing choline acetyltransferase, glutamic acid decarboxylase and
A1 and A2A receptor mRNA
mentor neuron.
J Neurophysiol • doi:10.1152/jn.00528.2011 • www.jn.org


ADENOSINE PRESYNAPTIC EFFECT IN BASAL FOREBRAIN

Rosner S, Schliebs R, Bigl V. Intracerebroventricular infusion of CH005, a rat monoclonal antibody directed against mouse low-affinity nerve growth factor receptor (p75NTR), specifically labels basal forebrain cholinergic neurons in mouse brain. _Metab Brain Dis_ 15: 17–27, 2000.


_J Neurophysiol_ doi:10.1152/jn.00528.2011 • www.jn.org