Opioid-induced Decreases in Rat Brain Adenosine Levels Are Reversed by Inhibiting Adenosine Deaminase

Ariana M. Nelson, B.A.,* Alanna S. Battersby, B.S.,* Helen A. Baghdoyan, Ph.D.,† Ralph Lydic, Ph.D.†

Background: Opioids disrupt sleep and adenosine promotes sleep, but no studies have characterized the effects of opioids on adenosine levels in brain regions known to regulate states of arousal. Delivering opioids to the pontine reticular formation (PRF) and substantia innominata (SI) region of the basal forebrain disrupts sleep. In contrast, administering adenosine agonists to the PRF or SI increases sleep. These findings encouraged the current study testing the hypothesis that microdialysis delivery of opioids to the PRF or SI decreases adenosine levels in the PRF or SI, respectively.

Methods: A microdialysis probe was placed in the PRF of isoflurane anesthetized rats and perfused with Ringer's solution (control) followed by Ringer's solution containing morphine (0, 10, 30, 100, or 300 μM), fentanyl (100 μM), morphine (100 μM) and the adenosine deaminase inhibitor EHNA (100 μM), or naloxone (10 μM) and morphine (100 μM). Additional experiments measured adenosine levels in the SI before and during microdialysis delivery of morphine, fentanyl, and morphine plus EHNA.

Results: Morphine caused a significant ($P < 0.05$) concentration-dependent decrease in PRF adenosine levels. The significant decrease (−20%) in adenosine caused by 100 μM morphine was blocked by coadministration of naloxone. Fentanyl also significantly decreased (−13.3%) PRF adenosine. SI adenosine levels were decreased by morphine (−26.8%) and fentanyl (−27.4%). In both PRF and SI, coadministration of morphine and EHNA prevented the significant decrease in adenosine levels caused by morphine alone.

Conclusions: These data support the interpretation that decreased adenosine levels in sleep-regulating brain regions may be one of the mechanisms by which opioids disrupt sleep.

OPIOIDS and the purine nucleoside adenosine modulate pain and states of sleep and wakefulness. Opioids provide excellent pain management but cause the un-

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wanted side effect of sleep disruption.[1] Interrupted sleep heightens the perception of pain,[2,3] which increases opioid requirement.[4] Adenosine increases sleep,[5-8] and adenosine can contribute to pain management[9] in a manner that can be opioid sparing.[10]

Mice lacking the μ-opioid receptor gene show loss of analgesic response to morphine as well as decreased pain sensitivity.[11] These μ-opioid receptor knockout mice also have reduced binding at adenosine A_1 receptors,[12] suggesting a functional interaction between opioid and adenosine receptors. Whether opioids alter adenosine levels in brain regions that regulate sleep and nociception has not previously been investigated.

The pontine reticular formation (PRF) and the substantia innominata (SI) region of the basal forebrain contribute to the regulation of sleep and anesthesia.[13,14] Sleep is disrupted by delivery of opioids to the PRF[15,16] or to the SI.[17] In contrast, sleep is increased by adenosine agonists delivered to the PRF[18-20] and by increasing adenosine levels in the SI.[5,21] Therefore, the current study was designed to test the hypothesis that microdialysis delivery of opioids to the PRF or the SI decreases PRF or SI adenosine levels, respectively.

Materials and Methods

Animals

All studies were performed using adult male Sprague-Dawley rats (n = 42; mean body weight 300 g) purchased from Charles River Laboratories (Wilmington, MA) and housed in a 12-h light–12-h dark cycle. In International Genetic Standard (IGS®) nomenclature, these animals are Crl:CD(SD) rats. Procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, D.C., 1996) and all studies adhered to the guidelines established by the University of Michigan Committee on the Use and Care of Animals (Ann Arbor, Michigan).

Adenosine Measurement Using Microdialysis and High-performance Liquid Chromatography with Ultraviolet Detection

Microdialysis probes (cuprophane membrane: 1 mm long, 0.24 mm in diameter, 6-kDa cutoff; CMA Microdialysis, North Chelmsford, MA) were connected to a CMA/100 pump set at a constant flow rate of 2.0 μl/min. Before the *in vivo* portion of each experiment, *in vitro* microdialysis of a known concentration of adenosine

was used to determine the amount of adenosine recovered by each dialysis probe. Pre-experiment probe recovery values and post-experiment probe recoveries were compared by t test to ensure that changes in adenosine levels measured during in vivo experiments were not an artifact due to changes in dialysis probe recovery. Endogenous adenosine in each dialysis sample was expressed in nM. Each 30-μl dialysis sample was injected into a high-performance liquid chromatography system (Bioanalytical Systems, West Lafayette, IN) coupled to an ultraviolet detector (wavelength 254 nm) to measure adenosine. Chromatograms were digitized and analyzed using ChromGraph software (Bioanalytical Systems). Adenosine chromatograms obtained from dialysis samples were compared to a five-point standard curve produced with known concentrations of adenosine ranging from 10 to 200 nM. A standard curve was obtained before each experiment.

Drug Preparation

Drugs were dissolved in Ringer's solution (pH 5.8–6.2) composed of 146 mM NaCl, 4.0 mM KCl, 2.4 mM CaCl$_2$, and 10 μM of the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; Sigma-Aldrich, St. Louis, MO). Morphine sulfate (Hawkins Chemical, Minneapolis, MN) was prepared in concentrations of 10, 30, 100, and 300 μM. To antagonize the effects of morphine, adenosine levels were measured during dialysis with a mixture of 10 μM naloxone HCl (Tocris, Ellisville, MO) and 100 μM morphine. Fentanyl citrate (Cat. No. F3886; Sigma) was administered to the dialysis probe in a concentration of 100 μM. A final set of experiments was designed to determine whether inhibiting adenosine deaminase reverses the morphine induced decrease in adenosine levels. For these experiments, 110 μM EHNA was coadministered with 100 μM morphine. All drug concentrations listed above refer to solutions used to perfuse the microdialysis probes and do not indicate drug concentrations delivered to the brain. Based on calculations of in vitro adenosine recovery, approximately 4.5% of the dialyzed drug was delivered to the brain.

Experimental Design and Procedures

A within-subjects experimental design was used to quantify the effects of opioids on adenosine levels by comparing adenosine measures obtained during 60 min of dialysis with Ringer's solution (control) to adenosine measures obtained during 60 min of dialysis with Ringer's solution containing morphine, fentanyl, morphine plus naloxone, or morphine plus EHNA.

Experiments began by placing a rat in a Plexiglas chamber for induction of anesthesia with 4% isoflurane in 100% O$_2$. After 5 min, the anesthetized rat was removed from the chamber and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A rat anesthesia mask was used to deliver 2.5% isoflurane in 100% O$_2$. A midline scalp incision exposed lambda and bregma landmarks, and the skull was marked above either the PRF (stereotaxic coordinates: 8.4 mm posterior to bregma, 1.0 mm lateral to the midline, 9.2 mm below bregma) or the SI (stereotaxic coordinates: 1.6 mm posterior to bregma, 2.5 mm lateral to the midline, 8.7 mm below bregma).[22] The oral and caudal portions of the pontine reticular nucleus comprise the PRF.[22] A small craniotomy was made in the skull using a Dremel tool (Racine, WI), and the microdialysis probe was lowered into the brain. Delivered isoflurane was measured continuously (Cardiocap/5; Datex-Ohmeda, Louisville, CO) and maintained at 1.5% throughout the experiment. Body temperature was maintained at 37°C using a water blanket and a recirculating heat pump (Gaymar Industries, Orchard Park, NY) for the duration of the experiment and during recovery from anesthesia.

Dialysis samples were collected every 15 min. Four samples were collected during dialysis with Ringer's solution to quantify control levels of adenosine. A liquid switch was then activated to dialyze with Ringer's solution containing morphine, fentanyl, morphine and naloxone, or morphine and EHNA. After collection of four dialysis samples during drug delivery, the microdialysis probe was withdrawn from the brain and the scalp incision was closed. Isoflurane delivery was stopped, and the animal was removed from the stereotaxic frame. Rats were monitored until ambulatory and returned to their home cages.

Histologic Localization of Microdialysis Sites

Two to 4 days after the dialysis experiment, each rat was deeply anesthetized and decapitated. The brain was removed, and 40-μm-thick coronal sections were cut serially using a cryostat (Leica Microsystems, Nussloch, Germany). Sections were mounted on chrome alum–coated slides, fixed in paraformaldehyde vapor (80°C), and stained with cresyl violet. The histologic sections were then digitized using a Nikon Super Coolscan 4000 scanner (Tokyo, Japan). Verification that the probe was located in the PRF or SI was achieved by comparison of the digitized histology sections to plates in a rat brain atlas.[22]

Statistical Analysis

Adenosine levels measured during microdialysis delivery of opioids were compared with adenosine levels measured during microdialysis with Ringer's solution (control). Each set of data was tested for goodness of fit to a normal distribution. SAS software (release 9.1.3; SAS Institute, Cary NC) was used to determine drug effects on adenosine levels. A linear mixed model analysis of variance was used for each of the outcome variables with drug as a fixed effect and rat as a random effect (a random intercept was used for each rat). This analysis
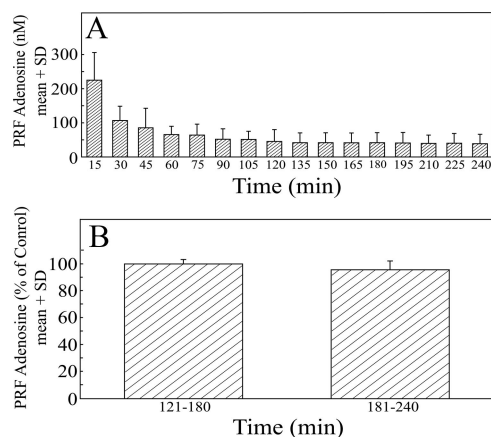
Fig. 1. Time course of stable adenosine measures. (A) Time course of pontine reticular formation (PRF) adenosine levels across 16 sequential brain dialysis samples collected during control dialysis with Ringer's solution. Adenosine levels stabilized 120 min after dialysis probe placement in the PRF and remained stable for an additional 120 min. (B) There was no significant difference between average PRF adenosine levels during minutes 121–180 and minutes 181–240.

resulted in a compound symmetric covariance structure that made it possible to take into account the correlations among observations from the same animal. This statistical approach is described in detail elsewhere.[23] In addition to analysis of variance and *post hoc* test, regression analyses showed that the slope of the declining adenosine levels during dialysis with Ringer's solution (control) (fig. 1) was not significantly different from zero slope and that the slope of the adenosine functions during minutes 121–180 was not different from the slope during minutes 181–240. *Post hoc* comparisons of drug effects were conducted using the Dunnett multiple comparisons method and *t* test to compare each drug condition to control. *P* values less than 0.05 were considered significant. Data are reported as mean ($\pm$SD).

Results

Stability of Adenosine Measurement

After brain insertion of a microdialysis probe, levels of adenosine are initially high and decline over time before stabilizing.[6] Therefore, to test the hypothesis that opioids decrease brain adenosine levels, the first step was to demonstrate stability of adenosine levels during the time course of the current experiment. Figure 1A illustrates the time course of adenosine levels measured every 15 min in the PRF during dialysis with Ringer's solution (control). Each bar summarizes the mean adenosine concentration for three rats at a given time point after the microdialysis probe was inserted into the brain. As shown previously for the neurotransmitter γ-aminobutyric acid, the stability of adenosine levels was confirmed by slope analysis.[14] After microdialysis probe insertion into the brain, the first 7 samples (minutes 15–105) showed a consistent decrease in adenosine. For minutes

120–240, however, regression analyses revealed that the slope of adenosine levels was not significantly different from zero. These data confirmed the appropriateness of the current experimental design in which microdialysis samples obtained during minutes 121–180 were used to quantify control levels of adenosine and samples collected during minutes 181–240 were used to quantify opioid effects on adenosine levels. Figure 1B shows that during microdialysis with Ringer's solution, the average adenosine level during minutes 121–180 was not significantly different by *t* test from the average adenosine level during minutes 181–240. These results confirmed that time was not a confounding variable and that opioids caused the decreased adenosine levels in PRF and SI.

Morphine Delivery to the PRF Caused a Concentration-dependent Decrease in PRF Adenosine Levels That Was Blocked by Naloxone

Figure 2A shows that dialysis delivery of morphine to 14 rats caused a concentration-dependent decrease in PRF adenosine ($P < 0.0001$). The linear mixed model with Dunnett *post hoc* analysis indicated that each concentration of morphine caused a significant decrease in PRF adenosine. The mean percent decrease ($\pm$SD) in adenosine levels caused by 10, 30, 100, and 300 μM morphine was 11.53 ($\pm$19.99), 15.31 ($\pm$16.19), 20.2 ($\pm$9.47), and 16.91 ($\pm$11.28), respectively. Data from five rats (fig. 2B) analyzed using the linear mixed model showed that the decrease in adenosine caused by morphine (100 μM) was blocked by coadministration of naloxone (10 μM). Figure 2C summarizes data from three rats showing that fentanyl (100 μM) significantly ($P = 0.0003$) decreased PRF adenosine levels by 13.3% ($\pm$9.1%). All dialysis sites were histologically confirmed to be within the PRF (fig. 2D). The mean PRF dialysis site was 8.3 ($\pm$0.3) mm posterior to bregma, 1.2 ($\pm$0.2) mm lateral to the midline, and 9.1 ($\pm$0.1) mm below the surface of the skull.

Adenosine Levels in SI of Basal Forebrain Were Decreased by Opioids

Previous studies using intact, unanesthetized cats report comparable basal levels of adenosine in SI and pontine brain stem, but different rates of change during 6 h of sleep deprivation.[6] Therefore, we also quantitatively evaluated the stability of adenosine levels in SI of isoflurane-anesthetized rats during microdialysis with Ringer's solution alone. Three rats were used to quantify SI adenosine levels every 15 min for 240 min after brain insertion of the microdialysis probe. Mean ($\pm$SD) SI adenosine levels were 24.3 ($\pm$5.8) nM during minutes 121–180 and 20.9 ($\pm$4.4) nM during minutes 181–240. There was no significant difference in SI adenosine levels as a function of time as confirmed by repeated-measures analysis of variance, *t* tests, and regression analyses. Power calculations using the mean and SD values above
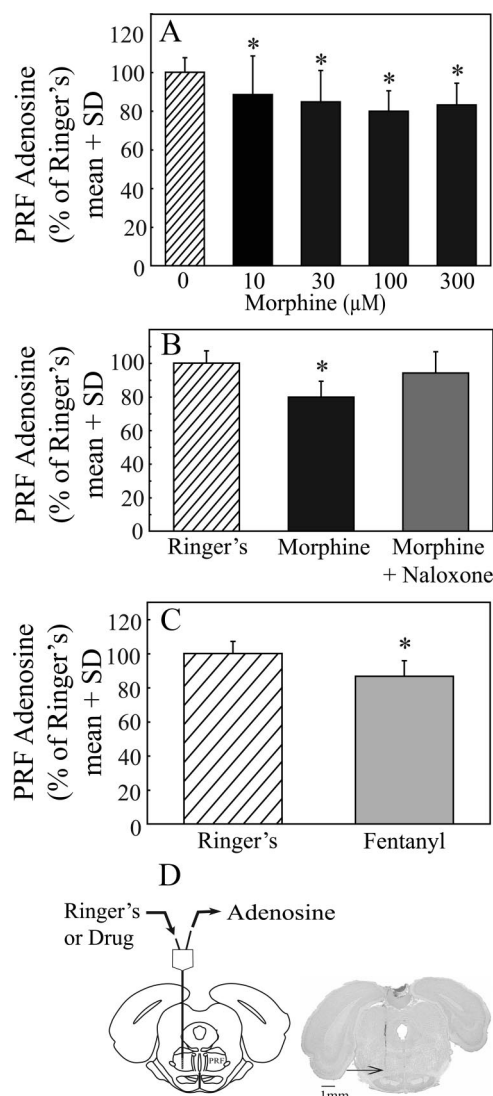
Fig. 2. Opioids decrease pontine reticular formation (PRF) adenosine levels. (*A*) Data summarize PRF adenosine levels during dialysis with Ringer's solution and 10, 30, 100, and 300 μM morphine. * Significant ($P < 0.05$) decrease from Ringer's control (0 μM). (*B*) The morphine (100 μM)–induced decrease in PRF adenosine was blocked by coadministration of naloxone (10 μM). These data illustrate that adenosine levels during coadministration of morphine and naloxone were not statistically different from adenosine levels during dialysis with Ringer's solution. (*C*) Dialysis with fentanyl (100 μM) caused a decrease in PRF adenosine levels. (*D*) A plate from a rat brain atlas[22] illustrates a microdialysis probe placed in the PRF for drug delivery and collecting endogenous adenosine. The digitized rat brain stem section at *right* shows a representative microdialysis site (*arrow*) in the PRF.

indicate that 33 rats would be required to demonstrate a significant difference in SI adenosine as a function of time alone.

Figure 3A summarizes data from five rats showing that dialysis of the SI with morphine (100 μM) significantly ($P < 0.0009$) decreased SI adenosine levels. The linear mixed model showed the mean percent decrease ($\pm$SD) was $26.8 \pm (10.5)$. In a second series of studies, fentanyl (100 μM) was delivered by dialysis to the SI of
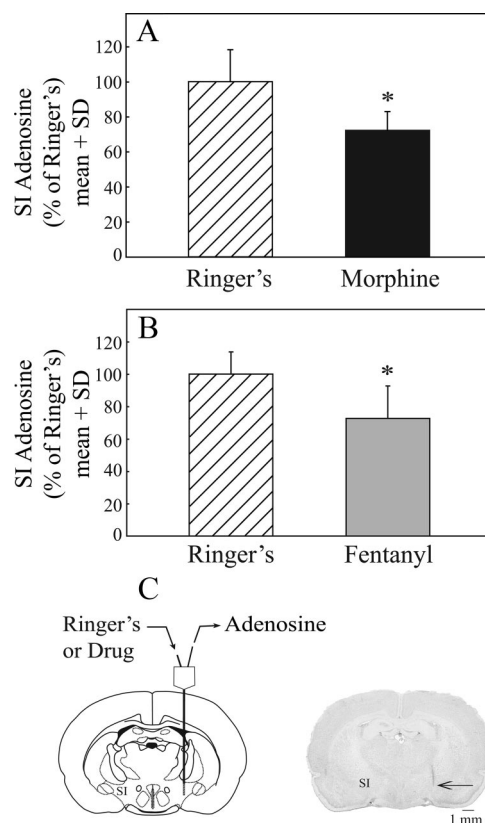

Fig. 3. Morphine and fentanyl decreased adenosine levels in the substantia innominata (SI). (*A*) Microdialysis with morphine (100 μM) decreased SI adenosine levels. (*B*) Similarly, the Student *t* test revealed a significant decrease in SI adenosine levels during dialysis with fentanyl (100 μM). (*C*) A coronal brain plate (*left*) illustrates placement of a microdialysis probe in the SI. A cresyl violet–stained brain section (*right*) confirms that the microdialysis site was localized to the SI. * $P < 0.05$.

three additional rats. The results (fig. 3B) revealed that fentanyl significantly ($P = 0.0004$) decreased adenosine levels by 27.4% ($\pm$20.11%). Mean ($\pm$SD) stereotaxic coordinates for microdialysis probe placement in the SI (fig. 3C) was 1.6 ($\pm$0.04) mm posterior to bregma, 2.6 ($\pm$0.1) mm lateral to the midline, and 8.5 ($\pm$0.2) mm below the surface of the skull. The morphine and fentanyl results, considered together with the control experiments, support the interpretation that opioids delivered to the SI cause a significant decrease in SI adenosine levels.

Coadministration of an Adenosine Deaminase Inhibitor with Morphine Reversed the Decrease in Adenosine Levels Caused by Morphine Alone

Figure 4A shows that coadministering EHNA with morphine to the PRF caused a 26.35% ($\pm$15.11%) increase in adenosine ($P = 0.0005$), relative to dialysis with Ringer's solution. Similarly, figure 4B summarizes results from three additional rats showing that coadministration of morphine and EHNA to the SI caused a 17.8% ($\pm$24.9%) increase in SI adenosine ($P = 0.02$).
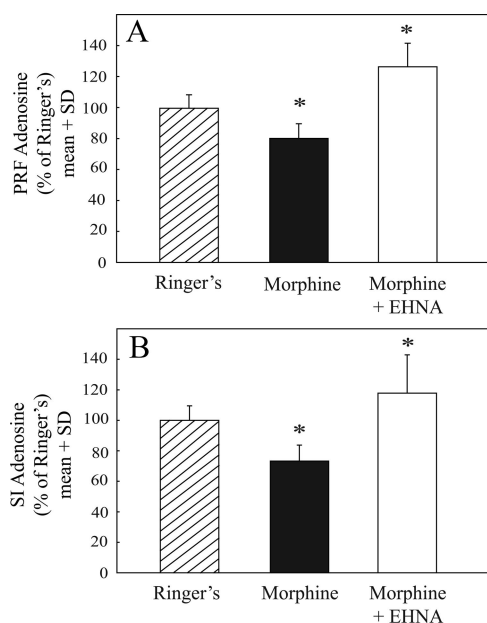
Fig. 4. The adenosine deaminase inhibitor EHNA prevented the morphine-induced decrease in adenosine. In both the pontine reticular formation (PRF; *A*) and the substantia innominata (SI; *B*), coadministration of EHNA, which prevents the degradation of endogenous adenosine, overcame the decrease in adenosine caused by morphine alone. * *P* < 0.05.

Discussion

The results add novel data to a growing body of evidence[14,17,24–34] supporting the hypothesis that anatomically distributed neural networks regulating states of sleep and wakefulness are also involved in generating states of anesthesia.[13,35] Agreement between preclinical and clinical studies illustrates the relevance of this line of investigation for anesthesiology. For example, increasing brain acetylcholine reverses isoflurane anesthesia in rats[36] and reverses propofol-induced unconsciousness in humans.[37] The endogenous neuropeptide hypocretin (orexin) promotes wakefulness in humans[38] and shortens recovery time from isoflurane anesthesia in mice.[33] The current focus on adenosine and opioids stems from long-standing evidence that adenosine modulates acute and chronic effects of opioids[39] and from the potential for adenosine to contribute to pain management.[9,10] The results are discussed in relation to pontine and basal forebrain regions through which opioids and adenosine significantly alter levels of behavioral arousal, nociception, and cognition.

Pontine Reticular Formation Adenosine Promotes Sleep, whereas Opioids Inhibit Sleep and Decrease Adenosine Levels

Converging lines of evidence encouraged the current study to begin by characterizing how opioids delivered to the PRF alter levels of adenosine (fig. 2). The hypothesis that adenosine promotes sleep[40,41] is now widely accepted[42] and is consistent with the fact that adenosine

antagonists such as theophylline and caffeine enhance wakefulness. Previous studies show that microdialysis delivery of an adenosine A_1 receptor agonist to cat PRF significantly delays recovery time from halothane anesthesia.[43] Likewise, microdialysis delivery of an adenosine A_{2A} receptor agonist to the PRF of intact, unanesthetized mice increases sleep.[19] There are four subtypes (A_1, A_{2A}, A_{2B}, and A_3) of G protein–coupled adenosine receptors,[44] and adenosine A_1 and A_{2A} receptors are present in the PRF.[19,45] The findings that morphine caused a concentration-dependent decrease in adenosine levels (fig. 2A), that this decrease was blocked by naloxone (fig. 2B), and that fentanyl also decreased adenosine (fig. 2C) all indicate opioid receptor modulation of adenosine in the PRF (fig. 2D). The reticular formation is also part of an ascending pathway that transduces nociceptive input into traits of behavioral and autonomic arousal comprising the psychophysiological experience of pain.[46] In this context, it is relevant that administering adenosine agonists into the PRF, in doses that do not eliminate wakefulness, also significantly decreases nociception.[47,48]

Basal Forebrain Adenosine Decreases Wakefulness and Opioids Diminish Sleep, Cognitive Function, and Adenosine Levels

Previous studies have shown that delivery of exogenous adenosine to the basal forebrain decreases wakefulness.[49] The concept that adenosine contributes to the homeostatic regulation of wakefulness and sleep is supported by the finding that endogenous adenosine levels in basal forebrain increase during prolonged intervals of wakefulness.[5] Adenosine inhibits wake-active neurons in the basal forebrain of rats[50] and cats,[51] consistent with the suggestion that behavioral state altering effects of adenosine result, in part, from inhibition of cholinergic input to cortex.[5]

Adenosine and acetylcholine interact to modulate arousal states, as clearly indicated by the finding that systemic administration of the adenosine receptor antagonist caffeine increases cortical acetylcholine.[52] Opioids cause brain region–specific changes in acetylcholine release[1,53] and have been shown to decrease acetylcholine release in the basal forebrain.[17] The figure 3 data indicate for the first time that opioids in the basal forebrain decrease adenosine levels. Cortical acetylcholine is essential for normal cognition and morphine decreases cortical acetylcholine release when delivered systemically or locally to the basal forebrain.[17] Postoperative cognitive dysfunction is a clinically significant problem for anesthesiology,[54] and patients with cognitive dysfunction at time of hospital discharge are those with greater opioid use.[55] The discovery that cortical cholinergic neurotransmission is decreased by opioids delivered to the basal forebrain suggested the potential for opioids to contribute to cognitive dysfunction.[17] The figure 3 data show further that opioids also decrease

adenosine levels in the SI region of the basal forebrain. In addition to replicating findings in the PRF (fig. 2), the figure 3 data identify the SI as another brain region, and adenosine as another molecule, through which opioids have the potential to disrupt sleep and cognition.

Potential Clinical Relevance: Adenosine as an Adjunctive Agent for Managing Pain while Minimizing Sleep Disruption

Similar to the paradoxical pain-enhancing effects of opioids,[56] sleep disruption by opioids adds to the conundrum of clinically managing pain with opioids.[57–59] Poor sleep is a major complaint of patients experiencing pain,[60,61] and sleep disruption, even in the absence of opioids, causes cognitive dysfunction.[62] Clinically relevant doses of opioids increase lighter stage 2 nonrapid eye movement sleep, decrease deeper stage 3 and 4 nonrapid eye movement sleep, and decrease rapid eye movement sleep.[1,63–66] Opioids slow the cortical electroencephalogram and create an obtunded state of wakefulness characterized by lethargy and cognitive slowing.[17,67,68] Sleep and electroencephalographic data recorded from rats[16] fit well with clinical evidence showing that opioids increase electroencephalogram power in the delta (0.35–3.5 Hz) and theta (3.5–8 Hz) frequency ranges.[69,70] Opioids disrupt sleep even in pain-free human volunteers.[71] Sleep disruption reduces emotional well-being,[72] causes hyperalgesia,[73] and exacerbates pain.[74]

For more than 10 yr, adenosine has been investigated as a potential adjunctive tool for pain management.[9,75–78] Therefore, a final set of neurochemical experiments was designed to determine whether the opioid-induced decrease in adenosine (figs. 2 and 3) could be reversed during opioid administration. The results show that within both the PRF (fig. 4A) and the SI region of the basal forebrain (fig. 4B), decreasing the enzymatic degradation of adenosine by coadministering morphine plus an adenosine deaminase inhibitor prevented any opioid induced decreases in adenosine. The finding that sleep can be increased by microdialysis delivery of an adenosine deaminase inhibitor,[79] combined with the figure 4 results, encourages continuing efforts to develop adjunctive therapies to counter opioid-induced disruptions of sleep and wakefulness.

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