Microinjection of an Adenosine A_1 Agonist into the Medial Pontine Reticular Formation Increases Tail Flick Latency to Thermal Stimulation

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Background: **Both pain and the pharmacologic management of pain can cause the undesirable effect of sleep disruption. One goal of basic and clinical neuroscience is to facilitate rational drug development by identifying the brain regions and neurochemical modulators of sleep and pain. Adenosine is thought to be an endogenous sleep promoting substance and adenosinergic compounds can contribute to pain management. In the pontine brain stem adenosine promotes sleep but the effects of pontine adenosine on pain have not been studied. This study tested the hypothesis that an adenosine agonist would cause antinociception when microinjected into pontine reticular formation regions that regulate sleep.**

Methods: **The tail flick latency (TFL) test quantified the time in seconds for an animal to move its tail away from a thermal stimulus created by a beam of light. TFL measures were used to evaluate the antinociceptive effects of the adenosine A_1 receptor agonist N^6-p-sulfophenyladenosine (SPA). Pontine microinjection of SPA (0.1 μg/0.25 μl, 0.88 mM) was followed by TFL measures as a function of time after drug delivery and across the sleep–wake cycle.**

Results: **Compared with saline (control), pontine administration of the adenosine agonist significantly increased latency to tail withdrawal ($P < 0.0001$). The increase in antinociceptive behavior evoked by the adenosine agonist SPA was blocked by pretreatment with the adenosine A_1 receptor antagonist 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX, 0.75 ng/0.25 μl, 10 μM).**

Conclusions: **These preclinical data encourage additional research on the cellular mechanisms by which adenosine in the pontine reticular formation contributes to the supraspinal modulation of pain.**

SLEEP disruption is a leading complaint of patients experiencing untreated and treated pain.[1–3] In addition, many pain medications disrupt the normal sleep cycle. Patients receiving opioids give subjective reports of sleepiness, yet objective polygraphic data demonstrate that opioids inhibit the rapid eye movement (REM) phase of sleep. Clinical[4,5] and preclinical[6–8] studies concur that opioids inhibit REM sleep even in the absence of pain. Thus, both pain and the pharmacologic treatment of pain can disrupt normal sleep. The sleep disrupting side effect of opioids is of particular relevance for postsurgical care because sleep deprivation impairs immune function and host defense.[9,10]

Adenosine is an important signaling molecule that is found throughout the nervous system.[11] Preclinical studies show that adenosine causes spinal antinociception.[12,13] Clinical studies also demonstrate that adenosine produces prolonged pain relief.[14–19] Recently, a multicenter, double-blind, placebo-controlled study found that intravenous infusion of adenosine reduces neuropathic pain.[20] The sleep-inducing effects of prolonged wakefulness may involve adenosinergic modulation of brain regions regulating sleep.[21,22] The medial pontine reticular formation is a brain region contributing to both REM sleep generation[23] and cholinergically modulated antinociception.[24] The current article presents preclinical data showing for the first time that an adenosine agonist, applied to REM sleep-regulating regions of the medial pontine reticular formation, causes antinociception. Portions of these data have been presented in abstract form.[25]

Materials and Methods

Measures of Antinociceptive Behavior as a Function of Drug and Sleep

All experimental protocols were reviewed and approved by the University of Michigan Committee for the Use and Care of Animals and this study strictly adhered to the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences Press, Washington, D.C., 1996). Using standard techniques,[26] adult male cats were deeply anesthetized with isoflurane (2 to 3% in oxygen) and implanted with electrodes for recording sleep and wakefulness. A permanent craniotomy was created to enable pontine drug administration during subsequent experiments. After the animals recovered from surgery, they were trained to sleep in a head-restrained position in the laboratory.

Acute thermal stimulation produced by a focused beam of light was applied to the shaved portion of the tail using an IITC Life Sciences (Los Angeles, CA) tail flick meter. An electronic timer and the beam of light were activated simultaneously. When the animal moved its tail the photocell was triggered to stop the timer. The measurement in seconds represented the tail flick latency (TFL) to movement and provided an objective measure of antinociceptive behavior. To avoid habituation and to
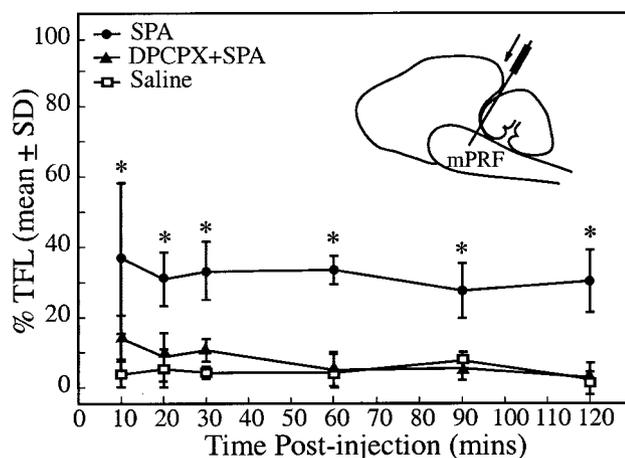
Fig. 1. Pontine administration of N⁶-p-sulfophenyladenosine (SPA) increased latency to tail withdrawal. Time-course for mean tail flick latency (TFL) following medial pontine reticular formation (mPRF) drug administration (*inset*). *Post hoc* analyses revealed that microinjection of SPA into the medial pontine reticular formation significantly (*$P < 0.0001$) increased TFL over saline (control) at each of the six time points for up to 120 min postinjection. The antagonist DPCPX blocked the antinociceptive behavior induced by SPA.

prevent tissue damage, the stimulus cutoff time was set at 8 s and stimuli were delivered to different areas of the tail.[27] Therefore, 8 s represented the maximal possible latency to respond to the thermal stimulus. These methods have been adapted from use with rodents[27] and have been shown to be appropriate for use with cats.[24,28]

During testing sessions the implanted electrodes were connected *via* a cable to a Grass Model 7 polygraph so that TFL measures could be obtained during objectively defined states of sleep and wakefulness.[29] The tail was positioned above the light sensor and five baseline TFL measurements were obtained while the animal was in a state of quiet wakefulness. These five measurements were averaged to create the mean baseline TFL. During each experiment, the animal was randomly assigned to receive one of three drug treatments. These included unilateral pontine microinjections (Fig. 1, inset) of either saline (0.25 μl, control), the adenosine A_1 receptor agonist N⁶-p-sulfophenyladenosine (SPA; Research Biochemicals International [RBI], Natick, MA) (0.1 μg/0.25 μl, 0.88 mM), or the adenosine A_1 receptor antagonist 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX; RBI, Natick, MA) (0.75 ng/0.25 μl, 10 μM) followed 15 min later by 0.88 mM SPA (0.1 μg/0.25 μl). Only one previous study[24] has demonstrated antinociceptive responses evoked from the medial pontine reticular formation. These data showed that cholinomimetics significantly increased TFL across the sleep-wake cycle. The 0.88 mM SPA concentration used in the current study was one log unit less than the concentration of cholinomimetics shown previously to be antinociceptive when microinjected into the medial pontine reticular formation.[24] The 0.88 mM SPA concentration also was within a range shown to significantly activate guanylyl nucleotide

binding proteins (G proteins) in the pontine reticular formation.[30] After drug administration, the animal was free to sleep or wake *ad libitum*, and five TFL measures were obtained at each of six time points. These six measurement points were 10, 20, 30, 60, 90, and 120 min postinjection. Each minute of the 120 min polygraphic recording was scored as wakefulness, non-rapid eye movement (NREM) sleep, or REM sleep.

Injection Sites within the Brain Stem

The medial pontine reticular formation was chosen as the brain region for study because of the compelling evidence that the pontine reticular formation contributes to sleep cycle control.[23,31] The trapezoid body was used for control microinjections to determine whether the effects of SPA on antinociception were site-specific to the pontine reticular formation. The nucleus of the trapezoid body is located below the pontine reticular formation and is not thought to play a role in regulating sleep or nociception. Upon completion of experiments, the animals were deeply anesthetized and perfused with saline followed by formalin (10%). Brains were removed for histologic evaluation of microinjection sites.

Experimental Design and Statistical Analysis

This study used an intensive, within-subjects design. Each animal (N = 4) received three pontine microinjections of each drug in randomized order. Each injection trial was separated by at least 1 week from the preceding trial. This made it possible to collect 1,079 TFL measures during approximately 4 months. Tail flick latency was expressed as a percent change from maximal possible effect by the formula: %TFL = (experimental TFL − mean baseline TFL)/(cutoff time − mean baseline TFL) × 100. This formula takes into account the 8 s cutoff and is frequently used for pain studies in animals.[24,28] Thus, for each measurement a value identical to the experimental baseline was equal to 0% change in TFL and a value of 8 s was equal to 100% of the maximal possible response.

As shown previously,[24] the lack of independence between TFL measures requires a repeated measures analysis of variance (ANOVA). Analyses were performed using SAS (SAS Institute Inc., Cary, NC) Proc Mixed Software (Release 8.2) and taking into account drug, time postinjection, and sleep–wake state. Tukey–Kramer *post hoc* multiple comparison statistics were used to evaluate differences in TFL between drug treatments, postinjection time intervals, and states of wakefulness and NREM sleep.

Results

Pontine Administration of the Adenosine A_1 Agonist Caused Antinociceptive Behavior

Figure 1 shows TFL as a function of time following pontine microinjection of the adenosine agonist (SPA),

Table 1. Average Tail Flick Latency (s) as a Function of Time Postinjection

Treatment	Animal	10	20	30	60	90	120
SPA	1	6.11	3.90	4.57	4.13	3.40	3.77
	2	2.80	2.81	2.88	3.58	2.79	3.69
	3	3.51	4.05	3.81	4.05	3.80	4.19
	4	4.35	4.42	4.32	3.94	4.33	3.37
DPCPX + SPA	1	2.76	2.68	3.21	2.53	2.59	2.77
	2	3.07	2.90	2.55	2.13	2.53	2.19
	3	3.27	3.25	3.15	3.05	2.87	2.50
	4	3.44	2.53	2.84	2.78	2.61	2.52
Saline	1	3.14	2.65	2.85	2.55	3.16	2.53
	2	1.93	2.06	2.09	2.11	2.29	2.01
	3	2.48	2.58	2.60	2.72	2.78	2.73
	4	2.54	3.21	2.73	2.86	2.78	2.55

Values give the mean latency in seconds for fifteen tail flick measures at each of six time points (10, 20, 30, 60, 90, and 120 min) after pontine injection. Drug treatment conditions (SPA, DPCPX + SPA, Saline) itemize the responses for the four animals (1–4). Preinjection baseline latencies averaged 2.42 s for the first animal, 1.73 s for the second animal, 2.2 s for the third animal, and 2.41 s for the fourth animal.

SPA = N^6-p-sulfophenyladenosine, an adenosine A_1 receptor agonist; DPCPX = 8-cyclopentyl-1, 3-dipropylxanthine, an adenosine A_1 receptor antagonist.

the adenosine antagonist followed by the agonist (DPCPX + SPA), and vehicle control (saline). ANOVA revealed a significant (F = 57.44; df = 2,6; P = 0.0001) main-effect of drug administration, no significant main-effect of time postinjection, and no significant interaction between time and drug. Microinjection of SPA into the medial pontine reticular formation increased TFL within 10 min postinjection in all animals (table 1). Histologic analyses confirmed that all SPA injection sites were within the medial pontine reticular formation, which is part of the gigantocellular tegmental field defined by Berman.[32] SPA caused more than a 30% enhancement of TFL that was maintained for 120 min postinjection (fig. 1). Pretreatment with the adenosine antagonist DPCPX 15 min prior to SPA administration blocked the SPA-induced increase in TFL. After saline microinjections, the mean change in TFL from baseline ranged between 3.7% at 10 min and 2.2% at 120 min (fig. 1). Administration of the antagonist alone did not cause a significant change in TFL compared with saline (data not shown). The antinociceptive effect of supraspinal SPA was site-specific within the pons. Microinjection of SPA at stereotaxic coordinates corresponding to the nucleus of the trapezoid body[32] did not elicit a significant increase in TFL compared with baseline.

State-dependent Enhancement of Antinociception

The data shown in figure 1 were analyzed without considering the sleep–wake state of the animal at the time points when the TFL measures were obtained. To determine whether the antinociceptive behavior produced by the adenosine agonist varied with sleep, TFL measures were analyzed as a function of both the sleep–wake state and the drug condition (fig. 2; table 2). ANOVA showed that TFL varied significantly during wakefulness and NREM sleep (F = 91.93; df = 1,3; P = 0.002). Tukey–Kramer statistic revealed two findings summarized by figure 2. First, the adenosine agonist SPA

increased TFL over control (saline) level during wakefulness (28%) and during NREM sleep (25%) (fig. 2, differences between solid bars and open bars). Second, pretreatment with the adenosine antagonist DPCPX completely blocked the agonist-induced antinociceptive behavior (fig. 2, hatched bars). Only a limited number of TFL measures were obtained during the REM phase of sleep. Because of this small sample size, the REM sleep data were not included in the ANOVA evaluating arousal state main-effect on TFL. During REM sleep, SPA increased TFL by 15% over saline (fig. 2, solid bar *vs.* open bar).

Discussion

The finding that pontine administration of SPA increased tail flick latency to nociceptive heat stimulation
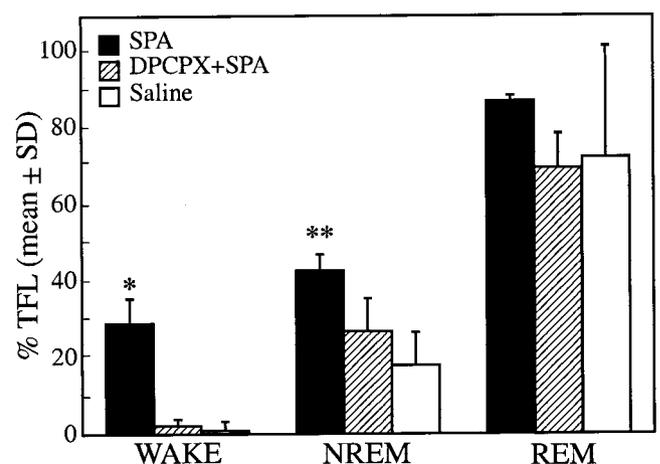

Fig. 2. N^6-p-sulfophenyladenosine (SPA) significantly increased antinociceptive behavior during wakefulness and non-rapid eye movement (NREM) sleep. Mean tail flick latency (TFL) is plotted as a function of arousal state and drug treatment. During wakefulness, SPA significantly (*P = 0.0001) increased TFL compared with saline (control) injection. During NREM sleep, TFL also was significantly increased (**P = 0.002) by SPA compared with saline.

Table 2. Average Tail Flick Latency (s) as a Function of the Sleep–Wake State

Animal	Baseline	Treatment	Wakefulness	NREM sleep
1	2.42	SPA	4.25	4.58
		DPCPX + SPA	2.61	3.91
		Saline	2.63	3.41
2	1.73	SPA	2.76	3.97
		DPCPX + SPA	2.27	3.43
		Saline	1.91	2.37
3	2.2	SPA	3.63	4.86
		DPCPX + SPA	2.57	3.35
		Saline	2.51	3.08
4	2.41	SPA	4.03	4.78
		DPCPX + SPA	2.53	4.61
		Saline	2.54	3.94

Values are mean tail flick latencies averaged by drug treatment condition (SPA, DPCPX + SPA, Saline) and arousal state (wakefulness and NREM sleep). The preinjection (baseline) latency for tail movement away from the heat source is shown for each animal (1–4) in the left column. Mean baseline latency across animals was 2.19 s.

SPA = N^6-p-sulfophenyladenosine, an adenosine A_1 receptor agonist; DPCPX = 8-cyclopentyl-1, 3-dipropylxanthine, an adenosine A_1 receptor antagonist; NREM = non-rapid eye movement.

(figs. 1 and 2) suggests pontine adenosine A_1 receptors as modulators of antinociceptive behavior during wakefulness and sleep. There is good agreement that opioids significantly inhibit REM sleep.[2,4,6,7] Pontine administration of SPA produced increased tail flick latency without blocking or enhancing REM sleep. These results can be contrasted with the finding that REM sleep was enhanced by microinjection of an adenosine agonist into homologous regions of rat brain[33] and by dialysis delivery of adenosine into the cholinergic laterodorsal tegmental nucleus of cat.[34] Potential factors accounting for differences in the amount of REM sleep reported here compared with previous studies include differences in drug, brain region, and species. Both preclinical and clinical data show that the medial region of the pontine reticular formation plays a key role in regulating sleep.[23,35,36] The medial pontine reticular formation has not traditionally been considered part of ascending or descending pain pathways[1], and few studies have characterized the neuronal mechanisms by which pain disrupts sleep. Excitability of spinoreticular tract neurons has been shown to vary with sleep–wake state[37] *via* mechanisms that may include primary afferent depolarization and postsynaptic inhibition.[38]

Efforts to understand the mechanisms that drive sleep propensity have been stimulated by the working hypothesis that endogenous adenosine promotes one function of sleep: the restoration of brain energy metabolism.[39] The postulated link between sleep and cellular energy requirement logically is related to adenosine triphosphate because hydrolysis of adenosine triphosphate to adenosine diphosphate provides cellular energy. Dephosphorylation of adenosine triphosphate yields adenosine, and accordingly all cellular activities that increase metabolic demand will increase adenosine. Substantial

data now suggest adenosine as a modulator of both sleep drive[21] and slow-wave cortical electroencephalographic activity characteristic of NREM sleep.[22] Rat brain levels of adenosine are greatest following prolonged intervals of activity.[40] Adenosine inhibits neurons that are known to promote arousal, and systemic administration of adenosine agonists increases NREM sleep.[11,41]

Limitations of the current study include the fact that only one concentration of SPA was tested. The SPA enhancement of TFL was blocked by the adenosine A_1 receptor antagonist DPCPX, and these results encourage future studies aiming to characterize antinociceptive behavior across a range of SPA concentrations. The present pontine microinjection data are focused on supraspinal antinociception and behavioral state control.[23] Clearly, intrathecal delivery is the most practical route for clinical administration of adenosine.[42,43] Basic studies, however, provide an essential first step in the development of efforts to diminish pain without disrupting sleep.[44] The translational link between preclinical and clinical studies of pain has been successfully demonstrated. For example, it was the intrathecal injection of opioids in rats by Yaksh *et al*. that led to the development of epidural opioids for clinical pain management.[45]

In the current study, fewer TFL measures were obtained during REM sleep than during wakefulness or NREM sleep because the animals were aroused from REM sleep by the thermal stimulation. In an effort to be conservative with the ANOVA, TFL measures obtained during REM sleep are summarized by descriptive statistics alone (fig. 2). Consistent with previous TFL measures obtained across the feline sleep cycle,[24] the results show a significant sleep state effect. Data from human volunteers also show that polysynaptic nociceptive reflexes are reduced in sleep, with the greatest reduction occurring during REM sleep.[46] After saline injection, the TFL increase during REM sleep compared with NREM sleep and wakefulness (fig. 2) is consistent with previous TFL measures obtained across the feline sleep cycle.[24] Comparisons across animals reveal a similar response to the independent variables (tables 1 and 2) and the intensive, within-subjects design demonstrated statistically robust drug effects.

At the cellular level, adenosine actions are mediated primarily *via* A_1 and A_2 high-affinity receptors.[47] The adenosine A_1 receptor is coupled to an inhibitory G protein[48] and the adenosine A_1 agonist SPA, used in the present study, activates G proteins in the medial pontine reticular formation.[30] Of particular interest in these previous studies was the finding of G protein activation caused by a partially additive interaction between SPA and a μ-specific opioid agonist.[30]

In clinical practice a wide range of drugs have been combined with opioids in an effort to improve epidural analgesia.[49] Recently, a phase 1 safety assessment clinical trial suggested that intrathecal adenosine does not

produce a high incidence of severe side effects,[42,43] further supporting research efforts toward the application of adenosinergic compounds for pain control. Considered together, the previous G protein data[30] and the functional evidence presented here raise the question of whether adenosinergic compounds can be developed as adjunctive therapies for supraspinal pain control that reduce or eliminate the unwanted sleep disrupting effects of opioids.

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