Cholinomimetics, But Not Morphine, Increase Antinociceptive Behavior from Pontine Reticular Regions Regulating Rapid-eye-movement Sleep

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Summary: Sleep disruption is a significant problem associated with the subjective experience of pain. Both rapid-eye-movement (REM) sleep and nociception are modulated by cholinergic neurotransmission, and this study tested the hypothesis that antinociceptive behavior can be evoked cholinergically from medial pontine reticular formation (mPRF) regions known to regulate REM sleep. The foregoing hypothesis was investigated by quantifying the effect of mPRF drug administration on tail flick latency (TFL) of cat during polygraphically defined sleep/wake states. The mPRF was microinjected with 0.25 ml saline, carbachol (4.0 µg), neostigmine (6.7 µg), or morphine sulfate (14.7 µg), and TFL measures were obtained in response to radiant heat. During wakefulness TFL (% increase) was not increased by morphine or saline, but was significantly increased by mPRF administration of carbachol (42.4%) and neostigmine (35.2%). Cortical somatosensory potentials (SSEPs) were reliably evoked by tail stimulation before and after mPRF microinjections of carbachol. The results show for the first time that mPRF administration of cholinomimetics significantly increased TFL. During NREM sleep and REM sleep, TFL was significantly increased compared to waking TFL (110% and 321%, respectively). The finding of sleep-dependent alterations in TFL demonstrates that mPRF regions known to regulate REM sleep can modulate supraspinal cholinergic antinociceptive behavior.

Key Words: Supraspinal cholinergic antinociception; tail flick latency; opioids; pain

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SLEEP DISRUPTIONS are among the most common complaints of patients experiencing pain, yet the neurobiological links between disrupted sleep and pain remain unspecified. Melzack and Wall proposed that nociceptive input (via A-delta and C fibers) is modulated by non-nociceptive, large-diameter (A-beta) fibers and by supraspinal descending pathways. The neurochemical identity of spinal and supraspinal systems modulating pain is complex and incompletely understood. Multiple lines of evidence, however, demonstrate that acetylcholine (ACh) plays a key role in the modulation of nociception. Preclinical studies in rat and cat showed that intrathecal administration of cholinomimetics has antinociceptive actions, and that nonopioid analgesia can be produced by administration of cholinergic agonists directly into the brainstem. Cholinomimetics also have been used for human pain management, and recent clinical advances with cholinergic antinociception have been highlighted elsewhere.

Acetylcholine is known to play a key role in sleep cycle control. Rapid-eye-movement (REM) sleep is reliably triggered and enhanced by administering microgram doses of carbachol, bethanechol, or neostigmine into specific regions of the medial pontine reticular formation (mPRF). Cholinomimetic evocation of the REM-sleeplike state is anatomically site-dependent within the pons, dose-dependent, and blocked by atropine (reviewed in 14). Microdialysis of the mPRF reveals significantly increased ACh release during REM sleep and during the cholinergic...
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The fact that ACh modulates both nociception and sleep led us to test the hypothesis that antinociceptive behavior can be evoked cholinergically from the mPRF. This hypothesis was examined by quantifying the effect of mPRF administration of morphine, carbachol, and neostigmine on tail flick latency during wakefulness, nonREM (NREM) sleep, and REM sleep. Portions of these data have been presented in abstract form.16

METHODS

Surgical Procedure

Five adult male cats (3.5 to 5 kg) were anesthetized with isoflurane (1-2% in O2) and implanted with electrodes for polygraphically recording the electroencephalogram (EEG), electrooculogram (EOG), electromyogram (EMG), and ponto-geniculo-occipital (PGO) waves from the lateral geniculate body of the thalamus. This constellation of electrographic recordings was used to obtain an objective assessment of wakefulness, NREM sleep, and REM sleep.17 The animals also were implanted with bilateral stainless steel guide tubes (24 gauge), stereotaxically positioned to permit repeated drug or vehicle microinjection into the mPRF (P=2, L=1.5, H=-5).18 These surgical and experimental procedures have been described and illustrated in detail,13,14,19,20 and strictly adhered to National Institutes of Health regulations specified in the Guide for the Care and Use of Laboratory Animals (7th edition, National Academy of Sciences Press, Washington, DC, 1996).

Pontine Microinjections and Tail Flick Latency

Approximately 1 month after surgery, cats were trained to sleep in a head-restrained position in the laboratory. Electrodes were connected to a Grass Model 7 polygraph by an electrically shielded cable. After the cats demonstrated normal sleep patterns, the pontine microinjection and tail flick latency experiments were begun. Each cat had a distal 10 cm portion of the tail shaved immediately prior to each experiment. A tail flick meter (IITC Life Sciences) focused a beam of light on a shaved portion of the tail. An electronic timer was started, coinciding with the onset of the light stimulus. As the thermal nociceptive threshold was reached, the animal moved the tail and the electronic timer automatically stopped. The recording in seconds represented the tail flick latency (TFL) to movement. The maximum time of exposure to the radiant light was set at 8 seconds and provided a cutoff time that prevented any tissue damage. In addition, thermal stimulus episodes were separated by at least 60 seconds and delivered to different portions of the tail, since repeated stimulation at one site has been shown to cause habituation in rats.21 Using a technique demonstrated to be appropriate for quantifying antinociceptive behavior in cats,22 TFL was expressed as a percent change from premicroinjection baseline by the formula:

\[
\frac{\text{experimental TFL} - \text{baseline TFL}}{\text{cutoff time} - \text{baseline TFL}} \times 100.
\]

At the beginning of each experiment, when the animals were in a state of quiet wakefulness, baseline TFL measurements were obtained prior to pontine drug administration. After the baseline TFL measures, 0.25 μl of saline or drug was administered into the mPRF via a 31-gauge microinjector connected to polyethylene tubing (PE 20) and a microdrive activated Hamilton syringe. This mPRF microinjection protocol has been shown to provide a useful experimental tool for elucidating the cholinergic modulation of sleep,10,14 anesthesia,23 and breathing.13,24 Compounds microinjected unilaterally into the mPRF, the concentration, amount injected, and number of injections (n) included saline (0.09%; 0.25 μl; n=12), the cholinergic agonist carbachol (88 mM; 4.0 μg/0.25 μl; n=12), the acetylcholinesterase inhibitor neostigmine bromide (88 mM; 6.67 μg/0.25 μl; n=12), and morphine sulfate (88 mM; 14.71 μg/0.25 μl; n=12). TFL measures were obtained at 10, 20, 30, 60, 90, and 120 minutes after each microinjection. Every minute of each 120-minute polygraphic recording was scored as wakefulness, NREM sleep, or REM sleep, making it possible to quantify TFL in relation to electrographically defined states.

In addition to the TFL studies, a Sentinel Axon Systems (Hauppauge, NY) computer was used to record cortical somatosensory evoked potentials (SSEPs) during electrical stimulation of posterior tibial nerve (hindlimb) and sacrococcygeal nerve (tail) (500 repetitions, 100 ms analysis time). Repeated evoked potential studies were conducted in one animal before and after mPRF carbachol administration in order to determine whether sensory input was transmitted to the cortex during the cholinergically induced REM-sleeplike state.

Statistical Analyses

Analysis of variance (ANOVA) for repeated measures was used to quantify TFL as a function of drug, time postinjection, and to evaluate time-by-drug interaction. ANOVA also was used to determine whether TFL varied significantly as a function of wakefulness, NREM sleep, and REM sleep. Comparisons of TFL across the four drug treatments and three sleep/wake states were evaluated statistically using Dunnett’s and Tukey’s multiple comparison tests with probability (p) value of p≤0.05 chosen as an index of statistical significance.
RESULTS

The pontine region where drugs were microinjected for the TFL studies is shown by Fig. 1. All microinjection sites were localized within the mPRF region of the gigantocellular tegmental field where application of cholinomimetics is known to cause a REM-sleeplike state (reviewed in 14).

The time course of antinociceptive behavior caused by mPRF microinjection is illustrated by Fig. 2. Two-way ANOVA for repeated measures revealed that TFL was significantly (p<0.0001) altered as a function of time postinjection (F=16.8; d.f.=5, 1080), drug (F=185.2; d.f.=3, 1062), and a time-by-drug interaction (F=6.7; d.f.=15, 1439). Analyses of individual drug effects showed that TFL was not significantly enhanced by mPRF administration of saline or morphine sulfate. In contrast, TFL was significantly increased (p<0.0001) following pontine administration of carbachol (F=34.8; d.f.=5, 356) and neostigmine (F=10.5; d.f.=5, 358). Dunnett’s procedure, statistically comparing TFL measures after cholinomimetics to TFL following mPRF saline, revealed that neostigmine significantly increased TFL at every timepoint throughout the experiment, and that carbachol significantly increased TFL at all timepoints except for 120 minutes postinjection (Fig. 2, asterisks). Maximal TFL enhancement by carbachol occurred at 10 minutes postinjection, when TFL was increased by 84% compared to premicroinjection baseline values. Dunnett’s procedure, statistically comparing TFL measures after cholinomimetics to TFL following mPRF saline, revealed that neostigmine significantly increased TFL at every timepoint throughout the experiment, and that carbachol significantly increased TFL at all timepoints except for 120 minutes postinjection (Fig. 2, asterisks). Maximal TFL enhancement by carbachol occurred at 10 minutes postinjection, when TFL was increased by 84% compared to premicroinjection TFL values. Following mPRF administration of neostigmine, maximal TFL enhancement occurred at 20 minutes postinjection, when TFL was 77% greater than premicroinjection TFL values.

Simultaneous polygraphic recording of sleep and wakefulness made it possible to evaluate the state-dependent nature of the TFL response. The electrographic features of REM sleep and the neostigmine-induced REM-sleeplike state (REM-Neo) are illustrated by Fig. 3. Injection of carbachol and neostigmine caused REM sleep enhancement and decreased latency to REM sleep onset (Fig. 4), as previously quantified by many laboratories (reviewed in 14). The pharmacologic control of behavioral state also is illustrated in Fig. 4 by comparing the temporal organization of waking, NREM sleep, and REM sleep following mPRF microinjection of saline, morphine, neostigmine, and carbachol. Saline administered to the mPRF caused no alteration in wakefulness, NREM sleep, or REM sleep (Fig. 4A). Consistent with earlier findings, mPRF administration of morphine caused REM sleep inhibition (Fig. 4B). The amount of time spent in wakefulness following mPRF morphine was increased and was not qualitatively different from the waking state associated with mPRF saline administration. As documented previous-
ly, no behavioral excitement or enhanced motor activity was observed after mPRF opioid administration. Administration of cholinomimetics into the mPRF reliably caused REM sleep enhancement (Fig. 4C and D). In no case did mPRF administration of carbachol or neostigmine cause the cholinergic side effects of excessive salivation or motoric tremor.

Figure 5 summarizes the effects of mPRF drug administration on TFL for each behavioral state. During wakefulness (Fig. 5, WAKE), there was a significant drug main-effect on TFL (F=69.4; d.f.=3, 767; p<0.0001). Compared to TFL following mPRF saline administration, Dunnett’s statistic revealed that carbachol and neostigmine, but not morphine, caused a significant increase in TFL. During NREM sleep (Fig. 5, NREM), ANOVA revealed a statistically significant drug-main effect on TFL (F=69.2; d.f.=3, 811; p<0.0001). Dunnett’s comparison showed that TFL during NREM sleep was significantly increased by mPRF administration of both carbachol and neostigmine and significantly reduced by morphine. Analyses of TFL during REM sleep (Fig. 5, REM) revealed a statistically significant effect of pontine drug administration (F=173.8; d.f.=3, 803; p<0.0001). The cholinomimetics significantly increased TFL, whereas morphine significantly decreased TFL as compared with TFL after mPRF saline injections.

The ANOVA model also made it possible to quantitatively evaluate the ability of arousal states to alter TFL (Fig. 5). These analyses revealed a statistically significant arousal state effect on TFL following saline (F=19.1; d.f.=2, 449; p<0.0001; Fig. 5, open bars), morphine (F=8.4; d.f.=2, 426; p<0.0003; Fig. 5, hatched bars), neostigmine (F=38.4; d.f.=2, 404; p<0.0001; Fig. 5, densely hatched bars), and carbachol (F=41.0; d.f.=2, 413; p<0.0001; Fig. 5, solid bars). For all mPRF-administered drugs, TFL was of shortest duration during wakefulness. The percent change in TFL during NREM sleep compared to waking for the microinjection conditions was saline (110%), morphine (-15%), neostigmine (23%), and carbachol (11%). During REM sleep, TFL values increased significantly over NREM values for saline (100%), morphine (212.7%), neostigmine (63%), and carbachol (60%). Relative to wakefulness, the greatest percent increase in TFL occurred during REM sleep following mPRF administration of saline (321%), morphine (167%), neostigmine (100%), and

Figure 3.—Polygraphic recordings illustrating the sleep/wake states during which it was possible to measure tail flick latency as a function of arousal state. Each trace shows a 60-second recording of wakefulness, NREM sleep, REM sleep, or the REM-sleeplike state caused by mPRF administration of neostigmine (REM-Neo). Tracings from top to bottom show respiratory rate measured by a thermistor placed at the nares, electromyogram (EMG) recorded from dorsal neck muscles, thalamic field potentials recorded from lateral geniculate bodies of the thalamus (LGB), electroocculogram (EOG) recordings of eye movements, a second LGB recording from the contralateral thalamus, a cortical electroencephalogram (EEG), and time in seconds (s).

Figure 4.—Histograms illustrating the time course of wakefulness (W), NREM sleep, and REM sleep as a function of mPRF drug administration. These plots represent the minute by minute scoring of polygraphic recordings (Fig. 3) into different arousal states. Sleep/wake states of cat are unaltered by microinjection of saline into the mPRF (A). Morphine microinjected into the mPRF suppressed REM sleep (B), whereas carbachol (C) and neostigmine (D) administration into the mPRF caused significant REM sleep enhancement.
During wakefulness and NREM sleep, when prominent muscle tone was present (Fig. 3), neostigmine and carbachol continued to cause a significant increase in TFL (Fig. 5, REM). These data demonstrate that the increase in TFL was not simply an artifact of cholinergically induced motor atonia. Although the present study was not designed to quantify sensory and motor contributions to the tail flick response, the results demonstrate that even during cholinergic REM sleep enhancement, nociceptive input could evoke activation of both antigravity and respiratory muscles (Fig. 5). Recordings of the cortical somatosensory evoked potentials (amplitude 0.32 µV; latency 15.7 ms) demonstrated that sensory input from tail was not blocked by mPRF microinjection of carbachol.

**DISCUSSION**

The present data show for the first time that antinociceptive behavior can be evoked cholinergically from mPRF regions (Fig. 1), also known to regulate electroencephalographic arousal and REM sleep. Neostigmine and carbachol caused significantly greater antinociceptive behavior during wakefulness, NREM sleep, and REM sleep than similar microinjections of morphine or saline. The results are discussed in relation to the mPRF as a modulator of supraspinal cholinergic antinociceptive behavior and in relation to the possible mechanisms through which opioids inhibit both REM sleep and mPRF ACh release.

**Cholinergic Modulation of REM Sleep: Relevance for Pain**

Acetylcholine plays a key role in REM sleep generation,26,27 and the present data support the assertion made nearly 20 years ago that “sleep and analgesia are two of the most prominent physiological functions in which ACh may play a role.”28 A large number of studies from many laboratories now demonstrate the similarities between REM sleep and the cholinergically induced REM-sleeplike state (reviewed in 14 and 24). The present quantitative data parallel a phenomenological report published 30 years ago, which noted that midbrain administration of carbachol produced REM sleep and a level of diminished responsiveness permitting incision followed by wound closure with surgical clips.29 Thus, the present data support the view that the cholinergic model of REM sleep may also contribute to the mechanistic understanding of pain.

REM sleep enhancement caused by mPRF administration of cholinergic agonists and acetylcholinesterase inhibitors (Figs. 3 and 4) is believed to involve activation of postsynaptic M2 muscarinic cholinergic receptors (mAChRs).30,31 Brain mAChRs of the m2/m4 subtype are coupled to inhibitory guanine nucleotide binding (G) proteins, and mPRF application of pertussis toxin, which blocks receptor-G protein interaction, prevents cholinergic REM sleep enhancement.32 Within the mPRF, transmembrane signal transduction elements shown to significantly modulate cholinergic REM sleep include enzyme amplifiers adenylyl cyclase and protein kinase A, and the second messenger cyclic AMP.32,33 Most recently, direct measurements of transducer proteins have shown that carbachol causes dose-dependent activation of G proteins in the rat homologue of mPRF and in the cholinergic pedunculopontine tegmental (PPT) and laterodorsal tegmental (LDT) nuclei.34 Many lines of evidence suggest that cholinergic LDT/PPT nuclei play a key role in REM sleep generation.35 For example, cholinergic projections to the mPRF originate in the LDT/PPT.36,37 Electrical stimulation of LDT/PPT neurons causes a monotonic increase in ACh release from LDT/PPT terminals in the mPRF38 and REM sleep.39 ACh release in the mPRF increases during both spontaneous REM sleep13 and during the cholinergically induced REM-sleeplike state.15 ACh release in the mPRF is modulated by presynaptic muscarinic autoreceptors of the M2 subtype.40 Considered together, these data demonstrate that cholinergic LDT/PPT neurons and mAChRs in the mPRF contribute to REM sleep generation.

The present finding that cholinergic mechanisms in the mPRF contribute to antinociceptive behavior may prove relevant for efforts to localize and characterize supraspinal and spinal mechanisms of opioid action. Opioids and volatile anesthetics exert differential effects at the brainstem and spinal cord.42-44 Recent evidence suggests that the analgesic effects of systemic opioids involve α-adrenergic and cholinergic interaction in the spinal cord.45 These spinal opioid data parallel a conceptual model, familiar to sleep neurobiology, hypothesizing that cholinergic and...
monoaminergic interactions contribute to sleep cycle control (reviewed in 26).

**Cholinergic Modulation of Nociception: Relevance for Sleep**

It has been known since 1933 that human pain threshold can be increased by cholinergic agonists and acetylcholinesterase inhibitors (reviewed in 8 and 46).

Intrathecal administration of neostigmine to humans is antinociceptive, and spinal neostigmine administration produces analgesia similar in duration to analgesia following administration of morphine. Midbrain modulation of antinociceptive behavior is attenuated by systemic administration of scopolamine, and spinal mAChRs mediate the descending inhibition of antinociceptive behavior arising from the medullary brainstem.

Well-defined cholinergic projections within the pontomedullary brainstem also contribute to hyperpolarization of spinal alpha motoneurons, producing the skeletal muscle atonia of REM sleep. These facts raised the question in the present study as to whether mPRF cholinomimetics could enhance latency to tail flick during wakefulness and NREM sleep when muscle atonia was not present. The results (Fig. 5) revealed significant cholinergic enhancement of TFL during wakefulness and NREM sleep when there was no motor atonia (Fig. 3). The even greater enhancement of TFL during REM sleep (Fig. 5) suggests a possible contributing role of motor atonia. The wake and NREM sleep TFL data, however, demonstrate that the cholinergic enhancement of TFL is not simply an artifact of motor atonia. Furthermore, during REM sleep accompanied by muscle atonia, the ability to initiate a tail-withdrawal response was not completely eliminated. For example, Fig. 6 illustrates that the thermal stimulus also could evoke vigorous movements of the tail, neck, and respiratory musculature during the neostigmine-induced REM-sleeplike state. In contrast, during thermal stimulus trials in which there was no tail flick response, there also were no electrographic or behavioral signs of awareness and no autonomic signs of pain, such as increased rate of breathing.

Finally, the finding of a state-dependent increase in TFL is consistent with cellular-level data documenting sleep-dependent decrements in the excitability of lumbar ascending sensory pathways, dorsal spinocerebellar tract neurons, and trigeminal sensory neurons. In the present study, cortical SSEPs were recorded before and after mPRF microinjections of carbachol. These data indicate that nociceptive input is relayed to somatosensory cortex during cholinergic REM-sleep enhancement. These sensory data are consistent with the respiratory motor and skeletal motor response (Fig. 6) showing the ability to overcome motor atonia during cholinergic REM sleep. An exciting opportunity for future studies will be to quantify the relative contributions made by sensory vs motor pathways to enhancement of TFL caused by mPRF cholinomimetics.

**Opioids Inhibit Cholinergic Neurotransmission and REM Sleep**

Opioid administration into mPRF suppresses REM sleep and promotes wakefulness. This enhancement of

Figure 6.—Polygraphic recording during the neostigmine-induced REM-sleeplike state showing recruitment of somatic and respiratory muscle activity by the thermal stimulus. Tracings from top to bottom indicate respiratory rate, electromyogram (EMG) recorded from dorsal neck muscles, thalamic field potentials recorded from lateral geniculate bodies of the thalamus (LGB), electro-occulogram (EOG) recordings of eye movements, a second LGB recording from the contralateral thalamus, electroencephalogram (EEG), and time in seconds (s). Onset of thermal stimulus is indicated by arrow at 20-second time mark. With stimulus onset, note increased rate and volume of airflow recorded from nasal thermistor, burst of EMG activity, abolition of PGO waves in LGB recording, and 10-15 seconds of EEG desynchrony indicating arousal.
wakefulness may account, in part, for the finding that tail flick latencies following mPRF morphine were shorter than latencies following mPRF saline administration (Figs. 2 and 5). Systemically administered opioids depress REM sleep in humans, and opioids microinjected into the mPRF inhibit REM sleep via mu but not delta or kappa opioid receptors. Dialysis-delivery of morphine to the LDT and mPRF recently has been shown to significantly decrease ACh release in both LDT and mPRF. The foregoing data are consistent with the possibility that opioid-induced depression of human REM sleep is due, in part, to decreased cholinergic neurotransmission in the LDT/mPRF network.

Opioids are known to alter cholinergic neurotransmission in a site-dependent manner. For example, opioids administered systemically stimulate ACh release in the dorsal horn of the spinal cord, and inhibit mPRF ACh release. A persistent problem for sleep disorders medicine and pain management is the fact that both nociceptive input and antinociceptive opioid therapy can cause dysphoric emotional states and disrupted arousal states. In humans, opioid inhibition of REM sleep increases the occurrence of parasomnias, and exacerbates the sleep deprivation and delirium known to contribute to intensive care unit (ICU) syndrome.

LIMITATIONS AND CONCLUSION

As reviewed elsewhere, a quarter of a century of data demonstrate that measurement of tail flick latency remains a useful tool for studies of pain. A limitation, however, is that enhancement of tail flick latency alone does not guarantee diminished nociception. The finding that cortical SSEPs were not abolished during cholinergic REM sleep enhancement is consistent with the view that the tail flick response was not merely a spinal reflex and that the TFL measures do represent antinociceptive behavior. Previous findings and the present data encourage continued studies of the mPRF using multiple measures of nociception and different sensory modalities. It also is possible that the enhanced antinociceptive behavior caused by mPRF cholinomimetics resulted, in part, from activation of additional, noncholinergic mechanisms. For example, during carbachol-induced REM sleep, the immediate-early gene c-fos is significantly increased in raphe dorsalis, magnus, and pallidus. Stimulation of these raphe nuclei long has been known to have significant analgesic effects. The present data support the conclusion that the tail flick response is state-dependent, and suggest that mPRF regions known to regulate REM sleep can modulate supraspinal cholinergic antinociceptive behavior. The results encourage future studies aiming to understand the contribution of pontine cholinergic neurotransmission to antinociceptive behavior and efforts to elucidate the mechanisms by which opioids inhibit mPRF ACh release and REM sleep.

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