

Morphine Inhibits Acetylcholine Release in Rat Prefrontal Cortex When Delivered Systemically or by Microdialysis to Basal Forebrain

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Background: Cortical acetylcholine originates in the basal forebrain and is essential for maintaining normal cognition and arousal. Morphine impairs these cholinergically mediated cortical functions. The current study tested the hypothesis that morphine decreases prefrontal cortical acetylcholine release by acting at the level of the basal forebrain.

Methods: Adult male Sprague-Dawley rats ($n = 18$) were anesthetized with isoflurane. One microdialysis probe was placed in the substantia innominata region of the basal forebrain and perfused with Ringer's solution (control) followed by one concentration of morphine (1, 10, 100, or 1,000 μM) or morphine (1,000 μM) plus naloxone (100 μM). A second microdialysis probe was placed in the prefrontal cortex for measuring acetylcholine. In a second series of experiments, rats ($n = 6$) were implanted with electrodes for recording states of arousal, a guide cannula positioned above the prefrontal cortex for inserting a microdialysis probe, and an indwelling jugular vein catheter. The effects of administering intravenous morphine (30 mg/kg) versus normal saline (0.9%) on prefrontal cortical acetylcholine release, cortical electroencephalographic power, and behavior were quantified.

Results: Dialysis delivery of morphine to the substantia innominata caused a concentration-dependent, naloxone-sensitive decrease in acetylcholine release within the prefrontal cortex. The maximal decrease in acetylcholine was $36.3 \pm 11.5\%$. Intravenous morphine administration significantly decreased cortical acetylcholine release, increased electroencephalographic power in the 0.5- to 5-Hz range, and eliminated normal wakefulness.

Conclusion: Morphine causes obtundation of arousal and may cause cognitive impairment by acting at the level of the substantia innominata to disrupt cortical cholinergic neurotransmission.

OPIOIDS are the main antinociceptive agents used for treating chronic and acute pain.¹ The therapeutic potential of opioids must be balanced against unwanted side effects such as respiratory depression, diminished arousal, impaired cognition, and delirium. Among intensive care unit patients² and nondemented elderly patients,^{3,4} delirium is common and is associated with delayed recovery and increased morbidity.^{2,5} The neuronal mechanisms by which opioids impair arousal and mental status remain poorly understood but are likely to involve cortical acetylcholine.

The prefrontal cortex comprises the most anterior cortical region and in humans represents approximately one third of the entire cortex.^{6,7} There is good agreement that major functions of the prefrontal cortex include regulation of attention,^{8,9} working memory,¹⁰ cardiopulmonary control,¹¹ and sleep.^{12,13} Cortical acetylcholine also is essential for normal cognition and arousal,^{14–16} and anticholinergic drugs can contribute to delirium.^{2,5} The substantia innominata region of the basal forebrain contains cholinergic neurons that project to cortex.¹⁷ Opioids decrease acetylcholine release in regions of the pontine reticular formation regulating arousal.^{18,19} No studies have localized neurons through which morphine might alter acetylcholine in the prefrontal cortex. Therefore, this study was designed to evaluate the hypothesis that morphine decreases acetylcholine release in prefrontal cortex and slows cortical activity by disrupting cholinergic neurotransmission from basal forebrain. Portions of these data have been presented in abstract form.²⁰

Materials and Methods

Chemicals and Drug Solutions

Chemicals used for Ringer's solution, mobile phase, acetylcholine standards, and histology were purchased from Sigma-Aldrich (St. Louis, MO). Morphine sulfate was obtained from Sigma-Aldrich. Naloxone hydrochloride came from Mallinckrodt, Inc. (St. Louis, MO). Sterile saline (0.9%) was purchased from Abbott Laboratories (North Chicago, IL). Drug solutions used for microdialysis or for systemic administration were made immediately before use.

Experimental Procedures and Design

All experiments were approved by the University of Michigan Committee on Use and Care of Animals and conducted in accordance with the Public Health Service Policy on Humane Use and Care of Laboratory Animals (NIH Publication 80-23, National Academy of Sciences Press, Washington, DC, 1996). The two sets of experiments reported here used adult male CrI:CD[®](SD)IGS BR (Sprague-Dawley) rats ($n = 24$) purchased from Charles River Laboratories (Wilmington, MA). The first set of studies measured acetylcholine release from prefrontal cortex of isoflurane anesthetized rat before and after dialysis delivery of morphine to basal forebrain. In the second set of experiments, prefrontal cortical acetylcholine release was measured from intact, unanesthetized

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rats before and after administering morphine or saline (control) *via* a permanently implanted jugular cannula.

Studies during Isoflurane Anesthesia

Rats ($n = 18$) were anesthetized with isoflurane in 100% O₂ and placed in a Kopf Model 962 stereotaxic frame (David Kopf Instruments, Tujunga, CA) equipped with a Kopf Model 920 rat adaptor, anesthesia mask, and ear bars. A Cardiacap/5 monitor (Datex-Ohmeda, Madison, WI) was used to measure delivered isoflurane concentration, which was held at 1.5%. An isoflurane concentration of $1.38\% \pm 0.02$ corresponds to a minimum alveolar concentration of 1 for rats.²¹ Spontaneous ventilation was maintained throughout the experiment, and continuous monitoring ensured that there was no depression of respiration. Core body temperature was measured by a rectal thermometer (YSI 400 Series; Harvard Apparatus, Holliston, MA) and maintained at 36° – 37.5° C by a T/Pump Heat Therapy System (TP-500 model; Gaymar, Orchard Park, NY). A midline longitudinal scalp incision was made to expose the skull, and a drill (Dremel, Racine, WI) was used to make two small craniotomies for placing the microdialysis probes according to the rat brain atlas.²² Microdialysis probes (CMA/11; CMA Microdialysis, North Chelmsford, MA) had a membrane of 1 mm in length, 0.24 mm in diameter, and a molecular weight cutoff of 6,000 Da. One probe was aimed for the substantia innominata (stereotaxic coordinates: 1.6 mm posterior to bregma, 2.5 mm lateral to the midline, and 8.7 mm below bregma). A second microdialysis probe was aimed for the ipsilateral prefrontal cortex (stereotaxic coordinates: 3.0 mm anterior to bregma, 0.5 mm lateral to the midline, and 5.0 mm below bregma). Dialysis probes were perfused continuously with Ringer's solution (147 mM NaCl, 2.4 mM CaCl₂, 4.0 mM KCl, pH 5.8–6.2) at a flow rate of 2 μ l/min using CMA/100 syringe pumps. The Ringer's solution used to perfuse the prefrontal cortex probe also contained neostigmine bromide (10 μ M) to prevent enzymatic degradation of acetylcholine. Dialysis probes were placed in the prefrontal cortex and after 45–60 min six dialysis samples (25 μ l/sample) were collected from the prefrontal cortex while the substantia innominata probe was perfused with Ringer's (control). A CMA/110 liquid switch was then used to deliver Ringer's containing one concentration of morphine (1, 10, 100, or 1,000 μ M) or a mixture of morphine (1,000 μ M) and naloxone (100 μ M) to the substantia innominata dialysis probe. The concentrations of morphine were based on previous studies.^{18,19} Six dialysis samples were collected from the prefrontal cortex during drug delivery to the substantia innominata. Only one concentration of morphine was tested per experiment, and separate experiments were performed for combined administration of morphine and naloxone. Morphine interferes with the electrochemical detection of acetylcholine by producing a large peak in the chromatogram that

occludes the acetylcholine peak. Therefore, it was not possible to measure acetylcholine in the substantia innominata while delivering morphine to the substantia innominata.

Studies of Unanesthetized, Behaving Animals

Rats ($n = 6$) were implanted with CMA/11 guide cannula (Plastics One, Roanoke, VA) stereotaxically positioned 1 mm above the prefrontal cortex (guide tube coordinates: 3.0 mm anterior to bregma, 0.5 mm lateral to the midline, and 4.0 mm below bregma).²² Three skull electrodes were implanted for recording the cortical electroencephalogram. Electrodes were positioned 1 mm anterior and 1.5 mm lateral to bregma, 2 mm posterior and 1.27 mm lateral to bregma, and 2 mm posterior and 1.5 mm lateral to bregma. Additional recording electrodes were implanted in the dorsal neck muscles for measuring the electromyogram. Electrode pins were inserted into an electrical connector (Plastics One). Two stainless steel anchor screws (Small Parts Inc., Miami Lakes, FL) were placed in the skull, and dental acrylic was used to secure the dialysis guide tube, electrodes, and electrical connector to the skull.

This group of six rats also was implanted to permit jugular vein access for systemic injections during quiet wakefulness. The jugular vein was exposed and catheterized with a 12-cm length of Micro-Renathane tubing (MRE-040; Braintree Scientific, Braintree, MA). The tube was tunneled subcutaneously and led through the skin between the scapulas. This exposed end of the tube was fitted with a back-mounted flange guide cannula (C315GFL45UPB; Plastics One) and removable cap (C313CAC; Plastics One) and secured to be accessible for subsequent intravenous delivery of saline or morphine without handling the animal.

After postsurgical recovery, rats were conditioned to a Plexiglas recording chamber (Bioanalytical Systems, Inc. [BAS], West Lafayette, IN). This enclosure permitted recording of the electroencephalogram and electromyogram, *in vivo* microdialysis, and drug administration *via* a cannula connected to a syringe outside the chamber. The implanted electrodes were attached *via* a cable (Plastics One) to the digital polygraph. While in the recording chamber, rats could ambulate freely and had free access to bedding, food, and water. During the conditioning interval, rats spent 6–8 h/day in the recording chamber and then were returned to their home cages. Electroencephalographic recordings were amplified and filtered at 0.3 and 30 Hz. Signals were digitally captured using a Grass model 15LT digital polygraph (Astro-Med., Inc., Quincy, MA).

After at least 5 days of conditioning, experiments were initiated to measure acetylcholine release in prefrontal cortex of awake animals before and after systemic administration of either morphine or saline (control). A microdialysis probe was placed in the prefrontal cortex

via the implanted guide tube. A syringe containing either morphine (30 mg/kg) or saline (0.9%) was connected by a 55.5 cm length of Micro-Renathane tubing (MRE-040; Braintree Scientific, Braintree, MA) to the jugular vein catheter. Tubing from the dialysis probe and the injection cannula were led out of the recording chamber. This made it possible to record the electroencephalogram, the electromyogram, and acetylcholine release before and after systemic drug delivery *via* the jugular cannula. States of arousal were scored in 10-s epochs based on the electroencephalogram, the electromyogram, and behavior. The dialysis probe was perfused continuously with Ringer's solution at a flow rate of 2 $\mu\text{l}/\text{min}$ using a CMA/100 syringe pump.

One hour after microdialysis probe insertion, six control dialysis samples (25 $\mu\text{l}/\text{sample}$) were collected during quiet wakefulness. At the end of the sixth sample, 1 ml of either morphine or saline was injected over a 4-min period *via* the jugular vein catheter. After these injections, another six microdialysis samples were collected from the prefrontal cortex. The prefrontal cortex probe was perfused with Ringer's solution throughout the experiment. Preexperimental and postexperimental *in vitro* probe recoveries were performed and compared using a *t* test. These measures of probe recovery ensured that alterations in measured acetylcholine were not an artifact of changes in the dialysis membrane.

Acetylcholine Measurement Using Microdialysis and High-performance Liquid Chromatography with Electrochemical Detection

For quantification of acetylcholine, each dialysis sample was injected into a high-performance liquid chromatography system (CC-5 electrochemical detector; BAS). As previously described,²³ samples were carried through the system by a 50 mM Na_2HPO_4 mobile phase (pH 8.5) at a flow rate of 1.0 ml/min and a pressure of 3,000–3,500 psi. An analytical column (MF-6150; 2.1 mm diameter, 100 mm length; BAS) separated choline and acetylcholine, and an immobilized enzyme reactor column (MF-6151; BAS) containing acetylcholinesterase converted acetylcholine into hydrogen peroxide. The hydrogen peroxide produced from this reaction was proportional to the amount of acetylcholine in the dialysis sample and was measured at a 500-mV applied potential on a platinum electrode referenced to a Ag^+/AgCl electrode. Chromatograms were digitized using ChromGraph software (BAS), and the area under the chromatographic peak was referenced to the standard curve to express acetylcholine as pmol/12.5 min. The limit of detection for acetylcholine was 0.05 pmol/25 μl .

Histologic Analyses

All microdialysis sites were identified histologically according to the following procedure. Animals were deeply anesthetized and decapitated. Brains were imme-

diately frozen and serially sectioned at 40 μm using a Leica cryostat (CM 3050S; Leica Microsystems, Nussloch, Germany). Coronal slide-mounted sections were dried, fixed in hot (80°C) paraformaldehyde vapor, and stained with cresyl violet. All tissue sections containing glial scars caused by microdialysis probes were compared to the rat brain atlas²² to localize probe placement. The deepest point of each probe site was assigned stereotaxic coordinates in millimeters relative to bregma. Histologically confirmed placement of dialysis probes in substantia innominata and prefrontal cortex was required for inclusion of acetylcholine measures in the results.

Statistical Analyses

Measures of acetylcholine were evaluated using descriptive statistics, one-way repeated-measures analysis of variance, and the Dunnett *post hoc* multiple comparisons test. Power calculations were used to confirm the appropriate sample size. A probability (*P*) value of 0.05 or less was considered statistically significant. Analyses of the cortical electroencephalogram using fast Fourier transformation quantified the dominant frequencies from 0.5 to 25 Hz. The total power for each frequency interval of cortical electroencephalogram was computed before and after intravenous injection of saline or morphine. Two-way analysis of variance for repeated measures and the Tukey-Kramer multiple comparisons test were used to evaluate the contributions of injection condition and electroencephalographic frequency to total electroencephalographic power.

Results

Histologic Analyses Confirmed Correct Dialysis Probe Placement

Figure 1 summarizes the histologically localized dialysis sites in prefrontal cortex and basal forebrain. Figure 1A schematically illustrates the relation between the two dialysis probes. Dialysis membranes are indicated by hatched lines at the end of the probe and are drawn to scale relative to the brain schematic. A distance of 7.3 mm separated the average microdialysis sites localized within the substantia innominata and prefrontal cortex. Figure 1B plots the deepest point of the cortical probe sites, confirming that all measures of acetylcholine were obtained from the prefrontal cortex. Figure 1C plots the deepest point of probes used for drug delivery, confirming that morphine and morphine plus naloxone were delivered to the substantia innominata region of the basal forebrain.

Acetylcholine in Prefrontal Cortex Was Decreased by Morphine in Substantia Innominata

Acetylcholine measured in prefrontal cortex during one representative experiment is plotted in figure 2 as

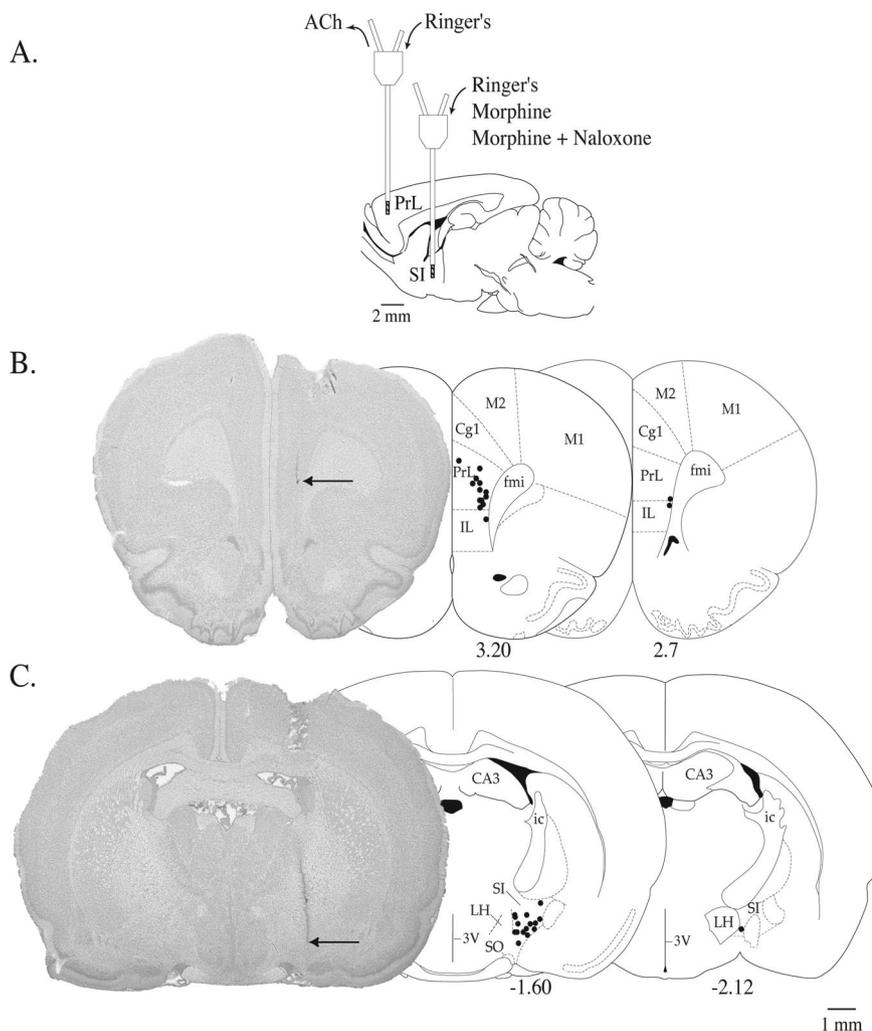


Fig. 1. Histologic confirmation of dialysis sites. (A) Sagittal schematic view of the rat brain illustrating the anatomical relationship between the prefrontal cortex (PrL) and substantia innominata (SI). The dialysis probe placed in the substantia innominata was used to deliver drugs. The probe placed in the prefrontal cortex was used to collect acetylcholine (ACh). The arrow on each histologic section indicates a microdialysis site localized to the prefrontal cortex (B) and substantia innominata (C). Drawings of sagittal and coronal rat brain sections were modified from a stereotaxic atlas.²² The anterior-posterior axis coordinate is indicated in millimeters relative to bregma at the bottom of each coronal drawing. The calibration bar at lower right applies to all sections in B and C. Filled circles localize the most ventral point of 18 dialysis sites in the prefrontal cortex (B) and 18 dialysis sites in the substantia innominata (C). Overlapping dialysis sites from different animals are represented by a single filled circle. 3V = third ventricle; CA3 = hippocampus; Cg1 = cingulate cortex; fmi = forceps minor of the corpus callosum; ic = internal capsule; IL = infralimbic cortex (part of the prefrontal cortex); LH = lateral hypothalamus; M1 and M2 = motor cortex; PrL = prelimbic cortex (part of the prefrontal cortex); SO = supraoptic nucleus.

function of time and drug delivery to the substantia innominata. Prefrontal cortex acetylcholine was quantified every 12.5 min. Thus, the raw data for each experiment was comprised of six acetylcholine measures (75 min) obtained during perfusion of the substantia innominata dialysis probe with Ringer's (control) and six acetylcholine measures obtained during dialysis delivery of morphine (75 min). The data acquisition plan illustrated by figure 2 made it possible for a within-subjects design to quantify acetylcholine in the prefrontal cortex before and after dialysis delivery of morphine to the substantia

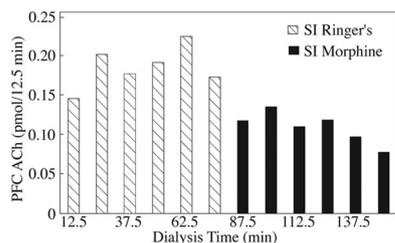


Fig. 2. Acetylcholine (ACh) in the prefrontal cortex (PFC) plotted as a function of the time that the substantia innominata (SI) probe was perfused with Ringer's (control) and Ringer's containing morphine (1,000 μM). Each bar plots acetylcholine in pmol/12.5 min of dialysis for a single experiment.

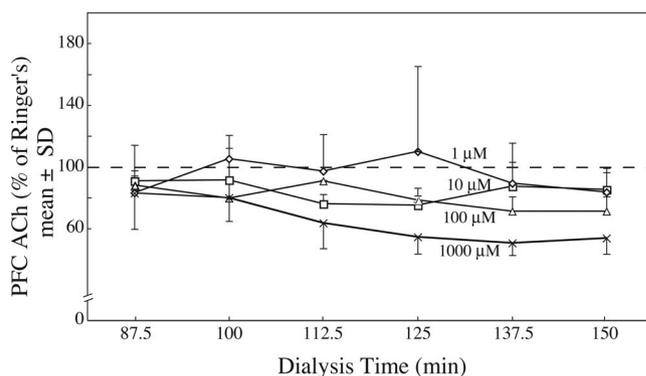


Fig. 3. Time course of prefrontal cortical acetylcholine (ACh) release during dialysis delivery of morphine to the substantia innominata region of the basal forebrain. The figure plots prefrontal cortical acetylcholine release for each 12.5-min sample bin during the 75 min when morphine was delivered by dialysis. The four functions represent four morphine concentrations (1, 10, 100, and 1,000 μM) delivered to the substantia innominata dialysis probe. The dashed horizontal line at 100% indicates acetylcholine release in prefrontal cortex during perfusion of the SI dialysis probe with Ringer's (control). Each of the four drug concentrations was administered to three animals, and acetylcholine was measured in 72 dialysis samples. PFC = prefrontal cortex.

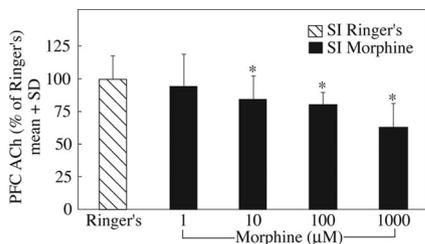


Fig. 4. Morphine delivered by dialysis to the substantia innominata (SI) caused a concentration-dependent decrease in acetylcholine (ACh) within the prefrontal cortex (PFC). Repeated-measures analysis of variance revealed a statistically significant concentration main effect of morphine on acetylcholine ($F = 16.76$; $df = 4, 143$; $P < 0.0001$). The Dunnett statistic indicated that dialysis with 10, 100, and 1,000 μM morphine significantly (*) decreased acetylcholine compared with Ringer's (control). Data from 12 rats summarize 72 measures of acetylcholine release during dialysis with Ringer's alone and 72 measures of acetylcholine during dialysis with Ringer's containing morphine.

innominata. Figure 3 summarizes the time course of acetylcholine release from prefrontal cortex of 12 rats during dialysis delivery of morphine to substantia innominata. Two-way analysis of variance indicated a significant effect of morphine concentration on cortical acetylcholine ($F = 10.6$; $df = 3, 71$; $P < 0.0001$), no significant effect due to time alone, and no concentration by time interaction.

Figure 4 summarizes group data showing that morphine delivered to the substantia innominata caused a concentration-dependent decrease in prefrontal cortex acetylcholine. Regression analyses revealed that the concentration of morphine delivered to the substantia innominata accounted for 85% of the variance in acetylcholine measured from the prefrontal cortex. The mean (\pm SD) percent decreases in acetylcholine release caused by the different concentrations of morphine were $-16.1 (\pm 14.06)$ for 10 μM , $-19.53 (\pm 2.37)$ for 100 μM , and $-36.28 (\pm 11.51)$ for 1,000 μM .

The uptake and distribution properties of isoflurane predict that partial pressure of isoflurane in rat brain would be constant within 10–15 min of isoflurane delivery. Given that isoflurane has been shown to decrease cortical acetylcholine release relative to waking levels of acetylcholine,^{24,25} additional experiments in three rats were performed to insure that the observed decreases in acetylcholine could be attributed only to morphine. For these experiments, 75 min of prefrontal cortex dialysis with Ringer's was followed by an additional 75 min of dialysis with Ringer's. Figure 5 results show that acetylcholine release in prefrontal cortex was not altered by 150 min of isoflurane anesthesia.

The next set of experiments was designed to test the hypothesis that the morphine-induced decrease in acetylcholine (figs. 3 and 4) could be blocked by coadministration of the μ -opioid antagonist naloxone. Dialysis delivery of morphine to the substantia innominata was followed by dialysis delivery of morphine and naloxone to the substantia innominata. Figure 6 shows that acetyl-

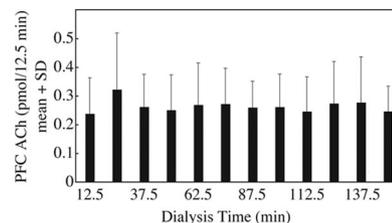


Fig. 5. Prefrontal cortex acetylcholine (ACh) release did not change as a function of time during 150 min of 1.5% isoflurane. Histograms emulate the time course of the morphine studies but show acetylcholine during 75 min of substantia innominata dialysis with Ringer's (first six bars) followed by a second 75-min interval of substantia innominata Ringer's dialysis (second six bars). Regression analyses revealed best fit by a linear model ($Y = 0.2714 + (-0.001) \times X$). The regression analysis of variance was not significant, and the adjusted r^2 showed that dialysis time accounted for 0% of the variance in acetylcholine release. PFC = prefrontal cortex.

choline levels in prefrontal cortex during dialysis delivery of morphine and naloxone were not significantly different from acetylcholine levels during dialysis with Ringer's.

The data summarized by figures 1–6 show that morphine significantly decreases prefrontal cortical acetylcholine release by actions on basal forebrain neurons that project to cortex. These results obtained during isoflurane anesthesia encouraged the second set of experiments using intact, unanesthetized rats to quantify the effect of systemically administered morphine on acetylcholine release and electroencephalographic power (fig. 7). Comparison of figures 7A and C show that intravenously administered morphine, but not saline, significantly decreased acetylcholine release in the prefrontal cortex of unanesthetized rats. During these microdialysis studies, simultaneous recordings were obtained of the electroencephalogram. Figures 7B and D show that morphine, but not saline, altered the electroencephalogram by significantly increasing power in the

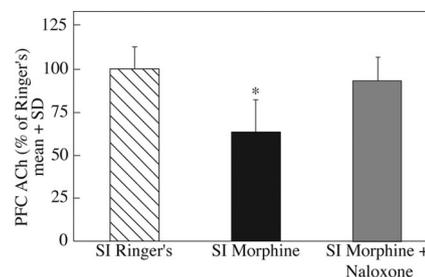


Fig. 6. The morphine-induced decrease in prefrontal cortex (PFC) acetylcholine (ACh) was blocked by dialysis delivery of naloxone to substantia innominata (SI). Analysis of variance revealed a significant drug treatment main effect ($F = 38.15$; $df = 2, 71$; $P < 0.001$). In these data from three rats, the histograms summarize 36 dialysis samples during Ringer's and 18 dialysis samples during each of the two drug conditions. The significant decrease (*) in prefrontal cortical acetylcholine caused by SI morphine (1,000 μM) was antagonized by coadministering naloxone (100 μM) and morphine (1,000 μM). The Dunnett test showed that acetylcholine in prefrontal cortex during dialysis with Ringer's only (control) was not statistically different from acetylcholine during coadministration of morphine and naloxone.

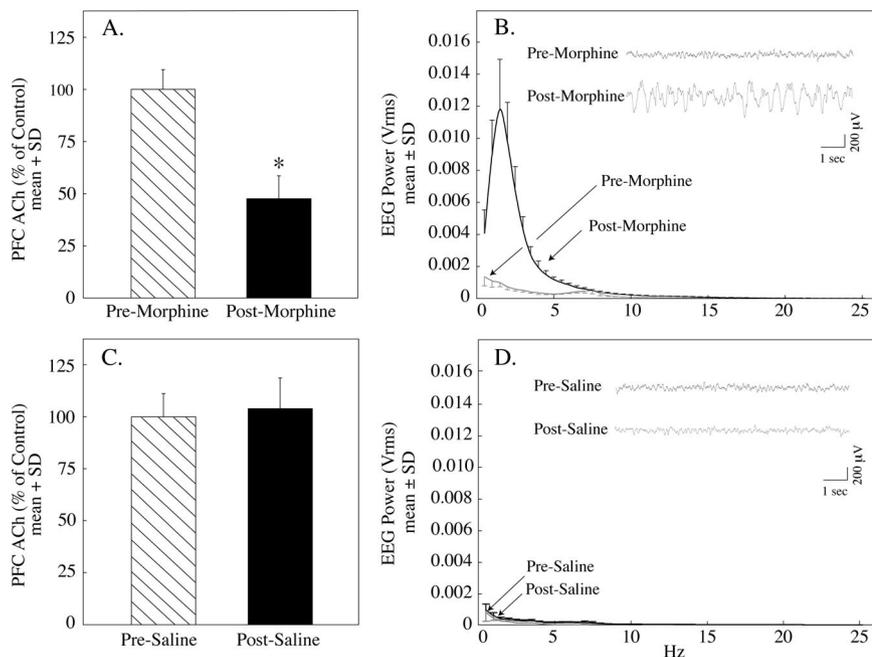


Fig. 7. Intravenous morphine administered to awake rats significantly decreased acetylcholine (ACh) release and altered power in the prefrontal cortex (PFC) electroencephalogram (EEG). Morphine (A) but not saline (C) injected *via* a jugular cannula significantly decreased acetylcholine release in PFC of these unanesthetized animals ($t = 4.15$; $df = 34$; $P < 0.0001$). Quantification of electroencephalogram power before (Pre) and after (Post) morphine injection (B) showed that morphine caused a significant slowing of the electroencephalogram ($F = 256$; $df = 1, 13$; $P < 0.0001$). In contrast, there was no change in electroencephalogram power before (Pre) and after (Post) intravenous injection of saline (D). Insets in B and D show 10-s recordings of representative electroencephalogram before and after administration of morphine (B) or saline (D).

0.5- to 4-Hz range. During the morphine-induced decrease in electroencephalographic activation, rats stopped all movements and displayed a frozen posture. The eyes remained open, and muscle tone was pronounced.

Discussion

The results are discussed in relation to the arousal-related role of cholinergic neurotransmission in the basal forebrain and prefrontal cortex. Opioid-induced delirium is a clinically significant problem defined by cognitive dysfunction, fluctuating levels of consciousness, and inability to maintain focused attention.^{2,5,26} The prefrontal cortex is involved in regulating all of the foregoing functions.

Morphine in Basal Forebrain Decreases Acetylcholine in Prefrontal Cortex

Neuroanatomical data demonstrate that basal forebrain neurons synthesize acetylcholine and send cholinergic projections to the cortex.¹⁷ These structural details encouraged the present use of microdialysis for site-directed opioid delivery to the basal forebrain. A major advantage of drug delivery by microdialysis is the potential to identify the role of restricted neuronal networks. Drug diffusion is not likely to account for the current finding of morphine-induced decrease in cortical acetylcholine. Dialysis delivery of morphine restricted to the substantia innominata region of the basal forebrain was more than 7 mm from the prefrontal cortex, where acetylcholine was measured (fig. 1). The large distance separating the two dialysis probes, and the finding that cortical acetylcholine was decreased with the onset of morphine delivery to the substantia innominata (fig. 2),

are consistent with the interpretation that the effects of morphine were synaptically mediated.

Compared with waking levels of acetylcholine, isoflurane is known to decrease acetylcholine release in cat pontine reticular formation²⁷ and in cerebral cortex and striatum of rat.^{24,25} Four lines of evidence indicate that in the current study, decreased acetylcholine release in prefrontal cortex was caused by morphine, independent of the effects of isoflurane. First, isoflurane was present in the control (Ringer's-alone) condition, and the decrease in cortical acetylcholine release was always caused by onset of morphine administration. Second, time course data (fig. 3) show that acetylcholine release during dialysis delivery of the lowest concentration of morphine was not significantly different from acetylcholine release during Ringer's control. In contrast, the highest concentration of morphine caused a significant decrease in cortical acetylcholine release (figs. 3 and 4). Third, in a series of positive control experiments, 75 min of dialysis with Ringer's was followed by a second 75-min interval of dialysis with Ringer's alone rather than with Ringer's containing morphine. The results (fig. 5) showed that there was no progressive decrease in cortical acetylcholine release caused by isoflurane. Fourth, the results obtained from isoflurane-anesthetized rats (figs. 2-6) were corroborated by studies conducted without isoflurane anesthesia (fig. 7). The foregoing results and interpretation also are consistent with data showing that 150 min of isoflurane anesthesia did not cause a progressive decrease in acetylcholine release from prefrontal cortex of C57BL/6J mouse.²⁸ Therefore, the current results do not contradict the fact that isoflurane decreases acetylcholine release relative to waking levels of acetylcholine.^{24,25,27} The current results dem-

onstrate that morphine alone decreases acetylcholine release in prefrontal cortex.

The effects of opioids on cholinergic neurotransmission and states of arousal are site specific within the brain. Opioids disrupt sleep²⁹⁻³¹ and, when delivered by microdialysis to the pontine reticular formation, inhibit acetylcholine release in the pontine reticular formation.^{18,19} In contrast, morphine increases acetylcholine release in rostral ventrolateral regions of the medulla³² and in the hippocampus,³³ and morphine enhances slow wave sleep when microinjected into the nucleus of the solitary tract.³⁴ The discrete actions of opioids within the central nervous system¹ require site-specific studies to make inferences about the effects of morphine on cholinergic neurotransmission. Electrophysiologic data have shown that systemic administration of morphine causes a naloxone-reversible inhibition of the majority of neurons in the medial prefrontal cortex.³⁵ In view of the fact that cholinergic input to the prefrontal cortex arises from the basal forebrain, the current results extend these previous findings by the discovery that morphine delivered to the basal forebrain causes a naloxone-reversible inhibition of acetylcholine release in the prefrontal cortex (figs. 4 and 6).

Prefrontal Cortical Functions Relevant to Anesthesia

Orientation to person, place, and time requires intact cholinergic neurotransmission at the level of the cortex.^{15,16} The prefrontal cortex has particular significance for regulation of arousal, autonomic control, and cognitive processing.^{6,11,13} The primate prefrontal cortex is commonly divided into dorsolateral and orbitomedial regions.¹¹ The orbitomedial prefrontal cortex receives viscerosensory information.³⁶ The dorsolateral prefrontal cortex receives information from all five senses, consistent with its role in integrating spatial and object information.⁶ Together, these two major divisions integrate somatosensory and visceral-sensory input with short- and long-term memories. This integration contributes to arousal state control and to behavioral planning that links past experiences with changing behavioral objectives.³⁷

There is good agreement between preclinical and clinical data showing that opioids obtund wakefulness and disrupt sleep.^{38,39} Sleep deprivation impairs working memory,⁴⁰ and prefrontal cortical function is disrupted by sleep deprivation.^{12,41} The current finding that morphine delivered to the substantia innominata caused a concentration-dependent (fig. 4), naloxone-reversible (fig. 6) decrease in prefrontal cortical acetylcholine encouraged studies using intact animals without anesthesia. Systemic delivery of morphine to these behaving rats also significantly decreased acetylcholine release in prefrontal cortex (fig. 7A) and significantly increased low-frequency electroencephalographic power (fig. 7B).

Slow wave activity in the cortical electroencephalogram is unique to the postmorphine state and is accompanied by behavioral stupor and muscle rigidity.⁴² The figure 7 data also fit well with clinical studies showing that opioid anesthesia increases electroencephalographic power in the δ (0.35–3.5 Hz) and θ (3.5–8 Hz) frequency range.^{43,44} In the current control experiments, systemically administered saline had no effect on acetylcholine release (fig. 7C), electroencephalographic power (fig. 7D), or behavior. Considered together, these data demonstrate that morphine-induced changes in prefrontal cortical acetylcholine release caused functionally significant changes in the electroencephalogram and behavior. These preclinical findings support the hypothesis that in susceptible patients, opioids may induce delirium by disrupting cholinergic neurotransmission in prefrontal cortex. This speculation is supported by data showing that cholinergic neurotransmission is particularly relevant for activating the electroencephalogram⁴⁵ and for regulating behavioral states of sleep,⁴⁶ anesthesia,⁴⁷ and sedation.⁴⁸

Limitations and Conclusions

Much of the information about the prefrontal cortex has come from studies of primates, and the homology between rodent and primate prefrontal cortex has been questioned.⁴⁹ The prefrontal cortex in rats is not as differentiated as it is in primates, but the homology between rodent and primate prefrontal cortex is supported by both structural and functional evidence.⁵⁰ The finding that cholinergic mechanisms also modulate cortical excitability in prefrontal cortex of mice^{23,51,52} suggest that the current results are generalizable, rather than species specific.

Acetylcholine release in the cortex⁵³ and in the substantia innominata region of the basal forebrain⁵⁴ varies significantly as a function of arousal state. Therefore, the first set of studies was designed to limit fluctuations in levels of arousal by holding arousal state constant with isoflurane anesthesia. The current results cannot discount the possibility that additional neurotransmitters and neuromodulators also contribute to the opioid-induced decrease in acetylcholine. In some brain regions, morphine increases dopamine,⁵⁵ and dopaminergic mechanisms contribute to decreasing acetylcholine.³³ In cat basal forebrain, there are approximately twice as many γ -aminobutyric acid-containing neurons as there are cholinergic neurons, and γ -aminobutyric acid significantly modulates acetylcholine release.⁵⁶

Although microdialysis has revolutionized neurochemistry, it has a number of limitations. The biophysical interface between microdialysis probe and brain tissue can vary as a function of brain region, time, analyte being measured, dialysis fluid composition, dialysis flow rate, temperature, anesthesia, and dialysis membrane.⁵⁷ These potential confounding variables emphasize the power of

the present within-subjects experimental design. Rather than assuming that all of the foregoing variables were normal, the within-subjects design distributes any potential effect of these variables across both control (Ringer's-alone) and experimental (Ringer's-plus-drug) conditions. The finding that morphine caused a statistically significant decrease in acetylcholine release (figs. 3, 4, and 7) means that the drug effect was large relative to all other potential sources of variability. These effects of morphine on cortical acetylcholine release agree with microdialysis studies in pons^{18,19} and with the established view⁵⁸ that these microdialysis measures accurately reflect relative extracellular fluid levels of acetylcholine in the prefrontal cortex.

In contrast to studies that microinject nanoliter volumes of drug directly into the brain, microdialysis studies typically are unable to quantify the amount of drug delivered. The selectivity of dialysis probe membranes means that only a fraction of endogenous analyte is recovered and, during dialysis drug delivery, only a fraction of the drug is delivered. The recovery of analyte standards can provide a useful index of the percent of a drug concentration delivered. In the current experiments, dialysis probe recoveries ranged from 3 to 5%. Therefore, although the morphine concentrations were high, it is likely that only a small fraction (< 5%) of the morphine was transferred across the dialysis probe membrane into the brain.

Even with the foregoing limitations, the results support the conclusion that morphine significantly decreases acetylcholine release in prefrontal cortex. The decreased acetylcholine release was functionally significant because it was accompanied by significant changes in electroencephalographic power (fig. 7) and elimination of normal waking behaviors. The effects of morphine on acetylcholine release were concentration dependent and blocked by naloxone, consistent with the interpretation that morphine activates μ -opioid receptors in the basal forebrain.^{59,60} μ -Receptor activation would be expected to hyperpolarize cholinergic projection neurons,⁶¹ leading to decreased acetylcholine release in prefrontal cortex. The decreased acetylcholine release caused by dialysis delivery of morphine to the basal forebrain was replicated by systemic administration of morphine. This finding implies, for the first time, that some of the undesirable side effects of systemically administered morphine result from actions on basal forebrain cholinergic neurons projecting to prefrontal cortex. Diminished transmission in this cholinergic pathway may contribute to opioid-induced delirium and to delayed anesthetic recovery associated with the central anticholinergic syndrome.⁶² Even in the absence of neurodegenerative disorders,⁶³ these unwanted opioid side effects are especially problematic in older patients.^{3,5} The need to understand basic mechanisms through which opioids alter cortical function is emphasized by

the anticipated increase in the number of patients with cognitive dysfunction.⁶⁴

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