Microdialysis Delivery of Morphine to the Hypoglossal Nucleus of Wistar Rat Increases Hypoglossal Acetylcholine Release

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**Study Objectives:** The medullary hypoglossal nucleus (XII) innervates the genioglossal muscles of the tongue, and opioid-induced alterations in tongue muscle tone contribute to airway obstruction. Previous studies have shown that morphine causes a significant decrease in acetylcholine (ACh) release in some brain regions, but the effects of morphine on ACh release in XII have not been quantified.

**Design:** A within-subjects design was used to test the hypothesis that morphine alters ACh release in XII of anesthetized Wistar rat. ACh release during microdialysis with Ringer’s solution (control) was compared to ACh release during dialysis delivery of opioids.

**Setting:** University of Michigan

**Patients or Participants:** N/A

**Interventions:** Microdialysis delivery of opioids to XII.

**Measurements and Results:** Morphine caused a statistically significant, concentration-dependent increase in XII ACh release. The increase in XII ACh release caused by 10 µM morphine was blocked by the mu opioid antagonist naloxone and not blocked by the kappa opioid antagonist nor-binaltorphimine.

**Conclusions:** The data comprise the first direct measures of ACh release in XII and support the conclusion that morphine depresses hypoglossal nerve activity, in part, by increasing ACh release in XII. Activation of mu opioid receptors on inhibitory neurons within XII likely disinhibits cholinergic terminals, causing increased ACh release. The results are consistent with previous studies showing that blocking the enzymatic degradation of ACh in XII significantly inhibited tongue muscle activity.

**Keywords:** Upper airway muscles, mu receptor, kappa receptor, GABAergic disinhibition

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**INTRODUCTION**

**OPIOIDS SUCH AS MORPHINE ARE EFFECTIVE FOR TREATING PAIN, BUT CAUSE THE UNWANTED SIDE EFFECT OF DOSE-DEPENDENT RESPIRATORY depression.** The accessory respiratory muscles of the upper airway are particularly sensitive to morphine, and during general anesthesia opioid-induced respiratory depression is often magnified. There is long-standing evidence that dysfunction of the genioglossus muscle may contribute to airway obstruction. The neurochemical mechanisms by which morphine disrupts control of the genioglossus muscle by the hypoglossal nucleus (XII) remain incompletely understood.

Acetylcholine (ACh) is present in XII, as demonstrated by XII nerve activity, in part, by increasing ACh release in XII. Activation of mu opioid receptors on inhibitory neurons within XII likely disinhibits cholinergic terminals, causing increased ACh release. The results are consistent with previous studies showing that blocking the enzymatic degradation of ACh in XII significantly inhibited tongue muscle activity.

Previous microdialysis data show that opioids cause decreased ACh release within the prefrontal cortex, nucleus accumbens, striatum, and pontine reticular formation. No studies, however, have quantified the effect of morphine on XII ACh release. The present study tested the hypothesis that microdialysis delivery of morphine to XII decreases ACh release in XII. Portions of these data have been presented in abstract form.

**METHODS**

**Chemicals and Drug Solutions**

Chemicals used for Ringer’s solution, mobile phase, ACh standards, and histology were purchased from Sigma-Aldrich (St. Louis, MO). Morphine sulfate was obtained from Sigma-Aldrich and naltrexone hydrochloride was supplied by Mallinckrodt, Inc., (St. Louis, MO). Nor-binaltorphimine (nor-BNI) was purchased from Tocris Bioscience (Ellisville, MO). Drug solutions used for microdialysis were made immediately prior to 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). Adult (mean weight = 260 g) male Wistar rats (n=19) were purchased from Charles River Laboratories (Wilmington, MA) and housed in a 12 h light-dark cycle with constant access to food and water. The Wistar rat was the strain used to develop both the Sprague-Dawley and the Long-Evans rat strains. Two key features of the Wistar rat made it the strain of choice for these studies. First, the brain atlas for Wistar rat greatly facilitates stereotaxic localization of small, medullary brain regions. Second, use of the Wistar rat makes it possible to compare the present neurochemical data with functional studies of XII that used Wistar rat.
Rats 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 Cardiopac/5 monitor (Datex-Ohmeda, Madison, WI) was used to hold delivered isoflurane concentration constant at 1.5% throughout the duration of the dialysis experiment. A rectal thermometer (Harvard Apparatus, YSI 400 Series, Holliston, MA) measured core body temperature which was maintained at ≈ 37°C by a T/Pump Heat Therapy System (Gaymar, TP-500 model, Orchard Park, NY). A longitudinal scalp incision was made along the midline to expose the skull, and a drill (Dremel, Racine, WI) was used to make a small craniotomy permitting insertion of the microdialysis probe. In accordance with a rat brain atlas, microdialysis probes were aimed for XII using stereotaxic coordinates 13.6 mm posterior to bregma, 0.1 mm lateral to the midline, and 8.9 mm ventral to bregma (Figure 1A). Microdialysis probes (CMA/11; CMA Microdialysis, North Chelmsford, MA) had a membrane length of 1 mm, a diameter of 0.24 mm, and a molecular weight cut-off of 6 kDa. Dialysis probes were perfused continuously with Ringer’s solution (147 mM NaCl, 2.4 mM CaCl₂, 4.0 mM KCl, 10 µM neostigmine, pH 5.8-6.2) at a flow rate of 2 µl/min using a CMA/100 syringe pump. Collection of dialysis samples (25 µl each) began 40 min after insertion of the microdialysis probe. A CMA/110 liquid switch was used to dialyze XII with Ringer’s solution (control), followed by Ringer’s containing the opioid receptor agonist morphine (1, 3, or 10 µM), Ringer’s containing a mixture of morphine (10 µM) and the mu opioid receptor antagonist naloxone (1 µM), or Ringer’s containing a mixture of morphine (10 µM) and the kappa opioid receptor antagonist nor-BNI (1 µM).

As previously described, recovery of ACh by the dialysis probe was tested in vitro before and after each experiment. ACh measures were included in the final data set only if dialysis probe recovery did not change significantly in the same direction as the drug-induced change in ACh. Dialysis probes used in this study had an average ACh recovery of 5 percent.

**ACh Measurement using High Performance Liquid Chromatography with Electrochemical Detection (HPLC-EC)**

For quantification of ACh, each dialysis sample was injected into an HPLC-EC system (CC-5 electrochemical detector; Bioanalytical Systems [BAS], West Lafayette, IN). As recently described, samples were carried through the HPLC-EC system by a 50 mM NaH₂PO₄, mobile phase (pH 8.5) at a flow rate of 1.0 ml/min and a pressure of 3,200-3,700 psi. ACh and choline were separated by an analytical column (MF-6150; BAS). An immobilized enzyme reactor column (MF-6151; BAS) containing acetylcholinesterase converted ACh into hydrogen peroxide. The amount of hydrogen peroxide produced from this reaction was proportional to the amount of ACh in the dialysis sample. Electrochemical detection was accomplished using a 500 mV applied potential on a platinum electrode referenced to an Ag⁺/AgCl electrode. Chromatograms were digitized using ChromGraph software (BAS). A standard curve (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 pmol ACh) was produced prior to each experiment. The area under the chromatographic peak was referenced to the 7-point standard curve and was proportional to the amount of ACh within the sample. Figure 1B illustrates ACh chromatography.

Histological Confirmation of Microdialysis Sites

Two to 4 days after each experiment, rats were deeply anesthetized and decapitated. Brains were immediately frozen and cut into 40 µm coronal sections on a Leica cryostat (CM 3050S; Leica Microsystems, Nussloch, Germany). Serial sections were taken at ≈ 37°C by a T/Pump Heat Therapy System (Gaymar, TP-500 model, Orchard Park, NY). A longitudinal scalp incision was made along the midline to expose the skull, and a drill (Dremel, Racine, WI) was used to make a small craniotomy permitting insertion of the microdialysis probe. In accordance with a rat brain atlas, microdialysis probes were aimed for XII using stereotaxic coordinates 13.6 mm posterior to bregma, 0.1 mm lateral to the midline, and 8.9 mm ventral to bregma (Figure 1A). Microdialysis probes (CMA/11; CMA Microdialysis, North Chelmsford, MA) had a membrane length of 1 mm, a diameter of 0.24 mm, and a molecular weight cut-off of 6 kDa. Dialysis probes were perfused continuously with Ringer’s solution (147 mM NaCl, 2.4 mM CaCl₂, 4.0 mM KCl, 10 µM neostigmine, pH 5.8-6.2) at a flow rate of 2 µl/min using a CMA/100 syringe pump. Collection of dialysis samples (25 µl each) began 40 min after insertion of the microdialysis probe. A CMA/110 liquid switch was used to dialyze XII with Ringer’s solution (control), followed by Ringer’s containing the opioid receptor agonist morphine (1, 3, or 10 µM), Ringer’s containing a mixture of morphine (10 µM) and the mu opioid receptor antagonist naloxone (1 µM), or Ringer’s containing a mixture of morphine (10 µM) and the kappa opioid receptor antagonist nor-BNI (1 µM).

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slide-mounted, dried, fixed in paraformaldehyde vapor (80˚C), and stained with cresyl violet. Stained sections containing a microdialysis probe site were analyzed microscopically. The dialysis site was localized relative to bregma using a rat brain atlas. Confirmed placement of dialysis probes within XII was required for inclusion of data in the results reported here.

Statistical Analyses

All measures of ACh were converted to a percent of control value with the ACh values measured during dialysis with Ringer’s solution serving as the control. Thus, 6 samples collected during dialysis with Ringer’s were averaged to yield a mean control ACh level for each rat. Power calculations were performed in order to determine required sample size. Analysis of variance followed by Dunnett’s multiple comparisons test were used to evaluate the effect of morphine concentration on XII ACh release. Student’s t-test was used to evaluate the effect of the mu antagonist naloxone and the kappa antagonist nor-BNI on changes in ACh release caused by morphine. A probability (P) value of ≤0.05 was considered statistically significant.

RESULTS

Figure 2 illustrates the location of microdialysis sites within XII and Figure 3 summarizes the effects of morphine on XII ACh release. Figure 3A illustrates the time course of ACh release during dialysis with Ringer’s solution followed by dialysis with Ringer’s containing morphine. Figure 3B shows that microdialysis delivery of morphine to XII caused a statistically significant (F = 35.4; df = 3, 124; P <0.0001), concentration-dependent, increase in XII ACh release. Compared to Ringer’s control, Dunnett’s analysis indicated a statistically significant (P <0.01) increase in ACh release caused by 3 µM and 10 µM morphine. Microdialysis delivery of morphine to XII did not significantly alter respiratory rate (data not shown).

Naloxone was delivered to XII in an effort to determine whether the morphine-induced increase in ACh release was mediated by mu opioid receptors. Figure 4 illustrates that coadministration of morphine (10 µM) and naloxone (1 µM) blocked the morphine-induced increase in XII ACh release. No significant alteration in respiratory rate occurred as a result of microdialysis with a mixture of morphine and naloxone.

As a further evaluation of the working hypothesis that the concentration-dependent increase in XII ACh release caused by morphine was mediated by mu opioid receptors, additional experiments delivered the kappa opioid receptor antagonist nor-BNI to XII. Figure 5 shows that coadministration of nor-BNI and morphine caused a significant increase (38.4%) in ACh release (t = 6.5; df = 34; P <0.0001) indicating that nor-BNI did not block the morphine-induced increase in ACh release.

DISCUSSION

Three novel findings emerged from this study. 1) Dialysis delivery of morphine to XII caused a significant increase in ACh...
release within XII. 2) The increase in XII ACh release varied significantly as a function of morphine concentration. 3) The morphine-induced increase in XII ACh release was blocked by naloxone and not blocked by nor-BNI. These results are discussed in relation to altered cholinergic neurotransmission in XII caused by opioids and sleep.

**Morphine Increased XII ACh Release**

Opioids decrease ACh release within many brain regions and opioid-induced decreases in ACh are consistent with the fact that opioids are antinociceptive, in part, because they inhibit neuronal activity by hyperpolarizing neurons. In spinal and caudal brain regions, however, there is precedent for morphine to increase ACh release. Intravenous morphine administration increases ACh release in the dorsal horn of the spinal cord and rostral ventrolateral medulla. The increased ACh release in rostral ventrolateral medulla caused by morphine was blocked by systemic administration of naloxone. Thus, the morphine-induced increase in XII ACh release (Figure 1B; Figure 3) is consistent with previous studies of the effects of morphine on medullary ACh. The contrast between morphine increasing ACh release at caudal levels of the neuraxis and decreasing ACh release in more rostral brain regions emphasizes the need to characterize opioid effects throughout the widely distributed neural networks that regulate sleep and breathing.

The present results are the first to demonstrate that morphine causes a concentration-dependent increase in XII ACh release (Figure 3) that is blocked by dialysis delivery of the mu opioid antagonist naloxone (Figure 4) but not blocked by the kappa opioid antagonist nor-BNI (Figure 5). Morphine acts primarily through mu receptors to produce analgesia. Morphine has affinity for both the mu and kappa opioid receptor subtypes but morphine is at least 3 times more selective for the mu subtype. Naloxone blocks mu, kappa, and delta opioid receptor subtypes but has greatest affinity for the mu opioid receptor. Considered together, the data (Figures 3-5) favor the interpretation that the opioid-induced increase in XII ACh release was mediated by mu opioid receptors. This interpretation is consistent with previous studies documenting the presence of mu opioid receptors in rat XII.

Given that opioids block nociception by decreasing cell excitability, what mechanisms in XII might account for the finding of a morphine-induced increase in ACh release? A schematic model (Figure 6) based on available data illustrates potential synaptic mechanisms through which dialysis delivery of morphine to XII could increase ACh release. Intrinsic cholinergic neurons are located within XII and XII also receives cholinergic...
input from the intermediate reticular formation and from the laterodorsal and pedunculopontine tegmental (LDT/PPT) nuclei (Figure 6A). Many studies concur that XII motoneurons receive GABAergic and glycine inhibitory input (Figure 6B). GABA and glycine are co-released from XII motoneurons and immunohistochemical studies show that cholinergic XII motoneurons possess GABA_A receptors, and glycine receptors (boxed G in Figure 6C). GABA and glycine inhibit XII neurons and endogenous GABA modulates synaptic transmission within XII. Genioglossus muscle tone is decreased by glycine, increased by the GABA antagonist bicuculline, and decreased by the GABA_A receptor agonist muscimol delivered to XII. These data demonstrate tonically active glycineergic and GABAergic input to XII (Figure 6B and C). If mu opioid receptors (boxed O in Figure 6B) activated by morphine are present on GABAergic neurons in or near XII, then the present results suggest that inhibiting GABAergic/glycinergic inhibitory neurons (i.e., disinhibition) would cause an increase in ACh release (Figures 1B and 3). This speculation is supported by evidence that endogenous and exogenous opioids can cause disinhibition by inhibiting GABAergic neurons. The present data cannot determine whether the receptors schematized by Figure 6 exist on presynaptic terminals or in postsynaptic locations. There is evidence that GABAergic processes schematized by Figure 6 are present in reticular regions known to modulate arousal and that these GABAergic processes project to XII. Furthermore, administering ACh to the pontine reticular formation causes increased GABA levels in XII.

Cholinergic input to XII can be excitatory or inhibitory, depending on activation of nicotinic (N) or muscarinic (M) cholinergic receptors (Figure 6D). Functional M2 muscarinic receptors are located within XII and excitatory glutamatergic input to XII is presynaptically inhibited by M2 muscarinic receptors. Nicotinic receptors are located in XII and application of nicotinic receptor agonists to XII increases excitability of XII motoneurons. Cholinergic receptor modulation of XII neuron excitability is functionally important, and tongue muscle tone is suppressed by activating muscarinic receptors but increased by activating nicotinic receptors. The finding that blocking acetylcholinesterase in XII suppressed tongue muscle activity strongly supports the conclusion that tongue muscle suppression is caused by increasing ACh in XII. These results are in line with the finding that decreases in XII nerve (Figure 6E) discharge are caused by enhancing cholinergic neurotransmission. Thus, the present measures of ACh release support the interpretation that morphine depresses hypoglossal nerve activity, in part, by increasing ACh release in XII.

**Opioids Disrupt the Cholinergic Regulation of Sleep and Breathing**

Opioids are widely used for pain management and the lack of alternative medications may contribute to the outdated, minority opinion that opioid-induced sleep disruption is not important. Sleep disruption is a major complaint of patients experiencing pain, and in 2007, the International Association for the Study of Pain will publish an entire volume focused on the topic of sleep and pain. Administering clinically relevant doses of opioids to otherwise healthy humans increases light NREM sleep (stage 2), decreases deep sleep (stage 4 NREM), and decreases REM sleep. Opioids also blunt wakefulness and slow the cortical electroencephalogram. The negative effect of opioids on wakefulness is relevant because the duration and quality of wakefulness significantly modulates subsequent sleep. Thus, pain and opioids disrupt sleep, and sleep disruption enhances perception of painful stimuli. For some individuals, the foregoing relationships can lead to a cycle of worsening or unrelenting pain. Reviews of available data document opioid-induced alterations in cholinergic neurotransmission as one mechanism contributing to the disruption of sleep and wakefulness.

The current focus on XII derives from early evidence that hypotonia of the genioglossus muscle can contribute to airway obstruction associated with sleep and opioids. Studies quantifying the effect of arousal state on genioglossus muscle tone of rat reveal sleep-dependent hypotonia that is homologous to the genioglossal hypotonia observed in humans. This remarkable homology is consistent with the convergent evolutionary perspective and supports the probability that preclinical studies can unmask neurobiological mechanisms leading to rationally based therapies for state-dependent respiratory depression.

ACh is a lower level phenotype that modulates the higher level phenotypes of sleep and breathing. Enhancing cholinergic neurotransmission by administering the cholinergic agonist carbachol into the medial pontine reticular formation of intact animals produces the physiological and behavioral traits characteristic of REM sleep. The increase in REM sleep traits caused by pontine carbachol is so pronounced that it can be demonstrated using anesthetized and surgically reduced preparations.

ACh release in the medial pontine reticular formation is maximal during REM sleep and during the REM sleep-like state
caused by carbachol.\textsuperscript{67} Pontine carbachol also causes postsynaptic inhibition of XII neurons\textsuperscript{48} and decreased levels of XII nerve activity.\textsuperscript{46} These actions of carbachol are consistent with REM sleep-dependent increases in ACh release causing postsynaptic inhibition of XII neurons. Carbachol activates muscarinic cholinergic receptors in XII and muscarinic receptor activation also causes presynaptic inhibition of XII neurons.\textsuperscript{46} Such inhibition is consistent with the decreased genioglossal muscle tone characteristic of REM sleep.\textsuperscript{59,69}

**Limitations and Future Directions**

Due to the small size of XII, it was a challenge to consistently place a microdialysis probe within the boundaries of XII. According to the brain atlas for Wistar rat\textsuperscript{16} XII measures approximately 0.70 mm in both medial-lateral and dorsal-ventral planes at its widest point, 13.30 mm posterior to bregma. Given the size of presently available CMA/11 microdialysis probe membranes (0.25 mm in diameter; 1 mm in length), it was not possible to have the probe membrane lie entirely within XII. These size constraints are a common methodological concern for studies of XII.\textsuperscript{69} In the present study, when >50% of the probe was determined to have been within XII (Figure 2), the respective neurochemical measures were included in the group data.

Three concentrations of morphine were delivered to XII (Figure 3), however it was not possible to perform microdialysis using morphine concentrations greater than 10 µM. As described previously,\textsuperscript{11,14,21} morphine concentrations greater than 10 µM produce a large chromatographic peak that interferes with the detection of ACh. Despite this technical limitation, it was possible to demonstrate that the morphine-induced increase in ACh release was concentration-dependent (Figure 3) and blocked by naloxone (Figure 4), indicating mediation by mu opioid receptors.

The present results are not likely to be confounded by cardiovascular effects of morphine. Due to limited diffusion across the microdialysis membrane, only about 5% of the 10 µM morphine was delivered to XII. Calculations using 0.05 µM morphine delivered to XII at a dialysis flow rate of 2 µl/min for 75 min indicate that rats in the present study received a total morphine dose of approximately 0.2 µg/kg. The 0.2 µg/kg dose delivered over 75 min to a restricted brain region is 50 times less than the lowest morphine dose causing altered cardiovascular function when administered systemically via bolus injection to anesthetized rat.\textsuperscript{70}

The neurobioloy of XII is complex,\textsuperscript{71} and although the present study focused on ACh, the neurochemical modulation of XII involves multiple neurotransmitters. Microdialysis delivery of serotonin or serotonin agonists to XII increases genioglossus muscle tone during sleep\textsuperscript{34,40} and during anesthesia.\textsuperscript{72} Data from anesthetized rat show that the serotonin 2A receptor excites XII motoneurons.\textsuperscript{75} Microinjection of cholinergic agonists into the medial pontine reticular formation decreases monoamines in XII.\textsuperscript{74} Metabotropic glutamate receptors also modulate respiratory drive to XII,\textsuperscript{75} and long-term exposure to intermittent hypoxia decreases XII responses to NMDA receptor activation.\textsuperscript{76} The present results, and the recent finding that morphine decreases GABA levels in rat pontine reticular formation,\textsuperscript{77} encourage future studies testing the hypothesis that the morphine-induced increase in XII ACh can be altered by microdialysis delivery of GABA agonists and antagonists to XII.

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