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SLEEP AND GABA LEVELS IN THE ORAL PART OF RAT PONTINE RETICULAR FORMATION ARE DECREASED BY LOCAL AND SYSTEMIC ADMINISTRATION OF MORPHINE

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Abstract

Morphine, a μ -opioid receptor agonist, is a commonly prescribed treatment for pain. Although highly efficacious, morphine has many unwanted side effects including disruption of sleep and obtundation of wakefulness. One mechanism by which morphine alters sleep and wakefulness may be by modulating GABAergic signaling in brain regions regulating arousal, including the oral pontine reticular formation (PnO). This study used in vivo microdialysis in unanesthetized Sprague-Dawley rat to test the hypothesis that μ -opioid receptors modulate PnO GABA levels. Validation of the high performance liquid chromatographic technique used to quantify GABA was obtained by dialyzing the PnO ($n=4$ rats) with the GABA reuptake inhibitor nipecotic acid (500 μ M). Nipecotic acid caused a $185 \pm 20\%$ increase in PnO GABA levels, confirming chromatographic detection of GABA and demonstrating the existence of functional GABA transporters in rat PnO. Morphine caused a concentration-dependent decrease in PnO GABA levels ($n=25$ rats). Coadministration of morphine (100 μ M) with naloxone (1 μ M), a μ -opioid receptor antagonist, blocked the morphine-induced decrease in PnO GABA levels ($n=5$ rats). These results show for the first time that μ -opioid receptors in rat PnO modulate GABA levels. A second group of rats ($n=6$) was used to test the hypothesis that systemically administered morphine also decreases PnO GABA levels. Intravenous morphine caused a significant ($p<0.05$) decrease (19%) in PnO GABA levels relative to control intravenous infusions of saline. Finally, microinjections followed by 2 h recordings of electroencephalogram and electromyogram tested the hypothesis that PnO morphine administration disrupts sleep ($n=8$ rats). Morphine significantly ($p<0.05$) increased the percent of time spent in wakefulness (65%) and significantly ($p<0.05$) decreased the percent of rapid eye movement (REM) sleep (-53%) and non-REM sleep (-69%). The neurochemical and behavioral data suggest that morphine may disrupt sleep, at least in part, by decreasing GABAergic transmission in the PnO.

Keywords

REM sleep; wakefulness; microdialysis; naloxone; nipecotic acid; high performance liquid chromatography

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Opioids such as morphine, that exert their effects primarily via the μ -opioid receptor, are the most efficacious and widely prescribed treatments for pain (Yaksh, 1997, Nicholson, 2003). Unfortunately, opioids have numerous side effects including sleep disruption (Kay et al., 1969, Kay, 1975). In healthy human volunteers, clinically relevant doses of opioids increase light sleep (stage 2), decrease deep sleep (stage 4), and decrease rapid eye movement (REM) sleep (Walder et al., 2001, Shaw et al., 2005). Negative consequences of disrupted sleep include an enhanced sensitivity to pain (Onen et al., 2001a, Onen et al., 2001b, Kundermann et al., 2004, Roehrs et al., 2006) and deficits in daytime performance leading to an increased risk of accidents (reviewed in Durmer and Dinges, 2005). Opioids disrupt wakefulness by causing impaired cognitive and psychomotor function (Clemons et al., 1996, Sjogren, 1997) and by increasing slow frequencies in the cortical electroencephalogram (EEG) (Volavka et al., 1970, Sebel et al., 1981, Wauquier et al., 1984, Scott et al., 1991, Phillips et al., 1994, Greenwald and Roehrs, 2005). Preclinical studies also show that systemic opioid administration disrupts deep sleep, inhibits REM sleep, and slows the cortical EEG during wakefulness (Colasanti and Khazan, 1973, King et al., 1981, de Andres et al., 1984, Mayo-Michelson and Young, 1993, Arankowsky-Sandoval and Gold, 1995, Osman et al., 2005).

The mechanisms underlying opioid-induced sleep disruption remain incompletely understood, but insights are emerging regarding brain regions and neurotransmitters altered by opioids (Baghdoyan, 2006). Direct administration of μ -opioid receptor agonists into cat pontine reticular formation inhibits REM sleep (Keifer et al., 1992, Cronin et al., 1995, Kshatri et al., 1998) and decreases the release of acetylcholine (ACh) in the pontine reticular formation (Mortazavi et al., 1999). Systemically administered morphine also decreases ACh release in cat pontine reticular formation (Lydic et al., 1993). Cholinergic transmission in the pontine reticular formation plays a key role in REM sleep generation, and decreasing pontine reticular formation ACh release may be one mechanism by which morphine inhibits REM sleep (reviewed in Lydic and Baghdoyan, 2005).

The mechanisms by which morphine alters arousal also may include modulation of γ -aminobutyric acid (GABA) levels in the pontine reticular formation. GABAergic and cholinergic transmission interact in the pontine reticular formation to regulate sleep and wakefulness (Vazquez and Baghdoyan, 2004). Microinjection data from cat (Xi et al., 1999), rat (Sanford et al., 2003), and mouse (Chang et al., 2005, Chang et al., 2006) concur in showing that GABAergic transmission in the pontine reticular formation promotes wakefulness. Therefore, the present study was designed to test the hypothesis that μ -opioid receptors in the oral portion of rat pontine reticular formation (PnO) modulate PnO GABA levels. This study also tested the hypothesis that microinjection of morphine into rat PnO disrupts sleep. Parts of this report have been presented as abstracts (Watson et al., 2005a, Watson et al., 2005b).

EXPERIMENTAL PROCEDURES

Animals and chemicals

All experiments followed the guidelines established by the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Institutes of Health Publication 80-23, National Academy of Sciences Press, Washington DC, 1996) and were approved by the University of Michigan Committee on Use and Care of Animals. Adult male Crl:CD® (SD) IGS BR (Sprague-Dawley) rats (n=48) weighing 235 to 250 g were purchased from Charles River Laboratories (Wilmington, MA, USA). Animals were housed in a temperature and humidity controlled environment with a 12 h light/dark cycle (lights on at 6:00 h) and had access to food and water ad libitum. Data collection for all experiments began between 9:00 and 11:00 h.

Rats were used for three experimental protocols. Group 1 rats (n=34) were each implanted with a microdialysis guide tube. These animals received drugs delivered by reverse dialysis and were used to test the hypothesis that μ -opioid receptors modulate PnO GABA levels. Group 2 rats (n=6) were each implanted with a microdialysis guide tube, electrodes for recording the cortical EEG and neck muscle electromyogram (EMG), and an indwelling jugular vein catheter. Group 2 rats were used to test the hypothesis that intravenous morphine administration alters PnO GABA levels and arousal state. Group 3 rats (n=8) were each implanted with a microinjection guide tube and EEG and EMG recording electrodes. Group 3 rats were used to test the hypothesis that PnO microinjection of morphine disrupts sleep. Details of the three experimental protocols are provided below.

Salts for Ringer's solution and 85% *o*-phosphoric acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acetonitrile, sodium tetraborate decahydrate, β -mercaptoethanol, nipecotic acid, and GABA came from Sigma-Aldrich (St. Louis, MO, USA). Morphine sulfate was obtained from Hawkins Inc., (Minneapolis, MN, USA). Sodium phosphate dibasic, *o*-phthalaldehyde (Fluoraldehyde), and naloxone hydrochloride were purchased from Mallinckrodt (St. Louis, MO, USA) and Pierce Biotechnology, Inc. (Rockford, IL, USA), respectively. High performance liquid chromatography (HPLC) grade methanol was purchased from both Sigma-Aldrich and Fisher Scientific.

Surgical preparation and conditioning of animals for in vivo microdialysis and intravenous drug delivery

To implant Group 1 rats with a microdialysis guide tube, anesthesia was induced with 3.5% isoflurane (Abbott Laboratories, North Chicago, IL, USA) in 100% O₂ and rats were placed in a Kopf Model 962 small animal stereotaxic instrument fitted with a Model 906 rat anesthesia mask (David Kopf Instruments, Tujunga, CA, USA). Delivered isoflurane concentration was measured continuously by spectrometry (CardiocardTM/5, Datex-Ohmeda, Louisville, CO, USA) and was adjusted to approximately 1.7% during surgery. Rats were kept on a hot water-filled pad connected to a re-circulating heat pump (Gaymar Industries, Orchard Park, NY, USA) in order to maintain a core body temperature of 37 °C. Aseptic surgical techniques were used to implant each rat with one CMA/11 guide cannula (CMA, North Chelmsford, MA, USA) aimed 1 mm above the PnO at stereotaxic coordinates 8.4 mm posterior to bregma, 1.0 mm lateral to bregma, and 8.2 mm ventral to skull surface (Paxinos and Watson, 1998). The cannula was held in place with the aid of four skull screws (MPX-080-2P, Small Parts, Inc., Miami Lakes, FL, USA) and dental acrylic (Jet Acrylic Self Curing Resin and Liquid; Lang Dental Manufacturing Company, Inc., Wheeling, IL, USA). Anesthesia was discontinued, rats were removed from the stereotaxic frame and kept warm using the heating pad and/or a heat lamp. Animals were returned to their home cages once they were ambulatory.

Group 2 rats were implanted with a microdialysis guide tube as explained above, electrodes for recording the EEG and EMG as explained below, and a permanent, intravenous catheter. As recently described (Osman et al., 2005), an incision was made to expose the jugular vein and Micro-Renathane tubing (MRE-040; Braintree Scientific (Braintree, MA) was inserted into the vein and tied in place. The other end of the 12 cm long piece of tubing was tunneled subcutaneously and externalized through the skin between the scapulas. This exposed end of the tubing was fitted with a back-mounted flange guide cannula (313-00BM-10; Plastics One) and closed with a removable cap (C313DC/CAC; Plastics One). The injection port was secured with sutures and was accessible for intravenous delivery of saline (control) or morphine to an unrestrained, freely moving rat while simultaneously measuring PnO GABA levels.

Following recovery from surgery, rats were conditioned to a Ratern® testing chamber (Bioanalytical Systems (BAS), West Lafayette, IN, USA) for a minimum of seven days before being used for an experiment. The Ratern® chamber permits freedom of movement and access

to food and water ad libitum. After at least eight h of conditioning per day, rats were returned to their home cages. The jugular vein catheter was flushed daily with saline (0.1 ml) to maintain patency.

In vivo microdialysis, intravenous drug delivery, and quantification of GABA

Each rat was used for only one microdialysis experiment. CMA/11 microdialysis probes (cuprophane membrane with a 1 mm length, 0.24 mm diameter, and 6 kDa cutoff) were used for all experiments and were perfused at a flow rate of 2.0 μ l/min using a CMA/100 or CMA/400 syringe pump. The percent of GABA recovered by the microdialysis probes was tested in vitro before each experiment by placing the probes in a GABA solution of known concentration. Mean $\pm$ SEM recovery of GABA for all microdialysis probes used in this study was 5.7 $\pm$ 0.2%.

Collection of dialysis samples began 17 to 18 h after probe insertion to minimize effects of tissue trauma on GABA levels. For Group 1 rats, each experiment consisted of collecting six sequential 14 μ l samples during each of three sequential treatment conditions, for a total of 18 dialysis samples per experiment. Treatment conditions included dialysis with: 1) Ringer's solution (147.0 mM NaCl, 2.4 mM CaCl₂, 4.0 mM KCl, 1.0 mM MgSO₄, pH 6.0; control); 2) Ringer's containing a drug; and 3) a return to Ringer's. A CMA/110 liquid switch was used to change between solutions delivered to the probe. The following drugs were administered by dialysis: nipecotic acid (500 μ M), morphine (10, 30, 100, 300, and 1,000 μ M), and morphine (100 μ M) mixed with naloxone (1 μ M). Animals were kept awake during the entire sample collection period (126 min) to ensure that observed changes in GABA levels were caused by drug administration and not by changes in levels of arousal (Lee et al., 2001, Marks et al., 2003, Thakkar et al., 2004, Vanini et al., 2006). Samples were collected on ice for subsequent quantification of GABA.

For Group 2 rats, each experiment involved collecting six sequential 14 μ l samples during each of two sequential treatment conditions, for a total of 12 dialysis samples per experiment. EEG and EMG signals were recorded during sample collection according to the procedure explained below. The dialysis probe was perfused continuously with Ringer's solution. Following collection of the first six samples, rats received a 1.0 ml intravenous injection of either morphine (30 mg/kg) or saline (0.9%) and six additional dialysis samples were collected. Injection duration was 4 min. Rats were kept awake during the sample collection period (84 min).

Dialysis samples were analyzed using an ESA (Chelmsford, MA, USA) HPLC system consisting of the following components: 582 solvent delivery system, 542 autosampler, and a Coulochem® II or III electrochemical detector equipped with a 5020 guard cell (700 mV potential) and a 5011A analytical cell (channel 1 = 250 mV potential, channel 2 = 650 mV potential). Analytes were separated using a Shiseido CAPCELL PAK C-18 separation column held at 33 °C (3 μ m particle diameter, 3 mm inner diameter, 5 cm length; JM Science Inc., Grand Island, NY). The separation method used was modified from a previous report (Donzanti and Yamamoto, 1988). Briefly, 12.5 μ l of each dialysis sample were loaded into the autosampler. The autosampler delivered 6.0 μ l of the derivatization solution (5.0 mM o-phthalaldehyde, 1.8 mM β -mercaptoethanol, 97.1 mM borate buffer, 2.5% (v/v) methanol, pH 9.3), mixed the derivatization solution with the dialysis sample three times, waited 1 min for the derivatization reaction to complete, and then injected 10 μ l of the derivatized dialysate onto the separation column. GABA was isolated using a mobile phase consisting of 100 mM sodium phosphate buffer, 25% (v/v) methanol, and 3% (v/v) acetonitrile (pH 6.75, adjusted using 85% o-phosphoric acid). The mobile phase flow rate was 0.6 ml/min. Automation of the HPLC instrument and determination of the GABA peak were accomplished using EZChrom Elite™ chromatography data system (Scientific Software, Inc, Pleasanton, CA). GABA peak determination in brain dialysis samples was based on the retention time of GABA standards.

Standard curves were generated at the beginning and end of each experiment to assure that instrument sensitivity did not decrease with time. The seven concentrations of GABA used to construct the standard curves bracketed the dialysate GABA concentrations, and ranged from 0.011 pmol/10 μ l to 0.913 pmol/10 μ l injection volume.

In vivo microinjection and electrographic recording of sleep and wakefulness

Group 3 rats were used to evaluate the hypothesis that PnO microinjection of morphine disrupts the sleep-wakefulness cycle. Group 3 animals were surgically prepared with a guide tube as described above and with EEG and EMG recording electrodes. To record the EEG, three screw electrodes (8IE36320SPCE; Plastics One, Roanoke, VA) were implanted above the cortex at the following coordinates (from bregma): 1.00 mm anterior, 1.50 mm lateral; 2.00 mm posterior, 1.50 mm lateral; and 2.00 mm posterior, 1.27 mm lateral. Recording electrodes also were implanted bilaterally in the dorsal neck muscle for monitoring the EMG. A sixth electrode was implanted under the skin flap and served as an indifferent (ground) electrode for EMG recording. EMG electrodes were constructed by soldering AS632 biomed wire (5.5 cm length; Cooner Wire Company, Chatsworth, CA) to a Plastics One electrical socket contact (8IE3630XXXXE). The six electrode leads were positioned in a 6-pin multi-channel electrode pedestal (MS363, Plastics One) that was fixed to the skull by dental acrylic and three anchor screws.

For a minimum of seven days post-surgery, each rat was conditioned to the Rturn® chamber as described above for the microdialysis studies. For conditioning and for experiments, rats were tethered via the electrode pedestal to a 6-channel cable (363-441/6 80CM 6TCM, Plastics One). Rats received one microinjection of Ringer's solution (0.1 μ l; vehicle control), followed at least one week later by one microinjection of morphine (7.6 μ g/0.1 μ l; 10 nmol; 100 mM). Microinjections were made using a Model 7001 microliter syringe (Hamilton Company, Reno, NV, USA) held in a manually driven microdrive. Injection duration was 60 s.

EEG and EMG signals were amplified, filtered (EEG, 0.3 to 30 Hz; EMG, 10 to 100 Hz), and recorded at 128 Hz for 2 h post-injection using Icelus Setup and Acquisition software (Opp, 1998). For all recordings, the 6-channel cable was plugged into a 16-channel mini electrode board (F-15EB/B1, Grass Instruments, Warwick, RI) connected to a Grass model 15LT physiodata amplifier system. The amplifier was connected to a 12-bit data acquisition card (6024E, National Instruments (NI), Austin, TX) via a NI BNC 2110 connector. States of wakefulness, non-REM (NREM) sleep, and REM sleep were scored using Icelus Analysis software and previously described criteria (Opp, 1998). EEG, EMG, and cage motion signals were evaluated in 10 s epochs. For fast Fourier Transform (FFT) analysis of the EEG, the power density distribution signal was analyzed in 0.5 Hz increments every 2 s, and five consecutive 2 s bins were averaged to obtain one FFT for each 10 s epoch.

Histological localization of microdialysis and microinjection sites

Microdialysis probe locations and microinjection sites were determined according to the following procedure. After a minimum of two days post-experiment, animals were deeply anesthetized and decapitated. Brains were rapidly removed, frozen, and serially sectioned (40 μ m thick) with a Leica CM 3050S cryostat (Leica Microsystems, Nussloch, Germany). Coronal sections spanning the pontine brainstem were mounted on chrome alum-coated slides, dried overnight, fixed in paraformaldehyde vapor (80° C), and stained with cresyl violet. All sections containing microdialysis probe or microinjection sites were compared to a rat brain atlas (Paxinos and Watson, 1998) to determine the three-dimensional coordinates (mm) of the site relative to bregma. Only experiments in which the microdialysis membrane or the tip of the microinjection cannula was located in the PnO were included in the group data.

Statistical analyses of microdialysis and microinjection data

GABA levels were expressed as percent of control (dialysis with Ringer's) and averaged by microdialysis condition for each experiment. The effects on PnO GABA levels of PnO dialysis with nipecotic acid or morphine were analyzed by descriptive and inferential statistics (GBStat™ v.6.5.6, Dynamic Microsystems, Inc., Silver Spring, MD, USA; and SPSS v.11.0.3, SPSS Inc., Chicago, IL, USA), including either completely randomized or repeated measures one-way analysis of variance (ANOVA) and Dunnett's or Tukey-Kramer post hoc multiple comparisons tests. A sigmoid curve was fit to the concentration-response data using non-linear regression analysis (GraphPad Prism software v4.0a for Macintosh, GraphPad Software Inc., San Diego, CA). Regression analysis provided the coefficient of determination (r^2), as well as the concentration of morphine that produced a half-maximal decrease in GABA levels (IC_{50}). The effects of intravenous morphine administration on PnO GABA levels were assessed using the nonparametric Wilcoxon Rank Sum/Mann-Whitney U test (GBStat™). Microinjection data were analyzed using paired t-test (GBStat™). A probability (p) value of <0.05 was considered statistically significant. All data are reported as mean $\pm$ standard error of the mean (SEM).

RESULTS

Dialysis administration of nipecotic acid to the PnO significantly increased GABA levels in the PnO

Fig. 1A shows typical chromatograms obtained from brain samples collected during dialysis with Ringer's (control) and Ringer's containing nipecotic acid. The nipecotic acid-induced increase in peak height confirmed the presence of GABA. Fig. 1B summarizes the results of four experiments. Repeated measures ANOVA demonstrated a significant treatment main effect on PnO GABA levels ($F=7.6$; $df=2,6$; $p=0.04$). The Tukey-Kramer multiple comparisons test indicated that nipecotic acid significantly ($p<0.05$) increased GABA levels. When nipecotic acid administration was discontinued (Post-NPA Ringer's), GABA in the PnO returned to control levels (Pre-NPA Ringer's). Histological analyses (Fig. 1C) confirmed that all four dialysis sites used to obtain the Fig. 1B data were localized to the PnO (Fig. 1D). The mean $\pm$ SEM stereotaxic coordinates for the microdialysis probes were 8.24 ± 0.21 mm posterior to bregma, 1.45 ± 0.08 mm from the midline, and 8.95 ± 0.17 mm below the skull surface (deepest part of the dialysis membrane).

Dialysis administration of morphine to the PnO decreased PnO GABA levels in a concentration-dependent and naloxone-sensitive manner

Fig. 2 shows the average time course of GABA levels in the PnO during sequential dialysis with Ringer's (control, hatched bars), Ringer's containing morphine (solid bars), and Ringer's (hatched bars). Dialysis with $10 \mu\text{M}$ morphine did not alter GABA levels (Fig. 2A). The Fig. 2A data also demonstrate that GABA levels in the PnO were stable for the entire 126 min dialysis period, during which time animals were kept awake. Dialysis with $100 \mu\text{M}$ or $1,000 \mu\text{M}$ morphine caused GABA levels to decrease within the first seven min of treatment (Figs. 2B and 2C, min 49) and to remain decreased during the 42 min treatment period (Figs. 2B and 2C, min 49-84). GABA then returned to pre-morphine control levels within seven min of discontinuing morphine administration (Figs. 2B and 2C, min 91) and remained stable for the rest of the sample collection period (Figs. 2B and 2C, min 91 to 126).

Fig. 3 shows that the morphine-induced decrease in PnO GABA levels was concentration-dependent. ANOVA demonstrated a significant main effect of morphine concentration on GABA levels ($F=9.46$; $df=4,20$, $p=0.0002$). Significant ($p<0.05$) decreases in GABA levels were caused by dialysis with $100 \mu\text{M}$ (-28%), $300 \mu\text{M}$ (-28%), and $1,000 \mu\text{M}$ (-34%) morphine. The regression equation was a sigmoidal concentration-response as follows: $Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{(\log EC_{50} - X) * (\text{Hill slope})})$. Y was the percent of control GABA levels, bottom

was the maximal decrease in GABA levels induced by morphine, top was the percent of control GABA levels during dialysis with Ringer's, and X was the logarithm of the morphine concentration. Non-linear regression analysis revealed that 98% of the variance in GABA levels was accounted for by the concentration of morphine, and the IC_{50} for the morphine-induced decrease in GABA levels was 58 μ M.

In a separate series of experiments, naloxone (1 μ M), a μ -opioid receptor antagonist, was administered with morphine (100 μ M) and GABA levels in the PnO were quantified. Fig. 4 shows that coadministration of naloxone blocked the morphine-induced decrease in PnO GABA levels. Time course data (Fig. 4A) demonstrate that PnO GABA levels were stable throughout the 84 min of dialysis. Animals were kept awake during the entire sample collection period.

Microdialysis probes used for the concentration-response study (Fig. 3) and the naloxone blocking study (Fig. 4) were all localized to the PnO. Fig. 5 shows a series of coronal rat brainstem drawings (modified from Paxinos and Watson, 1998) with shaded cylinders representing the dialysis membranes. The mean $\pm$ SEM stereotaxic coordinates for the 30 microdialysis sites were 8.25 $\pm$ 0.06 mm posterior to bregma, 1.19 $\pm$ 0.04 mm from the midline, and 8.93 $\pm$ 0.06 mm below the skull surface (deepest part of the 1 mm long dialysis membrane).

Intravenous morphine administration decreased GABA levels in the PnO and caused an increase in EEG slow wave activity

Fig. 6 summarizes the effects of morphine administered intravenously to unanesthetized rat on PnO GABA levels and cortical EEG activity. PnO GABA was measured for 42 min before intravenous injection to establish control levels. Rats then received an intravenous injection of either morphine or saline, and PnO GABA levels were measured for an additional 42 min. Fig. 6A shows that intravenous morphine administration significantly decreased ($p=0.025$) PnO GABA levels below those measured after intravenous saline injection. Behaviorally, morphine caused rats to maintain a rigid, immobile posture that lasted throughout the entire 42 min recording period. During this morphine-induced period of postural immobility, the normal sleep-wakefulness cycle was obliterated. Fig. 6B illustrates that the cortical EEG following morphine administration was characterized by an increase in slow wave activity (0.5-5 Hz) and a loss of the normal theta (5-8 Hz) pattern that occurs when rats explore their environment. The Fig. 6B inset also shows the abnormally high muscle tone caused by intravenous morphine administration. The rat brainstem schematic in Fig. 6C indicates the anatomical localization of the dialysis sites from which PnO GABA levels were measured following intravenous delivery of morphine or saline. Mean $\pm$ SEM stereotaxic coordinates for the six microdialysis sites were 8.32 $\pm$ 0.14 mm posterior to bregma, 1.22 $\pm$ 0.10 mm lateral to the midline, and 8.98 $\pm$ 0.10 mm ventral to the surface of the skull for the deepest part of the dialysis membrane (Paxinos and Watson, 1998).

Microinjection of morphine into the PnO increased wakefulness and suppressed sleep

Having demonstrated that both intravenous and PnO morphine administration decrease GABA levels in the PnO, this study then tested the hypothesis that microinjecting morphine into the PnO disrupts sleep. The results are summarized by Figs. 7 and 8. Fig. 7A shows that morphine significantly increased the amount of time spent in wakefulness ($t=3.8$, $df=7$, $p=0.003$) and significantly decreased the time spent in NREM sleep ($t=3.9$, $df=7$, $p=0.003$) and REM sleep ($t=2.6$, $df=7$, $p=0.019$). PnO microinjection of morphine also significantly increased REM latency (Fig. 7B; $t=3.3$, $df=7$, $p=0.006$) without significantly altering NREM latency.

The morphine-induced increase in the amount of wakefulness (Fig. 7A) occurred due to an increase in the average duration of waking episodes (Fig. 8B; $t=1.9$, $df=7$, $p=0.049$) and a

concomitant decrease in the number of waking episodes (Fig. 8A; $t=3.2$, $df=7$, $p=0.008$). Morphine reduced the amount of NREM sleep (Fig. 7A) by decreasing the number (Fig. 8A; $t=3.4$, $df=7$, $p=0.005$) and duration (Fig. 8B; $t=2.3$, $df=7$, $p=0.027$) of NREM sleep episodes. PnO microinjection of morphine reduced REM sleep (Fig. 7A) by significantly decreasing the number of REM sleep episodes (Fig. 8A; $t=3.5$, $df=7$, $p=0.005$) with no effect on the average duration of REM sleep (Fig. 8B).

All microinjection sites were histologically confirmed to be in the PnO (Fig. 8C). The mean $\pm$ SEM stereotaxic coordinates for the eight microinjection sites were 7.89 ± 0.05 mm posterior to bregma, 1.22 ± 0.11 mm from the midline, and 8.50 ± 0.11 mm below the skull surface (Paxinos and Watson, 1998).

DISCUSSION

The PnO, which is the rostral portion of the rodent pontine reticular formation, is a key component of the ascending reticular activating system (Moruzzi and Magoun, 1949) and is involved in generating states of sleep (Steriade and McCarley, 2005) and anesthesia (Lydic and Baghdoyan, 2005). Three novel findings emerged from this study, which focused on the PnO. First, dialysis delivery of morphine to the PnO of unanesthetized rat decreased GABA levels in the PnO. The morphine-induced decrease in GABA levels was concentration-dependent and blocked by coadministration of naloxone, indicating mediation by μ -opioid receptors. Second, intravenous morphine administration also decreased GABA levels in the PnO. As shown previously (Osman et al., 2005), intravenous morphine caused a dissociated behavioral state characterized by a suppression of normal sleep, increased muscle tone, increased slow wave activity in the cortical EEG, and behavioral immobility. The third novel finding was that PnO microinjection of morphine caused a significant increase in wakefulness and a significant decrease in both NREM sleep and REM sleep, with no change in muscle tone or EEG activity. Thus, both systemic and intrapontine routes of morphine administration caused a decrease in PnO GABA levels and a disruption of sleep. The motor tone and EEG responses to morphine differed depending upon the route of administration, as did the effects of morphine on wakefulness. These data extend the understanding of arousal state modulation by pontine GABAergic transmission to the clinically relevant molecule morphine.

Nipecotic acid increased GABA levels in the PnO

Nipecotic acid is a GABA reuptake inhibitor known to increase GABA levels (Kehr and Ungerstedt, 1988). The Fig. 1 finding that PnO GABA levels increased during dialysis delivery of nipecotic acid indicates the presence of functional GABA transporters in the PnO. Nipecotic acid is not selective for neuronal or astrocytic transporters (Krogsgaard-Larsen et al., 2000), and the cellular location of the transporters in rat PnO remains unknown. As reviewed elsewhere, the source of basal GABA levels measured with microdialysis is likely to include extrasynaptic and/or non-neuronal pools, and GABA can be released into the extracellular space by astrocytes (Timmerman and Westerink, 1997, Araque et al., 1999, Del Arco et al., 2003, Drew et al., 2004, Watson et al., 2006a). Glial cells surrounding synapses are very efficient at taking up neuronally released GABA and may act as buffers between the microdialysis probe and the synapses. This effect of glia suggests that the GABA measured in microdialysis samples may be an indirect reflection of synaptically released GABA (Rea et al., 2005). Thus, the present report refers to measures of GABA levels, rather than measures of GABA release. For the purpose of the present study, the increase in GABA levels observed in response to dialysis administration of nipecotic acid (Fig. 1) confirms the chromatographic method used to detect GABA.

Morphine decreased PnO GABA levels by actions at μ -opioid receptors

Morphine delivered to the PnO caused a concentration-dependent (Figs. 2, 3), naloxone-sensitive (Fig. 4) decrease in GABA levels within the PnO (Fig. 5). These findings indicate that the morphine-induced decrease in GABA levels was mediated by μ -opioid receptors. Morphine acting at μ -opioid receptors has been shown to decrease GABA levels in other regions of rat brain, including the periaqueductal gray (Stiller et al., 1996) and ventral tegmental area (Klitenick et al., 1992, Sotomayor et al., 2005), and functional μ -opioid receptors are present in rat PnO (Capece et al., 1998).

Following the discovery that opioids inhibit cell excitability by activating potassium conductance (North and Tonini, 1977), opioids were shown to alter a variety of ion channels and signal transduction cascades (reviewed in Williams et al., 2001, Samways and Henderson, 2006). GABAergic neurons in rat brainstem (Kalyuzhny and Wessendorf, 1997, Kalyuzhny and Wessendorf, 1998) and nucleus accumbens (Svingos et al., 1997) express μ -opioid receptors, and μ -opioid receptors are expressed by rat pontine reticular formation neurons (Kalyuzhny et al., 2000). In the present study, the mechanisms by which morphine decreased GABA levels are not known. The simplest explanation is that morphine activated μ -opioid receptors localized to the cell bodies of GABAergic interneurons or GABAergic neuronal terminals within the pontine reticular formation. This interpretation is consistent with evidence that opioids can decrease GABA release from axon terminals (Cohen et al., 1992). The present study also provides direct evidence that intravenous delivery of morphine decreases GABA levels in rat PnO (Fig. 6).

Morphine disrupted sleep when administered systemically or directly into the PnO

PnO microinjection of morphine caused a significant increase in wakefulness and a significant decrease in both NREM sleep and REM sleep (Figs. 7, 8). These effects are similar to the increase in wakefulness and decrease in sleep caused by microinjecting morphine into homologous regions of cat pontine reticular formation (Keifer et al., 1992, Cronin et al., 1995). During the increase in wakefulness that followed morphine microinjection, rats behaved normally and exhibited normal muscle tone. Power spectral analysis of the EEG (data not shown) revealed no differences in EEG frequencies following microinjection of morphine and Ringer's. Intravenous delivery of morphine also inhibited NREM sleep and REM sleep. These findings suggest that the pontine reticular formation is one site where morphine may act to cause the sleep disruption known to occur in humans (Shaw et al., 2005).

This study also revealed differences in the response to morphine that depended upon route of administration. Intravenous morphine administration eliminated normal wakefulness whereas pontine microinjection of morphine increased normal wakefulness. Intravenous morphine caused a dissociated state characterized by an increase in slow cortical EEG frequencies, immobility, and increased muscle tone (Fig. 6B), whereas pontine microinjection of morphine caused neither immobility nor increased muscle tone. Systemically administered morphine has been suggested to slow the cortical EEG by inhibiting the basal forebrain cholinergic neurons that release ACh in the neocortex (Osman et al., 2005). Considered together, the previous (Osman et al., 2005) and present results support the conclusion that the slowing of the cortical EEG and suppression of normal wakefulness caused by systemically administered morphine are not mediated at the level of the pontine reticular formation. However, the present findings that both intravenous and pontine morphine administration decreased sleep suggest that sleep suppression caused by systemic morphine may be mediated, at least in part, at the level of the pontine reticular formation.

Limitations and conclusions

GABAergic transmission at GABA_A receptors in the pontine reticular formation has been shown to regulate arousal levels (Camacho-Arroyo et al., 1991, Xi et al., 1999, Sanford et al., 2003) and ACh release (Vazquez and Baghdoyan, 2004). Microdialysis administration of bicuculline into cat pontine reticular formation causes a concentration-dependent increase in ACh release and triggers episodes of REM sleep (Vazquez and Baghdoyan, 2004). Microinjecting the GABA_A receptor agonist muscimol into cat pontine reticular formation increases wakefulness and decreases sleep, whereas microinjecting the GABA_A receptor antagonist bicuculline decreases wakefulness and increases REM sleep (Xi et al., 1999). Bicuculline also has been shown to increase REM sleep when microinjected into homologous regions of rat pontine reticular formation (Sanford et al., 2003). Consistent with the above findings, muscimol increases wakefulness and decreases NREM sleep when microinjected into rat pontine reticular formation (Camacho-Arroyo et al., 1991). Microinjection of bicuculline into the PnO of B6 mouse causes a significant, concentration-dependent decrease in wakefulness and increase in REM sleep (Chang et al., 2006). Preliminary data indicate that PnO microinjection of the GABA reuptake inhibitor nipecotic acid causes a significant increase in wakefulness and a significant decrease in sleep (Watson et al., 2006b). Taken together, these data are consistent with the interpretation that one functional role of GABA in the pontine reticular formation is to promote wakefulness.

Although dialysis administration of morphine into rat PnO in the present study decreased PnO GABA levels (Figs. 2, 3), microinjection of morphine into the PnO increased wakefulness and decreased both NREM sleep and REM sleep (Figs. 7, 8). These data may initially appear to conflict with the previous data outlined above, which showed that microinjection of the GABA_A receptor antagonists bicuculline into the pontine reticular formation decreased wakefulness and increased REM sleep (Xi et al., 1999, Sanford et al., 2003, Xi et al., 2004, Chang et al., 2006). However, it is premature to conclude that the present results contradict previous evidence that GABAergic mechanisms in the pontine reticular formation promote wakefulness and inhibit REM sleep. The present findings with morphine cannot be compared orthogonally to previous studies, because morphine decreased GABA levels whereas the previous studies blocked GABAergic transmission at the GABA_A receptor. Decreasing levels of a neurotransmitter is not functionally the same as pharmacologically blocking the receptor activated by that transmitter. For example, a decrease in GABAergic tone (caused by morphine) would affect both GABA_A and GABA_B receptors. In contrast, GABA_B receptors would not be affected by exogenous GABA_A antagonists (bicuculline) or agonists (muscimol).

GABA collected by in vivo microdialysis is likely to represent mainly extrasynaptic levels that contribute to volume transmission, rather than only synaptically released levels (reviewed in Timmerman and Westerink, 1997, Del Arco et al., 2003, Watson et al., 2006a). In addition to the classical rapid signaling that occurs at postsynaptic GABA_A receptors as a result of presynaptic release, GABA also signals in a mode that is temporally dissociated from synaptic events via GABA_A receptors localized outside the synaptic cleft (reviewed in Mody, 2001, Farrant and Nusser, 2005). These extrasynaptic GABA_A receptors are suggested to signal in a more tonic manner (i.e., volume transmission) than the synaptic GABA_A receptors that mediate phasic responses (i.e., synaptic, or point-to-point, transmission) (Mody, 2001, Farrant and Nusser, 2005). Although morphine caused a significant decrease in PnO GABA levels (Figs. 3, 6A), there remained measurable levels of GABA (Figs. 2, 3) available to activate PnO GABA receptors. In contrast, endogenous GABA is ineffective at GABA_A receptors blocked by direct administration of bicuculline.

The present results support the interpretation that the behavioral responses to a decrease in PnO GABA levels differ from the behavioral responses to blocking GABA_A receptors in the pontine reticular formation. The data reported here establish that GABA levels in the PnO are

modulated by μ -opioid receptors in the PnO and by systemically administered morphine. Finally, the findings from this study suggest that morphine-induced sleep disruption may be mediated, at least in part, by pontine reticular formation GABAergic transmission.

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ABBREVIATIONS

ACh, acetylcholine
ANOVA, analysis of variance
EEG, electroencephalogram
EMG, electromyogram
FFT, fast Fourier Transform
GABA, γ -aminobutyric acid
HPLC, high performance liquid chromatography
NREM, non rapid eye movement
p, probability
PnO, pontine reticular formation, oral part
REM, rapid eye movement
SEM, standard error of the mean

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Figure 1.

Dialysis administration of nipecotic acid to the pontine reticular nucleus, oral part (PnO) significantly increased GABA levels in the PnO. **A.** Representative chromatograms demonstrate excellent signal-to-noise ratios obtained during control dialysis (Ringer's, gray line) and during dialysis administration of the GABA reuptake inhibitor nipecotic acid (NPA, black line). The increase in peak size during nipecotic acid administration confirms the presence of GABA. The inset shows a sagittal schematic modified from a rat brain atlas (Paxinos and Watson, 1998) and depicts placement of a microdialysis probe in the PnO. Arrows indicate dialysis administration of nipecotic acid via one port on the dialysis probe, and simultaneous collection of dialysis samples containing GABA via the second port on the

dialysis probe. The unfilled area at the tip of the probe represents the 1 mm long dialysis membrane and is drawn to scale. **B.** Dialysis administration of nipecotic acid to the PnO of awake rat increased GABA levels (solid bar) to $185 \pm 20\%$ of control (Pre-NPA Ringer's). Control GABA levels (Pre-NPA Ringer's) were normalized to 100% for each experiment. The number of samples analyzed for each dialysis condition was: pre-NPA Ringer's=23, NPA=24, and post-NPA Ringer's =22. **C.** A digitized image of one cresyl violet-stained coronal section illustrates a typical PnO microdialysis site located approximately 8.30 mm posterior to bregma. The arrow marks the most ventral part of the dialysis site. Note that this section was cut in a different plane from the atlas (Paxinos and Watson, 1998), and that the dorsal part of the section is more caudal than the ventral part. **D.** Coronal diagrams are modified from a rat brain atlas (Paxinos and Watson, 1998). Shaded cylinders represent the dialysis membranes, are drawn to scale, and show that the four dialysis sites used for the nipecotic acid study were localized to the PnO. Numbers at the top right of each brainstem schematic indicate stereotaxic coordinates in mm from bregma.

Figure 2.

Time course of the decrease in GABA levels caused by administering morphine to rat (n=15) pontine reticular nucleus, oral part (PnO). **A.** Five experiments tested 10 μ M morphine, and resulted in n=30 samples collected during dialysis with pre-morphine Ringer's, n=29 samples collected during dialysis with Ringer's containing morphine, and n=30 samples collected during post-morphine dialysis with Ringer's. Morphine (10 μ M) did not alter GABA levels. **B.** Five additional experiments demonstrated that GABA levels were significantly (*, p<0.01) decreased below the mean Ringer's (control) level by 100 μ M morphine; n=29 pre-morphine Ringer's samples, n=29 morphine samples, n=27 post-morphine Ringer's samples. **C.** Five additional experiments revealed that GABA levels were significantly (*, p<0.05) decreased below the average control level by 1,000 μ M morphine; n=28 pre-morphine Ringer's samples, n=27 morphine samples, n=29 post-morphine Ringer's samples.

Figure 3.

Dialysis administration of morphine to the pontine reticular nucleus, oral part (PnO) caused a concentration-dependent decrease in PnO GABA levels. Every concentration of morphine was tested using five animals, for a total of 25 animals. GABA levels as a percent of control were 98.9 ± 5.1 (10 μM morphine), 97.0 ± 4.4 (30 μM), 72.3 ± 2.7 (100 μM), 71.9 ± 6.4 (300 μM), and 66.3 ± 5.6 (1,000 μM).

Figure 4.

The morphine-induced decrease in GABA levels was blocked by coadministration of naloxone to the pontine reticular nucleus, oral part (PnO). **A.** Time course plot of average GABA levels from five rats. Samples were collected during dialysis with Ringer's (control) followed by dialysis with Ringer's containing both morphine and naloxone. **B.** GABA levels were averaged by dialysis condition for each experiment. GABA levels are based on 30 samples collected during Ringer's dialysis and 29 samples collected during coadministration of morphine and naloxone.

Figure 5.

Histological localization of dialysis sites to the pontine reticular nucleus, oral part (PnO). Numbers at the top right of each coronal brainstem diagram indicate mm from bregma (modified from Paxinos and Watson, 1998). Shaded cylinders (n=30) drawn to scale represent the 1 mm long microdialysis membranes used to deliver five concentrations of morphine (n=25 experiments) and morphine plus naloxone (n=5 experiments). Dialysis sites were plotted on the closest existing atlas drawing, and spanned from 7.47 to 8.80 mm posterior to bregma, 0.75 to 1.65 mm from the midline, and 8.15 to 9.60 mm ventral to the skull surface. Inset: Vertical lines on the sagittal diagram (modified from Paxinos and Watson, 1998) delineate the anterior-to-posterior extent of the PnO microdialysis sites.

Figure 6.

Intravenous delivery of morphine to unanesthetized rat decreases PnO GABA levels and slows the cortical EEG. **A.** GABA levels in the PnO are expressed as a percent of control. Control levels of GABA (100%) were measured during perfusion of the dialysis probe with Ringer's prior to intravenous administration of saline or morphine. PnO GABA levels following intravenous morphine (solid bar) were significantly decreased below GABA levels measured following intravenous saline administration (hatched bar). **B.** Power spectral analysis of the cortical EEG revealed that intravenous morphine administration increased EEG power in the delta (0.5-5 Hz) range. The insets show raw EEG and EMG signals and illustrate the high amplitude slow frequency EEG waves and relatively high neck muscle tone caused by

intravenous delivery of morphine. **C.** All six microdialysis sites were localized to the PnO. Dialysis membranes are drawn to scale as shaded cylinders. Coronal diagrams are modified from a rat brain atlas (Paxinos and Watson, 1998). On the right side of each brainstem plate, the stereotaxic coordinate is indicated in mm from bregma.

Figure 7.

Microinjection of morphine into the pontine reticular nucleus, oral part (PnO) increased wakefulness and decreased sleep. Histograms summarize the percent of recording time spent in sleep and wakefulness during the 2 h recording period following PnO microinjection of morphine (solid bars) or Ringer's solution (hatched bars). Mean+SEM values are based on data from eight rats. Each rat received one microinjection of Ringer's (0.1 μ l) and one microinjection of Ringer's containing morphine (7.6 μ g/0.1 μ l). **A.** Morphine caused a significant (*) 65% increase in wakefulness. **B.** NREM sleep was significantly (*) decreased by 69%. **C.** PnO microinjection of morphine significantly (*) decreased REM sleep by 53%.

Figure 8.

The number and average duration of sleep and wakefulness episodes was altered by microinjection of morphine into the pontine reticular nucleus, oral part (PnO). Histograms summarize the number of episodes of sleep and wakefulness during the 2 h recording period that followed microinjection of morphine (solid bars) or Ringer's solution (hatched bars) into the PnO. Mean+SEM values are based on data from eight rats. Each rat received one microinjection of Ringer's (0.1 μ l) and one microinjection of Ringer's containing morphine (7.6 μ g/0.1 μ l). **A.** Morphine caused a significant (*) decrease in the number of episodes of wakefulness (-53%), NREM sleep (-55%), and REM sleep (-65%). **B.** Morphine caused the average duration of waking episodes to significantly (*) increase (614%) and the average duration of NREM sleep episodes to decrease (-47%). There was no effect of morphine on the duration of REM sleep episodes. **C.** Microinjection sites (filled circles) are plotted at 7.80 mm

posterior to bregma on a coronal diagram modified from the rat brain atlas (Paxinos and Watson, 1998). All eight microinjection sites were in the PnO.