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Pontine Nitric Oxide Modulates Acetylcholine Release, Rapid Eye Movement Sleep Generation, and Respiratory Rate

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Pontine cholinergic neurotransmission is known to play a key role in the regulation of rapid eye movement (REM) sleep and to contribute to state-dependent respiratory depression. Nitric oxide (NO) has been shown to alter the release of acetylcholine (ACh) in a number of brain regions, and previous studies indicate that NO may participate in the modulation of sleep/wake states. The present investigation tested the hypothesis that inhibition of NO synthase (NOS) within the medial pontine reticular formation (mPRF) of the unanesthetized cat would decrease ACh release, inhibit REM sleep, and prevent cholinergically mediated respiratory depression. Local NOS inhibition by microdialysis delivery of N^G-nitro-L-arginine (NLA) significantly reduced ACh release in the cholinergic cell body region of the pedunculopontine tegmental nucleus and in the cholinoceptive mPRF. A second series of experiments demonstrated that mPRF microinjection of NLA significantly reduced the amount of REM sleep and the REM sleep-like state caused by mPRF injection of the acetylcholinesterase inhibitor neostigmine. Duration but not frequency of REM sleep epochs was significantly decreased by mPRF NLA administration. Injection of NLA into the mPRF before neostigmine injection also blocked the ability of neostigmine to decrease respiratory rate during the REM sleep-like state. Taken together, these findings suggest that mPRF NO contributes to the modulation of ACh release, REM sleep, and breathing.

Key words: acetylcholine; nitric oxide; pons; reticular formation; respiratory control; halothane anesthesia; REM sleep

Pontine cholinergic neurotransmission is involved in regulating the rapid eye movement (REM) phase of sleep (Steriade and McCarley, 1990; Jones, 1993; Lydic and Baghdoyan, 1994; McCarley et al., 1995). Both anatomical (Mitani et al., 1988; Shiromani et al., 1988) and functional (Lydic and Baghdoyan, 1993) studies have shown that cholinergic neurons of the laterodorsal and pedunculopontine tegmental (LDT/PPT) nuclei project axon terminals to the medial pontine reticular formation (mPRF), where acetylcholine (ACh) is released. Electrical stimulation of the LDT/PPT causes a monotonic increase in mPRF ACh release (Lydic and Baghdoyan, 1993). Lesions of the LDT/PPT disrupt REM sleep, and the amount of REM sleep reduction is correlated positively with LDT/PPT cell destruction (Webster and Jones, 1988; Shouse and Siegel, 1992). Microinjection of cholinergic agonists into the mPRF elicits a state with the behavioral and electrophysiological traits of REM sleep (Baghdoyan et al., 1984; Baghdoyan et al., 1989; Vanni-Mercier et al., 1989; Yamamoto et al., 1990; Baghdoyan et al., 1993). This cholinergically evoked REM sleep-like state also is characterized by upper airway muscle hypotonia and depressed rate of breathing (Lydic and Baghdoyan, 1989; Lydic et al., 1989). ACh release in the mPRF increases during the cholinergically evoked REM sleep-like state (Lydic et al., 1991) and during natural REM sleep (Leonard and Lydic, 1995). Therefore, multiple lines of evidence have established the involvement of cholinergic LDT/PPT cells and noncholinergic, cholinoceptive mPRF neurons in the generation of REM sleep and state-dependent respiratory depression. An important question for understanding the cellular and molecular regulation of REM sleep concerns the mechanisms by which pontine cholinergic neurotransmission is controlled.

Nitric oxide (NO) is a modulator of neuronal function (Garthwaite and Boulton, 1995; Zhang et al., 1995) and has been shown to alter ACh release (Prast and Philippu, 1992; Guevara-Guzman et al., 1994; Ohkuma and Kuriyama, 1994; Leonard and Lydic, 1995; Ohkuma et al., 1995; Prast et al., 1995). LDT/PPT cholinergic neurons in cat stain positively for NADPH diaphorase (Vincent et al., 1983; Mizukawa et al., 1989), which has been identified as a neuronal nitric oxide synthase (NOS) (Dawson et al., 1991; Hope et al., 1991). In rat (Kapas et al., 1994a) and rabbit (Kapas et al., 1994b), systemic inhibition of NOS alters sleep. The presence of NOS protein and mRNA in LDT/PPT neurons has been confirmed (Bredt et al., 1991), and mPRF administration of a NOS inhibitor reduces mPRF ACh release (Leonard and Lydic, 1995). These findings suggested that mPRF levels of NO might modulate pontine cholinergic neurotransmission and possibly participate in the regulation of arousal states. Therefore, the present study has expanded these earlier findings by testing the hypothesis that stereoselective inhibition of NOS in the mPRF would decrease local pontine ACh release, inhibit REM sleep, and prevent cholinergically evoked respiratory rate depression.

MATERIALS AND METHODS

Animal model

Electrodes for polygraphic monitoring of sleep and wakefulness were implanted during halothane anesthesia (1–2% in O_2) in 10 adult male cats. Each cat was used for either microinjection or microdialysis experiments. For microinjection studies ($n = 5$ cats), 24 gauge stainless steel guide tubes were implanted 5 mm above the mPRF using the stereotaxic

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coordinates [2.0 mm posterior (P); 1.5 mm lateral (L); −5.0 mm horizontal (H)] of Berman (1968). For experiments involving microdialysis ($n = 5$ cats), the cranial acrylic encasement surrounding the sleep scoring electrode array was equipped with a plastic well that permitted subsequent placement of microdialysis probes into the mPRF. After recovery from surgery and before beginning microdialysis or microinjection experiments, all cats were trained for 1–2 months to sleep in the laboratory in a head-stable position. Animals were studied in this head-restrained position and all experiments strictly adhered to the National Institutes of Health guidelines for the care and use of laboratory animals (National Institutes of Health Publication No. 85-23, 1985).

mPRF ACh measurement

Microdialysis. Before *in vivo* mPRF dialysis, a microdialysis probe (CMA/10, Acton, MA) with a polycarbonate membrane of 20 kDa pore size was placed in a vial containing a known concentration of ACh and was perfused (CMA/100 microinjection pump) with a modified Ringer's solution, pH 6.0, 147 mM NaCl; 4.0 mM KCl; 2.4 mM $CaCl_2$; 10 μM neostigmine bromide (Sigma, St. Louis, MO). As demonstrated previously, (Lydic et al., 1991) 10 μM neostigmine does not alter arousal state. This procedure was used to determine the preexperiment recovery of ACh by the microdialysis probe. At the conclusion of each mPRF dialysis experiment, probe recovery of ACh from a standard solution verified that *in vivo* measurement of changes in mPRF ACh release was not attributable to mechanical alteration of the probe membrane. Only data from experiments in which preexperiment *in vitro* probe recovery did not differ from postexperiment ACh recovery are included in this report.

For each experiment, a microdialysis probe was placed in the mPRF using stereotaxic coordinates: P = 1.5–3.0 mm; L = 0.8–1.5 mm; H = −5.0 to −6.5 mm; probe angle = 30° P. The dialysis probe was perfused continuously with Ringer's solution (control) at 3 μl/min, and endogenous ACh was recovered in 30 μl dialysate samples. Each 10 min mPRF dialysate sample was collected during unambiguously scored states of wakefulness, non-REM (NREM) sleep, or REM sleep. After 2–3 hr of sample collection during Ringer's perfusion, the probe was perfused with 10 mM N^G-nitro-L-arginine (NLA; RBI, Natick, MA) dissolved in Ringer's. Dialysate samples again were collected during wakefulness, NREM sleep, and REM sleep for determination of mPRF ACh release in the presence of the NOS inhibitor NLA. After collection of samples during Ringer's dialysis, the mPRF was dialyzed with Ringer's containing 10 mM N^G-nitro-D-arginine (NDA; Bachem, Torrance, CA) instead of NLA. NDA is a stereoisomer of NLA that has been shown to be less potent in its ability to inhibit NOS (Wang et al., 1993). Because NDA is a relatively inactive enantiomer of NLA, it has been suggested that NDA can serve as an effective pharmacological control for NLA administration (Griffith and Stuehr, 1995). Multiple experiments using different mPRF sites in the same animal were separated by at least 5 d.

High performance liquid chromatography (HPLC). Each 30 μl mPRF dialysate sample was injected into an HPLC system (Bioanalytical Systems, West Lafayette, IN) and carried in a 50 mM Na_2HPO_4 mobile phase, pH 8.5, at 1.0 ml/min (pressure = 13–15 MPa). Samples passed through an analytical separation column before entering an immobilized enzyme reactor column, where H_2O_2 was produced from ACh in stoichiometric amounts. H_2O_2 was detected at a platinum electrode with an applied potential of 500 mV relative to an Ag^+/AgCl reference electrode. The generated current created a chromatogram peak that was recorded on a flat-bed recorder and processed by a computer software program (Inject). Mean retention time for the ACh chromatogram was 5.30 min. The chromatogram peak areas are proportional to the ACh content in each dialysis sample. Chromatogram areas were compared with a series of ACh standards (0.1–3.0 pmol) to express ACh values as pmol/10 min for each brain sample.

PPT ACh measurement

Additional microdialysis experiments in two cats examined the effect of PPT NLA delivery on ACh release within the PPT. The animals were anesthetized with halothane (1–2% in O_2) delivered through a mask. Once anesthetized, cats were intubated with a #4 cuffed endotracheal tube and placed in stereotaxic head restraint. A microdialysis probe was placed in the PPT according to the coordinates of Berman (1968): P = 0.8 mm; L = 3.0 mm; H = −2.5 mm; angle = 30° P. The probe was constantly perfused at 3 μl/min with Ringer's solution. A Raman spectrophotometer sampled expired gas from the endotracheal tube and measured end tidal CO_2 and halothane concentration. Halothane anesthesia was maintained at 1.2% (in O_2). End tidal CO_2 was maintained at 20–25 mmHg by adjusting minute ventilation. During 1.2% halothane anesthesia, 30 μl dialysate samples were collected and ACh content was measured as pmol/10 min. After termination of halothane anesthesia, ACh released into the PPT was measured during wakefulness. Wakefulness was determined by (1) measurement of end tidal halothane; (2) polygraphic recordings (EEG desynchrony, return of muscle tone, conjugate eye movements); and (3) behavioral observation (limb movements, tracking eye movements). Finally, the microdialysis probe was perfused with 10 mM NLA and samples were analyzed for ACh content as a result of delivery of a NOS inhibitor during a state of quiet wakefulness. From these experiments, it was possible to quantify the effects of both 1.2% halothane anesthesia and PPT NOS inhibition on ACh release within the PPT.

mPRF microinjections

Drug administration. Because the brain parenchyma is devoid of nociceptors, it was possible to make repeated microinjections into the mPRF of unanesthetized cats while they were in a state of quiet wakefulness. Microinjections were given through 31 gauge stainless steel tubing placed in the implanted guide tube. A 250 nl volume of saline (vehicle control) or drug was injected into the mPRF over a 30 sec period using a 1 μl Hamilton syringe (Thomas Scientific, Swedesboro, NJ) and a manual microdrive assembly. For 2 hr after the microinjection, states of sleep and wakefulness were recorded on a Grass polygraph. A thermistor placed at the nares also permitted polygraphic quantification of respiratory rate. In this way, effects of mPRF drug administration on sleep/wake states were determined for the following six microinjection conditions: (1) saline; (2) NLA (22.8 mM: 1.25 μg/0.25 μl); (3) NDA (22.8 mM: 1.25 μg/0.25 μl); (4) neostigmine bromide (40.0 mM: 3.0 μg/0.25 μl saline); (5) 22.8 mM NLA microinjected 15 min before a 40.0 mM neostigmine injection; and (6) 22.8 mM NDA microinjected 15 min before a 40.0 mM neostigmine injection. In addition, respiratory rate was quantified after mPRF injection of saline; 40.0 mM neostigmine alone; 22.8 mM NLA 15 min before neostigmine; and 22.8 mM NDA before neostigmine. All experiments in which a drug (NLA, NDA, or neostigmine) were microinjected into the mPRF of the same animal were separated by at least 3 d.

State and breathing quantification. For each experiment, 2 hr polygraphic recordings were divided into 120 bins, and each min was scored as wakefulness, NREM sleep, or REM sleep. Polygraphic variables recorded from the implanted electrodes were used to objectively score states of wakefulness, NREM sleep, and REM sleep according to standard criteria (Ursin and Sterman, 1981). For each recording, 10 min of each of the three states was randomly selected and respiratory rate (breaths/min) was tabulated.

Data analysis

For microdialysis and microinjection experiments, descriptive statistics and ANOVA were used to quantify drug effects on the following dependent measures: mPRF and PPT ACh release (pmol/10 min); percent wakefulness, NREM sleep, and REM sleep; REM sleep latency; REM sleep epoch frequency and duration; and rate of breathing. *Post hoc* multiple pairwise comparisons were performed using Tukey's tests for analysis of state effect on mPRF and PPT ACh release. For mPRF microdialysis experiments, *a priori* independent *t* tests were used to test for statistical significance in the difference between ACh release during control (Ringer's) or drug (10 mM NLA or 10 mM NDA) dialysis within states of wakefulness, NREM sleep, and REM sleep. To test the effects of mPRF microinjection on the percent time spent in REM sleep, NREM sleep, and wakefulness; REM sleep epoch duration, frequency, and latency; as well as injection effect on respiratory rate within each state, multiple pair-wise comparisons were made using independent *t* tests with Bonferroni correction factors (p_{actual} = 0.05/number of comparisons). From four of the five animals, it was possible to obtain three measures of breathing and arousal state in each of the six microinjection conditions. From the fifth animal, three measures of breathing and arousal state were obtained for four of the six microinjection conditions. For all statistical comparisons, a significance level of $p = 0.05$ was chosen.

Histological analysis

At the completion of mPRF microdialysis or microinjection experiments, cats were deeply anesthetized with sodium pentobarbital and transcardially perfused with isotonic saline followed by 10% phosphate buffered formalin, pH 7.0, (Fisher Scientific, Houston, TX). Brains were removed and soak-fixed first in the buffered formalin and then in 30% sucrose-formalin for 1–2 weeks. Brainstems were sectioned (40 μm thick) on a

freezing microtome, mounted on chrome-alum-coated slides, stained with cresyl violet, and coverslipped.

RESULTS

The results were obtained from a total of 1870 min of mPRF microdialysis, 330 min of PPT microdialysis, and 82 mPRF microinjection experiments. These data are the first to show the ability of NOS inhibition within specific brain regions to alter simultaneously (1) neurotransmitter release, (2) states of sleep and wakefulness, and (3) state-dependent respiratory depression. Portions of these mPRF microdialysis data were published previously in a brief report (Leonard and Lydic, 1995). The present results describe for the first time an increased number mPRF dialysis samples, ACh release from PPT brain regions, the stereoselective effects of NLA, and the ability of mPRF NOS inhibition to alter REM sleep and breathing during REM sleep.

Identification of microdialysis and microinjection sites

For all microdialysis experiments, confirmation of dialysis probe placement in the mPRF and the PPT was achieved by successful *in situ* recovery of ACh and histological visualization of probe-induced lesions. Figure 1, *A* and *B*, demonstrates visualization and localization of probe-induced lesions within the mPRF and the PPT, respectively. Likewise, stereotaxic placement of microinjectors in the mPRF was verified by histological analyses. For all microinjection studies, a 3 μg injection of neostigmine was able to cause a REM sleep-like state (Baghdoyan et al., 1984) during at least 50% of a 2 hr recording period. A typical mPRF microinjection lesion is illustrated in Figure 1*C*.

mPRF ACh release varied across states

mPRF ACh release during states of sleep and wakefulness

To test the hypothesis that ACh release in the mPRF would increase during REM sleep relative to NREM sleep and wakefulness ACh levels, 10 min (30 μl) mPRF dialysate samples (n) were collected during objectively defined states of wakefulness (n = 41), NREM sleep (n = 33), and REM sleep (n = 18) from five different cats. Quantification of these samples revealed state-dependent differences in mPRF ACh release ($F_{(2,92)} = 42.10$; $p < 0.0001$) (Fig. 2*A*, *hatched bars*). During REM sleep, mPRF ACh release increased significantly, rising 100% over waking levels and 124% above ACh release during NREM sleep. In every experiment in each animal, mPRF ACh release measured during REM sleep was at least 80% greater than ACh release during either wakefulness or NREM sleep. There was no significant difference in ACh levels recovered from the mPRF comparing wakefulness and NREM sleep (*post hoc* Tukey's test).

NLA decreased mPRF ACh release

To test the hypothesis that NO regulates pontine ACh release, the mPRF of intact, unanesthetized cats was dialyzed with the NOS inhibitor NLA while simultaneously measuring ACh release during wakefulness, NREM sleep, and REM sleep. Figure 2*A* shows that during every state, NLA caused a significant reduction in mean mPRF ACh release. Compared with Ringer's dialysis, NLA caused decreases in average mPRF ACh release of 39% during wakefulness ($t = 6.01$; df = 74; $p < 0.0001$); 44% decrease during NREM sleep ($t = 4.47$; df = 61; $p < 0.0001$); and 45% during REM sleep ($t = 3.52$; df = 27; $p = 0.0016$).

During NLA dialysis, state-dependent changes in mPRF ACh release also were observed ($F_{(2,76)} = 12.82$; $p < 0.0001$) (Fig. 2*A*, *solid bars*). REM sleep ACh release (n = 11) was significantly greater than wakefulness ACh release (82% increase) and NREM

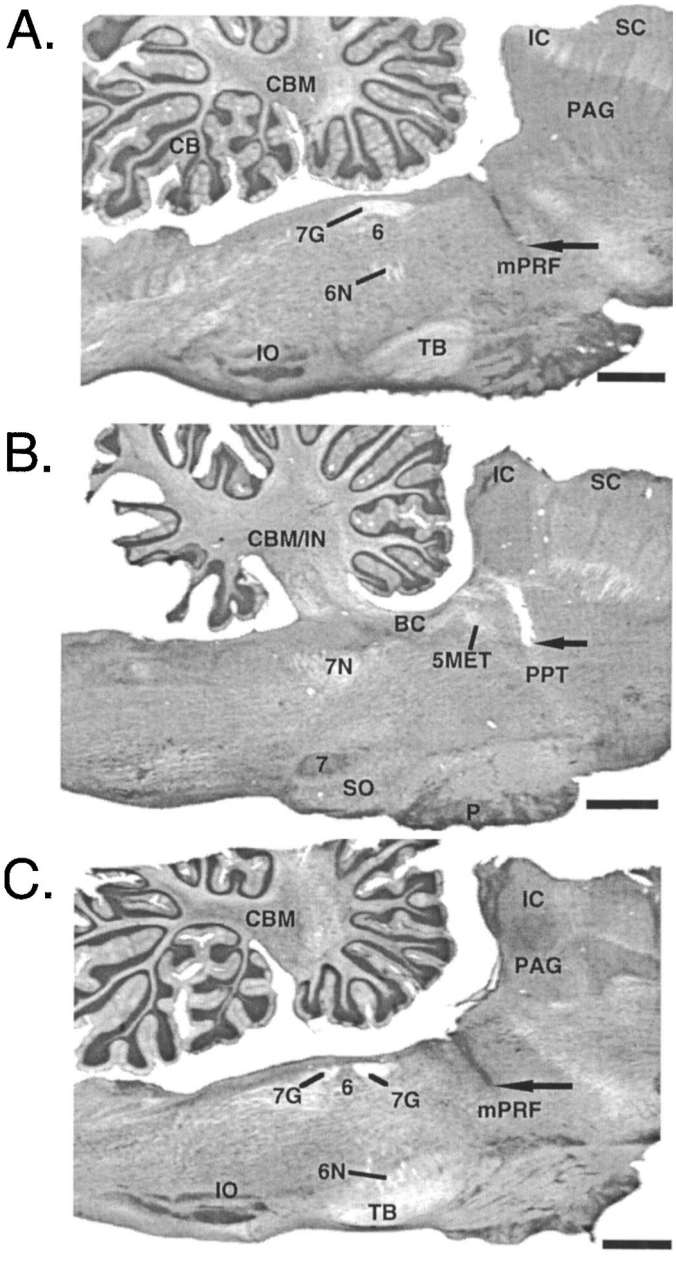

Figure 1. Sagittal sections of cat brainstem stained with cresyl violet and showing histological localization of representative microdialysis and microinjection sites. Rostral is to the *right*. *A*, The *black arrow* marks the tip of the lesion in the mPRF (also referred to as the gigantocellular tegmental field, or FTG, by Berman, 1968) caused by the microdialysis probe. The tip of the lesion was localized to the coordinates P = 3.0 mm; L = 1.5 mm; H = −5.5 mm. *B*, Cat brainstem cut through the PPT nuclei at 2.9 mm lateral to midline. The tip of the microdialysis probe lesion is located at P = 0.5 mm and H = −2.5 mm and is indicated by the *black arrow*. *C*, Brainstem section illustrating a microinjection site in the cat mPRF (*black arrow*) localized to P = 2.0 mm; L = 1.6 mm; H = −6.0 mm. Scale bars (*lower right corners*), *A–C*, 2 mm. *5MET*, Mesencephalic trigeminal tract; *6*, abducens nucleus; *6N*, abducens nerve; *7*, facial nucleus; *7G*, genu of facial nerve; *7N*, facial nerve; *BC*, brachium conjunctivum; *CB*, cerebellar cortex; *CBM*, medial nucleus of the cerebellum; *CBM/IN*, medial and interpositus nuclei of the cerebellum; *IC*, inferior colliculus; *IO*, inferior olive; *mPRF*, medial pontine reticular formation (or FTG); *P*, pyramidal tract; *PAG*, periaqueductal gray; *SC*, superior colliculus; *SO*, superior olive; *TB*, trapezoid body.

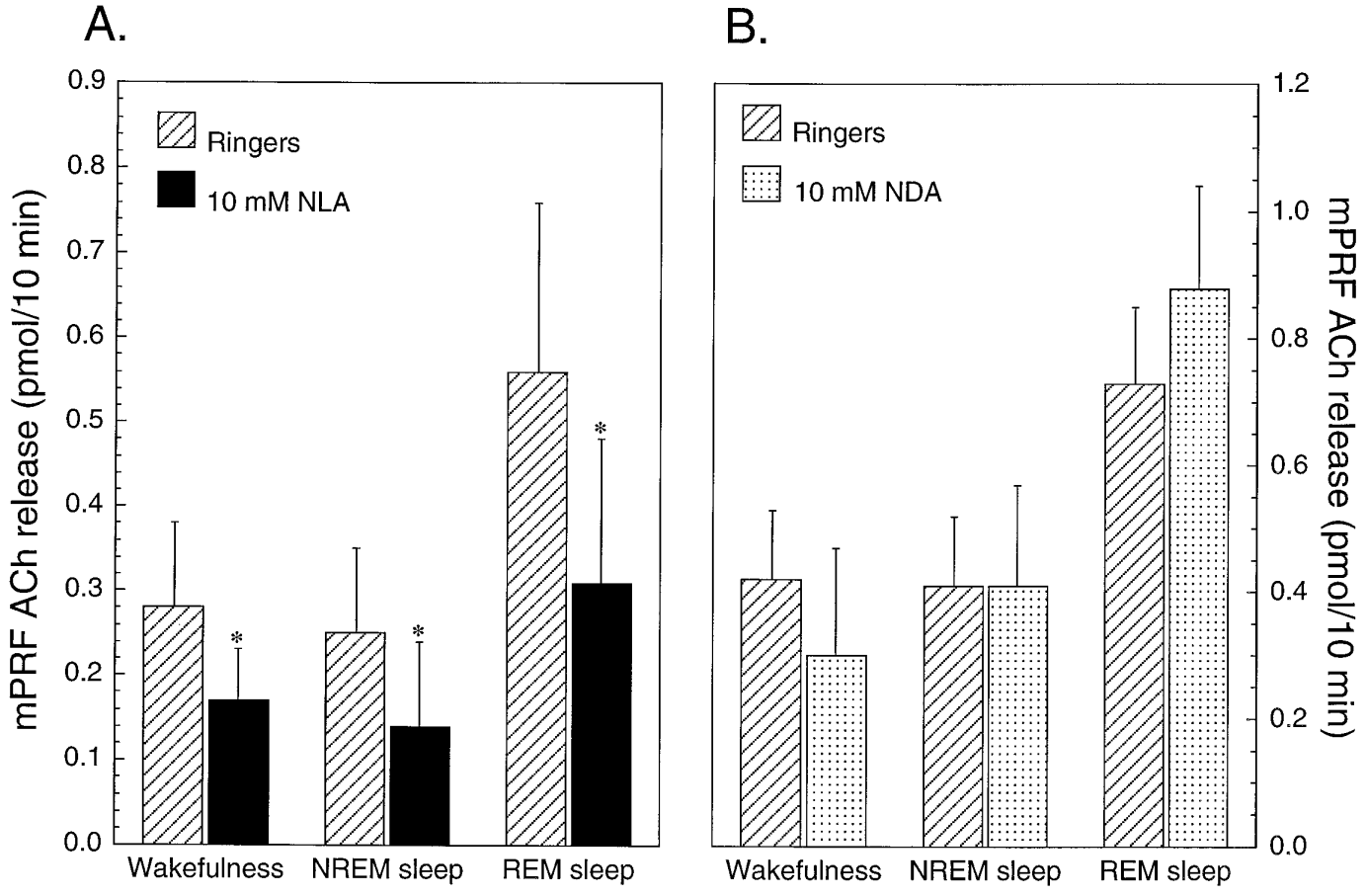

Figure 2. NLA dialysis significantly reduced mPRF ACh release compared with Ringer's control. *A*, mPRF ACh release while dialyzing the mPRF with Ringer's (control, *hatched bars*) or 10 mM NLA (*solid bars*) during wakefulness, NREM sleep, and REM sleep. Values on the ordinate are expressed as mean + SD pmol of ACh recovered from the mPRF per 10 min of dialysis. *Asterisks* designate a significant difference ($p < 0.05$; independent *t* tests) in mean mPRF ACh release between NLA dialysis and Ringer's dialysis within each state. *B*, Mean + SD mPRF ACh release from separate experiments dialyzing the mPRF with either Ringer's solution (control, *hatched bars*) or 10 mM NDA (*stippled bars*) during states of wakefulness, NREM sleep, or REM sleep. Note that NDA, the less active stereoisomer of NLA, had no statistically significant effect on mPRF ACh release compared with Ringer's control.

sleep ACh values (121% increase). Average ACh release during wakefulness ($n = 35$ samples) was not significantly different from NREM sleep ACh release ($n = 30$).

mPRF ACh release was not altered by dialysis with NDA

Additional microdialysis experiments were designed to confirm that mPRF administration of NLA decreased mPRF ACh release because of specific enzymatic inhibition of NOS. These experiments involved dialyzing the mPRF with 10 mM NDA, the less active stereoisomer of NLA. Figure 2*B* shows that compared with control, NDA did not significantly alter mPRF ACh release during wakefulness, NREM sleep, or REM sleep. Dialysis with NDA also did not produce any observable behavioral or electrographic effects on states of arousal.

PPT ACh release was decreased by halothane anesthesia and PPT NOS inhibition

Halothane anesthesia has been shown to decrease ACh release from cholinergic terminals in the mPRF (Keifer et al., 1994). Additionally, stereoselective NOS inhibition now has been shown to decrease ACh release within the mPRF (Fig. 2). Because the mPRF is known to contain PPT axon terminals, these results encouraged experiments designed to test the hypothesis that halothane and NLA would decrease ACh release in the cholinergic cell body region of the PPT. Figure 3*A* illustrates chromatogram peaks representative of PPT ACh release during 1.2% halothane anesthesia (*left*), during quiet wakefulness with Ringer's dialysis (*middle*), and during quiet wakefulness while dialyzing the PPT with 10 mM NLA (*right*). Figure 3*B* shows that both 1.2% halothane and delivery of the NOS inhibitor NLA during wakefulness caused a significant decrease in PPT ACh release compared with ACh levels of release during quiet wakefulness with Ringer's dialysis. Mean (± SD) ACh release (pmol/10 min) in the PPT was reduced 15% ($p < 0.05$) by 1.2% halothane anesthesia ($n = 11$) compared with wakefulness ($n = 10$). PPT ACh release was decreased by 36% ($p < 0.01$) with NLA dialysis ($n = 11$) during quiet wakefulness compared with ACh levels of release during Ringer's dialysis. The reduction in PPT ACh release caused by NLA was greater than the reduction caused by 1.2% halothane anesthesia ($p < 0.01$, *post hoc* Tukey's test).

mPRF microinjection of NOS inhibitor decreased REM sleep

Having demonstrated that NLA significantly decreased mPRF ACh release (Fig. 2), and knowing that REM sleep is generated, in part, by cholinergic stimulation of the mPRF, this study also tested the hypothesis that mPRF NLA microinjection would de-

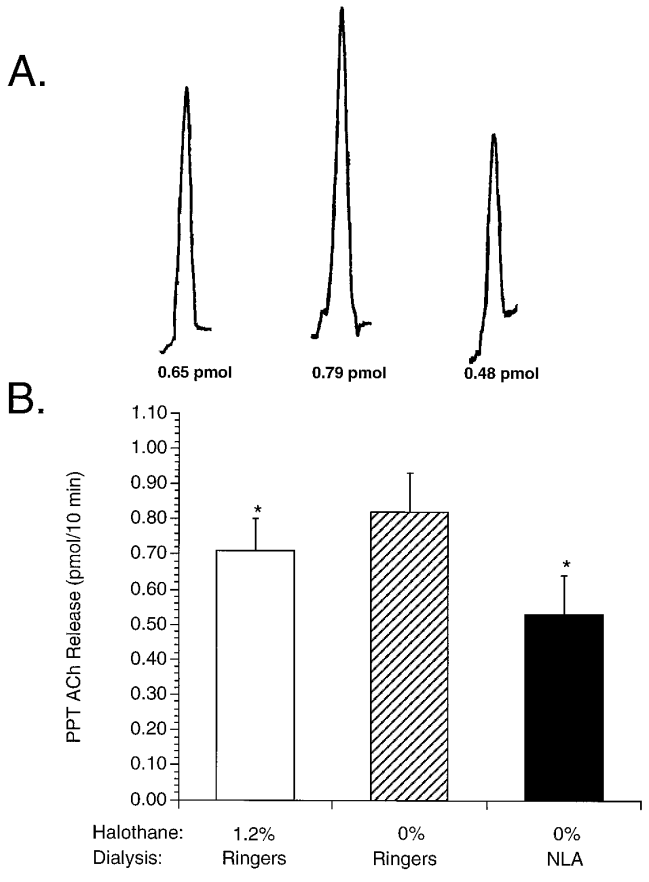

Figure 3. Both 1.2% halothane and PPT NLA dialysis decreased PPT ACh release. *A*, Chromatograms show peak areas proportional to ACh content present in 30 μl (10 min) dialysate samples indicating PPT ACh release under three different conditions. The chromatogram to the *far left* is representative of PPT ACh release during 1.2% halothane anesthesia while dialyzing with Ringer's. The *center peak* shows ACh release during quiet wakefulness with Ringer's dialysis. The effect of NLA dialysis on ACh release during quiet wakefulness is illustrated on the *far right*. The *numbers below* each chromatogram indicate the amount of ACh (pmol/10 min). *B*, Mean + SD PPT ACh release is shown on the ordinate during administration of 1.2% halothane with Ringer's dialysis (*open bar*) during wakefulness (0% halothane) with Ringer's dialysis (*hatched bar*) and during wakefulness in the presence of NLA dialysis (*solid bar*). There was a significant main effect of dialysis and anesthetic condition on PPT ACh release ($F_{(2,32)} = 23.59$; $p < 0.0001$). *Asterisks* indicate significantly decreased ($p < 0.05$; Tukey's test) ACh release during 1.2% halothane and NLA dialysis.

crease natural REM sleep and would block the neostigmine-induced REM sleep-like state (Baghdoyan et al., 1984). Figure 4 shows polygraphic recordings obtained from the present experiments demonstrating the electrographic traits of wakefulness, NREM sleep, REM sleep, and the REM sleep-like state induced by 3 μg neostigmine mPRF injection (REM-Neo). Figure 5 illustrates the typical patterns of waking, NREM sleep, and REM sleep states during 120 min after mPRF microinjection for six different microinjection conditions. The Figure 5 data also show the ability of neostigmine and NLA to alter the temporal organization of REM sleep

mPRF microinjection of NLA, but not NDA, inhibited REM sleep

Figure 6 illustrates the effect of mPRF microinjection of saline (control), NLA, or NDA on the percent of time spent in states of sleep and wakefulness during the first 2 hr after mPRF microinjection. The NOS inhibitor NLA significantly reduced the time spent in REM sleep (Fig. 6*A*) compared with saline ($t = 4.22$; df = 19; $p < 0.01$; Bonferroni correction applied) and compared with NDA ($t = 4.11$; df = 19; $p < 0.01$). NDA microinjection, however, had no effect on REM sleep percentage compared with control. There was no significant effect of mPRF microinjection of NLA or NDA on the percent time spent in NREM sleep (Fig. 6*B*) or wakefulness (Fig. 6*C*).

mPRF microinjection of NLA, but not NDA, blocked the REM-Neo state

Figure 7*A* shows the ability of mPRF NLA to block the REM-Neo state. Compared with saline control, neostigmine injection caused a 408% increase in the percent time occupied by the REM sleep-like state ($t = 13.36$; df = 26; $p < 0.01$). NLA injected 15 min before neostigmine significantly reduced the neostigmine-induced REM sleep-like state by 64.2% ($t = 6.71$; df = 28; $p < 0.01$ compared with Neo alone). NDA pretreatment, however, had no effect on neostigmine's ability to cause an increase in REM sleep percentage. Figure 7, *B* and *C*, shows that the REM sleep-enhancing effect of Neo occurred with a concomitant decrease in NREM sleep, not wakefulness. Figure 7*B* shows that significantly less time was spent in NREM sleep (−49.2%) after Neo compared with saline ($t = 21.96$; df = 26; $p < 0.01$). NLA injected before neostigmine significantly blocked (47.2%; $t = 4.10$; df = 28; $p < 0.01$) the neostigmine-induced decrease in NREM sleep. NREM sleep time after NLA/Neo injections still was significantly less (−26%) than after saline injection ($t = 4.71$; df = 26; $p < 0.01$). NDA pretreatment did not alter the neostigmine-induced decrease in NREM sleep. Figure 7*C* shows that there was no effect of Neo, NLA/Neo, or NDA/Neo injections on time spent in wakefulness.

mPRF microinjection of NLA, but not NDA, altered the temporal organization of REM sleep

Figure 8 illustrates the effect of mPRF microinjection on both the frequency and duration of REM sleep epochs. There was a statistically significant main effect of mPRF microinjection on the mean duration of REM sleep epochs ($F_{(5,404)} = 8.30$; $p < 0.0001$) and the number of REM sleep epochs over a 2 hr period ($F_{(5,73)} = 5.25$; $p = 0.0004$). Independent *t* test comparisons revealed that mean REM sleep epoch duration after NLA injection was reduced significantly (−60%) compared with saline (NLA vs saline), whereas NDA had no effect on REM sleep duration (NDA vs saline). Neostigmine injection significantly increased REM sleep epoch duration (+103%) compared with saline (Neo vs saline). NLA administration before neostigmine completely blocked the epoch duration enhancement induced by neostigmine (NLA/Neo vs Neo) and returned the REM sleep epoch duration to control levels (NLA/Neo vs saline). NDA pretreatment had no effect on the REM sleep epoch duration enhancement caused by neostigmine (NDA/Neo vs Neo). Compared with saline, neostigmine injection significantly increased (+155%) the number of REM sleep epochs (Neo vs saline). Neither NLA nor NDA pretreatment affected the ability of neostigmine to enhance the number of REM sleep epochs (NLA/Neo or NDA/Neo vs Neo). Injection of NLA or NDA alone did not alter the frequency of naturally occurring REM sleep epochs (NLA or NDA vs saline). Taken together, these data show that mPRF NLA injection had selective effects on the temporal organization of REM sleep, causing a significant decrease in the duration, but not the number, of REM sleep and REM-Neo episodes.

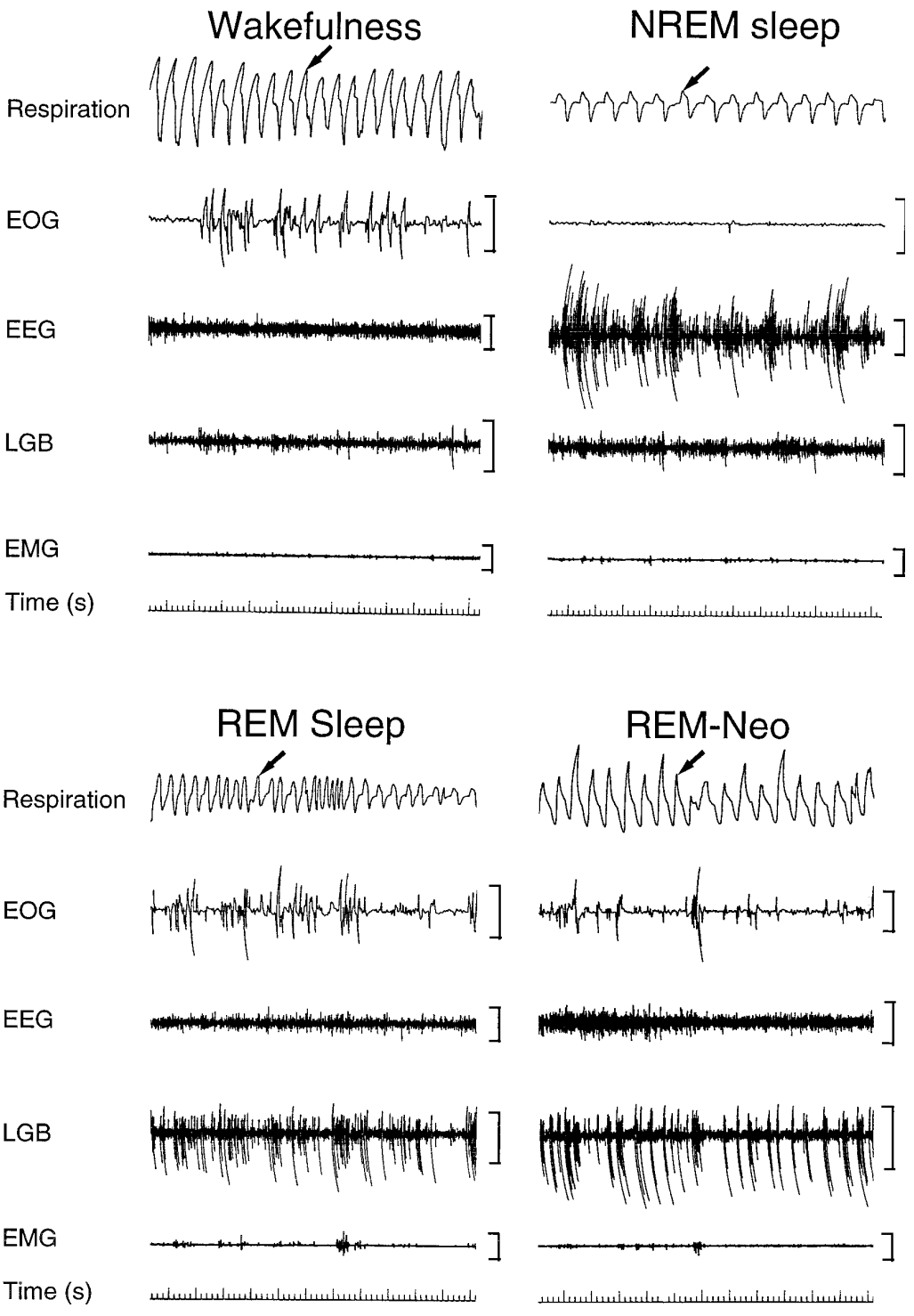

Figure 4. One minute samples of polygraphic recordings during states of wakefulness, NREM sleep, REM sleep, and the REM sleep-like state induced by mPRF microinjection of neostigmine (*REM-Neo*). During each state, polygraphic tracings record respiration (*arrow* marks a point of peak inspiratory airflow), eye movements (*EOG*), cortical electroencephalogram (*EEG*), field potentials from the lateral geniculate body of the thalamus (*LGB*), and neck muscle electromyogram (*EMG*). Time scale (each tick equals 1 sec) is shown at the *bottom* of each 1 min polygraphic record. Calibration bars show amplitude of pen deflection equal to 100 μV. Note that during REM-Neo, the REMs, EEG activation, presence of ponto-geniculo-occipital waves in the LGB recording, and muscle atonia interrupted by periodic bursts of muscle activity are similar to those seen during natural REM sleep.

mPRF microinjection of NLA, but not NDA, altered respiratory rate

The ability of mPRF NLA injection to block the cholinergically induced decrease in respiratory rate during the REM sleep state is illustrated in Figure 9. In agreement with previously described effects of mPRF carbachol (Lydic and Baghdoyan, 1989) and bethanechol (Lee et al., 1995) on respiratory rate, mPRF injection of neostigmine caused a significant reduction in respiratory rate

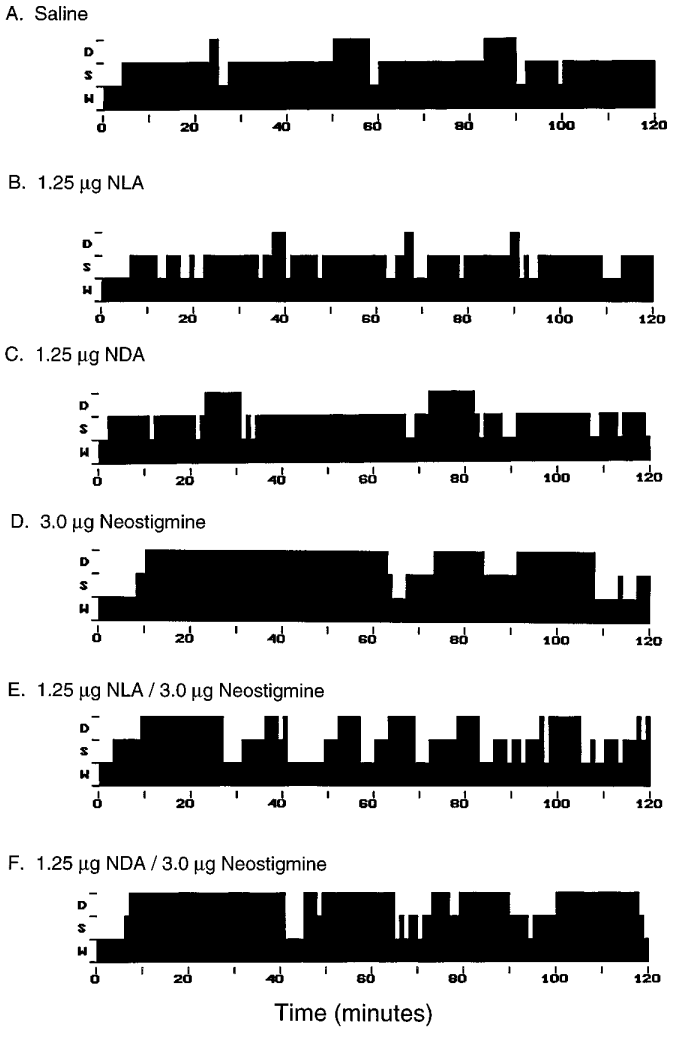

Figure 5. Time course of sleep and wakefulness after mPRF microinjection. For each minute of the 2 hr polygraphic recording (shown on the *abscissa*), the behavioral state is indicated as wakefulness [*W* (lowest level), NREM sleep (*S* = EEG *S*ynchronization, middle level), or REM sleep (*D* = EEG *D*esynchronization, highest level)] on the ordinate. These plots illustrate typical sleep/wake patterns for 120 min after each of six different mPRF microinjection conditions (*A–F*). Note the increase in REM sleep time evoked by Neo injection (*D* vs *A*). Note also the ability of the NOS inhibitor NLA to decrease both natural REM sleep (*B* vs *A*) and the REM sleep-like state induced by neostigmine (*E* vs *D*). NDA had no effect on natural (*C* vs *A*) or neostigmine-induced (*F* vs *D*) REM sleep. These plots also illustrate how the temporal organization of REM sleep was quantified for (1) REM sleep latency (the time from mPRF injection at min 0 to the onset of the first REM sleep episode: 22 min in plot *A*); (2) REM sleep epoch frequency (the number of REM sleep epochs that occurred over 2 hr: 3 for plot *A*); and (3) duration of individual REM sleep epochs (2, 4, and 3 min for plot *A*).

during REM sleep compared with saline control ($t = 6.50$; df = 262; $p < 0.01$; Bonferroni correction). NLA injection before neostigmine completely blocked the ability of neostigmine to decrease respiratory rate. NDA injection before neostigmine did not alter the neostigmine-induced decrease in respiratory rate.

DISCUSSION

The results demonstrate that mPRF administration of the NOS inhibitor NLA stereoselectively (1) decreased ACh release within the mPRF, (2) inhibited REM sleep, and (3) prevented the cholinergically induced decrease in respiratory rate during the REM sleep-like state. These are the first data to suggest that NO, produced within a specific brain region, the mPRF, altered sleep/wake states by modulating the release of a specific neurotransmitter, ACh.

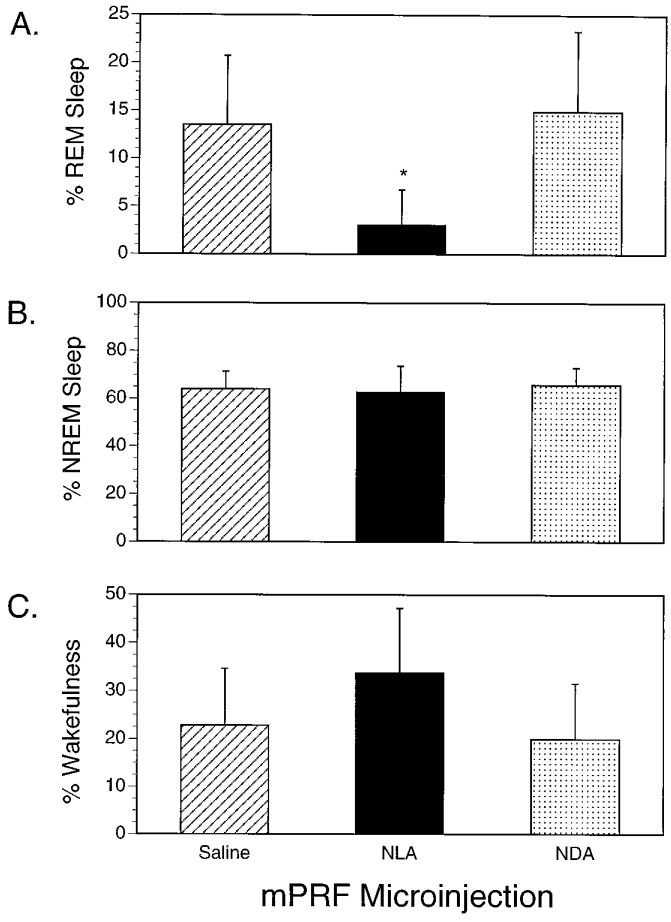

Figure 6. Effect of mPRF microinjection of 22.8 mM NLA or 22.8 mM NDA on percent time spent in REM sleep (*A*), NREM sleep (*B*), or wakefulness (*C*) over a 2 hr polygraphic recording period. Percent time (mean + SD) spent in each state after microinjection of saline (control, *hatched bars*), NLA (*solid bars*), or NDA (*stippled bars*) is shown on the ordinate. NLA microinjection caused a 71% decrease in REM sleep time but did not significantly alter the percent time spent in either NREM sleep or wakefulness. Microinjection of NDA had no effect on the amount of time spent in REM sleep, NREM sleep, and wakefulness; $^*p < 0.01$ (independent *t* tests).

Local inhibition of NOS reduced pontine ACh release

Delivery of NLA to the mPRF by microdialysis caused a stereoselective decrease in mPRF ACh release. Although stereoisomers of NOS inhibitors have been shown to produce weakly some of the effects of NOS inhibition (Wang et al., 1991, 1993, 1994a), many investigators have used the D-enantiomer of NLA (referred to here as NDA) to demonstrate that the biological activity of NLA is attributable to specific interaction with NOS and inhibition of NO production (Liu et al., 1991; Iadecola, 1992; Khalil and Helme, 1992; Khanna et al., 1993; Tanaka et al., 1994; Wang et al., 1994b; Fukuto and Chaudhuri, 1995; Griffith and Stuehr, 1995). Therefore, the ability of NLA, but not the enantiomer NDA, to significantly decrease ACh release within the mPRF (Fig. 2) suggests that NO produced in the mPRF plays a role in regulating mPRF ACh release.

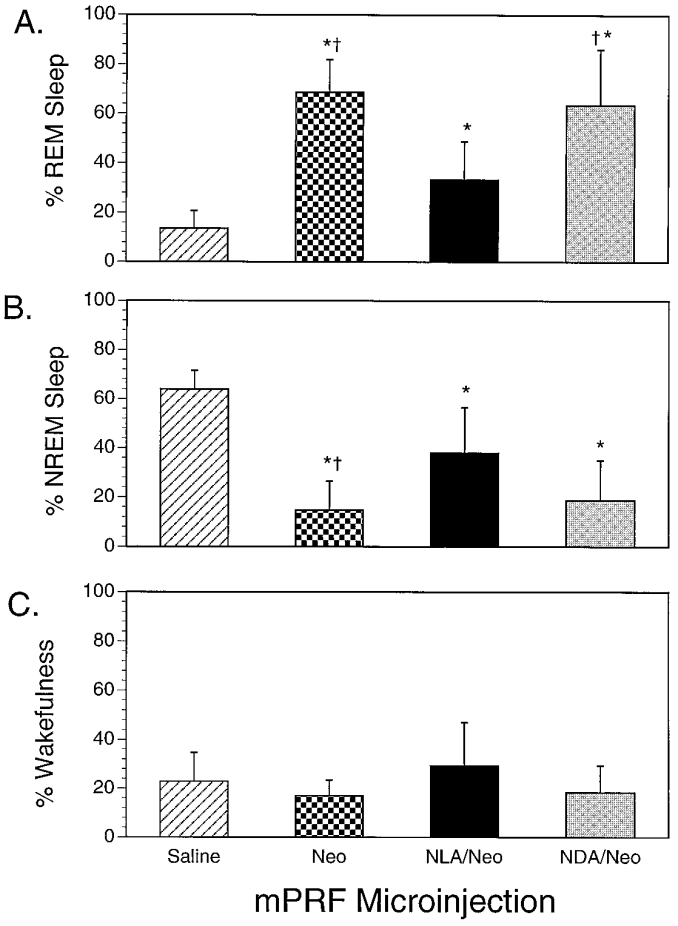

Figure 7. Effect of NLA or NDA mPRF microinjections on the ability of mPRF neostigmine (*Neo*) microinjection to increase the amount of time spent in a REM sleep-like state over a 2 hr period. The ordinate shows percent of time (mean + SD) spent in a polygraphically defined REM sleep state (*A*), NREM sleep (*B*), or wakefulness (*C*) after microinjection of saline, 40 mM Neo, 22.8 mM NLA pretreatment to Neo (*NLA/Neo*), or 22.8 mM NDA pretreatment to Neo (*NDA/Neo*). Neo microinjection increased the amount of time spent in REM sleep (*A*), while decreasing NREM sleep time (*B*). NLA pretreatment (*NLA/Neo*) significantly attenuated the ability of Neo to enhance REM sleep time (*A*) and decrease NREM sleep percentage (*B*). NDA pretreatment did not alter the effect of Neo on REM sleep and NREM sleep time. (*C*) None of the mPRF microinjections produced a significant effect on the percent time spent in wakefulness compared with saline control. *Asterisks* indicate significant difference compared with saline ($p < 0.01$; independent t tests, Bonferroni correction); †, significant difference from NLA/Neo injections ($p < 0.05$).

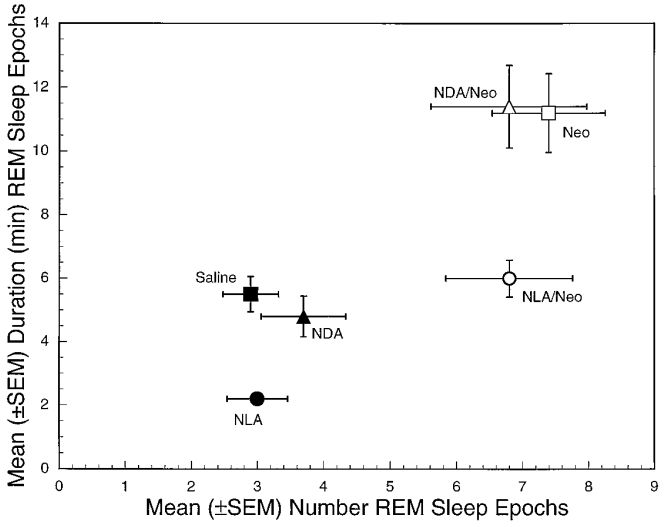

Figure 8. mPRF NLA injection disrupted the maintenance of REM sleep but not the initiation of REM sleep. This graph shows the mean ± SEM duration of REM sleep epochs after mPRF microinjection (*y*-axis) versus the mean ± SEM number of REM sleep epochs which occurred in the 2 hr period after mPRF microinjection (*x*-axis). The mPRF microinjection conditions are indicated by the following symbols: ■, saline; ●, 22.8 mM NLA; ▲, 22.8 mM NDA; □, 40 mM Neo; ○, 22.8 mM NLA/40 mM Neo; △, 22.8 mM NDA/40 mM Neo. Notice that mPRF NLA administration reduced the duration of REM sleep epochs both for naturally occurring REM sleep (*solid symbols*) and for the REM sleep-like state (*open symbols*). The number of REM sleep epochs that occurred both naturally and after Neo injection, however, was not altered by NLA injection. These data suggest that there was an NLA-specific effect on the ability to maintain REM sleep episodes but no effect on the ability to generate REM epochs.

ACh is released in the mPRF from terminals of cholinergic LDT/PPT neurons (Lydic and Baghdoyan, 1993), and the activity of these neurons is known to be important in the generation of cortical activation characterizing both REM sleep and waking states (Webster and Jones, 1988; El Mansari et al., 1990; Steriade et al., 1990; Kayama et al., 1992). Synaptically mediated, inhibitory modulation of cholinergic LDT/PPT neurons is effected by the neurotransmitters serotonin (Luebke et al., 1992; Leonard and Llinas, 1994), norepinephrine (Williams and Reiner, 1993), and ACh (Luebke et al., 1993; Leonard and Llinas, 1994). The presence of reciprocal cholinergic innervation by cholinergic LDT/PPT neurons (Semba and Fibiger, 1992; Steininger et al., 1992) suggests that the release of ACh in the LDT/PPT cholinergic cell body region might serve to modulate the activity of these cholinergic neurons and hence participate in REM sleep regulation. Anatomical evidence has shown that NOS is present in the axon terminals and the cell bodies of LDT/PPT cholinergic neurons (Vincent et al., 1983; Mizukawa et al., 1989; Bickford et al., 1993). In addition to decreasing ACh release in the mPRF, NOS inhibition decreased ACh release in the cholinergic PPT cell body region (Fig. 3). The ability of NO production to modulate ACh release in the PPT nuclei suggests that levels of NO, by altering ACh release, may influence the activity of cholinergic neurons known to be important in the generation of REM sleep.

NOS inhibition and anesthesia induced alterations in arousal

The present study also used halothane anesthesia as an additional tool for examining the relationship between levels of arousal and NOS modulation of ACh release in the mPRF. The results indicate that halothane anesthesia, like PPT NOS inhibition, reduced ACh release in the cholinergic PPT cell body region (Fig. 3). The finding that both PPT NOS inhibition and halothane anesthesia diminished ACh release in the PPT is consistent with results showing that halothane anesthesia significantly reduces mPRF ACh release (Keifer et al., 1994). Volatile anesthetics, including halothane, have been shown to inhibit NOS in rat cerebellum (Tobin et al., 1994). In addition, halothane has been shown to interfere with the stability of NO (Rengsamy et al., 1995) and the ability of NO to cause vasodilation (Blaise et al., 1994). The idea that NO contributes to the regulation of arousal states also has been supported by data showing that in mice and rats, inhibition of NOS augmented anesthesia, analgesia, and sedation caused by isoflurane and halothane anesthesia (Johns et al., 1992; Ichinose

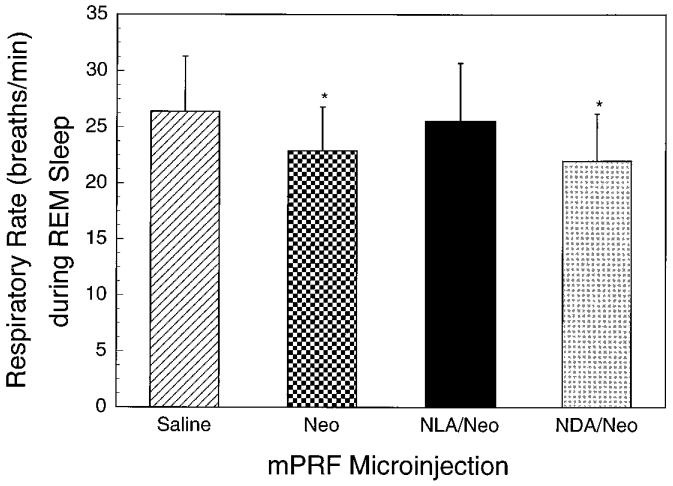

Figure 9. Injection of the NOS inhibitor NLA into the mPRF blocked the cholinergically induced, state-dependent decrease in respiratory rate. Mean + SD respiratory rate (breaths/min) is shown on the ordinate for each of four different mPRF microinjection conditions (*abscissa*): saline; neostigmine (*Neo*); NLA/Neo; and NDA/Neo. Compared with saline control, REM sleep respiratory rate was significantly reduced by mPRF injection of neostigmine (*$p < 0.01$). Injection of NLA before neostigmine (*NLA/Neo*) prevented the neostigmine-induced decrease in respiratory rate. NDA pretreatment had no significant effect on the cholinergically induced decrease in respiratory rate.

et al., 1995). The finding that NOS inhibition decreased ACh release in the mPRF (Fig. 2) and PPT (Fig. 3) is consistent with reports that both pontine cholinergic stimulation (Keifer et al., 1996) and brain NO (Nistico et al., 1994) contribute to generation of electrocortical (EEG) arousal. The present results (Fig. 3) demonstrating that both NOS inhibition and halothane anesthesia decreased ACh release in the PPT, therefore, are consistent with the notion that NO and volatile anesthetics may have antagonistic effects on cholinergic modulation of EEG and behavioral arousal.

mPRF NOS inhibition interfered with the maintenance of REM sleep

Injection of NLA into the mPRF caused a reduction in the time spent in natural REM sleep and attenuated the ability of mPRF neostigmine injection to produce the REM sleep-like state illustrated by Figure 4. More specifically, mPRF NOS inhibition diminished the duration of individual REM sleep epochs but did not alter the latency to REM sleep onset or the frequency of REM sleep episodes (Figs. 5–8). These data suggest that NO production in the mPRF is important for maintaining REM sleep once it has been initiated. NLA in the mPRF inhibited both naturally occurring REM sleep (Fig. 6*A*) and the cholinergically induced REM sleep state (Fig. 7*A*). Furthermore, NLA administration decreased the epoch duration of both natural REM sleep and neostigmine-induced REM sleep (Fig. 8).

The similarity between the effects of NOS inhibition on natural REM sleep and the cholinergically induced REM sleep state supports two conclusions. First, these data suggest that mPRF NO production participates in natural REM sleep regulation by modulating pontine cholinergic neurotransmission. Second, these data lend additional support to the premise that endogenous cholinergic neurotransmission plays a major role in natural REM sleep generation. McCarley et al. (1995) noted that REM sleep generation requires the coordinated activation of pools of cholinergic LDT/PPT and noncholinergic, cholinoceptive mPRF neurons. The present data suggest the possibility that NO may contribute to the recruitment of both cholinergic and cholinoceptive neurons (Fig. 10).

The absence of a significant difference in ACh levels recovered from the mPRF during wakefulness compared with NREM sleep is consistent with previous microdialysis studies of mPRF (Lydic et al., 1991, 1993; Lydic and Baghdoyan, 1993). Wakefulness is the most heterogeneous of behavioral states, and ACh release in the mPRF also may vary during specific waking behaviors. To the best of our knowledge, all currently available data on pontine ACh release in cat has been obtained from head-restrained animals. Measures of ACh release from the pons of freely moving dog, however, note that motor activity did not significantly alter ACh levels (Reid et al., 1994).

It is interesting to note that microdialysis of large areas of rat thalamus revealed increased ACh release during wakefulness and REM sleep, compared with NREM sleep (Williams et al., 1994). This suggests the possibility that cholinergic LDT/PPT neurons, which selectively increase their discharge rates during REM sleep, project to the mPRF, whereas LDT/PPT neurons with discharge rates that are highest in waking and REM sleep project to various thalamic nuclei (Steriade et al., 1990). Cholinergic LDT/PPT neurons have been noted to be good candidates for disrupting the synchronized spindle oscillations in thalamocortical systems during both arousal and REM sleep (Steriade et al., 1990). Recently, it has been shown that mPRF microinjection of the cholinergic agonist carbachol significantly decreased cortical EEG spindles that normally accompany halothane anesthesia (Keifer et al., 1996).

mPRF NOS inhibition blocked cholinergically mediated respiratory rate depression

Microinjection of neostigmine into the mPRF is known to produce a REM sleep-like state (Baghdoyan et al., 1984). Presumably, the REM-Neo state results from the accumulation of endogenously released ACh. This assumption is supported by recent evidence showing that mPRF microinjection of vesamicol-like compounds that inhibit vesicular packaging of ACh inhibit REM-Neo (Lydic et al., 1996). The present study is the first to show that REM-Neo also is characterized by respiratory rate depression (Fig. 9). The data also show that NLA administration into the mPRF prevented the neostigmine-induced depression in respiratory rate. These data suggest that a reduction in NO production, caused by NLA, resulted in diminished ACh levels within the mPRF and eliminated the neostigmine-induced reduction in respiratory rate. This conclusion is supported by previous studies indicating that pontine cholinergic neurotransmission contributes to respiratory rate depression during the REM sleep-like state caused by mPRF administration of cholinomimetics (Lydic and Baghdoyan, 1992). Both neuroanatomical (Lee et al., 1995) and electrophysiological (Gilbert and Lydic, 1994) data demonstrate pathways whereby the mPRF may influence respiratory rate. The specific mechanisms through which NO, ACh, and mPRF neurons alter breathing remain unknown, but state-dependent respiratory modulation has been shown to involve pertussis toxin-sensitive G-proteins and adenylate cyclase (Shuman et al., 1995) and cAMP signal transduction systems (Capece et al., 1995, 1996).

Limitations and conclusions

In the present study, the inferences regarding the role of NO in modulating ACh release, REM sleep, and respiratory rate are based on the effects of NLA and NDA administration. NOS

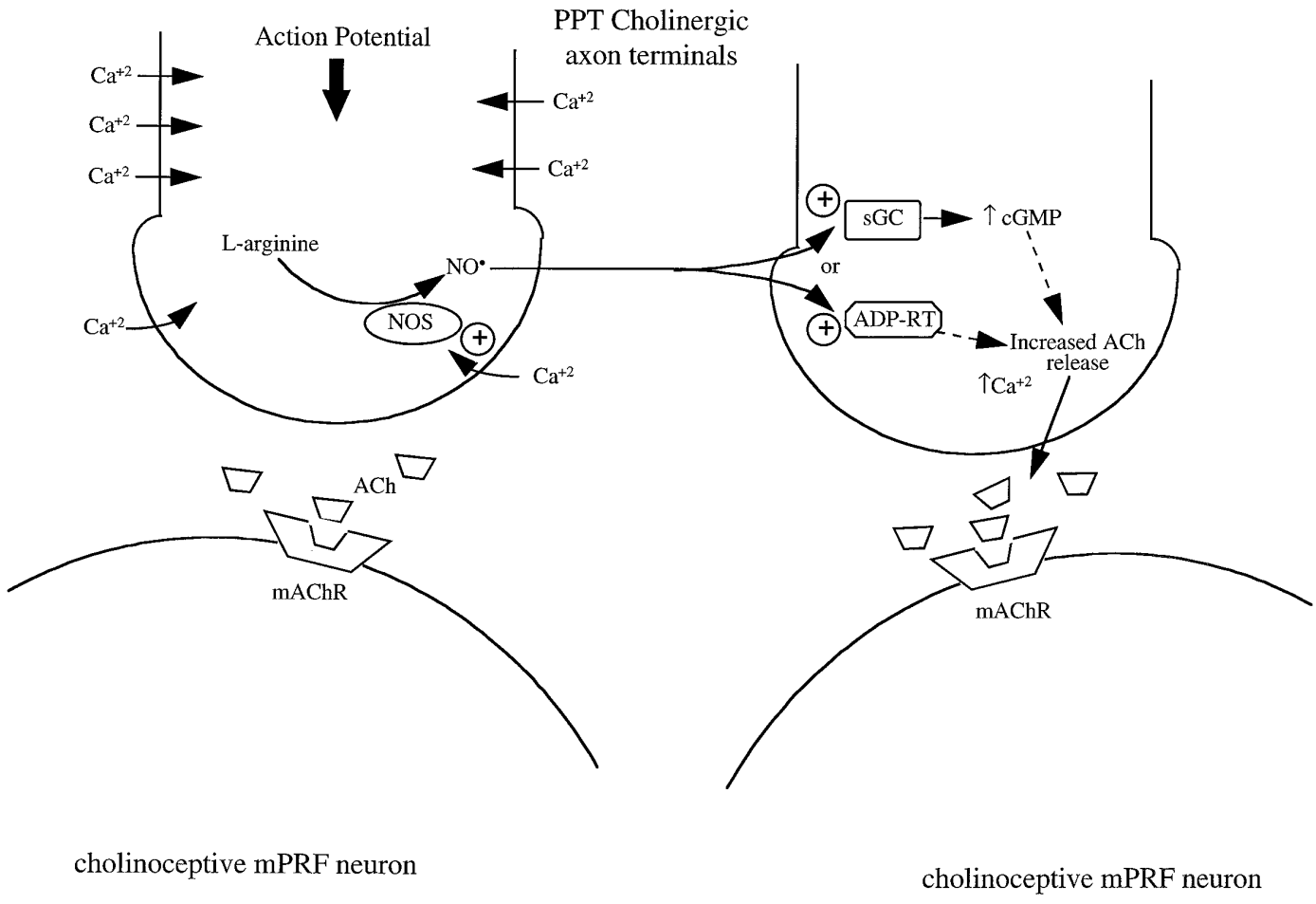

Figure 10. Possible mechanism by which NOS enhances ACh release in regions of the mPRF. Previous studies have presented anatomical (Shiromani et al., 1988) and functional (Lydic and Baghdoyan, 1993) data demonstrating that cholinergic terminals from LDT/PPT neurons regulate ACh release in the mPRF. Normally, the propagation of an action potential into an LDT/PPT neuron terminal would cause an influx of calcium (Ca^{+2}), which stimulates NOS known to be present in PPT axon terminals (Vincent et al., 1983; Mizukawa et al., 1989; Bickford et al., 1993). Increased production of NO (*NO•*) stimulates target proteins such as soluble guanyl cyclase (*sGC*) or ADP-ribosyltransferase (*ADP-RT*). Activation of these proteins would lead to increased ACh release thereby stimulating muscarinic cholinergic receptors on postsynaptic mPRF neurons (Baghdoyan et al., 1994). Each terminal contains all the molecules schematized in the left and right terminals, and inhibition of NOS by NLA in these cells would decrease ACh release (Fig. 2), REM sleep (Fig. 6), and state-dependent changes in respiratory rate (Fig. 9).

inhibitors currently represent one of the most widely used research tools for investigating the role of NO in biological systems (Griffith and Stuehr, 1995). It is acknowledged that the conclusions drawn from the results of these experiments would be strengthened by the use of NO-generating compounds and the *in situ* electrochemical measurement of NO. The use of NO scavengers such as hemoglobin recently have been shown to provide a technically difficult but promising technique for measuring levels of NO (Williams et al., 1995). Additional studies measuring ACh release in the LDT/PPT during REM sleep also are needed. Such studies are technically difficult, and stereotaxic access to the LDT/PPT in the cat is limited by the presence of an ossified tentorium. Nonetheless, the data shown in Figure 3 represent the first measurements of ACh release in the pontine cholinergic cell body region in the awake and anesthetized cat.

Data presented here provide evidence for the role of NO in facilitating pontine ACh release, maintaining REM sleep once it has been initiated, and participating in the cholinergic modulation of respiratory rate. It is likely that NO influences REM sleep and breathing during REM sleep via modulation of ACh release. NO also may serve to potentiate and prolong the duration of ACh release from presynaptic axon terminals within the mPRF. The production of NO within the mPRF may be clinically relevant in the cholinergic modulation of state-dependent respiratory depression (Pack, 1995), narcolepsy (Reid et al., 1994; Nishino et al., 1995), and REM behavior disorder (Mahowald and Schenck, 1992).

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