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Nitric Oxide Production in Rat Thalamus Changes with Behavioral State, Local Depolarization, and Brainstem Stimulation

Julie A. Williams, Steven R. Vincent, and Peter B. Reiner

Kinsmen Laboratory of Neurological Research, Department of Psychiatry, Graduate Program in Neuroscience, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3

Since its discovery as a putative neurotransmitter in the CNS, several functional roles have been suggested for nitric oxide (NO). However, few studies have investigated the role of NO in natural physiology. Because NO synthase (NOS) has been localized in regions believed to be important for attention and arousal, we hypothesized that NO production would be state-dependent. To test this hypothesis, we used *in vivo* microdialysis, coupled with the hemoglobin-trapping technique, to monitor extracellular NO concentrations in rat thalamus during wake, slow-wave sleep (SWS), and rapid eye movement (REM) sleep. The thalamus is known to receive a massive innervation from the NOS/cholinergic neurons in the mesopontine brainstem, which have been suggested to play a key role in EEG desynchronized states. To test whether thalamic NO output was sensitive to neuronal-dependent changes in the mesopontine brainstem, we measured thalamic NO concentration in response to electrical stimulation in the laterodorsal tegmentum (LDT) of anesthetized rats. Finally, the calcium dependence of NO release was tested by local depolarization with a high potassium dialysate or by addition of a calcium chelator. The results showed that (1) extracellular NO concentrations in the thalamus were high during wake and REM sleep and significantly lower during SWS, (2) thalamic NO release increased in response to LDT stimulation in both a site-specific and tetrodotoxin (TTX)-dependent manner, and (3) NO production was calcium-dependent. These data suggest that thalamic NO production may play a role in arousal.

Key words: nitric oxide; sleep; arousal; microdialysis; mesopontine cholinergic neurons; EEG

After the identification of nitric oxide (NO) as a potential neurotransmitter in the CNS (Garthwaite et al., 1988), a large body of work has implicated NO in a variety of functional roles in the brain. Some examples include modulation of neurotransmitter release (Lonart et al., 1992; Lorrain and Hull, 1993; Guevara-Guzman et al., 1994; Strasser et al., 1994; Silva et al., 1995), regulation of local cerebral blood flow (Adachi et al., 1992; Northington et al., 1992), synaptic plasticity (O'Dell et al., 1991; Schuman and Madison, 1991; Shibuki and Okada, 1991), resetting of the circadian clock (Ding et al., 1994; Amir et al., 1995; Weber et al., 1995), and arousal (Bagetta et al., 1993; Nistico et al., 1994).

It has been well established that NO synthase (NOS) is highly expressed in cholinergic neurons in the mesopontine tegmentum (Vincent et al., 1983, 1986), which project heavily to the thalamus (Sofroniew et al., 1985; Satoh and Fibiger, 1986; Woolf and Butcher, 1986; Hallanger and Wainer, 1988). Acetylcholine (ACh) release from these cells is thought to play a key role in rapid eye movement (REM) sleep and in cortical arousal. This hypothesis has its origins in the studies of Moruzzi and Magoun (1949), who showed that brainstem stimulation in the region of the mesopontine tegmentum elicited a desynchronized EEG. On the basis of acetylcholinesterase histochemistry, Shute and Lewis (1967) suggested that brainstem cholinergic neurons were prime candidates for this ascending reticular activating system. Recently, we demonstrated that ACh output in the rat thalamus is high during wake and REM sleep and is significantly lower during slow-wave sleep (SWS) (Williams et al., 1994). This finding was consistent with *in vivo* electrophysiological recordings in the mesopontine tegmentum, which showed that the majority of cells fired during both wake and REM sleep (El Mansari et al., 1989; Steriade et al., 1990a), although Kayama et al. (1992) found that such neurons were in the minority. These data suggest that thalamic ACh release from the terminals of mesopontine cholinergic neurons may be an important component of EEG desynchronized states.

To date, few studies have addressed the role of neurotransmitters that colocalize in brainstem cholinergic neurons in the regulation of sleep and wakefulness. The data available on the role of NO are limited to the use of NOS inhibitors. Dzoljic and De Vries (1994) found that systemic injection of N^{ω}-nitro-monomethyl-L-arginine (L-NMMA) reduced wakefulness in rats. In contrast, administration of L-NAME (intracerebroventricularly; Kapás et al., 1994) or 7-nitroindazole (intraperitoneally; Dzoljic et al., 1996) reduced sleep in rats. Consistent with a modulatory role of NO in neurotransmission, local infusion of the NOS inhibitor N^{ω}-nitro-L-arginine (*N*-ARG) into the medial pontine reticular formation reduced both ACh release (Leonard and Lydic, 1995), and REM sleep (Leonard and Lydic, 1996).

On the basis of the evidence for the state-dependent activity of mesopontine cholinergic neurons (Williams et al., 1994), we hypothesized that NO release from mesopontine cholinergic terminals in the thalamus would be state-dependent. To test this hypothesis, we used *in vivo* microdialysis, combined with the

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Correspondence should be addressed to Dr. Peter B. Reiner, Kinsmen Laboratory of Neurological Research, Department of Psychiatry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3.

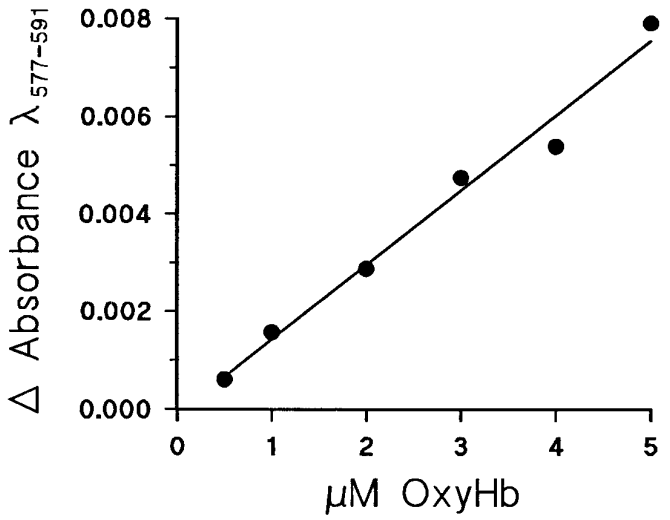

Figure 1. Calibration curve obtained by quantitative oxidation of increasing concentrations of HbO_2 to metHb (0.5–5 μM) by aqueous NO. Values along the *y*-axis are the absorbance differences between 577 and 591 nm obtained for metHb subtracted from that obtained from corresponding concentrations of HbO_2. NO concentrations were estimated by using the slope of the *line*.

hemoglobin-trapping technique, to monitor extracellular NO across the sleep–wake cycle in the rat thalamus.

MATERIALS AND METHODS

Assay of nitric oxide. The hemoglobin-trapping technique, based on the conversion of the ferrous form of hemoglobin (oxyhemoglobin, or HbO_2) into the ferric form, methemoglobin (metHb), by NO (Murphy and Noack, 1994) was used for the detection of NO. Others have shown previously a linear relationship between NO production and the conversion of HbO_2 to metHb by NO *in vitro* (Feelisch and Noack, 1987; Noack et al., 1992). This technique has been used for both *in vitro* and *in vivo* applications (Murphy et al., 1991; Mayer et al., 1992; Balcioglu and Maher, 1993).

HbO_2 was prepared from bovine double-crystallized hemoglobin (Sigma, St. Louis, MO) as follows. A Sephadex G-25 column (Pharmacia, Piscataway, NJ) was equilibrated with oxygenated sodium phosphate buffer (50 mM, pH 7.4). Sodium dithionite of excess molar concentration was added to the column (12 mg in 1 ml of PO_4 buffer), followed by 1 mM hemoglobin (1 ml). The conversion of metHb to HbO_2 was monitored by the change of color from brown to purple as the metHb was reduced by the sodium dithionite to deoxyhemoglobin and then from purple to a bright orange-red as the deoxyhemoglobin reacted with the oxygen contained in the PO_4 buffer (Dixon and McIntosh, 1967). To protect HbO_2 from auto-oxidation, we added superoxide dismutase (500 U/ml) and catalase (2000 U/ml) immediately to the eluent.

Because catalase has a strong absorbance in the Soret region, the conversion of HbO_2 to metHb was measured at 577 and 591 nm with a Bio-Rad (Richmond, CA) diode-array spectrophotometer. Because HbO_2 exhibits an absorbance maximum at 577 nm and is isosbestic with metHb at 591 nm (where the absorbance does not change as HbO_2 is converted to metHb; Van Assendelft, 1970), a decrease in absorbance at 577 is indicative of an increase in the concentration of metHb. NO concentrations were determined from calibration curves obtained by quantitative oxidation of increasing concentrations of HbO_2 to metHb (0.5–5 μM) by aqueous NO (Fig. 1). Aqueous NO solutions were prepared as described previously by Feelisch (1991). The detection limit of the assay was 10 pmol/100 μl sample.

Surgery. Male Wistar rats weighing 285–320 gm were anesthetized with 50–60 mg/kg pentobarbital intraperitoneally and supplemented as needed. For EEG recordings, animals were chronically implanted with three screw electrodes (2 for cortical EEG and 1 over the cerebellum as a reference) and depth electrodes for the recording of hippocampal theta (AP, −3.1; DV, −3.0; ML, −2.4 from bregma; Paxinos and Watson, 1982). Electrode pins were held in place with an Amphenol strip connector and fixed to the skull with dental acrylic.

Transverse microdialysis probes were constructed and implanted as described in detail elsewhere (Damsma and Westerink, 1991). Briefly, probes were made of a cellulose ester microdialysis membrane [inner diameter (i.d.), 0.20; outer diameter (o.d.), 0.21 mm; molecular weight cutoff, 10 kDa] with an active surface of 7 mm delimited by application of epoxy resin. A sharpened length of tungsten wire was threaded through the membrane for support, and a stainless steel cannula (22 gauge, 15 mm) was attached to one end. To prevent the membrane from buckling during placement, we glued the free end to the tungsten support. For implantation, the probe was secured horizontally to a micromanipulator on the stereotaxic apparatus and gently inserted through the brain through a hole drilled into the temporal bone at stereotaxic coordinates from bregma: AP, −3.3; DV, −5.8 to −5.6 (Paxinos and Watson, 1982). The probe was advanced until it protruded from the hole drilled on the opposite side, and the active surface was centered to include the ventroposterolateral, ventroposteromedial, centromedial, mediodorsal, interomediodorsal, and posterior thalamic nuclei using reference points marked directly on the probe. The glued tip was cut, the tungsten wire was removed, and a second cannula was attached to the loose end of the membrane. Both cannulae were secured to the parietal bone with screws and dental acrylic and served as the probe inlet and outlet.

Microdialysis. Microdialysis and sample collection procedures were similar to those used by Williams et al. (1994). After surgery, rats were housed individually in 35 × 35 × 25 cm Plexiglas cages and were given food and water *ad libitum*. On the first day after surgery, each animal was moved into a secluded recording room and attached to a model 8-10 Grass polygraph for several hours for adaptation. Experiments were performed on the second day after surgery during the light phase of a 12:12 hr light–dark cycle (from 8:00 A.M. to 8:00 P.M.) between 10:00 A.M. and 5:00 P.M. The samples were collected as follows. The dialysis probes were perfused with artificial cerebrospinal fluid (ACSF) containing (in mM): 147 NaCl, 3 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 1.0 $NaPO_4$ buffer, pH 7.4, and 1 μM HbO_2 for the assay of NO. The perfusion rate was 5 μl/min, controlled by a syringe pump (Harvard Apparatus, South Natick, MA), and samples were collected in volumes of 100 μl or 20 min fractions for on-line experiments. For each sample, absorbance differences measured at 577 and 591 nm were compared against an equal volume of a control dialysate solution. To ensure the stability of HbO_2, we replaced the dialysate each hour. Both the probe inlet and outlet consisted of polyethylene tubing (800 × 0.28 mm), which contained an inner fused silica tubing (100 μm i.d., 200 μm o.d.; Polymicro Technologies) to maintain an air-tight environment. Before sample collection, animals were dialyzed for 60 min to allow the brain to equilibrate with the perfusion solution.

For the behavioral experiments, all dialysate samples were collected into ice-cold microcentrifuge tubes marked "wake," "SWS," or "REM." Samples that were collected in the wake vials included periods in which the animal was clearly alert with a desynchronized EEG and engaged in some sort of waking behavior such as grooming, eating, drinking, or exploring. SWS vials included periods in which the animal was in a sleeping position, eyes closed, and the EEG was synchronized for >30 sec at a time. REM vials included the sleep state, in which the animal exhibited muscle twitches, EEG desynchrony, and theta activity. All samples from ambiguous and transition states were discarded. Samples were collected during 5–6 hr sessions and were stored on dry ice for off-line analysis immediately after the session.

For animals used in the stimulation experiments, the dialysis probe and screw electrodes were implanted 2 d before the experiment. On the day of the experiment, animals were anesthetized with urethane (1.5–2.0 gm/kg), and a burr hole was drilled over the cerebellum for a concentric stimulating electrode, which consisted of a Teflon-coated silver wire inside stainless steel tubing (30 gauge). Electrodes aimed at the laterodorsal tegmental (LDT) nucleus were lowered at a 30° angle to avoid the venous sinus (Kayama et al., 1992). For all other on-line experiments, animals were implanted with a dialysis probe only. Body temperature in urethane-anesthetized rats was maintained by a water-heated pad (American Medical Systems, Indianapolis, IN) controlled by a rectal probe (Yellow Springs Instrument, Ohio).

On completion of experiments, animals were killed with an overdose of sodium pentobarbital, and probe placement was verified by standard techniques (Fig. 2*A*). Placement of stimulation electrodes was verified by using NADPH-diaphorase histochemistry, a reliable marker of the mesopontine NOS-positive cholinergic neurons (Vincent et al., 1983). Briefly, fixed brains were trimmed to a block containing the pontine brainstem, sliced into 40 μm sagittal sections on a microtome, and mounted onto chrome-alum-coated slides. Slides were incubated at 37° in

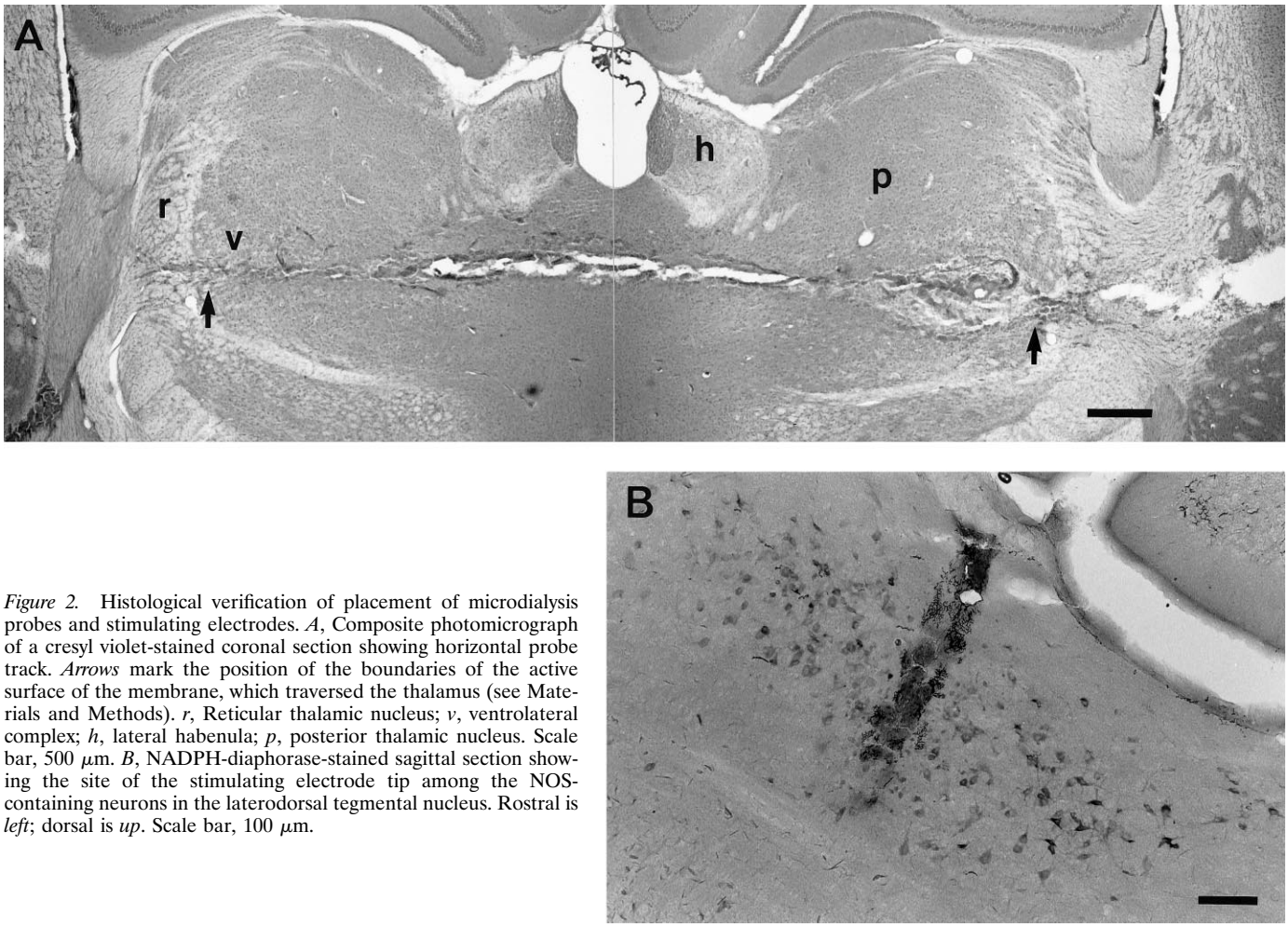

Figure 2. Histological verification of placement of microdialysis probes and stimulating electrodes. *A*, Composite photomicrograph of a cresyl violet-stained coronal section showing horizontal probe track. *Arrows* mark the position of the boundaries of the active surface of the membrane, which traversed the thalamus (see Materials and Methods). *r*, Reticular thalamic nucleus; *v*, ventrolateral complex; *h*, lateral habenula; *p*, posterior thalamic nucleus. Scale bar, 500 μm. *B*, NADPH-diaphorase-stained sagittal section showing the site of the stimulating electrode tip among the NOS-containing neurons in the laterodorsal tegmental nucleus. Rostral is *left*; dorsal is *up*. Scale bar, 100 μm.

a solution containing 1 mg/ml NADPH and 0.1 mg/ml nitro blue tetrazolium in a 0.3% Triton X-100/0.1 M PBS for 45–60 min. Slides were observed under a light microscope for determining whether electrode tips were in or near the diaphorase-positive neurons in the mesopontine tegmentum (Fig. 2*B*).

Statistical analyses. ANOVA with repeated measures was used to analyze the effects of various treatments on NO concentrations, including behavioral state, electrical stimulation of LDT and cerebellum, applications of perfusates containing either high potassium, BAPTA [1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid (tetrapotassium salt); Molecular Probes, Eugene, OR], or tetrodotoxin (TTX; Sigma), and systemic injections of NOS inhibitors. Scheffe's test was used for the comparison of the NO concentrations between each of the different behavioral states as well as between the means obtained for the treatment conditions listed above and those for the corresponding baselines. NO concentrations are reported as mean ± SEM in picomoles per minute (pmol/min).

RESULTS

Pharmacological properties of thalamic NO release

The first set of experiments was designed to determine whether the HbO_2 oxidation measured in the microdialysis samples was sensitive to predictable alterations in NOS activity. When animals were treated with 50 mg/kg of the NOS inhibitor, N^{ω}-nitro-L-arginine (*N*-ARG) baseline concentrations of NO in awake, freely moving animals were reduced significantly from 1.31 ± 0.07 to 0.86 ± 0.06 pmol/min ($p < 0.001$, $n = 4$; Fig. 3*A*).

We next sought to determine whether the changes in measured NO concentration were attributable to action potential-dependent activity. To address this question, we added 1 μM TTX, a compound that blocks voltage-dependent sodium channels, to the dialysate. Surprisingly, there was no change in NO concentration observed during TTX application ($n = 2$; data not shown), which suggested that action potentials were not necessary for thalamic NO production in awake, freely moving animals.

We therefore tested to see whether other manipulations of neuronal activity could affect NO release. We focused on calcium, because NOS is a calcium-dependent enzyme (Bredt and Snyder, 1990). Perfusion with a nominal Ca^{2+}-free dialysis solution did not affect thalamic NO release ($n = 2$); however, when 10 mM of the Ca^{2+} chelator BAPTA was added, baseline concentrations were reduced significantly in awake animals from 1.09 ± 0.05 to 0.80 ± 0.07 pmol/min ($p < 0.01$, $n = 3$; Fig. 3*B*). We next tested whether neuronal depolarization by perfusion of a solution containing a high potassium concentration could affect NO output. Potassium-induced depolarization should activate voltage-dependent calcium channels and thereby increase NOS activity. A solution containing 30 mM potassium (in place of an equimolar amount of NaCl to maintain osmolarity) significantly increased NO output in urethane-anesthetized animals from a baseline of 0.72 ± 0.06 to 1.15 ± 0.11 pmol/min ($p < 0.001$, $n = 4$; Fig. 3*C*). Finally, to determine whether the potassium-induced increase was Ca^{2+}-dependent, we added 30 mM potassium to a 10 mM BAPTA/Ca^{2+}-free solution. The BAPTA/Ca^{2+}-free solution prevented the effect of potassium ($n = 3$; Fig. 3*D*). Taken together, these

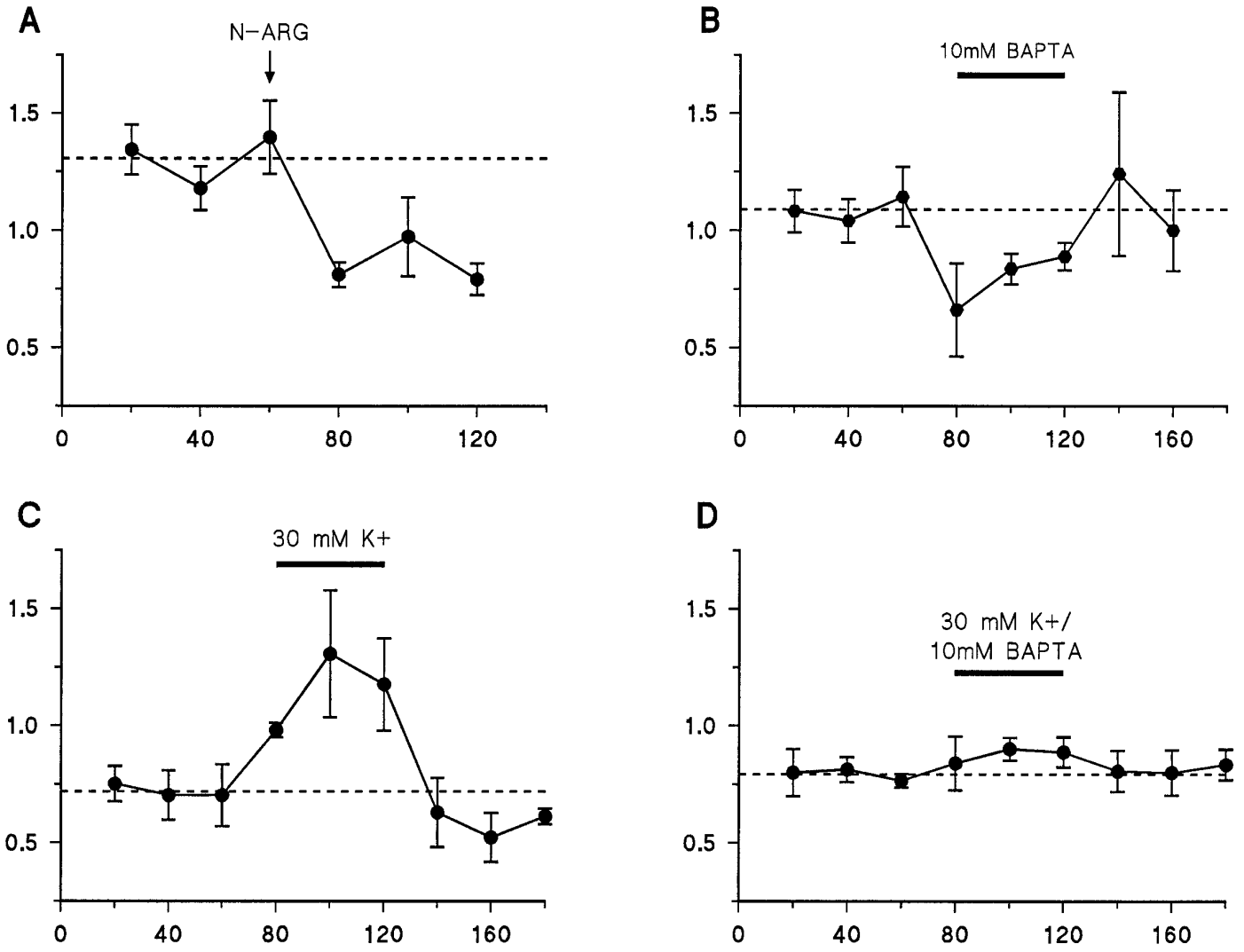

Figure 3. On-line experiments demonstrating that the HbO_2 oxidation measured in the samples was attributable to NO synthase (NOS) and Ca^{2+}-dependent activity. In each panel, the mean ± SEM concentrations of extracellular NO in pmol/min are shown versus time in minutes. *Dashed lines* represent the baseline average. *A*, Intraperitoneal injection of the NOS inhibitor *N*-ARG significantly reduced baseline concentrations in awake animals ($p < 0.001$; $n = 4$). *B*, Application of a Ca^{2+} chelator BAPTA (10 mM) in a Ca^{2+}-free dialysate solution significantly and reversibly reduced baseline concentrations in awake animals ($p < 0.01$; $n = 3$). *C*, Application of a solution containing 30 mM K^+ significantly increased NO release in urethane-anesthetized rats ($p < 0.001$; $n = 4$). *D*, Addition of 10 mM BAPTA to the high K^+ solution blocked the potassium-induced increase ($n = 3$), suggesting that the increase was attributable to Ca^{2+}-dependent mechanisms. Although baselines varied slightly among groups, these differences were not significant between the animals within the awake groups (*A, B*) or anesthetized groups (*C, D*), but the baselines in anesthetized animals were significantly lower than those in awake animals ($p < 0.001$).

data strongly suggest that thalamic NO output as measured by the hemoglobin-trapping technique was generated by a calcium-dependent isoform of NOS.

State dependence of thalamic NO release

The number of 100 μl dialysis samples collected from each behavioral state was consistent across the seven animals tested. After a 5–6 hr session, enough dialysate was collected to fill one sample for REM, one for active wake, and three ($n = 4$) or four ($n = 3$) for SWS. The average duration of the REM periods included in the sample collection was 101.77 ± 10.95 sec across the seven animals tested; durations of active wake or SWS periods ranged from 40 sec to a full 20 min. Values obtained from the SWS samples were averaged for each animal for the statistical analyses. The mean NO concentration during wake was 1.34 ± 0.07 pmol/min; during SWS, 0.97 ± 0.03 pmol/min; and during REM, 1.42 ± 0.12 pmol/min (Fig. 4). A repeated measures ANOVA showed that NO concentration varied significantly across state ($p < 0.04$ with the Greenhouse–Geisser correction). Scheffe's *post hoc* analysis showed that NO concentration did not vary significantly between wake and REM but was significantly less during SWS ($p < 0.01$).

The mesopontine NOS-containing cholinergic terminals are the major source of NOS input to the thalamus (Vincent and Kimura, 1992). To test whether changes in NO production were related to the activity of these NOS-containing mesopontine cholinergic neurons, we placed an electrode in the LDT of urethane-anesthetized rats (1.5–2.0 gm/kg), and thalamic NO release was measured in response to electrical stimulation. After collecting three baseline samples (1 hr), we stimulated the LDT for a 20 min period at 60 Hz (bipolar, sine-wave pulse) for 10 sec, with 30 sec

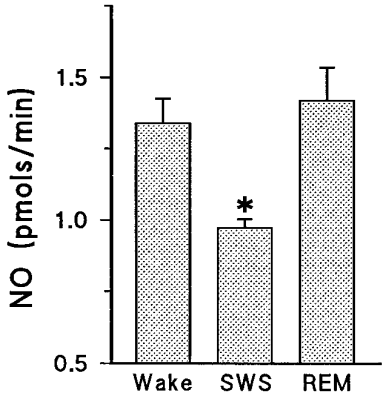

Figure 4. Extracellular NO concentrations across behavioral state in the rat thalamus. Mean NO concentrations (±SEM) are reported for wake (*Wake*), slow-wave sleep (*SWS*), and REM sleep (*REM*; $n = 7$). NO concentrations did not differ between wake and REM but were significantly lower during SWS. $^*p < 0.01$.

intervals. Cortical EEG during baseline in anesthetized rats was characterized by short bursts of high amplitude spikes and periods of high frequency, low amplitude waves, as described previously (Grahn et al., 1989). During the 10 sec stimulation periods, the EEG exhibited low amplitude waves at 6–8 Hz or theta activity, likely a reflection of synchronous hippocampal activity, similar to that during REM sleep. During the 30 sec interstimulus interval, the EEG exhibited high amplitude, slow-wave activity, similar to that observed during natural SWS (Fig. 5*A*). After the 20 min stimulation period, the cortical EEG gradually reverted to the burst-discharge state within 5 or 10 min. The 20 min sample collected during the stimulation period was compared against the baseline average. LDT stimulation significantly increased thalamic NO release in 10 of 12 rats from 1.09 ± 0.05 to 1.62 ± 0.15 pmol/min ($p < 0.001$, $n = 12$; Fig. 5*B*).

To determine whether the stimulation-induced increase was attributable to action potential-dependent activity, we added 1 μM TTX to the dialysate. Consistent with the observations in awake animals, addition of TTX had no significant effect on baseline concentrations of NO in anesthetized rats; however, the LDT-stimulated increase in NO production was abolished completely ($n = 3$; Fig. 5*B*).

Increases in thalamic NO output in response to stimulation occurred when the electrode tip was in or near (within 600 μm in the rostral direction) the LDT. However, when the electrode was placed in the cerebellum, stimulation had no effect on the cortical EEG or on thalamic NO release (Fig. 5*B*). Taken together, these results suggest that the NO release in the thalamus was sensitive to activity-dependent changes of LDT NOS-positive cholinergic neurons.

DISCUSSION

The principal finding in the present report is that NO is produced in the thalamus in a state-dependent manner, with the highest rate occurring during EEG desynchronized states, wake and REM, and at a significantly slower rate during SWS. These results support a role of NO in arousal mechanisms and are in accordance with several other findings discussed below. Ogasahara et al. (1981) showed that cGMP levels were elevated during EEG desynchronized states in the frontal cortex, midbrain, and pons. cGMP efflux measured *in vivo* has been reported to be dependent on NOS activity (Luo et al., 1994; Vallebuona and Raiteri, 1994).

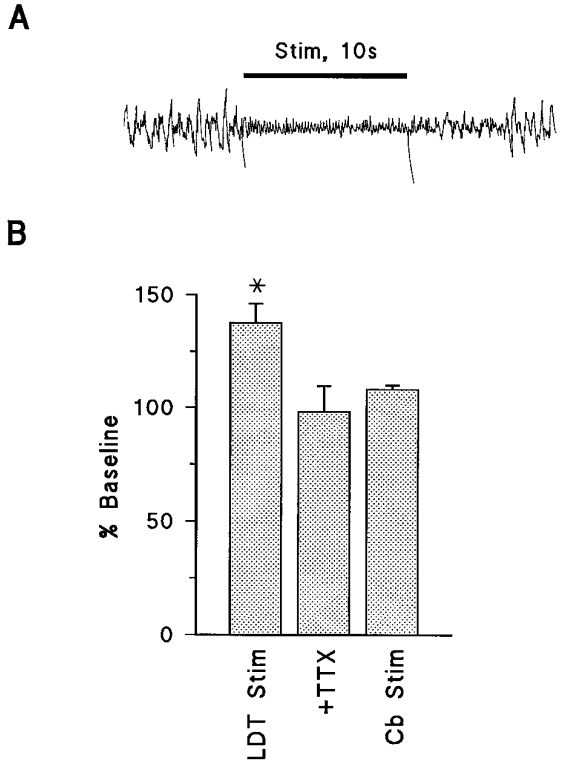

Figure 5. Effects of electrical stimulation of the laterodorsal tegmental nucleus (LDT) on cortical EEG and NO concentrations in the thalamus of anesthetized rats. *A*, EEG recording during a 10 sec stimulation pulse exhibits low amplitude waves (6–8 Hz), as compared with the high amplitude slow-wave activity during the interstimulus intervals (see Results). *B*, LDT stimulation significantly increased NO concentration (*LDT Stim*; $n = 12$; $^*p < 0.001$). This effect was blocked by addition of 1 μM TTX to the Ringer's solution (*+TTX*; $n = 3$). No change was observed when the stimulating electrode was placed in the cerebellum (*Cb Stim*; $n = 3$).

The soluble guanylyl cyclase, which forms cGMP, is known to be a major effector of NO in the CNS (Knowles et al., 1989). The observations of Ogasahara et al. (1981) may, therefore, reflect NO-dependent cGMP production. In contrast, using voltametry to detect NO in the cortex, Cespuglio et al. (1996) showed that NO concentrations were highest during wake, as compared with SWS and REM.

The origin of NO in the thalamus may arise from several brain regions, including the hypothalamus, tectum, dorsal raphe, and LDT/PPT, as well as from the cell bodies that stain for NOS in the ventrolateral geniculate nucleus of the rat (Vincent and Kimura, 1992; Gabbot and Bacon, 1994; J. A. Williams, unpublished observations). Based on these anatomical observations as well as the results from the present study, the majority of NOS inputs arise from the NOS-positive cholinergic neurons in the mesopontine tegmentum. Electrical stimulation in the LDT significantly increased thalamic NO output. This effect was both action potential-dependent, because it was blocked by TTX, and site-specific, because no change in concentration was observed when the stimulating electrode was placed in the cerebellum. These findings confirm those of Miyazaki et al. (1996), who found that electrical stimulation in the LDT increased thalamic NO output, as measured by differential pulse voltametry. Taken together with our previous findings, which showed that thalamic ACh release from mesopontine cholinergic terminals exhibited a similar behavioral

profile (Williams et al., 1994), these data support the hypothesis that the state dependence of thalamic NO release is derived mainly from the corresponding activity of the NOS-containing mesopontine cholinergic neurons.

NOS-dependent signaling

The second series of experiments verified the extent to which the assay for NO was sensitive to NOS-dependent activity. HbO_2 is susceptible to oxidation by a variety of compounds in the brain, including oxygen, hydrogen peroxide, superoxide, and carbon monoxide (CO). Protection from auto-oxidation as well as from superoxide and H_2O_2 was controlled to some extent by addition of superoxide dismutase and catalase to the HbO_2 stock solution (Murphy and Noack, 1994; see Materials and Methods). Although some thalamic nuclei are known to express heme oxygenase, the enzyme responsible for CO synthesis (Verma et al., 1993; Vincent et al., 1994), oxidation of HbO_2 by CO is unlikely for several reasons. First, the affinity of HbO_2 for NO is 1000-fold higher than that for CO (Cassoly and Gibson, 1975). Second, unlike NOS, heme oxygenase is constitutively active and is not known to be regulated by neuronal or calcium-dependent mechanisms (Maines et al., 1986; Cruse and Maines, 1988). HbO_2 oxidation in the dialysate was indeed sensitive to NOS activity, because peripheral injections of the NOS inhibitor *N*-ARG significantly reduced the amount of HbO_2 oxidation in the samples, reflecting a decreased concentration of NO. In addition, HbO_2 oxidation was sensitive to neuronal depolarization, as indicated by infusing a dialysate with a high potassium concentration through the probe. Presumably, the potassium-induced depolarization increases NO output by increasing the open probability of voltage-sensitive Ca^{2+} channels, thereby facilitating NOS activity. Indeed, the potassium-induced increase in NO was abolished by addition of a Ca^{2+} chelator, BAPTA, indicating the Ca^{2+} dependence of NO production. Baseline concentrations were also Ca^{2+}-sensitive, because a BAPTA/Ca^{2+}-free solution caused a significant decrease. Taken together, these observations suggest that the changes in HbO_2 oxidation directly reflect changes in thalamic NO output.

Neuronal-dependent NO production

The observation that both local depolarization and electrical stimulation of the LDT increased thalamic NO production suggests that the signal was attributable to neuronal-dependent activity. However, to our surprise, application of 1 μM TTX did not affect baseline concentrations in awake animals. We have found previously that this concentration of TTX effectively reduced baseline concentrations of thalamic ACh (Williams et al., 1994). Three possible scenarios may account for this discrepancy. First, NO may diffuse through brain tissue more readily than ACh (Lancaster, 1994; Wood and Garthwaite, 1994), allowing us to measure NO release from terminals unaffected by TTX. Second, this finding is consistent with the observation that in some structures basal concentrations of other neurotransmitters, such as glutamate or GABA, also are unaffected (or even increased) by TTX (Bradford et al., 1987; Tanganelli et al., 1994; Abarca et al., 1995; Hashimito et al., 1995; Hondo et al., 1995). The third possibility relates to the behavioral state of the animal during TTX application. Local application of TTX in the thalamus clearly induced a hyperaroused state, in which animals were continuously active for the duration of the application (Williams, unpublished observations). Any number of sequelae of this hyperaroused state may have increased thalamic NO production from either neuronal or non-neuronal sources, resulting in the apparent lack of effect of TTX.

It is possible that part of the measured NO signal was derived from non-neuronal sources, including endothelial NOS (eNOS), which is abundant in the brain (Gabbott and Bacon, 1993), and inducible NOS (iNOS) found in microglia, which may accumulate as an inflammatory response to the dialysis probe implant. eNOS is known to be Ca^{2+}-dependent, whereas iNOS is not (Nathan and Xie, 1994; Förstermann and Kleinert, 1995). Because we were able to detect Ca^{2+}-sensitive activity, the majority of the NO signal most likely was attributable to either eNOS or neuronal NOS activity. NO initially was recognized to be an endothelium-derived relaxing factor (Ignarro et al., 1987; Palmer et al., 1987), thus making it an important component in regulating vascular tone and blood flow (Ignarro, 1989). Koyama et al. (1994) demonstrated that LDT stimulation increased blood flow in the rat lateral geniculate nucleus of the thalamus. In addition, changes in blood flow are known to occur across the sleep–wake cycle, with the highest levels occurring during wake and REM and at progressively slower rates with each successive stage of SWS in humans (Hajak et al., 1994; Hoshi et al., 1994). The present results are consistent with these data and suggest that part of our signal may have derived from eNOS activity. On the other hand, several studies have suggested that neurons that release NO also may affect cerebral blood flow (Adachi et al., 1992; Northington et al., 1992; Kayama et al., 1995; Miyazaki et al., 1996). The study by Koyama et al. (1994) showed that the stimulated–induced increase in thalamic blood flow was mediated through cholinergic receptors. The authors concluded that mesopontine cholinergic innervation was important in controlling thalamic blood flow. Our data suggest that NO release from the same terminals also may contribute to this effect.

Role of NO in arousal mechanisms

A growing body of work has suggested a variety of other effects of NO in the thalamus. For example, Pape and Mager (1992) demonstrated that NO-releasing compounds and cGMP agonists depolarize thalamic relay cells *in vitro* by shifting the activation curve of the hyperpolarization-activated cation current (I_h) to more positive potentials. This change in the voltage dependence of I_h may be one mechanism by which thalamic neurons switch from bursting to a tonic mode of firing, which is known to underly EEG desynchronization (Steriade et al., 1990b). Thus, NO produced during wake or REM may bias relay cells toward a more tonic mode of firing, which is an underlying constituent of these states. Others have shown that NO facilitates responses of thalamic neurons to both visual (Cudeiro et al., 1996) and tactile (Do et al., 1994) stimuli. Taken together, these findings further support an excitatory role of NO in arousal mechanisms.

Recently, a type II cGMP-dependent protein kinase (cGKII) was identified and found to be expressed at high levels in the rat thalamus (El-Husseini et al., 1995). We found that LDT stimulation or exogenous application of NO to thalamic extracts increased the autophosphorylation of cGKII (El-Husseini et al., 1996; C. Bladen, J. A. Williams, P. B. Reiner, S. R. Vincent, A-E-D El-Husseini, unpublished results), which is indicative of an increase in its activity (Jarchau et al., 1994). Taken together, these data suggest that cGKII is a target for NO in the thalamus. Whether cGKII is involved in the facilitatory effects of NO on sensory responses or on the modulation of I_h remains to be determined.

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