Activity-Dependent Nitric Oxide Concentration Dynamics in the Laterodorsal Tegmental Nucleus In Vitro

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Leonard, C. S., E. K. Michaelis, and K. M. Mitchell. Activity-dependent nitric oxide concentration dynamics in the laterodorsal tegmental nucleus in vitro. J Neurophysiol 86: 2159–2172, 2001. The behavioral-state related firing of mesopontine cholinergic neurons of the laterodorsal tegmental nucleus appears pivotal for generating both arousal and rapid-eye-movement sleep. Since these neurons express high levels of nitric oxide synthase, we investigated whether their firing increases local extracellular nitric oxide levels. We measured nitric oxide in the laterodorsal tegmental nucleus with a selective electrochemical microprobe (35 μm diam) in brain slices. Local electrical stimulation at 10 or 100 Hz produced electrochemical responses that were attributable to nitric oxide. Stimulus trains (100 Hz; 1 s) produced biphasic increases in nitric oxide that reached a mean peak concentration of 33 ± 2 (SE) nM at 4.8 ± 0.4 s after train onset and decayed to a plateau concentration of 8 ± 1 nM that lasted an average of 157 ± 23.4 s (n = 14). These responses were inhibited by Nω-nitro-L-arginine-methyl-ester (1 mM; 92% reduction of peak; n = 3) and depended on extracellular Ca2+. Chemically reduced hemoglobin attenuated both the electrically evoked responses and those produced by authentic nitric oxide. Application of the precursor, L-arginine (5 mM) augmented the duration of the electrically evoked response, while tetrodotoxin (1 μM) abolished it. Analysis of the stimulus-evoked field potentials indicated that electrically evoked nitric oxide production resulted from a direct, rather than synaptic, activation of laterodorsal tegmental neurons because neither nitric oxide production nor the field potentials were blocked by ionotropic glutamate receptor inhibitors. Nevertheless, application of N-methyl-D-aspartate also increased local nitric oxide concentration by 39 ± 14 nM (n = 8). Collectively, these data demonstrate that laterodorsal tegmental neuron activity elevates extracellular nitric oxide concentration probably via somatodendritic nitric oxide production. These data support the hypothesis that nitric oxide can function as a local paracrine signal during the states of arousal and rapid-eye-movement sleep when the firing of mesopontine cholinergic neurons is highest.

INTRODUCTION

Mesopontine cholinergic cells of the laterodorsal tegmental (LDT) and the adjacent pedunculopontine tegmental (PPT) nuclei provide the major cholinergic input to the thalamus (Hallanger et al. 1987; Paré et al. 1988; Satoh and Fibiger 1986; Semba and Fibiger 1992; Sofroniew et al. 1985; Steriade et al. 1988; Woolf and Butcher 1986) and can profoundly influence thalamocortical processing (Munk et al. 1996; Ste-riade et al. 1991). Mounting evidence indicates these cells play an instrumental role in the induction of rapid-eye-movement (REM) sleep and arousal by releasing acetylcholine (Ach) in the thalamus and medial pontine reticular formation (for review, see Steriade and McCarley 1990). While the function of these neurons has been considered mainly in terms of acetylcholine release, they probably also release other chemical messengers including peptides (Standaert et al. 1986; Sutin and Jacobowitz 1988; Vincent et al. 1983b, 1986) and nitric oxide (NO) (Leonard and Lydic 1997; Williams et al. 1997). Indeed, virtually all mesopontine cholinergic neurons are intensely labeled by reduce β-nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemistry (Vincent et al. 1983a), which vividly reveals their somatic, dendritic, and axonal morphology (Leonard et al. 1995a; Vincent et al. 1983a). This staining results from the cytoplasmic distribution of high levels of the enzyme neuronal NO synthase (nNOS) (Dawson et al. 1991a; Hope et al. 1991), which catalyzes the formation of NO from L-arginine (L-Arg) in a Ca/calmodulin-dependent manner (Bredt and Snyder 1990; Mayer et al. 1991).

NO is an ubiquitous, membrane-permeant, intercellular signaling molecule that functions in several diverse physiological processes (for review, see Moncada et al. 1991), including the control of vascular tone, where it was first identified. Evidence suggests that NO is also an important molecule in the CNS (Garthwaite et al. 1988; Knowles et al. 1989), where it may modulate synaptic transmission and cellular excitability (for review, see Garthwaite and Boulton 1995) and may have both excitotoxic (Dawson et al. 1991b) and neuroprotective actions (Lipton et al. 1993). The control of neuronal NO production has been associated with Ca2+ entry following activation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors (Garthwaite 1991; Garthwaite et al. 1988; Kiedrowski et al. 1992). Because mesopontine cholinergic neurons both express voltage-dependent Ca2+ channels (Kamondi et al. 1992; Leonard and Llinás 1990; Takakuwaki and Kitai 1997) and display elevated somatodendritic [Ca2+]i following action potentials (Leonard et al. 1995b, 2000) and because excitatory synaptic input is mediated partly by NMDA receptors (Sanchez and Leonard 1994, 1996), NO may, in principle, be generated by these cells at both somatodendritic regions as well as axonal...
terminals during periods of activity. Evidence indicates that NO is released from the terminals of these neurons in the thalamus (Williams et al. 1997) and medial pontine reticular formation (Leonard and Lydic 1997) in relation to behavioral state. We have sought to directly determine whether extracellular NO is generated within the LDT in response to activity of LDT neurons.

Measurement of neuronal NO production has been limited to a few brain regions and has relied mainly on indirect methods, including bioassay (Garthwaite et al. 1988), cGMP assays (DeVente et al. 1990; East and Garthwaite 1991; Garthwaite et al. 1988; Morris et al. 1994), [3H] citrulline production (Kiedrowski et al. 1992; Toms and Roberts 1994), and the measurement of nitrates from brain microdialysis (Luo et al. 1993; Shintani et al. 1994), although direct measurement of NO by microdialysis has also been made (Williams et al. 1997). While these methods demonstrate NOS activity, they provide little information about the bioavailability or spatiotemporal patterns of NO in tissue. Recent electrochemical techniques have been developed (Bediou et al. 1997; Christodoulou et al. 1996; Fabre et al. 1997; Friedemann et al. 1996; Iravani et al. 1998; Malinski and Taha 1992; Meulemans 1993; Mitchell and Michaelis 1998; Park et al. 1998; Shibuki 1990) that directly measure NO at precise locations, and a growing number of studies have applied these methods to neural tissue (Burlet and Cespuglio 1997; Desvignes et al. 1997; Iravani et al. 1998; Kimura et al. 1998; Malinski et al. 1993a; Meulemans 1994; Meulemans et al. 1995; Rivet et al. 1997, 1999; Shibuki and Kimura 1997; Shibuki and Okada 1991; Zhang et al. 1995).

We have utilized a recently developed porphyrinic microprobe, modified for enhanced NO selectivity (Mitchell and Michaelis 1998), to investigate the activity-dependent production of NO within the LDT. We provide, for the first time, direct evidence that NO is produced in the LDT following electrical and chemical stimulation. This finding implies that the local [NO] is modulated by the behavioral state-dependent firing of mesopontine cholinergic neurons and therefore that NO may function as a paracrine signal within the mesopontine tegmentum.

METHODS

Brain slice preparation

Brain slices of the mesopontine tegmentum were prepared according to standard methods (Leonard and Llinás 1994) in accordance with the National Institutes of Health policy on humane care and use of laboratory animals (NIH Publication 80-23). The minimum number of animals were used to reach statistically meaningful results. Briefly, female guinea pigs (175–300 g) were anesthetized with pentobarbital sodium (75–100 mg/kg ip) and decapitated. The section of the brain female guinea pigs (175–300 g) were anesthetized with pentobarbital of laboratory animals (NIH Publication 80-23). The minimum number with the National Institutes of Health policy on humane care and use according to standard methods (Leonard and Llina´s 1994) in accordance

Electrochemical measurements

Electrochemical measurements in the slice and in vitro calibrations of the probe sensitivity and selectivity were made with a standard three-electrode cell configuration with Ag/AgCl reference and auxiliary electrodes. The probes were mounted on a micromanipulator and inserted into the LDT from either the ventral or dorsal aspect. The angle of the probe with respect to the slice surface was adjusted so the entire sensing surface of the probe was buried within the tissue. Measurements were performed using a PC-controlled amplifier and interface (Cypress Systems, Lawrence, KS) operated in the coulometric mode. The current resulting from NO oxidation at the electrode interface was integrated for either a 500-ms or 5-s period yielding a continuous on-line recording of the electrode response in picocoulombs versus time. These measurements were then converted via calibration of each individual probe to apparent NO concentration (typically nanomolar) versus time. Some additional amperometry experiments were also conducted using an Axopatch 200A amplifier (Axon Instruments) modified for an extended range of operation. Measurements with both systems were performed using an applied potential at the NO probe of +700 mV versus Ag/AgCl. Following placement of the NO probes, the potential was applied and the probes were allowed to equilibrate for ≥1 h before further experimentation. Acquired waveforms were imported into the computer program Igor Pro (WaveMetrics), for calibration, filtering and graphing. Amperometry records were integrated over a period of 480 ms off-line using Igor Pro.

Calibration of the NO probe was performed in an oxygenated and stirred standard slice solution before and then again after tissue measurements to account for any changes that may have resulted from exposure of the probe to tissue, e.g., adsorption of protein. Identical coulometry was employed to measure the electrode response to small volume aliquots of standard solutions of diethylamine NO complex (DEA-NO). The concentration of NO in the DEA-NO solution was established using a spectrophotometric method based on the conversion of oxyhemoglobin to met-hemoglobin by NO (Doyle and Hoekstra 1981). This assay was performed as described previously (Mitchell and Michaelis 1998). In some experiments, a second calibration was not performed and the data are expressed in picocoulombs.

Two additional tests were performed on these probes. First, because hemoglobin was used to scavenge tissue NO in some experiments (see following text), the sensitivity of the probe in the presence of hemoglobin was determined. A reduced hemoglobin (Hb) preparation (see following text) was quantitatively oxidized to methemoglobin (Di Iorio 1981) by preexposure to NO prior to testing the NO probe.
response. This oxidized Hb sample had no discernable effect on the NO or background response of the NO probes. Second, because carbon fibers electrodes can exhibit pH sensitivity under some conditions, e.g., with certain surface treatments and for measurements made in particular applied potential ranges (Rice and Nicholson 1989; Rummels et al. 1999), the pH sensitivity of the NO probe was also determined. Neuronal activity evoked by electrical stimulation is associated with extracellular pH changes of a few tenths of a pH unit (Chesler and Chan 1988; Kraig et al. 1983) and typically consist of a transient alkaline shift followed by an acidic shift of longer duration (~110 s to return to the basal pH). We found that the NO probe was insensitive to pH changes over the range pH 6.0 –7.8 when operated at the potential used for detection in these experiments.

**Extracellular and intracellular recordings**

Electrophysiological recordings were made in experiments separate from the NO measurements. Extracellular recordings of electrically evoked field potentials were made with borosilicate patch electrodes (6 MΩ) filled with Normal Ringer solution. Intracellular recordings were performed with sharp microelectrodes (80–100 MΩ) filled with 3 M KCl. Electrical measurements were obtained using an Axoclamp 2A amplifier (Axon Instruments) operated in bridge mode. Current and voltage traces were digitized using pClamp software (Axon Instruments) running on a personal computer. pClamp data were imported into Igor Pro (WaveMetrics) for graphing and measurement. For both of these sets of experiments, a stimulating electrode was positioned at the location used in the NO measurements.

**Electrical and chemical stimulation**

Electrical stimulation of the slice was accomplished with isolated constant-current pulses (Neurodata Instruments) applied to a Teflon-coated stainless steel bipolar stimulating electrode (114 μm OD) bored only at the tips. The electrodes were positioned at the ventral edge of the LDT as determined by inspection of the slice surface (see Fig. 1B). Chemical stimulation was accomplished by either superfusion of the compound dissolved at final concentration in normal Ringer solution or by pressure pulses (Picospritzer II; General Valve) applied to a patch pipette positioned near the surface of the slice at various distances from the electrochemical probe. In some experiments, which are indicated in the text, the pipette was positioned in the tissue for drug delivery. The drugs in the pipette were dissolved in either deionized water or in normal Ringer solution. All drugs were obtained from Research Biochemicals International unless otherwise indicated. The applied drugs were NMDA, Nω-nitro-1-arginine-methyl-ester (L-NAME), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 2-amino-5-phosphonopentanoic acid (APV) and l-Arg (tissue culture grade; Sigma). Chemically reduced Hb was prepared from a Ringer solution containing 1 mM hemoglobin (Sigma) in 10 mM sodium dithionite (Sigma). This solution was dialyzed in the dark against a total of 2,500 volumes of oxygenated Ringer at 4°C. The resulting solution was then diluted to its final concentration in Ringer. In some experiments a solution of authentic NO was pressure-ejected into the tissue. This NO solution was produced by bubbling double-distilled deoxygenated water with NO gas (99%, Matheson, Joliet, IL), which was purified of higher oxides of nitrogen prior to use as described previously (Mitchell and Michaelis 1998). The transients evoked by this solution were reproducible and were specifically related to the NO and not the hypotonic vehicle (see Fig. 6) (Mitchell and Michaelis 1998). Under similar recording and ejection conditions, comparable NO transients were also obtained by pressure ejection of DEA-NO in normal Ringer (data not shown). Tetrodotoxin (TTX) was purchased from Sigma and was dissolved in Ringer solution to a concentration of 1 μM.  

**Histochemistry**

To mark the recording sites, the probes were gently agitated to produce a small mechanical lesion after recordings were completed. The slice was then removed and immersion fixed in 4% paraformaldehyde for 12–36 h. After fixation, the slice was equilibrated with 20% sucrose in 0.1 M phosphate buffer (pH 7.4) for 12–24 h. The slice was then resectioned on a freezing microtome, and the tissue was processed for NADPH-diaphorase (NADPH-d) histochemistry by a method modified from (Hope and Vincent 1989) as previously described (Leonard et al. 1995a). The location of the NO probes were then determined with respect to the pattern of NADPH-d staining centered on the LDT and PPT.

![Image](https://journals.physiology.org/doi/10.2203/jn.2161ACTIVITY-DEPENDENT%20[NO]%20DYNAMICS%20IN%20THE%20LDT)
RESULTS

Probe locations and the distribution of NOS in the LDT and DR of the guinea pig

Histological examination of sections prepared from brain slices used for electrochemical measurement revealed that all LDT recording sites contained numerous NADPH-d cells (n = 11). To visualize the precise probe locations with respect to the NADPH-d-containing cells, it was necessary to make small mechanical lesions after recording (Fig. 1A) because positioning of the probes in the tissue did not result in observable damage (see LDT region, Fig. 1B). The example in Fig. 1A was taken from an experiment where an electrochemical probes was placed in the LDT (Fig. 1A, ↓). Labeled cells were observed within the LDT and ventral LDT (LDTv) as previously described in guinea pig (Leonard et al. 1995a). Numerous NOS-containing somata were within a few soma diameters of the probes located within the LDT. Moreover, these findings verified the absence of NADPH-d-containing somata from the DR of the guinea pig as was previously reported (Leonard et al. 1995a) and is different from the rat (Leger et al. 1998).

Electrical stimulation experiments were conducted with the NO probe located in the LDT and the stimulating electrodes placed on the surface of the slice ventral to the LDT in the underlying tegmentum. An example of the relation between the stimulating electrodes and the NADPH-d cells of the LDT region is shown in Fig. 1B (↑, lesions from stimulating electrodes). This location is effective in evoking local EPSPs in NADPH-d-labeled LDT cells (Sanchez and Leonard 1994).

Local electrical stimulation evoked a NOS-dependent electrochemical signal in the LDT

Electrical stimulation of the LDT resulted in electrochemical signals that can be attributed to increases in extracellular [NO]. Single pulses typically used to evoke synaptic input to LDT neurons (Sanchez and Leonard 1994, 1996) did not evoke detectable signals. However, trains of pulses delivered at either 10 or 100 Hz did evoke detectable signals. Pulses delivered at 10 Hz for 10 s evoked clear signals, but pulses delivered at 100 Hz for 0.1–1 s were preferred and were used in all experiments, unless otherwise indicated, because the shock artifacts subsided more rapidly. Electrochemical signals resulting from pulse trains of increasing current strengths (0.1 s at 100 Hz) are superimposed in Fig. 2A. With each successive train, the electrochemical signal became larger until it reached a maximal value for the highest stimulus strengths tested. Lengthening the train duration to 1 s at the highest current strength produced a striking increase in the amplitude and duration of the electrochemical response (Fig. 2B) revealing a biphasic time course (Fig. 2C). Responses to this stimulation paradigm often consisted of a rapid increase that peaked (33 ± 2 nM; n = 14) at 4.8 ± 0.4 s following stimulus onset and a variable duration plateau phase (8 ± 1 nM; measured at 90 s; n = 14), which

![image](http://jn.physiology.org/doi/10.1152/jn.00819.2001/full/ni307686d01c.png)

**FIG. 2.** Local electrical stimulation evoked an increase in the electrochemical signal in the laterodorsal tegmental nucleus. A: minimally detectable electrochemical responses resulting from constant-current pulse trains (10 pulses at 100 Hz) differing in amplitude. The small response is maximal with current pulses of 0.4- to 0.5-mA amplitude. B: an increase in the number of pulses in the train (to 100) evoked a larger increase in the electrochemical signal. C: the time course of the electrically evoked signal was biphasic and characterized by an early peak (single arrow) that was followed by a prolonged plateau (double arrow). Signals resulting from 2 stimuli that were separated by ~10 min are superimposed. The total response lasted between 200 and 300 s. Integration time was 0.5 s. The stimulation artifact was limited to the initial 3-5 s as determined by identical stimuli delivered in the absence of tissue (inset).
lasted until 157 ± 23.4 s from stimulus onset ($n = 14$). While such biphasic responses were common, other patterns were also observed. In some cases, responses decayed monophasically (cf. Fig. 4B), and in other cases, the late-phase was comparable to, or even larger than, the early peak (cf. Figs. 5B and 8B). Nevertheless, these electrochemical signals were not artifacts associated with polarization of the stimulating electrode or probe surface because identical shocks delivered in the absence of tissue, and with less distance separating the probe and stimulating electrodes, produced only a brief artifact (Fig. 2C, inset).

The electrochemical response to local electrical stimulation was also sensitive to NOS inhibitors as indicated in Fig. 3. Following the superfusion of solution containing 1 mM L-NAME, the electrically evoked signal progressively declined so that by 48 min the signal was virtually abolished (Fig. 3, A–C). The time course of L-NAME inhibition was measured in three slices (Fig. 3D). For two of the slices, the electrochemical signal was completely abolished with a time constant of ~20 min, while in the third case, the signal was reduced by 77% with a similar time constant. The average steady-state inhibition was 92.3% ($P < 0.01; n = 3$). This indicates that the electrochemical signals evoked by local electrical stimulation required activation of NOS and further supported the idea that these signals arose from endogenous NO production.

Electrochemical signal required extracellular Ca$^{2+}$ and was diminished by hemoglobin

nNOS is a Ca/calmodulin-dependent enzyme so we investigated the possibility that the electrically evoked electrochemical signals were Ca$^{2+}$ dependent. Superfusion of the slices with a Ringer solution containing no added calcium and 2.7 mM EGTA reversibly blocked these electrochemical signals (Fig. 4A). This was observed in two of three slices where the response was reduced by 89.5%. In the third slice, the response was reduced by 55%. These results indicate that most, if not all, of the electrically evoked signal arose from Ca-dependent processes. Because NO binds to the heme center of reduced hemoglobin (Hb) with high affinity and rapid kinetics (Doyle and Hoekstra 1981), we investigated whether the electrically
evoked electrochemical signals were also sensitive to Hb. Bath superfusion of 1 µM Hb produced a reversible inhibition of the electrochemical signal (54.0 ± 1.8%, n = 3), suggesting again that the electrochemical signal had properties attributable to NO (Fig. 4B). However, inhibition was not complete and occurred slowly following superfusion. Because our probe was positioned to sense NO throughout the thickness of the slice, one possible explanation for the slow and incomplete effect was that the high-molecular-weight Hb only entered the slice slowly. We tested this idea by pressure ejecting small volumes of authentic NO into the brain slice and measuring the effectiveness of superfused Hb in attenuating that signal. This showed that even when the Hb concentration was raised to 10 µM it took 30 min to attenuate the electrochemical signal by 65% (Fig. 4C). This experiment also demonstrated that superfusion of Hb reduced the basal electrochemical signal with a slow time course. We further examined this point by pressure ejecting Hb into the slice at a location situated between the NO source pipette and the microprobe as schematically illustrated in the inset of Fig. 5A. Under these conditions, four brief pulses of Hb (100 µM) produced small but rapid reductions in the baseline electrochemical signal and reduced the effect of a 60-ms pulse of NO by nearly 50% even when delivered ~3 min after the last Hb ejection (Fig. 5A1). Moreover, ejecting Hb near the peak of the response produced by a 60-ms pulse of NO rapidly reduced (within 1 s) the response by 50% (Fig. 5A2). These data illustrate that Hb can rapidly attenuate the NO signal if it is delivered into the slice between the source and the probe. We then determined if Hb ejection into the slice would also rapidly attenuate the electrically evoked electrochemical signal (Fig. 5B1). Two ejections of Hb delivered after the peak of the electrically evoked response rapidly attenuated the response by ~20% each. Moreover, responses to subsequent stimulation were attenuated by 50%. Following the third Hb ejection, the responses began to slowly recover. The rapidity of the Hb attenuation is illustrated on a faster time scale in Fig. 5B2. These data indicate that Hb has a similar effect on both the electrically evoked electrochemical signal and authentic NO, which strongly supports the idea that the microprobes were detecting tissue NO or a NO-related product.

**Electrochemical signal results from direct activation of NOS-containing processes**

The NO detected following electrical stimulation of the LDT may have arisen from several sources. These include NOS-containing axons and terminals that may have been directly stimulated to liberate NO, and the dendrites and somata of NOS-containing neurons, that may have been synthetically or directly stimulated to produce NO. Results from two types of experiments pertain to this point. First, we examined the possibility that activation of LDT neurons by glutamatergic EPSPs was necessary for the NO responses. To do this, we compared the electrically evoked NO signals from before and after inhibition of EPSPs with CNQX and APV. Because we previously had noticed that EPSPs recorded from LDT neurons undergo strong synaptic depression during high-frequency stimulation (Leonard, unpublished observations), we chose a stimulation paradigm of 10 Hz for 10 s to maximize the likelihood of observing excitatory postsynaptic potential (EPSP)-dependent signals for these experiments. However, as mentioned in the preceding text, this stimulation protocol produced a long-duration artifact which interfered with the early part of the electrochemical signal. We therefore measured the effect of ionotropic glutamate receptor antagonists in the late-phase of the evoked electrochemical signal. Bath superfusion of CNQX (10 µM) and APV (50 µM) produced no detectable suppression (107.6% of control; n = 5; P > 0.1) of these electrochemical responses (Fig. 6, A and B), indicating that fast EPSPs are not necessary for the stimulus-evoked increase in NO. Bath application of L-NAME verified these late signals were NOS dependent (Fig. 6C).
In another series of experiments, we examined the field potentials produced in the LDT by high-frequency stimulation. Results from these experiments indicated that substantial direct activation of LDT neurons occurred for the stimulus strengths used to evoke detectable electrochemical signals (Fig. 7). A short-latency, graded field potential (1.6 mV, maximum amplitude; 1.15 ms, width at half-maximum amplitude; $n = 2$ slices) was evoked with stimulus currents that ranged from 0.3 to 0.8 mA (Fig. 7A). The amplitude of this field potential decreased monotonically as the interval between the stimuli was shortened without evidence for paired-pulse facilitation (Fig. 7B) as expected if this field potential resulted from synchronized firing of LDT neurons rather than synaptic currents. Although the field potential was reduced by $\sim 50\%$ at a pulse interval of 10 ms, the field potential followed a stimulus frequency of 100 Hz (Fig. 7C). The field potential was insensitive to bath superfusion of 10 $\mu$M CNQX (Fig. 7, D and E), which always attenuated EPSPs (Sanchez and Leonard 1994) and indicated that the field was not synaptic. Intracellular recordings verified that local electrical stimulation could directly fire LDT cells (Fig. 7, F and G). Collectively, these results indicate that the electrically evoked electrochemical signal resulted from direct activation of LDT neurons.

FIG. 5. Rapid delivery of reduced-hemoglobin into the slice rapidly attenuated exogenous NO transients and the electrically evoked electrochemical signal. Inset: diagram of the recording arrangement used in A. The pressure ejection pipettes were placed at mid-depth of the slice. The NO pipette was $\sim 200 \mu$m and the Hb pipette was $\sim 100 \mu$m from the sensor. $A_1$: brief pressure-ejections (20, 40, and 60 ms; 10 psi) of authentic NO ($\sim 2$ mM) into the slice produced dose-dependent increases in the signal at the microprobe. †, times of NO application. Four pressure ejections (20, 40, 60, and 80 ms sequentially; 10 psi) of reduced hemoglobin (labeled Hb; 100 $\mu$M) produced small but rapid reductions in the baseline NO level and attenuated the subsequent response to NO (60 ms; 10 psi). ‡, time of Hb application. The same volume of Hb (80 ms; 10 psi) delivered while extracellular [NO] was elevated by a pulse of NO (60 ms; 10 psi), produced a large and rapid reduction in the [NO]. $A_2$: data from $A_1$ on a faster time scale shows the rapid effect of Hb on the NO transient. Traces prior to Hb application are labeled 20, 40, and 60 ms. ††, the first response evoked after the 4 Hb pulses in $A_1$ and was evoked by a 60-ms pulse of NO. Hb was ejected at the point-labeled Hb. The relative timing of the NO and Hb pulses is shown on the bottom trace. $B_1$: electrical stimulation (90 pulses; 100 Hz) evoked increases in NO that were rapidly reduced by pressure ejecting Hb (200, 400, 1,000 ms sequentially; 10 psi) into the slice. Delivery of Hb into the slice also reduced the subsequent electrically evoked transient and the basal levels of NO measured. ‡‡, the time of Hb ejection. $B_2$: data from $B_1$ on a faster time scale to better show the speed of the Hb action on the electrically evoked NO transient.
The previous data indicate that electrical stimulation resulted in a prolonged activation of NOS in the LDT. We investigated the possibility that this long-duration response was limited by substrate availability. We compared the electrochemical signals evoked by electrical stimulation in normal Ringer solution to those evoked in a solution containing l-Arg. As illustrated in Fig. 8A, l-Arg (1 mM) did not effect the early peak but the plateau phase was increased in amplitude (181.1% of control; n = 4; P < 0.01) and the response duration (423.5 ± 39.8 s; n = 4) was longer than controls (157.1 ± 23.4 s; n = 14; P < 0.01). The prolonging of the plateau phase was reversed after washing out the l-Arg (Fig. 8B), suggesting that the plateau phase of the response was sensitive to the availability of l-Arg. Moreover, application of TTX (1 μM), which blocks voltage-gated Na⁺ channel-dependent action potentials, also abolished both the peak and the enhanced plateau responses (Fig. 8A, right), indicating that both phases of the response depends on the generation of TTX-sensitive currents.

**NMWA evoked NO production in the LDT**

Since NMDA receptor activation has been specifically linked to NO production, we also examined the possibility that NMDA elicits NO production in the LDT, which is known to contain synaptic NMDA receptors (Sanchez and Leonard 1994, 1996). A 3-min superfusion of Ringer containing 100 μM NMDA produced a reversible increase in the electrochemical signal measured by a probe located within the LDT (Fig. 9A). Such an increase was also observed following local ejection of NMDA with a patch pipette placed above the slice near the probe. In this configuration, NMDA produced a dose-dependent increase in the signal (Fig. 9B) with an average time to peak of 42.1 ± 14.9 s. Such signals were observed following NMDA application either locally (n = 8) or by bath superfusion (n = 2) in 5/6 slices. These signals were reversibly inhibited (64.6 ± 4.1%; n = 2) by l-NAME (1 mM; Fig. 9C), indicating that a majority of the signal could be attributed to activation of NOS. Such partial inhibition of NMDA-evoked NO signals has been reported previously under similar recording conditions using a different NO-selective probe (Iravani et al. 1998). The average maximal increase in [NO] produced by local NMDA application in the LDT was 39 ± 14 nM (n = 8).

**DISCUSSION**

We have used an NO-selective electrochemical microprobe to measure changes in extracellular [NO] evoked by local electrical and chemical stimulation in a brain-slice preparation of the guinea pig LDT nuclei. Our central finding is that direct electrical stimulation of the LDT triggers a long-lasting electrochemical signal that is attributable to an increase in local [NO]. Moreover, this signal was increased in duration by exogenous l-Arg and was abolished by blocking voltage-gated Na⁺ channels with TTX. These findings, along with the observation that NMDA application increased tissue [NO] within the LDT, support the hypothesis that the activity-dependent production of NO by mesopontine cholinergic neurons functions as a local paracrine signal in the control of behavioral state.

**Technical consideration**

An important consideration relates to the specificity of the probe. Several lines of evidence indicate that the electrochemical signals recorded in these experiments arose from tissue concentration changes of authentic NO. First, the basis of these measurements was the use of a porphyrinic sensor that has been previously shown to be highly sensitive to NO (Malinski and Taha 1992). The probes used in our experiments were modified to reduce interference from molecules such as monoamines and their metabolites, which are expected to be found in brain tissue. The degree of interference from these and other possible interferants were previously quantified and these probes were shown to be highly selective for NO (Mitchell and Michaelis 1998). Second, the individual probes used in these experiments were tested for interference from monoamines and monoamines.
experimental reagents, including Hb and changes in pH, and calibrated for NO as described in METHODS to verify their selectivity under the current experimental conditions. Third, the electrochemical signals were sensitive to L-NAME indicating that NOS activity was necessary for the production of the signals. Fourth, the electrically evoked signals were Ca^{2+} dependent and were attenuated by reduced hemoglobin in a manner similar to signals produced by exogenous NO. Finally, an electrochemical signal was also evoked by NMDA application, indicating that it was not an artifact of electrical stimulation. Collectively, these data strongly support the view that the measured electrochemical signals arose from endogenous NO.

Possible sources of NO

Our data indicate that the measured NO signals most likely arose from the NOS-containing cells of the LDT. First, the probes were shown histochemically to have been positioned...
among the NADPH-d-containing cells of the LDT, and local electrical stimulation evoked NO production at these measurement sites. Second, tissue superfusion and local pressure application of NMDA increased the \([\text{NO}]\) at these LDT sites. Third, field potential measurements indicated that the electrical stimuli that evoked NO production also evoked firing of LDT neurons. Finally, electrical-stimulation-evoked NO signals were blocked by TTX but not by CNQX and APV, which abolish fast excitatory synaptic input to LDT neurons (Sanchez and Leonard 1994, 1996). This indicates that NO production was evoked by direct rather than by synaptic activation of action potentials in NOS-containing neurons and processes within the LDT.

Because nNOS is distributed throughout the cytoplasm of neurons, NO might be produced in axons, terminals, dendrites, and somata. Electrochemical measurements of NO in the molecular layer of the cerebellum (Shibuki and Kimura 1997), the substance gelatinosa of the spinal cord (Kimura et al. 1999), and dialysis measurements in the thalamus (Williams et al. 1997), which is innervated by axons of NOS-containing mesopontine cholinergic neurons, suggest that activity-dependent NO production in these structures arises from axon terminals.

However, given the high density of NOS-containing somata and dendrites within the LDT (Leonard et al. 1995a), it is more likely that the electrically evoked changes in \([\text{NO}]\) measured here were generated at these somatic and dendritic sites. The measurement of spike-evoked changes of \([\text{Ca}^{2+}]_i\) in NOS-containing LDT cells supports this view because during repetitive firing, somatodendritic \([\text{Ca}^{2+}]_i\), can readily achieve the levels reported to activate NOS (Leonard et al. 2000). This idea was further supported by our finding that NMDA application, which produces large increases in somatodendritic \([\text{Ca}^{2+}]_i\) in NOS-containing LDT cells, generates NO. Nevertheless, the available evidence does not exclude the possibility that at least some of the measured NO arose from NOS-containing axon terminals within the LDT, especially because some of these axons are local collaterals of the NOS-containing LDT neurons (Surkis et al. 1996). Furthermore, the possibility that some measured NO arose from blood vessel endothelial cells cannot be ruled out, although this seems unlikely, at least for NMDA receptor-mediated NO production because cerebrovascular microvessel endothelial cells appear to lack functional glutamate receptors (Morley et al. 1998).
from other mechanisms such as the firing of TTX-sensitive action potentials.

In some neurons, nNOS appears to be selectively regulated by NMDA receptor-evoked Ca\(^{2+}\) entry (Kiedrowski et al. 1992). This may be mediated by the association of nNOS with NMDA receptors and by their mutual targeting to postsynaptic densities (Brennan et al. 1996; Christopherson et al. 1999) and spines (Aoki et al. 1998) where Ca\(^{2+}\) influx through NMDA receptors mediates large increases in local [Ca\(^{2+}\)] (Yuste et al. 1999). However, the degree of functional compartmentalization in neurons remains unclear. The most intensely NOS-immunoreactive neurons in neocortex and those in the LDT are aspiny or sparsely spiny and have nNOS label distributed throughout their cytoplasm (Leonard et al. 1995a; Vincent and Kimura 1992). Elevation of cytoplasmic Ca\(^{2+}\) by several different pathways might activate nNOS. Our Ca\(^{2+}\) imaging studies from NOS-containing LDT neurons indicate that even weak activation of NMDA receptors stimulates large somatodendritic [Ca\(^{2+}\)] transients mediated by action potential-activated voltage-gated Ca\(^{2+}\) channels (Leonard et al. 2000) rather than by Ca\(^{2+}\) influx through NMDA receptors. Thus it seems unlikely that the observed NMDA-evoked changes in [NO] could be mediated by a restricted influx of Ca\(^{2+}\) through NMDA receptors. It is more likely that Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels played a common role in both the NMDA- and electrically evoked NO production we have measured. Perhaps, subthreshold activation of NMDA receptors produces the highly localized NO signals necessary for synapse-selective plasticity (Schuman and Madison 1994) while suprathreshold activation recruits NOS activity throughout the cytoplasm for a more general paracrine signaling function.

Kinetics of tissue [NO] changes produced by electrical stimulation

Electrical stimulation resulted in an average peak increase of 33 ± 2 nM, which is comparable to peak concentration changes reported in the molecular layer of the cerebellum following white matter stimulation (Shibuki and Okada 1991). Considering that NO is produced at sites removed from the immediate surroundings of the microprobe surface and that NO can react with tissue and molecular oxygen (Taha et al. 1992), consideration of the relation between NO production and the size of the NO signal is important. The onset of the NO signal following electrical stimulation was evident before the complete decay of the stimulus artifact, and the average time to peak was 4.8 ± 0.4 s (n = 14), which was comparable to the times of under five seconds reported for NO signals evoked by electrical stimulation in the molecular layer of the cerebellum (Shibuki and Kimura 1997) and cerebral cortex (Wakatsuki et al. 1998). Because our NO probes have response times of ~0.5 s (Mitchell and Michaelis 1998) and our integration time was 0.5 s, the time to peak accurately reflects the time course of the concentration change of extracellular NO. This indicates that the dynamics of signals carried by extracellular NO would be quite slow compared with conventional synaptic mechanisms.

The time course of the electrically evoked [NO] transients often had a form consisting of a peak followed by a plateau lasting over a minute. In contrast to our findings, the [NO] in the molecular layer of the cerebellum is reported to declined to

**FIG. 9.** N-methyl-D-aspartate increased the electrochemical signal in the laterodorsal tegmental nucleus. A: bath superfusion of 100 μM NMDA (3 min) produced a reversible increase in the electrochemical signal measured in the LDT. Current from the electrophysiological probe was integrated for 0.5 s. B: local NMDA application evoked a dose-dependent NO signal. NMDA (5 mM) was pressure ejected (at arrow) from a patch pipette positioned above the tissue near the LDT probe (~200 μm). These applications produced long-lasting increases in the electrochemical signal that were reversible. Integration time was 5 s for this experiment. C: superfusion (49 min) with Ringer solution containing 1 mM L-NAME reversibly inhibited the NMDA-evoked electrochemical signal. Integration time was 5 s. Calibration bar in B applies to C.
baseline within 10 s, following either white matter or molecular layer stimulation (Shibuki and Kimura 1997; Shibuki and Okada 1991). While this may reflect methodological differences, another consideration is the source of NO in these two studies. In the LDT, measured NO appears generated mainly by the somatodendritic pool of NOS while in the molecular layer. NO appears generated by NOS in the parallel fibers (see Shibuki and Kimura 1997). It is possible the differences in decay time course reflect a differential regulation of these two pools of NOS. One possibility is that the somatodendritic pool of nNOS remains activated by the residual elevation of [Ca\(^{2+}\)], which declines only slowly (τ = 134 s) after repetitive firing (Leonard et al. 2000). Of course, other factors might also have contributed to the plateau response and include a secondary activation of NOS by other cellular messengers released during electrical stimulation and/or the direct alteration of NOS activity through interactions of its numerous, but poorly understood, intracellular modulators (for review, see Michel and Feron 1997). Further work will be necessary to clarify the underlying mechanisms of these plateau responses.

**Functional implications of NO produced by mesopontine cholinergic neurons**

A role for NO in the control of behavioral state was initially suggested from the effects of central l-NAME administration (Kapas et al. 1994). Several lines of evidence now indicate that NO production by NOS-containing cholinergic neurons of the LDT and PPT plays a role in this control. Mesopontine cholinergic neurons provide extensive NOS-containing input to the thalamus (Bickford et al. 1993) and thalamic NO levels vary according to behavioral state with the highest levels during waking and REM sleep (Williams et al. 1997). The action of this NO appears to enhance visual and somatosensory activity (Cudeiro et al. 1994a,b; Do et al. 1994), perhaps by shifting the voltage dependence of the H-current in thalamic relay neurons (Pape and Mager 1992).

Evidence for local NO actions within the mesopontine tegmentum also supports a role for NO in behavioral state control. Potential targets of local NO action include the locus coeruleus (LC) where NOS inhibitors enhance EPSPs (Xu et al. 1994) and where NO donors produce a cGMP-dependent depolarization (Pineda et al. 1996). These neurons are closely adjacent to LDT in rat and are extensively interdigitated among LDT neurons in the guinea pig and other species including human (see discussion in Leonard et al. 1995a), suggesting that they might be influenced by the somatodendritic production of NO by mesopontine cholinergic neurons.

Mesopontine cholinergic neurons, themselves, may also be targets of their own NO production. NO donor compounds influence NOS-containing LDT cells in two ways (Leonard et al. 1995b). First, they reduce NMDA receptor-mediated excitation, which on average comprises ~10% of the excitatory synaptic current at ~60 mV (Sanchez and Leonard 1996). Second, exogenous NO inhibits EPSPs in NOS-containing LDT cells. Thus NO generated by mesopontine cholinergic neurons could function as an inhibitory feedback pathway by reducing excitatory synaptic input to the LDT. Strong feedback control of this system is also mediated by the postsynaptic inhibitory action of Ach on autoreceptors (Leonard and Lilnás 1994; Luebke et al. 1993). Interestingly, NO may also enhance this cholinergic feedback inhibition by promoting Ach release as suggested by the finding that NOS inhibition in the cat PPT reduces the local release of Ach (Leonard and Lydic 1997).

Although the specific roles need to be further elucidated, NO actions at LDT and nearby sites appear to be functionally important in REM sleep. NOS inhibitors applied to the cat PPT (Datta et al. 1997) and medial pontine reticular formation (Leonard and Lydic 1997) reduced REM sleep amounts. Microinjection of NOS inhibitors in the closely adjacent dorsal raphe nucleus reduced REM sleep in rat (Burlet et al. 1999) and a consolidation of REM episodes has been reported following local injection of l-NAME into the mPRF (Okabe et al. 1998).

In conclusion, our data indicate that neural activity evoked by electrical stimulation and NMDA receptor activation increases extracellular [NO] within the LDT. This implies that NO can act as a paracrine signal whose production is linked to the suprathreshold activity of LDT neurons. Given the evidence from single-unit studies (El Mansari et al. 1989; Kayama et al. 1992; Koyama et al. 1998; Steriade et al. 1990) indicating that putative NOS-containing cells of the LDT fire at their highest rates during waking and REM sleep (~10–20 spikes/s in rodent) and are either quiescent or fire at low rates during slow-wave sleep, our results and the work discussed in the preceding text, support the hypothesis that NO functions as a local paracrine signal within the mesopontine tegmentum in the control of behavioral state.

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