

PHYSIOLOGY OF THE MESOPONTINE CHOLINERGIC/NITRIC OXIDE SYSTEM:  
MECHANISMS OF BEHAVIORAL STATE CONTROL

by

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## Abstract

Historically, research on questions which address the neurobiological mechanisms of behavioral state control has focused upon identifying neuronal candidates for the ascending reticular activating system. Several lines of evidence now suggest that mesopontine cholinergic neurons indeed play a key role in EEG desynchronized states as well as in the triggering of rapid eye movement (REM) sleep. Using *in vitro* patch clamp and *in vivo* microdialysis techniques combined with histochemical and immunocytochemical staining, some of the intrinsic biophysical, pharmacological, and behavioral properties of mesopontine cholinergic/nitrgenic neurons were characterized.

Several lines of evidence had suggested that inhibition of brainstem cholinergic neurons by noradrenergic neurons of the locus ceruleus is a key mechanism of behavioral state control; however, this effect had never been directly demonstrated. The whole-cell patch clamp technique was used to investigate the effects of noradrenaline (NA) on mesopontine cholinergic neurons *in vitro*. The majority of identified cholinergic neurons hyperpolarized in response to NA, an effect mediated by an  $\alpha_2$  receptor via activation of an outward potassium current. In addition, cholinergic neurons expressed intrinsic ionic currents which predisposed them to burst-firing activity, a feature which may underly PGO activity.

To test the hypothesis that these cells would be selectively active during REM sleep, *in vivo* microdialysis was used to monitor acetylcholine (ACh) release across behavioral state in the rat thalamus, a major projection site of mesopontine cholinergic neurons. ACh was released at equal rates during wake and REM sleep, and significantly less during slow-wave sleep (SWS). Retrograde tracing combined with ChAT immunohistochemistry verified that

the majority of cholinergic projections in the vicinity of the dialysis probe originated in the mesopontine tegmentum.

Finally, a feature of mesopontine cholinergic neurons which has been addressed to a limited extent is the role of nitric oxide (NO) synthase, which is known to co-localize in these cells. Using the hemoglobin-trapping technique coupled with *in vivo* microdialysis, NO production in the thalamus was measured across the sleep-wake cycle. NO concentrations were high during wake and REM sleep as compared to SWS. Taken together, these data provide a broad profile of some of the properties of the mesopontine cholinergic/nitric oxide system.

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In Memory of  
Ann Louise Dougherty  
1967-1990

## I. INTRODUCTION

The mesopontine cholinergic system has long been suggested to play a key role in behavioral state regulation. This hypothesis originates from the series of experiments that were carried out in Horace Magoun's laboratory (Moruzzi and Magoun, 1949; Lindsley et al., 1949; 1950) long before brainstem cholinergic neurons had been identified (Shute and Lewis, 1967). Moruzzi and Magoun (1949) showed that stimulation of the brainstem reticular formation in cats under chloralosane-anesthesia or during spontaneous synchronized states in the unanesthetized *encéphale isolé* preparation (see below) elicited an activation of the cortical EEG. Taken together with the previous work of others who also observed similar effects upon stimulation of the basal encephalon or midbrain (Morison et al., 1941, Jasper, 1948; Ward, 1949), they suggested that the brainstem reticular formation, which extended from the lower medulla to the rostral midbrain, had an important ascending influence upon cortical activity, and thus coined the term *ascending reticular activating system* (ARAS). They posited that a threshold of activation of the ARAS was necessary for the maintenance of wakefulness and arousal, and that when activity fell below the threshold, the animal was predisposed to sleep. In a parallel experiment, Lindsley et al. (1949) found that destruction of the remaining afferent paths in the *encéphale isolé* preparation did not affect the EEG which suggested that this effect was due to activity of the reticular formation rather than from activation of sensory collaterals. The importance of this work derives from the integration of studies on cortical EEG, behavioral state, and functional anatomy of the reticular formation, as outlined below.

### *Electroencephalography*

The electroencephalogram (EEG) was first discovered in animals by Caton in 1875 (reviewed in Brazier, 1980) who observed that changes in currents measured in rabbits occurred

upon sensory stimulation, as well as between waking and sleep. Adolf Beck independently discovered EEG in animals in 1890, and reported that rhythmic oscillations disappeared upon sensory stimulation. The first published photographs of EEG activity by Pravdich-Neminsky (1913) clearly showed sensory-evoked responses in curarized dogs. Again, each sensory response was accompanied by a drop in amplitude or voltage. These findings were later confirmed in humans by recording brain potentials using epidural electrodes (Berger, 1930), as well as in animals (Adrian and Matthews, 1934; Adrian, 1936), however most of the latter studies were limited to anesthetized or curarized subjects, as chronic preparations were not widely used (Derbyshire et al., 1936; Rheinberger and Jasper, 1937). This issue was resolved to some extent when Bremer (1938) introduced the *encéphale isolé* preparation, which involved transection of the lower medulla. This preparation effectively paralyzed the animal, making the study of EEG possible in animals which continued to exhibit a relatively normal sleep-wake cycle. Thus a general consensus emerged such that the EEG was characterized by low-voltage fast activity during periods of arousal, and by high-voltage slow-wave activity during periods of drowsiness, sleep, or under anesthesia. Although the mechanisms underlying EEG desynchronization were not well understood, it was thought that slow-wave activity represented synchronous firing patterns (Derbyshire et al., 1936), and that interruption of slow waves by stimulation or arousal was due to cortical activation by sensory afferents (Rheinberger and Jasper, 1937; Dempsey and Morison, 1943; Morison et al., 1943).

### *Sleep and Arousal*

Sleep has long been a subject of formal investigation, with the earliest recorded theories dating back to Alcmeon, a contemporary of Pythagorus, in the sixth century B.C. (Kleitman, 1963) While many theories developed with respect to the mechanisms of sleep over the centuries, the

hypotheses advanced in 1949 and later emerged from concepts that were first introduced in the mid-nineteenth century. Preceding the studies of Magoun and co-workers (1949; 1950), there existed a controversy over whether sleep was a passive process involving sensory deafferentation, or if it was an active process, involving the activation of an anatomical substrate for sleep. Although not explicitly addressed by Magoun, these studies were directly relevant to this controversy.

A common theme among many hypotheses that were proposed during the mid-nineteenth century was that sleep was simply a consequence of sensory deafferentation. Several mechanisms for this process had been proposed. For example, Purkinje suggested in 1846 that an interruption of sensory input was caused by a hyperemia of the basal nuclei, which would compress the corona radiata of the thalamus and thereby block thalamocortical connections (reviewed in Kleitman, 1963). In the 1890's, a popular notion was that sleep was the result of dendritic retraction, where cortical cells would physically lose contact with each other and consequently cease activity, which corresponded to a loss of consciousness (Economo, 1930; Kleitman, 1963; Cajal, 1995). Cajal proposed that astrocytes might be involved in separating neurons by extending processes into the synapses, and normal activity restored upon their retraction (Cajal, 1995). Based upon clinical observations of patients with Encephalitis Lethargica or sleeping sickness, Mauthner postulated that the periventricular gray (PVG) of the hypothalamus was associated with sleep function. He suggested that inflammation of the PVG resulted in a break or an interruption between the cortex and lower brain structures. In normal individuals, he proposed that sleep was the result of a natural cessation of activity in PVG, which would prevent the cortex from receiving sensory signals. Interestingly, Mauthner also suggested that cortical activity could be maintained during sleep by dreaming. However, deafferentation prevented output commands from the cortex, which

explained the lack of movement during sleep (reviewed in Economo, 1930; Kleitman, 1963). Another hypothesis which had a major influence in later studies was that introduced by Pavlov. Based upon his observation that dogs became drowsy during a delayed conditioned stimulus, he maintained that sleepiness was the result of cortical inhibition. That is, localized inhibition in the cortex, whether from exhaustion or lack of sensory input, diffuses rapidly and causes sleep (Economo, 1930; Kleitman, 1963).

To test directly the hypothesis that sleep was associated with a decrease or inhibition of afferent input, Bremer transected the midbrain just posterior to the oculomotor nucleus, completely eliminating all sensory input to the cortex with the exception of olfactory and visual afferents. This *cerveau isolé* preparation produced animals that exhibited a non-reversible sleep-like state (Bremer, 1935). Bremer concluded that sleep was indeed a function of diminished sensory input to the cortex, and that olfactory and visual sensation alone were not sufficient to maintain wakefulness. In a subsequent experiment, Bremer observed that animals with a transection of the lower medulla exhibited sleep patterns similar to those of intact animals. Based upon this observation, he suggested that in this *encéphale isolé* preparation, sensory afferents from the face and head were sufficient to maintain wakefulness (Bremer, 1938). These findings were later replicated in other species by many other investigators, and became standard preparations for a variety of experimental paradigms. However, using these preparations, Lindsley et al. (1949) showed that sensory deafferentation, regardless of what the mechanism may be, was not an adequate explanation of the sleep state.

With respect to mechanisms of sleep, a second important issue addressed by Magoun and co-workers was the existence of a "sleep center." The underlying concepts of the studies described above assumed that sleep was a passive process, such that it was a physiological

condition caused by long periods of activity, where a change in the system no longer allowed sensory processing or consciousness. Few early investigators had suggested a sleep center or an anatomical locus which actively produced the state. One of the first advocates of this hypothesis was Constantin von Economo. Based upon his studies of the post-mortem brains of patients who died from sleeping sickness during the epidemic of the early 1900's, Economo associated the floor of the third ventricle with sleep function. He expanded upon the notion first introduced by Mauthner, such that destruction of the anterior portion resulted in complete insomnia, while the posterior portion was associated with hypersomnia, the more common symptom found in patients with the sleeping sickness (Economo, 1930). In normal subjects, he proposed that hypnotoxins (a concept first introduced in the 18th century) circulating in the blood stimulated the sleep center, subsequently producing inhibition of the thalamus and cortex in a manner described by Pavlov. In the 1920's, Hess found that electrical stimulation anywhere within the region delimited by the habenulo-interpeduncular tract as the caudal boundary and the mammillothalamic bundle as the rostral boundary, induced a reversible sleep-like state in cats (Hess, 1954). He suggested that activation of the diencephalic center corresponded to the onset of sleep and maintained the state by inhibition of cortical activity. With the discovery of the ARAS, Hess modified his model by proposing a reciprocal interaction between the diencephalic sleep center and the ARAS as the center for arousal. Maximal activation of either system produces a corresponding maximal response at the level of behavior--deep sleep in the case of the diencephalon, and alertness or readiness for aggressive action in the case of the ARAS. He suggested a reciprocal antagonism between the two centers, such that the balance of activation between the two systems determined intermediate states such as relaxation, drowsiness, or light sleep. Although part of Hess' sleep center overlapped with that proposed by Economo, it was later demonstrated that rhythmic

stimulation in a variety of brain regions, or even peripheral afferents, elicited sonorous effects (cf. Kleitman 1963; Jouvet, 1972). Hess' work therefore did not have any localizing significance, rather he was the first to propose that sleep was an *active* process in which inhibition was secondary to a sleep center activation.

The work of Hess had sparked the important controversy mentioned above. Bremer, even after the 1949 papers were published, continued to maintain that sleep was a passive process which involved primarily deafferentation. Interestingly, despite the overwhelming evidence for a brainstem mechanism for arousal derived even from his own experiments as well as from those of Moruzzi and Magoun (1949), he advocated a hypothesis which appeared to be influenced by the dendritic retraction theory described above:

The physiological process of falling asleep may be explained, without necessary recourse to the hypothesis of a hypnogenic centre, by the cumulative de-activation (de-facilitation) of the encephalic neuronal networks resulting from synaptic fatigue and favoured by a reduction in the exteroceptive and proprioceptive sensory afflux. In this process of neuronal de-activation which culminates in sleep, the functional slackening of the brain stem reticular formation, by reason of the latter's central situation in the nervous apparatus of arousal, plays without doubt an essential role. The hypothesis of a hypnogenic centre cooperating with the waking apparatus in the regulation of sleep is a logical assumption, but one whose adoption still raises theoretical and technical difficulties. (Bremer, 1954)

### *The Reticular Formation*

Finally, the third important issue addressed in Magoun's work was the function of the brainstem reticular formation. The morphology and some of the connecting pathways of the reticular formation had been well described by Cajal and others (Cajal, 1995; reviewed in Brazier, 1980; Jones, 1995). Although Cajal had reported ascending and descending projections of reticular neurons, many of their destinations were unknown. Because of the extensive inputs from sensory and motor collaterals to the reticular formation, it was believed that it served as a "coordinating apparatus" (Kappers et al., 1960) for incoming motor and sensory information. Based upon studies using electrical stimulation, others had previously reported ascending

influences of the brainstem upon cortical EEG (Morison et al., 1941; Dempsey and Morison, 1943; Jasper, 1948); however, this effect was attributed solely to the stimulation of sensory collaterals. The importance of this earlier work was rather a demonstration of the widespread origin of projections to the cortex. The aim of the study by Moruzzi and Magoun (1949) was therefore to describe the effect of the reticular formation, itself, upon the cortical EEG. They were the first to suggest that the reticular system was involved in controlling an arousal response. As reviewed in Steriade and McCarley (1990), Moruzzi and Magoun (1949) evaded the issue of whether sleep was an active or passive process. Rather, they suggested that an activation threshold of the ARAS determined the arousal state of the animal.

#### *Brainstem Mechanisms of Sleep and Arousal*

EEG desynchronization had been observed in waking as well as in normal sleep by several investigators as early as the 1930's (Derbyshire et al., 1936; Rheinberger and Jasper, 1937). This phenomenon was ignored until rapid-eye movement sleep (REM) and its associated features were described in both humans and animals in a series of papers that came out in the 1950's (Aserinsky and Kleitman, 1953; Dement and Kleitman, 1957; Dement, 1958; Jouvet and Michel, 1959). It became well established that there exist two kinds of sleep in mammals. The first type--quiet or slow-wave sleep (SWS)--was associated with EEG synchronization (high voltage, slow wave activity), spindle activity (bursts of high frequency, large amplitude waves), low muscle tone, and slow rolling eye movements. The second type (REM sleep) was associated with EEG desynchronization, muscle atonia, rapid eye movements, and phasic electrical events which could be recorded simultaneously in the pons, lateral geniculate nucleus, and occipital cortex (PGO waves; Brooks and Bizzi, 1963). The discovery of REM sleep led to the hypothesis that multiple brain structures were involved in controlling waking and the two-stage process of sleep.



Two approaches were used to address this hypothesis. One approach involved the identification of the locus of the controlling mechanism for sleep and wakefulness through brainstem transection and selective lesioning. An important discovery by Jouvet (1962) was that in the *cerveau isolé* preparation, not only were the signs of REM detected caudal to the transection, but REM sleep also occurred with the same periodicity as that observed in intact animals. On the other hand, when transections were placed more caudally at the level of the genu of nerve VII dorsally and ventrally at the trapezoid body, REM sleep could be detected as in intact animals, with the exception of muscle atonia. Taken together, these data suggested that brainstem structures delimited by the transections described above were responsible for controlling REM sleep, and that the more rostral structures were required for waking. In support of this hypothesis, Jouvet had observed that stimulation of the nucleus reticularis pontis caudalis could induce a state that was similar to REM. Data supporting a center for SWS were less conclusive. Batini et al. (1959) observed that transection posterior to the trigeminal nucleus resulted in an increase in wakefulness, and suggested that "synchronizing" centers were localized in the lower brainstem. However, more selective lesioning did not support this hypothesis (Bonvallet and Bloch, 1961; Camacho-Evangelista and Reinoso-Suárez, 1964).

The second approach involved the neurochemical aspects of sleep. Based upon the observations that behavioral states were determined by a multiplicity of physiological components such as cortical EEG, muscle tonus, eye movements, and PGO waves, that they occurred in a manner that approximated a circadian rhythm; and that selective deprivation of REM resulted in a rebound, Jouvet suggested that electrophysiological mechanisms alone were no longer sufficient to account for these phenomena (1962;1969;1972). He therefore strongly advocated the concept of "wet neurophysiology," as necessary to any theory of sleep such that drug application or more

selective lesioning should be aimed at delineating the precise roles of a specific neurochemical system. Humoral theories of sleep had existed for centuries (for review, see Kleitman 1963). Among the most widely accepted was that proposed by Piéron, who proposed that hypnotoxins accumulated in the blood with more time spent in waking, and were excreted during sleep. Subsequent searches for such “toxins” or sleep-inducing agents in the blood or cerebral spinal fluid have generated intriguing results, but they remain inconclusive (Cravatt et al., 1995 and references therein).

Identification of neurotransmitters in discrete neuronal populations provided major advances for a neuropharmacological approach. Using fluorescence histochemistry combined with pharmacology, Dahlström and Fuxe (1964) localized catecholaminergic and serotonergic cell groups in the brainstem. They determined that serotonin-containing neurons were in the dorsal and medial raphe; noradrenergic neurons were located in the medulla oblongata near the nucleus solitarius as well as in the lateral tegmentum of the pons in the locus ceruleus (LC); and that dopamine-containing neurons were located more rostrally in the substantia nigra and in the lateral and ventral tegmentum of the midbrain. Using acetylcholinesterase (AChE) histochemistry, Shute and Lewis (1967) identified an ascending cholinergic reticular system and suggested that it was a key component of the ARAS. The development of antibodies directed against choline acetyltransferase (ChAT), the enzyme responsible for the synthesis of acetylcholine (ACh), allowed more definitive mapping of cholinergic neurons, as AChE was contained in both cholinergic and noncholinergic neurons. Likewise, the histofluorescence used by Dahlström and Fuxe was limited to non-specific identification of monoamine-containing neurons, although they used a pharmacological approach to reduce stores of one or more of the monoamines to determine the localization of each type. Later development of antibodies directed against

enzymes involved in the synthetic pathway of each monoamine or against the transmitter itself confirmed localization of each of the monoamines (Nieuwenhuys, 1985). Nonetheless, these two papers offered pioneering steps in applying a neurochemical identity to the brainstem reticular formation and the ARAS.

A plethora of work has since suggested roles of both the monoaminergic and cholinergic systems in arousal mechanisms. Based upon a combination of lesion and pharmacological experiments, Jouvet (1962; 1969) proposed that serotonin (5-HT) was necessary for the induction and maintenance of SWS, while catecholamines (noradrenaline (NA) in particular) and ACh were necessary for REM sleep. Support for this hypothesis derived from the observation that lesion of the median and dorsal raphé nuclei had produced insomnia in cats; while intravenous or intraperitoneal injections of a metabolic precursor of 5-HT, 5-hydroxytryptophan, induced SWS. Reserpine, which depletes monoaminergic stores, also suppressed SWS. Finally, *p*-chlorophenylalanine, which also antagonizes serotonergic transmission, decreased both SWS and REM sleep. The evidence supporting ACh in REM sleep was derived from observations that 1) atropine, a cholinergic receptor antagonist, inhibited REM sleep (Jouvet, 1962); 2) eserine, an anti-AChE agent, enhanced REM sleep in a *cerveau isolé* preparation (Jouvet, 1962); and 3) microinjection of cholinergic agonists in the region of the mPRF triggered a state that was indistinguishable from REM sleep (Cordeau et al., 1963; George et al., 1964). Jouvet (1969) suggested, primarily on the basis that lesions of the LC suppressed REM sleep, that NA was important for the maintenance of REM sleep.

However, the monoaminergic hypothesis was later refuted by extracellular unit recordings in the raphé nuclei (McGinty and Harper, 1976; Trulson and Jacobs, 1979) and in the LC (Chu and Bloom, 1973; Hobson et al., 1975; Aston-Jones and Bloom, 1981), which showed that these

cells exhibited highest firing rates during wake. In addition, other investigators had reported increased PGO activity in raphé lesioned or in reserpinized animals, and suggested that 5-HT may play an inhibitory role upon this component of REM sleep (Simon et al., 1973). The role of ACh in REM became more firmly established, as several studies confirmed the finding that application of cholinergic agonists in the mPRF triggered a REM sleep-like state in both cats and rats (Baxter, 1969; Mitler and Dement, 1974; Amatruda et al., 1975; Gnadt and Pegram, 1986). That monoamines inhibit the cholinergic system, in particular, was first suggested by Karczmar et al. (1970) who showed that administration of eserine could promote REM sleep, but only after animals had been reserpinized. Based upon these findings, as well as upon the observation that neurons in the gigantocellular tegmental field (FTG), which contains AChE-positive cells, fire selectively during REM sleep (Hobson et al., 1975), McCarley and Hobson (1975) proposed the reciprocal interaction hypothesis of behavioral state control and further elaborated upon the monoaminergic inhibition of the brainstem cholinergic system (see next chapter).

In summary, by the mid-1970's at least three neuronal candidates of the ARAS had been identified, including the brainstem noradrenergic, serotonergic, and cholinergic systems. It had also become widely accepted that sleep was indeed an active process, as first suggested by Hess 50 years earlier, which involved the brainstem monoaminergic and cholinergic systems. Although no single "sleep center," as originally proposed by Economo and Hess, had been identified, multiple substrates for each of the different components which define behavioral state, including muscle tone, PGO activity, eye movements, and EEG have been suggested.

Historically, the approach to questions which address the neurobiological mechanisms of behavioral state control has focused upon *identifying* neuronal candidates for the ARAS. More recently, research has been directed toward delineating the precise anatomical, pharmacological,

and behavioral profiles of each of these systems using more advanced technology. The work presented in this thesis involves the use of both *in vitro* patch clamp and *in vivo* microdialysis techniques combined with histochemical and immunocytochemical staining to characterize some of the biophysical, pharmacological, and behavioral properties of mesopontine cholinergic neurons. An additional feature of this system which has only recently been addressed with respect to behavioral state, is the co-localization of nitric oxide synthase (NOS) in these cells (Vincent et al., 1983). Several other neurotransmitter substances are known to co-localize in some of these cholinergic neurons, or in non-cholinergic neurons as well. These include glutamate, substance P, corticotropin releasing factor, bombesin/gastrin releasing peptide, and the atrial and brain natriuretic peptides which are found in all cholinergic cells (Clements and Grant, 1990; Clements et al., 1991; Vincent et al., 1983a, Vincent et al., 1986; Sutin and Jacobowitz, 1988; Standaert et al., 1986; Saper et al., 1989). In order to address a potential role of nitric oxide (NO) in behavioral state, chapter IV is devoted to measuring NO production from the mesopontine NOS/cholinergic terminals in the rat thalamus using the hemoglobin trapping technique combined with *in vivo* microdialysis.

## II.

### **Noradrenaline Hyperpolarizes Identified Mesopontine Cholinergic Neurons *in vitro***

#### **INTRODUCTION**

The most widely-accepted model of rapid-eye movement (REM) sleep generation is that release of acetylcholine within the medial pontine reticular formation (mPRF) is one, if not the natural trigger for REM sleep generation. This is based upon the well-established observation that microinjection of cholinergic agonists into the mPRF induces a state indistinguishable from REM sleep (Amatruda et al., 1975; Baxter, 1969; Mitler and Dement, 1974). That amines inhibit this cholinergic trigger was suggested by the observation that systemic administration of the anticholinesterase agent eserine induces a state indistinguishable from REM sleep, but only after depletion of amines by reserpine (Karczmar et al., 1970). Based upon extracellular recordings of neurons selectively active during REM sleep in the gigantocellular tegmental field (FTG) where acetylcholinesterase-positive neurons are found, and of neurons which fall silent during REM sleep in the noradrenergic locus ceruleus (Hobson et al., 1975) and serotonergic dorsal raphe nuclei (McGinty and Harper, 1972), McCarley and Hobson (1975) proposed the reciprocal interaction hypothesis: that the sleep cycle is controlled by an inhibitory aminergic population and a reciprocally excitatory cholinergic population, in which the silence of aminergic neurons would disinhibit cholinergic neurons and thereby evoke REM sleep by release of acetylcholine in the mPRF.

The striking behavioral neurophysiological profile of noradrenergic and serotonergic neurons has been fully confirmed in numerous studies (Chu and Bloom, 1973; Aston-Jones and Bloom, 1981; McGinty and Harper, 1976; Trulson and Jacobs, 1979). However, two features of the original reciprocal-interaction model were subsequently shown to be invalid. First, the REM

selectivity of FTG neuronal activity was found to be an artifact of head restraint (Siegel and McGinty, 1977; Vertes, 1977). Second, the acetylcholinesterase-positive neurons of the gigantocellular tegmental field were found not to be cholinergic when studied with choline acetyltransferase immunohistochemistry (Jones and Beaudet, 1987; Vincent and Reiner, 1987; Shiromani et al., 1988). However, these same studies unambiguously demonstrated that the neurons of the laterodorsal (LDT) and pedunculopontine tegmental nuclei (PPT) represent the predominant group of brainstem cholinergic neurons, and these neurons give rise to a massive innervation of the thalamus (Hallanger and Wainer, 1988; Satoh and Fibiger, 1986; Sofroniew et al., 1985; Steriade et al., 1988; Woolf and Butcher, 1986) as well as direct projections to the mPRF (Mitani et al., 1988; Quattrochi et al., 1989; Shiromani et al., 1988; Jones, 1990; Semba et al., 1990; Semba, 1993). Unfortunately, the behavioral neurophysiology of mesopontine cholinergic neurons is still not known with certainty. A small percentage of neurons in this region exhibit REM-selective discharge patterns, but the majority do not (Steriade et al., 1990b; El Mansari et al., 1989). Based upon these data Steriade et al. (1990b) have concluded that cholinergic neurons are active during both wake and REM sleep, while other authors have argued otherwise (Kamondi et al., 1992; Sakai, 1988). Barring more direct evidence, the behavior of mesopontine cholinergic neurons across states cannot be stated with complete confidence (see following chapter). Nonetheless, both the LDT and PPT contain neurons which are at least active during REM, and are thus candidates for being the cholinergic neurons which trigger the state of REM sleep.

Given these observations, the original reciprocal-interaction hypothesis has been considerably modified by various groups (Hobson et al., 1986; Sakai, 1988; Steriade and McCarley, 1990). One explicit prediction of all these models is that noradrenaline (NA) and

serotonin (5-HT) inhibit mesopontine cholinergic neurons. A previous study showed that serotonin inhibits identified cholinergic neurons (Luebke et al., 1992). Utilizing whole-cell patch clamp recordings of LDT neurons in rat brain slices, we sought to test the other arm of this hypothesis, that noradrenaline hyperpolarizes mesopontine cholinergic neurons.

## **MATERIALS AND METHODS**

### *Brain Slice Preparation for Patch Clamp Recording in vitro*

Brain slices from rats were prepared using standard techniques. Rats 7-15 days old were anesthetized with halothane, decapitated, the brain rapidly removed and immersed in cold artificial cerebro-spinal fluid (ACSF). The brain was trimmed to a block containing the pontomesencephalic tegmentum and cut into 400  $\mu$ m coronal sections with a Vibratome. Usually, two slices containing the LDT were obtained from each brain. Slices were stored in a holding chamber for at least one hour before being transferred to a recording chamber where they were superfused with ACSF at 2 ml per minute. ACSF contained (in mM) 126 NaCl, 25 NaHCO<sub>3</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 2.5 KCl, 2.5 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 11 glucose, pH 7.4, when saturated with 95% O<sub>2</sub>, 5% CO<sub>2</sub>. All experiments were carried out at room temperature.

Drugs were applied by superfusing the slice in ACSF containing a given concentration of the drug. Except for idazoxan and barium, drugs were stored in frozen aliquots at high concentrations, and were thawed and diluted in ACSF prior to the experiment. NA, phenylephrine, and isoproterenol were obtained from Sigma Chemical Co.; idazoxan and UK-14,304 from Research Biochemicals, Inc., and clonidine from Boehringer Ingelheim, Ltd.

Patch pipettes were constructed from thin wall (1.5 mm o.d., 1.1 mm i.d.) borosilicate glass (Sutter Inst. Co.). The electrode solution contained (in mM) 15 NaCl, 10 Na-HEPES, 11



EGTA, 140 KOH, 1 CaCl<sub>2</sub>, 2 MgATP, 0.3 GTP, 2% biocytin, and was pH-balanced with methane-sulfonic acid to 7.4. Electrode resistance ranged from 4-6 M $\Omega$  in the recording chamber, typical electrode seals were 9-12 G $\Omega$ , and access resistance following establishment of the whole cell recording configuration ranged from 10 to 80 M $\Omega$ . Both bridge mode and continuous single electrode voltage clamp recordings were obtained with an Axoclamp-2A amplifier. For voltage clamp experiments, gains were from 5-10 nA/mV, and the output filter reduced from 30 to 1 kHz; neither capacitance or series resistance compensation were utilized. To block voltage-dependent sodium currents, 300 nM tetrodotoxin (TTX) was routinely added to the ACSF. Data were collected through an Axolab interface using pCLAMP computer software, version 5.0 (Axon Instruments) and recorded onto videotape in a digitized format. Data are reported as the mean  $\pm$  standard deviation.

#### *Histochemical Identification of Recorded Neurons*

In order to unambiguously identify biocytin-filled neurons, only one cell was obtained from each side of a slice. Slices were nicked on one side during preparation to differentiate one side from the other. After experiments were complete, each slice was fixed overnight in 2% paraformaldehyde and 15% picric acid in 0.1 M phosphate buffered saline (PBS), pH 7.4. The following day, slices were removed from the fixative and rinsed three times for twenty minutes in 0.1 M PBS. Slices were then incubated in PBS containing 20  $\mu$ g/ml Texas-red-conjugated avidin and 0.3% Triton-X for two hours, rinsed in PBS as before, and left in a Triton/PBS solution with 15% sucrose overnight. The next day slices were cut into 40-50  $\mu$ m sections with a cryostat and mounted onto coated slides.

Cholinergic neurons were identified using NADPH-diaphorase histochemistry, a reliable marker of LDT cholinergic neurons (Vincent et al., 1983). Slides were immersed in a solution

containing 1 mg/ml NADPH and 0.1 mg/ml nitroblue tetrazolium in PBS and incubated at 37° C for 45-60 minutes. Afterwards, slices were rinsed in PBS and stored in the dark to dry. Slides were coverslipped and examined under a microscope equipped with both brightfield optics and epifluorescence for identification of NADPH-positive and Texas-red-positive neurons, respectively.

## RESULTS

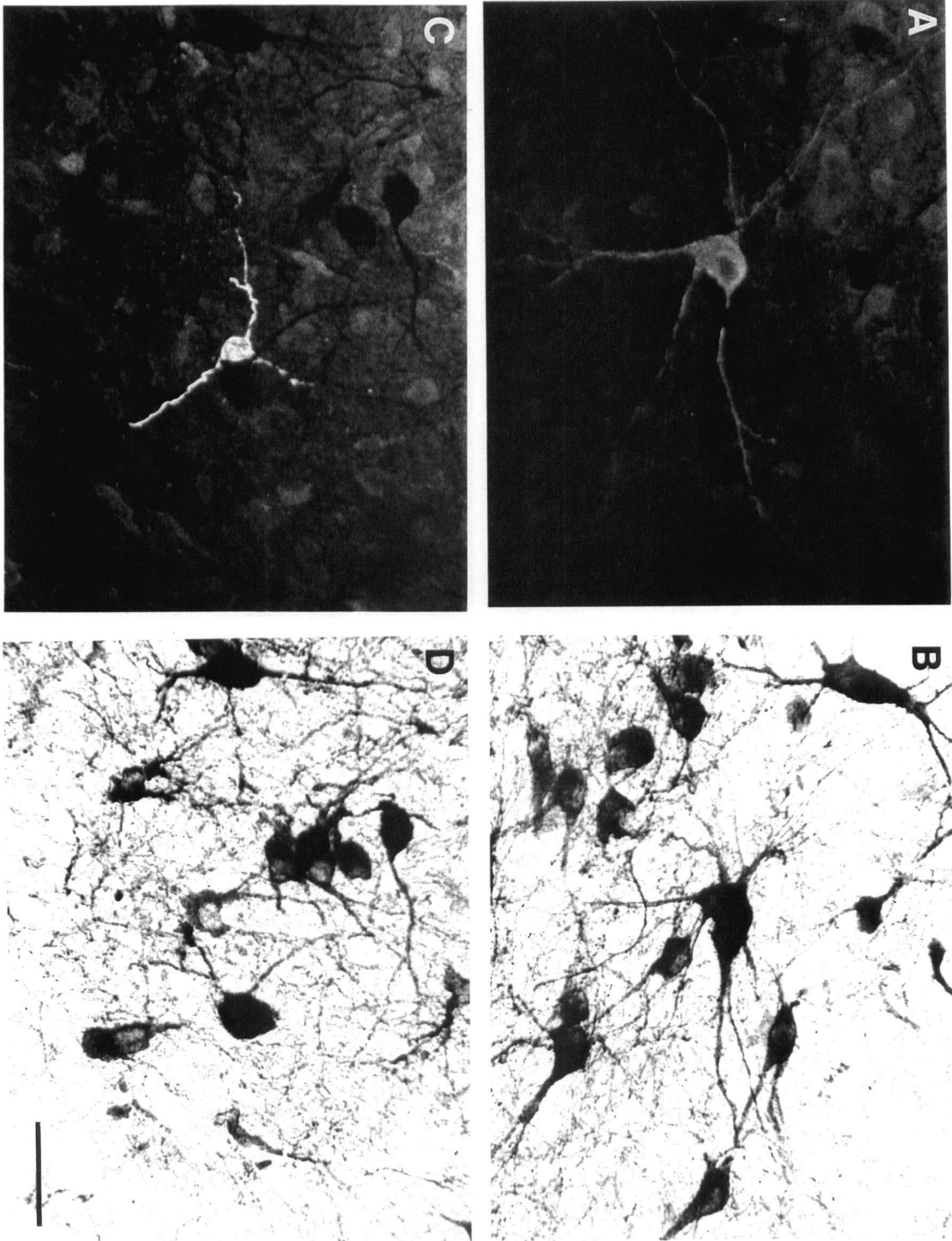
### *Identification of Cholinergic and Non-cholinergic Neurons.*

This study is based upon whole-cell patch clamp recordings of 175 cells in the LDT. Successful intracellular labelling with biocytin was obtained in 96 cells, and these were all processed for NADPH-diaphorase histochemistry. Forty-one percent (39 of 96) of biocytin-labelled cells were NADPH-diaphorase positive and thus identified as cholinergic. The remaining cells (57 of 96, or 59%) were non-cholinergic (Figure 1). Characterization of the membrane properties of LDT neurons are based upon the histochemical identification of the first 30 cells used in this study. The focus of the pharmacological results reported below is largely upon the identified cholinergic neurons (n=39).

### *Biophysical properties of cholinergic and noncholinergic LDT neurons*

Similar to previous observations (Luebke et al., 1992; Leonard and Llinás, 1990), some LDT neurons exhibited a burst of action potentials at the offset of a hyperpolarizing current pulse in bridge mode, while others did not. Based upon the intrinsic ionic conductances underlying these firing patterns, three classes of neurons were identified in the LDT (Kamondi et al., 1992). The classification scheme used is similar to that reported previously (Leonard and Llinás, 1990). Type I neurons exhibited a burst of action potentials on the crest of a depolarizing wave at the

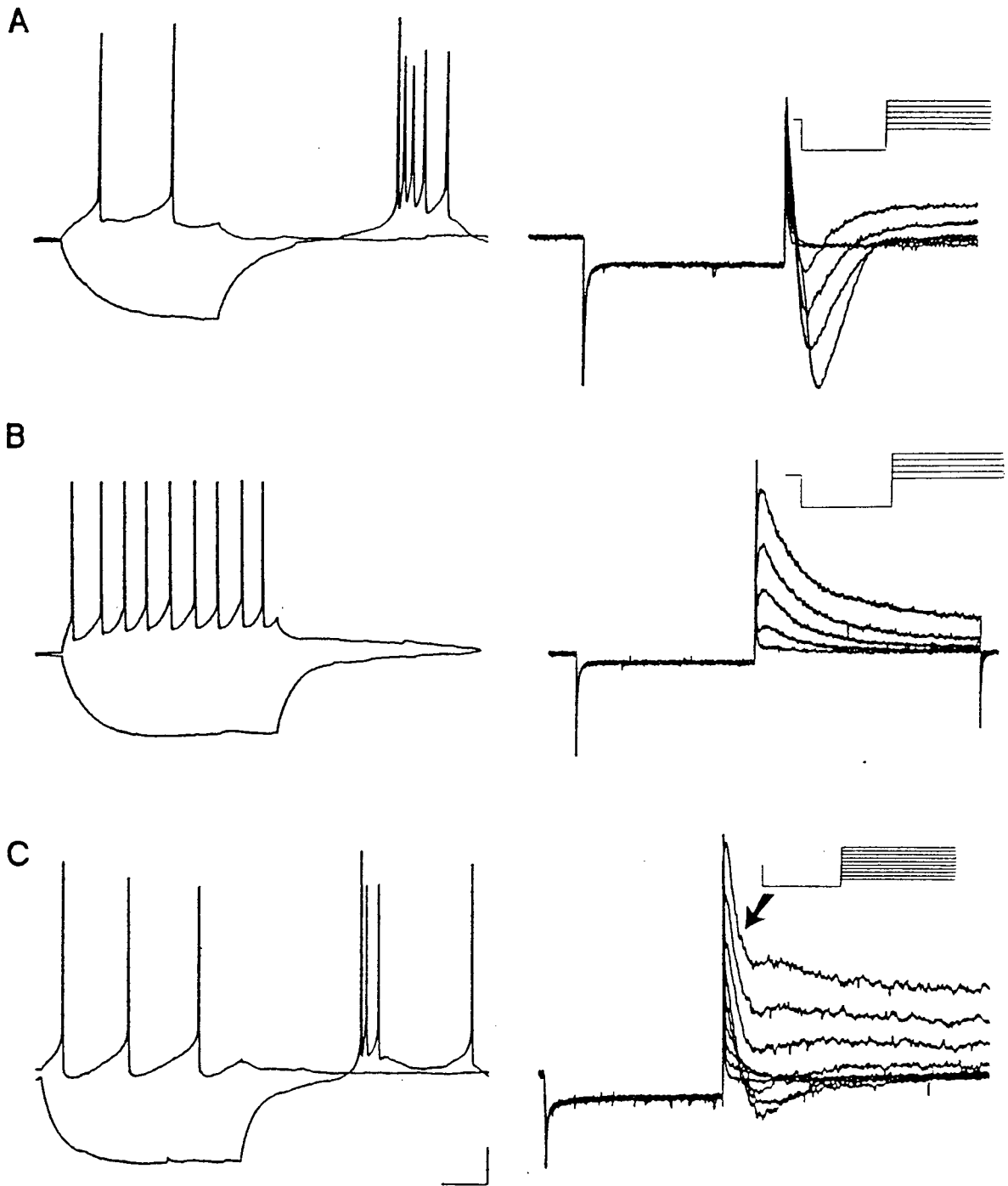
Figure 1. Histochemical identification of laterodorsal tegmental nuclei. A,C. Biocytin-filled neurons. B. NADPH-diaphorase histochemistry shows that the labelled neuron in A is NADPH-positive and therefore cholinergic. D. The labelled cell in C did not stain for NADPH and is therefore non-cholinergic. Scale bar = 25  $\mu$ m for all four plates.



offset of a hyperpolarizing current pulse (Figure 2A). When studied in voltage clamp, this depolarization was mediated by a rapidly inactivating inward current which exhibited both voltage and time-dependence for removal of inactivation as well as a steep voltage-dependent activation. In addition, this current was sensitive to changes in extracellular calcium and was blocked by addition of 100  $\mu$ M nickel to the ACSF. These properties are characteristic of the low-threshold, rapidly inactivating inward calcium current,  $I_T$  (Nowycky et al., 1985; Carbone and Lux, 1987). Type II neurons were the "non-bursting" cells, and exhibited a delay in the return to the resting membrane potential at the offset of a hyperpolarizing current pulse (Figure 2B). Voltage clamp recordings revealed a rapidly inactivating outward current at the offset of hyperpolarizing voltage steps. This current exhibited voltage and time-dependence for removal of inactivation as well as a voltage-dependent activation, and was blocked by addition of 2 mM 4-aminopyridine. These properties are characteristic of the transient outward potassium current,  $I_A$  (Rogawski, 1985; Rudy, 1988). Type III neurons also exhibited burst activity, but expressed both  $I_T$  and  $I_A$  (Figure 2C).

Out of 30 cells, combined staining using biocytin as an intracellular marker and NADPH-diaphorase revealed that 18 of the 30 cells studied were double-labelled and therefore cholinergic. As shown in Figure 3, 16/30 of LDT neurons were type III. This observation can be taken to suggest that type III neurons are the predominant cell type in the LDT. However, the possibility that this class of neurons is more easily recorded because of the use of the whole-cell patch-clamp technique per se cannot be discounted. Correlation of physiologically defined cell types with transmitter status revealed that 40% (2/5) of type I, 33% (3/9) of type II, and 81% (13/16) of type III neurons were cholinergic. Thus type III neurons represent the dominant cholinergic cell type in the LDT. These results are consistent with other findings which showed that the majority

Figure 2. Basic electrophysiological characteristics of 3 types of laterodorsal tegmental (LDT) neurons. A. Type I neurons studied in bridge mode (left) exhibited a burst of action potentials riding on the crest of a depolarizing wave at the offset of a 100-pA hyperpolarizing current command. A depolarizing current step of 100-pA evoked repetitive action potentials. Resting membrane potential, -56 mV. In voltage clamp (right), the current underlying the depolarizing wave seen on the offset of the hyperpolarization in bridge mode appeared as a transient inward current,  $I_T$ . B. In type II cells, at the offset of a sufficiently large hyperpolarizing current step (-30 pA), the membrane potential returned to the baseline with a delay. A depolarizing current step (30 pA) from the resting potential of -48 mV evoked a train of action potentials. In voltage-clamp mode (right), voltage commands from a conditioning hyperpolarized potential to more depolarized voltages resulted in a transient outward current,  $I_A$ . C. Type III LDT neurons exhibited both  $I_A$  and  $I_T$ . In bridge mode (left) this cell exhibited a delayed return to the resting potential of -57 mV followed by a depolarizing overshoot with a burst of action potentials after a hyperpolarizing current step of -80 pA. In voltage clamp (right), both a rapidly decaying transient outward current ( $I_A$ ) and a transient inward current could be observed. Calibration: 200 ms, 200 pA, 20 mV.



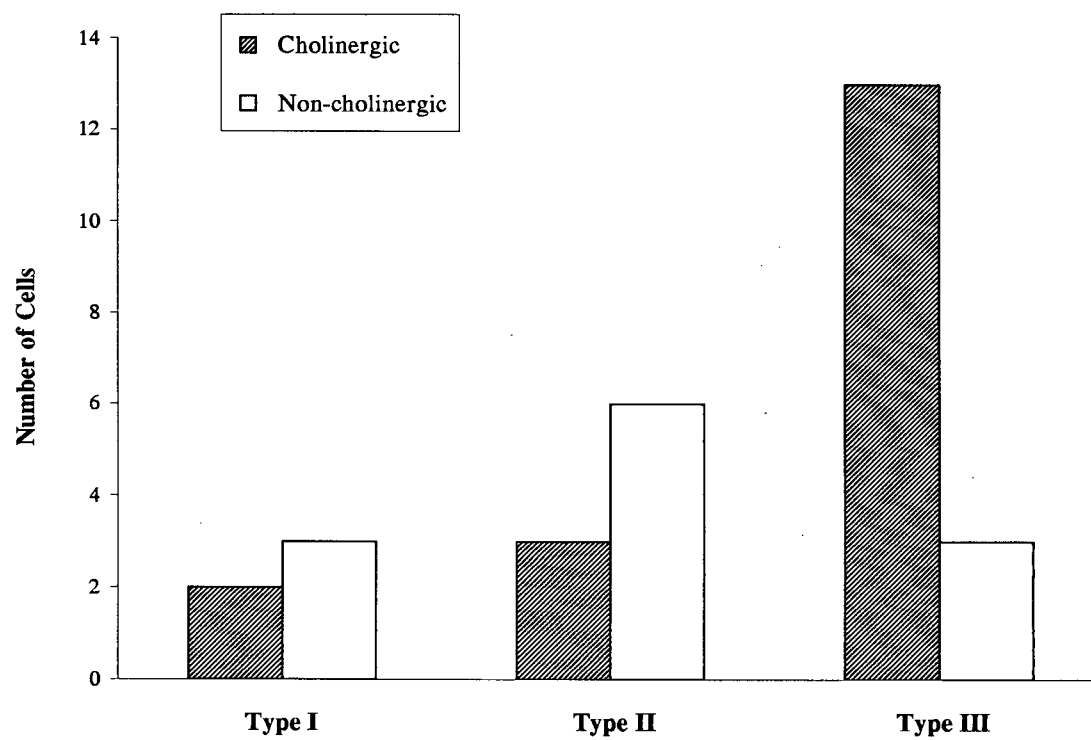


Figure 3. Frequency histogram of cholinergic and noncholinergic laterodorsal tegmental neurons correlated with physiological properties.



of identified cholinergic neurons recorded with the patch clamp technique *in vitro* exhibited burst firing properties (Luebke et al., 1992; 1993), as well as with the observation that populations of LDT neurons exhibited bursting activity *in vivo* (Steriade et al., 1990b; 1990c; El-Mansari et al., 1989; see General Discussion).

*NA Hyperpolarizes LDT Cholinergic Neurons.*

Ninety-two percent (36/39) of identified cholinergic neurons hyperpolarized by  $10.1 \pm 4.1$  mV in response to 50  $\mu$ M NA. In contrast, non-cholinergic neurons exhibited heterogeneous responses to NA--35% [20/57] hyperpolarized, 46% [26/57] depolarized, and 19% [11/57] did not respond (Figure 4). The hyperpolarization of cholinergic neurons was characterized by an increase in conductance, as illustrated in Fig. 5A and 5B. When the cell hyperpolarized by NA was returned to the resting potential by injection of depolarizing current, the input resistance was decreased as evidenced by smaller voltage deflections during the hyperpolarizing pulses as compared to control. Thus, NA hyperpolarizes cholinergic neurons by increasing an ionic conductance.

We next carried out experiments to determine if the noradrenergic effect was direct. To test this, two paradigms were utilized. First, in the majority of cases ( $n=27$ ), 300 nM TTX was included in the bath solution to block voltage-dependent sodium channels. The noradrenergic hyperpolarization always persisted under such conditions. Second, some slices ( $n=4$ ) were bathed in ACSF with low  $\text{Ca}^{2+}$  and high  $\text{Mg}^{2+}$  concentration (0.5 mM and 10 mM, respectively) in addition to TTX to abolish  $\text{Ca}^{2+}$ -dependent synaptic transmission. The low- $\text{Ca}^{2+}$  ACSF abolished the low threshold  $\text{Ca}^{2+}$  spike normally seen after a 500 ms hyperpolarizing pulse (Figure 5C), thus ensuring a minimal synaptic  $\text{Ca}^{2+}$  influx and thereby blocking synaptic transmission. Responses to

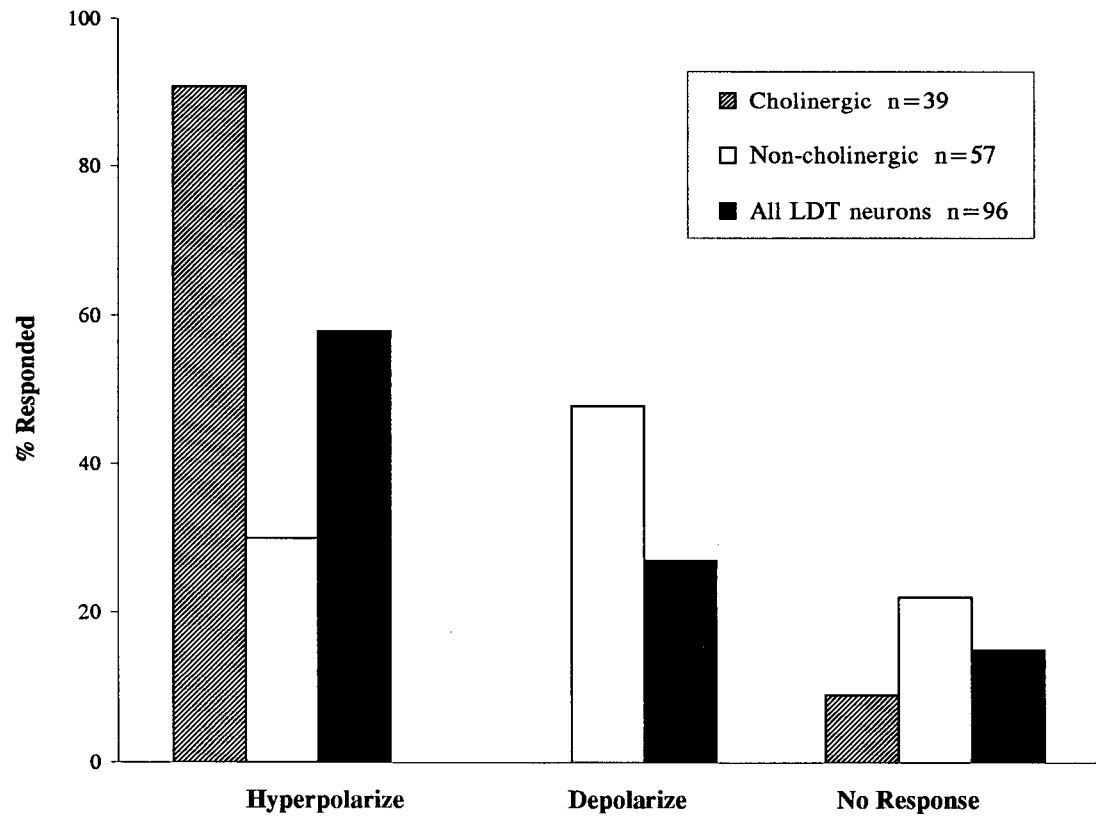


Figure 4. Distribution of effects of NA upon laterodorsal tegmental neurons. The majority of cholinergic neurons hyperpolarized to NA, while non-cholinergic neurons exhibited heterogeneous responses to NA. Approximately half (58%) of all LDT neurons studied hyperpolarized to NA.

NA in low  $\text{Ca}^{2+}$  were identical to those seen in the normal solution, which indicates that the noradrenergic effect is indeed direct (compare Fig. 5B with 5A).

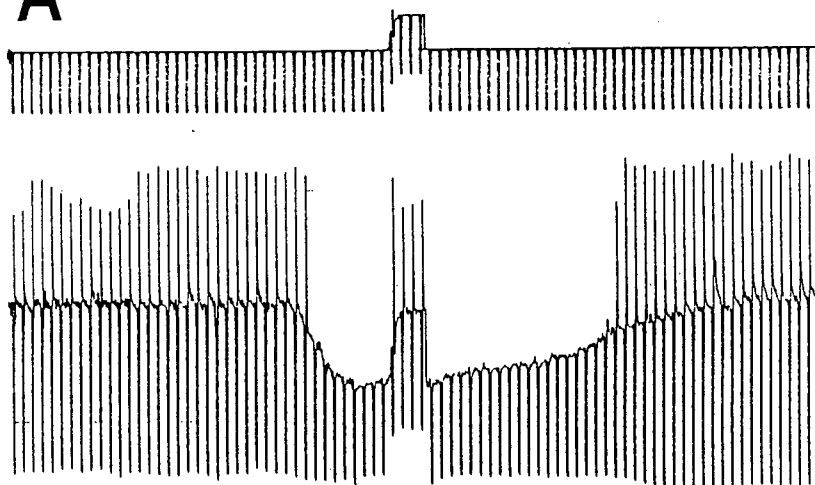
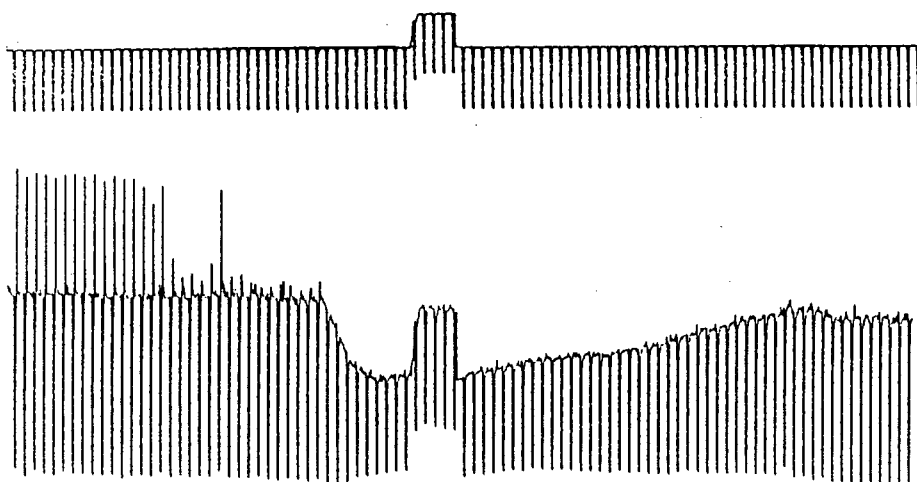
#### *Noradrenergic Hyperpolarization is Mediated by $\alpha_2$ Receptors*

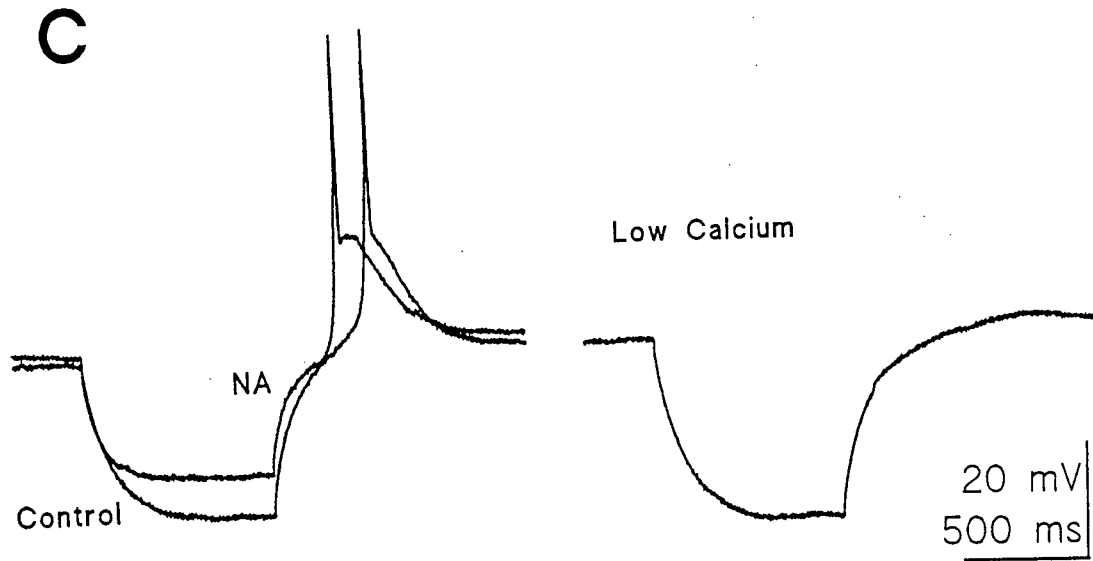
Application of 1  $\mu\text{M}$  idazoxan, an  $\alpha_2$  adrenergic antagonist, completely blocked the hyperpolarizing effect of NA on 5/8 cholinergic cells and reduced the hyperpolarization in the other three cells by  $2.3 \pm 0.5$  mV. Interestingly, in 3 of the 5 cells in which the hyperpolarization was abolished by idazoxan, NA induced a depolarization of  $5.0 \pm 1.6$  mV when  $\alpha_2$  receptors were blocked (Fig. 5D). Thus NA may have an additional effect on cholinergic neurons mediated by receptors other than the  $\alpha_2$  receptor. However, these experiments were not carried out in low  $\text{Ca}^{2+}$  ACSF, and therefore we do not know if the depolarization is a direct effect of NA. Both the  $\alpha_1$  agonist phenylephrine (10  $\mu\text{M}$ ,  $n=2$ ) and the  $\beta$  agonist isoproterenol (5  $\mu\text{M}$ ,  $n=1$ ) elicited no response from cells that exhibited a large hyperpolarization to NA. UK-14,304, a full  $\alpha_2$  adrenoceptor agonist (Cambridge, 1981), induced a hyperpolarization of  $5.8 \pm 2.3$  mV on cholinergic neurons (10  $\mu\text{M}$ ,  $n=3$ ). Clonidine, a partial  $\alpha_2$  adrenoceptor agonist (Medgett et al., 1978), had no effect on cholinergic neurons that were hyperpolarized by NA (1-10  $\mu\text{M}$ ,  $n=6$ ). Because clonidine has also been reported to act as a competitive  $\alpha_2$  antagonist, we tested its ability to block the noradrenergic hyperpolarization. It did not ( $n=3$ ). We therefore concluded that NA exerts its hyperpolarizing effect through a clonidine-insensitive  $\alpha_2$  adrenoceptor.

#### *NA Activates $I_{KG}$*

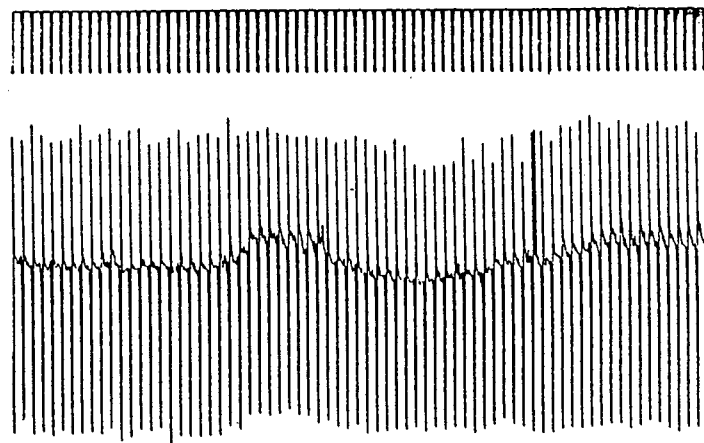
As described above, the bridge mode data indicated an increase in conductance during the noradrenergic hyperpolarization of cholinergic neurons. Based upon the calculated equilibrium potentials of each of the ions in solution, we hypothesized that the increase in conductance was due to the activation of an outward potassium current. We tested this hypothesis by obtaining

Figure 5. Bridge mode experiments on a single identified cholinergic neuron. Top traces in A, B, and D are hyperpolarizing current steps; lower traces reflect the voltage of the cell. A. Application of 50  $\mu$ M NA hyperpolarized the cell with a complete recovery after washout; B. NA hyperpolarization as in A, except in low  $\text{Ca}^{2+}$  ACSF solution, demonstrating that NA is acting directly on the cell. C. Left trace: individual traces of a single voltage deflection in response to a hyperpolarizing current pulse prior to NA application (control) superimposed on one obtained during NA. The smaller voltage deflection during NA demonstrates the increase in whole-cell conductance. Right trace: the same cell in a low  $\text{Ca}^{2+}$  solution, showing the absence of the calcium spike seen in the left trace. D. Application of NA after pretreatment with 1  $\mu$ M idazoxan. Idazoxan completely blocked the hyperpolarization (as in A and B), and demonstrated that NA also exerts a small depolarization through a non- $\alpha_2$  receptor.

**A**NA 50  $\mu$ M**B**NA 50  $\mu$ M



**D**



NA 50  $\mu$ M

20 mV

0.1 nA

1 min

current-voltage ( $I$ - $V$ ) ramps before and during application of NA ( $n=5$ ). As shown in Fig. 6A, the ramps crossed at a position which approximated the calculated potassium equilibrium potential of  $-101.5$  mV. When the control  $I$ - $V$  ramps were subtracted from ones obtained during NA, the resulting curves showed inward rectification (Fig. 6B). This phenomenon is characteristic of the  $K^+$  current directly activated by G-proteins,  $I_{KG}$  (Hille, 1992). Addition of  $100 \mu\text{M Ba}^{2+}$  (a non-specific  $K^+$  channel blocker) to the bath markedly reduced the current in 3/3 cells as illustrated in Fig. 6C, consistent with the hypothesis that noradrenaline is activating a  $K^+$  current. We conclude that the noradrenergic hyperpolarization of cholinergic neurons is mediated by the inwardly rectifying potassium current,  $I_{KG}$ .

## DISCUSSION

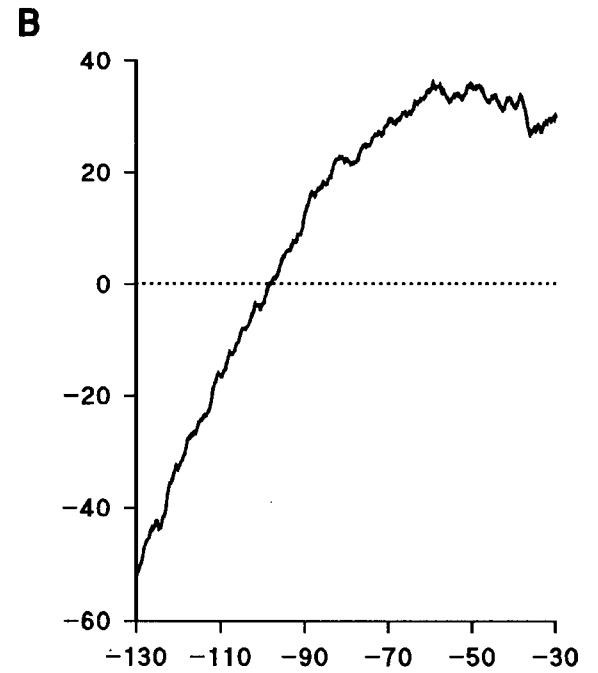
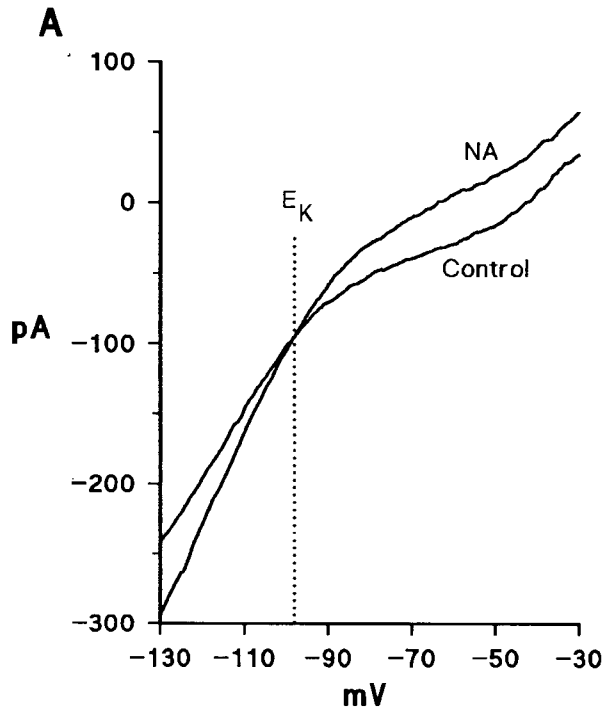
The principal findings of this study are that 1) NA powerfully and directly inhibits identified LDT cholinergic neurons; 2) NA exerts its hyperpolarizing effect through an  $\alpha_2$  adrenergic receptor; and 3) the noradrenergic hyperpolarization is the result of activation of the inwardly rectifying potassium current,  $I_{KG}$ .

### *Identification of Cells Studied with Whole-Cell Patch Clamp*

Identification of the neurotransmitter phenotype of a cell studied with the whole-cell patch clamp technique is essential for deriving conclusions about the significance of the results. We found that identified cholinergic neurons had a homogeneous (and unambiguous) response to NA (92% hyperpolarized), whereas the non-cholinergic cells, of which the phenotype is unknown, exhibited mixed responses to NA. Without histochemical identification of the cholinergic cells, we would have found that NA hyperpolarized only about half of LDT neurons (see Figure 4), and

Figure 6.  $I$ - $V$  curves obtained from voltage clamp experiments on an identified cholinergic neuron. A. Whole-cell currents obtained from control and during NA application approximate the potassium equilibrium potential ( $E_K$ ), indicating the activation of a  $K^+$  channel by NA. B. The NA-evoked current (obtained by subtracting the control curve from the NA curve in A) shows inward rectification. C. The same curve as in B superimposed on one obtained in a similar manner, except in a solution containing  $100\ \mu\text{M}\ \text{Ba}^{2+}$ . Barium completely blocked inward rectification as well as reduced the outward current. These results suggest that the NA hyperpolarization is due to the activation of the inwardly rectifying potassium current,  $I_{KG}$ .





to conclude that NA was inhibiting cholinergic neurons would have been only speculative. As it is, noradrenergic inhibition of cholinergic neurons is unequivocal.

### *The $\alpha_2$ Adrenergic Receptor*

That the noradrenergic hyperpolarization of LDT cholinergic neurons is mediated by the  $\alpha_2$  adrenergic receptor is supported by 1) blockade of the effect of NA by the  $\alpha_2$  adrenergic antagonist, idazoxan; 2) hyperpolarization induced by the  $\alpha_2$  adrenergic agonist, UK-14,304; and 3) the observation that the  $\alpha_1$  and  $\beta$  agonists phenylephrine and isoproterenol, respectively, failed to induce any effect.

Although a weak partial  $\alpha_2$  adrenergic agonist (Medgett et al., 1978), clonidine is remarkably effective in mimicking noradrenergic inhibition in several different regions in the nervous system, including the locus ceruleus (Cedarbaum and Aghajanian, 1977; Reiner, 1985; Williams et al., 1985), the rat sympathetic ganglion (Brown and Caufield, 1979), substantia gelatinosa (North and Yoshimura, 1984), and dorsal motor nucleus of the vagus (Fukuda et al., 1987). A notable exception is the submucous plexus of the guinea pig ileum. In these neurons, clonidine competitively antagonized the hyperpolarizing action of NA (Surprenant and North, 1988). Surprisingly, in LDT cholinergic neurons, clonidine was ineffective as either agonist or antagonist. These data suggest that LDT neurons may express a novel  $\alpha_2$  adrenergic receptor with a low affinity for clonidine. In support of this hypothesis, four  $\alpha_2$  receptor subtypes have been classified pharmacologically as well as identified through molecular cloning, however all subtypes exhibit a high affinity for clonidine (Bylund, 1995). An alternative hypothesis is that dialysis of the cell interior using whole-cell patch clamp recordings may modify the affinity of the receptor for clonidine. However, we found clonidine to powerfully hyperpolarize noradrenergic locus ceruleus neurons using the whole-cell patch clamp technique (Williams and Reiner,

unpublished observations), making it unlikely that the inability of LDT cholinergic neurons to respond to clonidine is due to experimental error. Further investigation is needed to determine precisely which  $\alpha_2$  adrenergic receptor subtype is involved in the noradrenergic inhibition of LDT cholinergic neurons.

Although we have deduced the mechanism of the NA hyperpolarization, the mechanism of the depolarizing effect of NA observed during  $\alpha_2$  blockade by idazoxan has yet to be delineated. Clearly, a second receptor that is coupled to a different ion channel is involved. Consistent with these findings, in a minority of cells the reversal potential of the NA-induced hyperpolarization was somewhat depolarized to  $E_K$  (-80 to -70). Whether this phenomenon is due to a voltage clamp error or to the activation of a separate set of ion channels is unclear. In any case, the hyperpolarization is unambiguously the predominant effect of NA and thus we did not investigate the "hidden" effect any further.

#### *Implications for Behavioral State Control*

For many years, several lines of evidence have implicated an inhibitory role of noradrenergic neurons of the locus ceruleus (LC) in REM sleep generation (reviewed in Steriade and McCarley, 1990). Karczmar et al. (1970) demonstrated that absence of aminergic transmission was required to systemically induce REM sleep with acetylcholinesterase inhibitors. Based upon these and other findings, many have hypothesized noradrenergic inhibition of brainstem cholinergic mechanisms (McCarley and Hobson, 1975; Sakai, 1988; Steriade and McCarley, 1990). Recent data have provided a plausible anatomical substrate for such effects: catecholaminergic fibers synapse onto LDT cholinergic neurons (Kubota et al., 1992), and LC neurons project to the LDT (Semba and Fibiger, 1992). The present data extend such findings into the functional realm by directly confirming the hypothesis that NA inhibits mesopontine

cholinergic neurons. Taken together with previous work these results allow us to define selected aspects of the cellular control of behavioral state with certainty.

Based upon electrophysiological data showing that the LC and dorsal raphe (DR) are most active during wake and silent during REM sleep (McGinty and Harper, 1976; Trulson and Jacobs, 1979; Hobson et al., 1975; Aston-Jones and Bloom, 1981), and upon previous reports that both NA and 5-HT hyperpolarize LDT cholinergic neurons (Luebke et al., 1992; present report), a reasonable hypothesis is that during wake cholinergic neurons would be under considerable inhibitory tone. At the transition to REM sleep when aminergic neurons fall silent (McGinty and Harper, 1976; Trulson and Jacobs, 1979; Hobson et al., 1975; Aston-Jones and Bloom, 1981), cholinergic neurons are disinhibited. Now capable of robust activity, they release acetylcholine in the mPRF and trigger the state of REM sleep.

In the past few years, considerable strides have been made in understanding the neuronal control of behavioral states. Key to the enterprise has been critical tests of explicit and implicit models based upon neuronal circuit analysis. The present experiments firmly establish a cellular basis for a noradrenergic-cholinergic interaction in the brainstem, with attendant implications for behavioral state control.

### III. State-dependent Release of Acetylcholine in the Rat Thalamus as Measured by *in vivo* microdialysis.

#### INTRODUCTION

Mesopontine cholinergic neurons are thought to have important roles in EEG desynchrony and in the generation of REM sleep. This notion has been supported by a large body of work originating with Moruzzi and Magoun (1949), who found that eliciting EEG desynchrony by electrical stimulation in the brainstem was most effective when the stimulating electrode was placed in the region of the mesopontine tegmentum. Early brainstem lesion studies showed that lesions at the midbrain-pontine junction abolished EEG desynchrony during wake (Lindsley et al., 1949, 1950; Batini et al., 1959; Jouvet, 1962; Hobson, 1965), and reduced the phasic activity normally observed during REM sleep, including rapid eye movements, PGO waves and muscle atonia (Hobson, 1965; Jouvet, 1962). It was later shown by means of acetylcholinesterase (AChE) histochemistry and choline acetyltransferase (ChAT) immunohistochemistry that the brainstem cholinergic neurons are localized in the laterodorsal and pedunculopontine tegmental nuclei (LDT, PPT, respectively; Shute and Lewis, 1967; Satoh et al., 1983; Jones and Beaudet, 1987; Vincent and Reiner, 1987). In addition, retrograde and anterograde tracing combined with ChAT immunohistochemistry revealed that mesopontine cholinergic neurons gave rise to a massive innervation of the thalamus in rats (Hallanger and Wainer, 1988; Sofroniew et al. 1985; Satoh and Fibiger, 1986; Woolf and Butcher, 1986; Semba and Fibiger, 1992), which is thought to control cortical EEG (Steriade et al., 1990a). Mesopontine cholinergic neurons thus became one of the neuronal candidates for Moruzzi and Magoun's "Ascending Reticular Activating System" (1949).

Recent brainstem lesion studies employing modern techniques to further localize damage to the LDT/PPT have suggested that mesopontine cholinergic neurons may be crucial to the

generation of REM sleep and its associated features. Shouse and Siegel (1992) found that lesion of the PPT reduces the amount of PGO activity in REM sleep. Webster and Jones (1988) demonstrated that lesion of the LDT/PPT cholinergic neurons eliminated REM sleep in some animals for up to several weeks, while in others, PGO activity and muscle atonia during REM sleep were reduced--the extent of the reduction of time spent in REM sleep and of PGO activity correlated with the extent of the loss of cholinergic neurons. Unlike earlier studies, in which lesions encompassed a much larger area (Lindsley et al., 1949, 1950; Batini et al., 1959; Jouvet, 1962; Hobson, 1965), waking and EEG desynchrony were not significantly altered, which suggested that other neuronal substrates were involved in the control of waking EEG.

The importance of LDT/PPT cholinergic neurons in the generation of REM sleep is further supported by the observation that microinjection of the cholinergic agonist, carbachol, into the medial pontine reticular formation (mPRF) induces a state that is indistinguishable from REM sleep (George et al., 1964; Baxter, 1969; Mitler and Dement, 1974; Amatruda et al., 1975; Gnadt and Pegram, 1986; Bourgin et al., 1995). Anatomical data have shown that the mPRF is innervated by cholinergic neurons originating in the LDT/PPT (Mitani et al., 1988; Shiromani et al., 1988; Quatrocchi et al., 1989; Jones, 1990; Semba et al., 1990; Semba, 1993). It has therefore been hypothesized that release of ACh in the mPRF is perhaps the natural trigger for REM sleep.

The interaction of cholinergic, noradrenergic, and serotonergic neurons in the brainstem has long been suggested to be a key factor in behavioral state control (Hernandez-Peon et al., 1963; Jouvet, 1969; Hobson et al., 1975). That amines inhibit ACh-induced triggering of REM sleep was first suggested by the observation that systemic injection of the AChE inhibitor eserine induced a state that was indistinguishable from REM sleep, but only after depletion of amines with

reserpine (Karczmar et al., 1970). Electrophysiological recordings in the locus ceruleus and in the dorsal raphe showed that noradrenergic and serotonergic neurons are very active during wake, less so during slow wave sleep (SWS), and silent during REM sleep (Hobson et al., 1975; McGinty and Harper, 1976; Trulson and Jacobs, 1979; Aston-Jones and Bloom, 1981). Based upon these findings, it was hypothesized that amines have an inhibitory influence on brainstem cholinergic mechanisms (Karczmar et al., 1970; McCarley and Hobson, 1975; Sakai, 1988; Steriade and McCarley, 1990). Indeed, recent studies have shown that noradrenaline (NA) and serotonin (5-HT) inhibit the majority of rat LDT neurons *in vivo* (Koyama and Kayama, 1993) as well as hyperpolarize identified mesopontine cholinergic neurons in brainstem slices *in vitro* (previous chapter; Williams and Reiner, 1993; Luebke et al., 1992; Leonard and Llinás, 1994). Taken together, these data suggest that brainstem cholinergic neurons would be powerfully inhibited during wake, when both serotonergic and noradrenergic neurons are most active.

On the other hand, while the data reviewed above may suggest that mesopontine cholinergic neurons are REM-specific, *in vivo* electrophysiological recordings in the mesopontine tegmentum have revealed that only a minority of cells fire selectively during REM sleep, and that a majority fire both during REM sleep and wake (Steriade et al., 1990b; El Mansari et al., 1989, Kayama et al., 1992). Thus the state-dependent activity of identified mesopontine cholinergic neurons remains unclear. In an attempt to resolve this issue, experiments using *in vivo* microdialysis were performed, in which ACh release was measured across behavioral state in the rat thalamus. Several anatomical investigations have shown that the rat thalamus receives cholinergic projections exclusively from the brainstem, with the exception of the reticular nucleus, which receives cholinergic innervation from both the basal forebrain as well as the mesopontine tegmentum (Hallanger and Wainer, 1988; Jourdain et al., 1989; Sofroniew et al. 1985; Satoh and

Fibiger, 1986; Woolf and Butcher, 1986). The medial portion of the thalamus, therefore, is an ideal region to test the extent to which there is state-dependent ACh release from brainstem cholinergic neurons.

## **MATERIALS AND METHODS**

### *Surgery*

Male wistar rats weighing 275-390 g were surgically implanted with EEG recording electrodes for monitoring behavioral state and with a transverse microdialysis probe in the thalamus for measuring ACh concentrations *in vivo*.

Animals were anesthetized with 50-60 mg/kg pentobarbital i.p., and supplemented as needed. For EEG recordings, animals were chronically implanted with 3 screw electrodes (2 for cortical EEG and 1 over the cerebellum as a reference) and with either nuchal electrodes for EMG or 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 Hospal Filtral AN69 microdialysis membrane (i.d. 0.22, o.d. 0.27, molecular weight cut off 60,000) with an active surface of 7 mm. Dialysis probes were placed at stereotaxic coordinates from bregma: AP -3.3, DV -5.8 to -5.6 (Paxinos and Watson, 1982), and the active surface centered to expose the ventroposterolateral, ventroposteromedial, centromedial, mediodorsal, interomediodorsal, and posterior thalamic nuclei.



### *Microdialysis*

Following surgery, rats were housed individually in 35x35x25 cm plexiglass cages and were given food and water ad libitum. On the first day after surgery, each animal was moved into a secluded recording room, where they were attached to a model 78 Grass polygraph for several hours for adaptation. Experiments were performed on the second and third days after surgery during the light phase of a 12:12h light:dark cycle (from 8:00 to 20:00) between 10:00 and 19:00. Because the different behavioral states of the rat are short-lived, the sample collection procedure (Damsma and Westerink, 1991) was modified in order to achieve the purest dialysis samples possible for each of the behavioral states of wake, SWS, and REM sleep. The samples were collected as follows. The dialysis probes were perfused with artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 3 KCl, 1.3 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, 23 NaHCO<sub>3</sub>, 1.0 PO<sub>4</sub> buffer, and pH balanced to 7.4 with HCl. In order to recover detectable levels of extracellular ACh, the reversible AChE inhibitor neostigmine bromide (Sigma) was added to the ACSF (100 nM). The dialysis membrane was perfused at a rate of 5 µl per minute, controlled by a syringe pump (Harvard Apparatus). The probe inlet was connected to the syringe by polyethylene tubing (800 cm x 0.28 mm; 50 µl in volume). The outlet polyethylene tubing was also cut to a 50 µl volume length and weighted at the end with a fitted short piece of tygon tubing.

For the behavioral experiments, all dialysate samples were collected into ice-cold microcentrifuge tubes marked "wake," "SWS," or "REM." Each vial contained 5 µl of 0.4% acetic acid in order to stabilize the ACh collected in the dialysate. No more than 100 µl of dialysate was allowed to accumulate in each vial in order to maintain equal concentrations of acetic acid for each of the behavioral states. As the animals shifted from wake to SWS to REM,

each state was timed and a 10 min. 30 sec. delay was added to allow the perfusate to move from the animal to the end of the output tubing.

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 synchronized for >30 s 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.

Prior to sample collection, animals were dialyzed for 40-60 minutes in order to allow the brain to equilibrate with the perfusion solution. Samples were collected during 5-6 hour sessions and were stored in an ice bucket for off-line analysis via HPLC-ECD immediately after the session.

To test for the sodium and calcium dependence of thalamic ACh release, animals were attached directly to the HPLC system for on-line analysis of ACh release. Once steady baseline levels were achieved, animals were perfused either with solution containing 1  $\mu$ M tetrodotoxin (TTX, Sigma) or with calcium-free perfusate for at least 30 minutes.

#### *Assay of Acetylcholine*

For the off-line experiments, dialysis samples were manually injected into a 50  $\mu$ l sample loop of the analytical system with a 100  $\mu$ l Hamilton syringe. For the on-line experiments, the output tubing was connected directly to the sample loop, and perfusate was injected at ten minute intervals controlled by an adjustable timer (Valco). Standards ( $10^{-7}$  M ACh) were injected hourly into a separate 20  $\mu$ l sample loop.

The assay of ACh via HPLC-ECD was performed in conjunction with an enzyme reactor (Damsma et al., 1987). Briefly, after separation of ACh and choline (Ch) with a reverse phase column (75 x 2.1 mm) pretreated with lauryl sulphate, the eluent was then passed through an enzyme reactor (10 x 2.1 mm) containing acetylcholinesterase (EC 3.1.1.7; Sigma, type VI-S) and choline oxidase (1.1.3.17; Sigma) covalently bound to glutaraldehyde-activated Lichrosorb NH<sub>2</sub> (10 mm; Merck). The reaction of ACh and Ch with these enzymes produced a quantitative yield of hydrogen peroxide, which was electrochemically detected at a platinum working electrode set at a potential of +500 mV versus an Ag/AgCl reference electrode (BAS-LC4B or ANTEC VT-03). An HPLC pump (LKB-2150 or Shimadzu LC-600) delivered the mobile phase (0.2 M aqueous potassium phosphate buffer pH 8.0, 1mM tetramethylammonium hydroxide) at a rate of 0.4 ml/min. Chromatograms were drawn on a chart recorder (Kipp); hydrogen peroxide peaks corresponding to ACh normally appeared 4-5 min. after the initial solvent front. The detection limit of the assay was 50 fmol per injection.

### *Immunohistochemistry*

Probe placement was verified in four rats using standard techniques, while in six rats, the source of cholinergic innervation to the probe was also verified using Fluoro-Gold (FG) as a retrograde tracer (4% in 0.9% saline) and ChAT immunohistochemistry. FG was manually injected into the dialysis probe and rinsed out with normal perfusion solution after 24h. After 5-14 days, animals were anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 100 ml of 0.9% saline followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). The brains were removed and postfixed at 4° C for 2h and then transferred to 15% sucrose in 0.1 M PBS and stored overnight at 4° C. The next day, brains were

cut into 30  $\mu\text{m}$  coronal sections on a freezing microtome and every 5th section separated into wells containing 0.1 M PBS.

Sections that contained the site of the probe were mounted onto coated slides immediately for verification of placement. Ten sections containing basal forebrain cholinergic neurons, and ten brainstem sections containing LDT/PPT cholinergic neurons were selected and processed for ChAT immunohistochemical staining as follows. The 20 sections were placed in a solution composed of 1:50 normal goat serum (Jackson ImmunoResearch Laboratories, Inc.), 0.02% sodium azide, and 1:50 rat anti-ChAT antibody (Boehringer Mannheim) in 0.1 M PBS containing 0.3% Triton-X-100 and incubated for two nights at 4° C. Sections were washed 3 x 20 min. in PBS and then incubated at room temperature for 2h in 1:20 texas-red conjugated goat-anti-rat antibody (Jackson ImmunoResearch Laboratories, Inc.) in 0.3% Triton-X/PBS. After the 2h period, slices were washed again for 3 x 20 min. in 0.1 M PBS and mounted onto coated slides for inspection using fluorescence microscopy.

For identifying and quantifying double-labelled cells, basal forebrain and brainstem sections were photographed twice: once using green light (530-560 nm) to visualize the texas-red labelled cholinergic neurons, and once using ultra-violet light (340-380 nm) to visualize the retrogradely labelled cells. Negatives obtained under the green light were projected onto 8.5" x 11" sheets of paper, onto which the individual cholinergic cells were traced. Afterwards, the corresponding exposures under ultra-violet fluorescence were projected onto the drawing for identification of double-labelled cells by the superimposed images of the retrogradely labelled neurons.

### *Statistical Analyses*

One-way analysis of variance (ANOVA) with repeated measures was used to analyze the effect of behavioral state on ACh concentrations. Scheffe's test was used for the comparison of the ACh concentrations between each of the states. The rate of ACh release is reported as femtomoles per minute (fmol/min).

## **RESULTS**

### *Thalamic Acetylcholine Release*

In order to determine whether ACh release in the thalamus was the result of action potential dependent neuronal activity two experiments were conducted. First, TTX (1  $\mu$ M) was added to the perfusion solution to block voltage-dependent sodium channels. TTX reduced extracellular ACh in 2/2 rats from an average baseline of  $18.21 \pm 5.82$  to  $2.12 \pm 1.31$  fmol/min. Second, two other rats were perfused with calcium-free ACSF in order to abolish  $\text{Ca}^{2+}$ -dependent synaptic transmission. The calcium-free solution reduced the baseline ACh output in 2/2 rats from  $15.75 \pm 7.25$  to  $3.51 \pm 0.75$  fmol/min. These results confirm that thalamic ACh release was due to sodium and calcium dependent mechanisms, as opposed to overflow of extracellular neurotransmitter caused by trauma from the probe implant (Westerink and De Vries, 1988).

The number of 50  $\mu$ l samples obtained on any particular day for each rat varied between the behavioral states of wake, SWS, and REM. An average of  $4.93 \pm 2.72$  samples were obtained for wake,  $5.13 \pm 2.00$  for SWS, and  $1.53 \pm 0.50$  for REM on each day. Because most of the samples within each state for each rat were very similar, within-state samples were averaged for each experimental day. A total of 15 sample values for each behavioral state were obtained in 10 rats. These values and their standard deviations are reported in Table 1.

The average rate of ACh release during wake was  $18.01 \pm 8.58$  fmol/min; during SWS,  $8.17 \pm 2.55$  fmol/min; and during REM,  $19.52 \pm 9.36$  fmol/min (Figure 7). Analysis of variance showed that ACh output varied significantly with behavioral state ( $F = 14.16$ ,  $df = 2, 28$ ,  $p < 0.001$ ). Further analysis with Sheffe's test showed that ACh release rates between wake and REM sleep were not significantly different ( $t = 1.44$ ,  $p = 0.173$ ), but were significantly higher than that in SWS ( $t = 5.25$ ,  $p < 0.0001$ ).

#### *In Vitro Recovery of Acetylcholine*

The average duration of the REM periods across the ten rats used in this study was  $92.28 \pm 57.87$  seconds. Because the REM periods were short-lived, we tested our sample collection technique *in vitro* in order to determine the extent to which the ACh output values obtained for REM sleep were accurate. Microdialysis probes of the type used in this study were connected to polyethylene tubing and perfused with ACSF at a rate of 5  $\mu$ l per min (see Methods). The probe was placed in a vial containing normal ACSF and manually transferred to another vial containing 100 nM ACh for either 30-60 sec (87% of the accumulated sample was obtained from intervals of 50 sec or less) or 2 min intervals. In order to measure total recovery rates, the probe was left in the 100 nM ACh solution for up to twenty min. Both solutions were maintained at 37° C. Samples were collected off-line as described in the Methods.

Dialysis probes had an *in vitro* recovery of  $22 \pm 3.39\%$  ( $n = 4$ ). Collection of 30-60 sec samples reduced the recovery in one probe from 21% to 12%. Collection of samples from 2-minute time periods reduced the recovery in another probe from 17% to 15%. These results indicate that our *in vitro* sampling is accurate to 89% within a 2-minute time period, but only 57% accurate for time periods of one minute or less, which suggests that the ACh output measured

Rat#	Wake	SWS	REM
3	14.73 (0.47)	7.87 (0.75)	16.4
4	10.53 (3.0)	7.00 (0.7)	10.36 (0.36)
	11.09 (1.70)	10.39 (1.27)	18.91 (0.73)
5	8.10 (1.22)	5.17 (0.69)	10.00 (1.00)
	8.13 (1.30)	6.00 (0.39)	11.56 (0.88)
8	19.19 (3.61)	13.66 (1.66)	22.99
	15.19 (1.38)	9.17 (1.13)	12.90
10	13.49 (1.23)	9.04 (0.37)	18.07
	7.83 (0.79)	4.35 (0.26)	7.68
15	21.74 (0.31)	8.40 (0.59)	23.94
	24.23 (0.67)	6.69 (1.72)	20.53 (0.52)
18	35.57 (0.60)	8.89 (1.00)	30.27
26	18.93 (0.89)	4.97 (0.58)	14.91 (1.42)
31	31.37	9.59 (0.62)	37.91
32	30.06 (1.71)	11.28 (0.37)	36.36

Table 1. Extracellular ACh in the thalamus across behavioral state. Mean ( $\pm$  S.D.) rates of ACh release are reported as fmol/min for each behavioral state in individual rats.

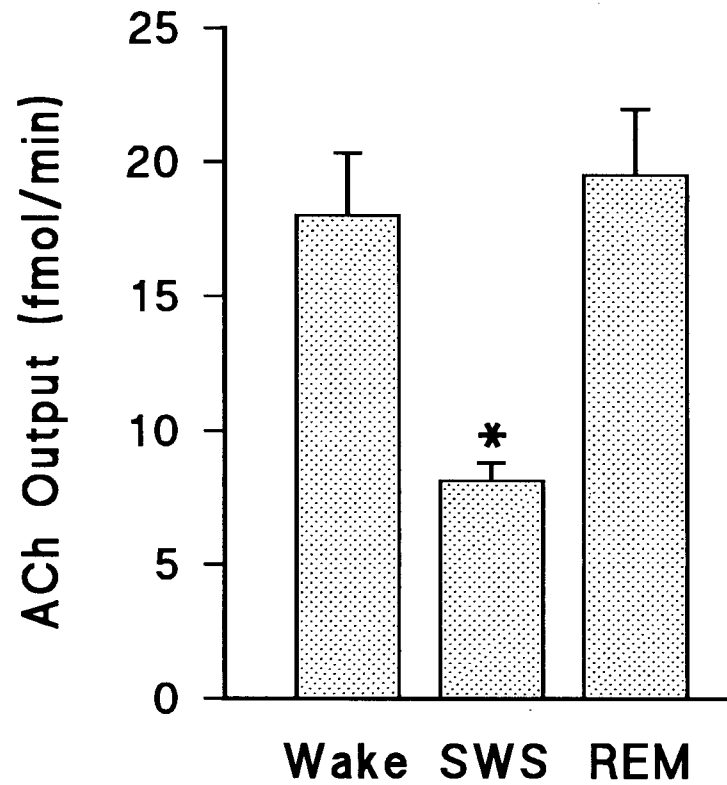


Figure 7. Extracellular acetylcholine across behavioral state in the rat thalamus. Mean rates of ACh release ( $\pm$  SEM, error bars) are reported for wake, slow wave sleep (SWS) and REM sleep ( $n = 15$  for each behavioral state). ACh release was significantly higher in REM sleep and wake than in SWS ( $p < .0001$ ).



during REM sleep periods may have been underestimated, as these samples were most likely contaminated with dialysate from SWS periods.

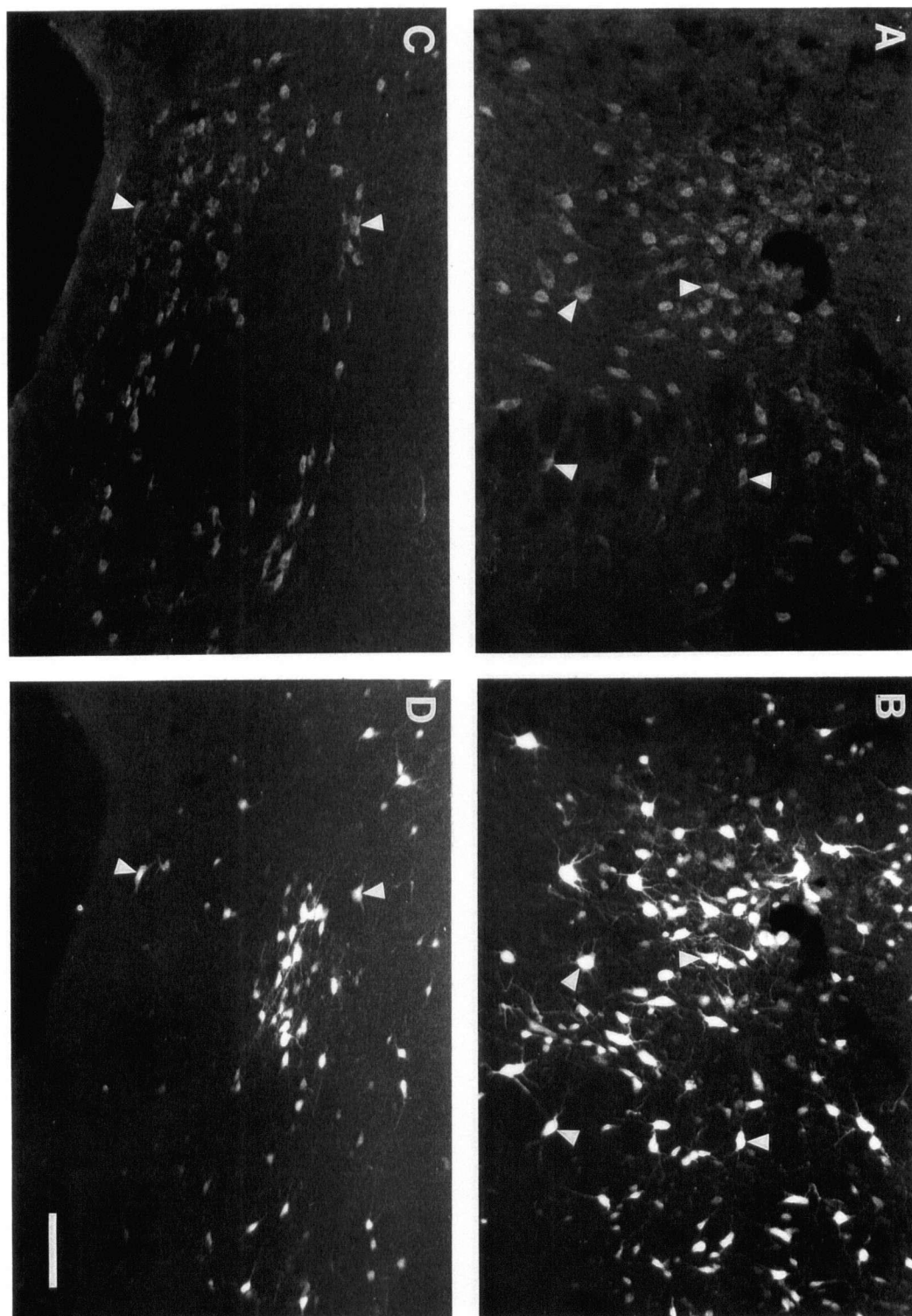
*Immunohistochemical verification of cholinergic innervation to the thalamus*

Retrograde tracing with FG resulted in many labelled cells throughout the cortex, hippocampus, basal forebrain, and brainstem. Few neurons were labelled in the medial septum, while a moderate number was found in the more caudal portions of the cholinergic basal nuclear complex, including the horizontal limb of the nucleus of the diagonal band of Broca and in the nucleus basalis of Meynert. Across six rats, a total of 4,679 cholinergic cells were counted in the basal forebrain. Of these cells, only 84 or 1.80% were found to be double labelled. In contrast, 2,784 ChAT positive cells were counted in the LDT and PPT. Of these cells, 1,632 or 58.62% were double labelled (Figure 8). Therefore, of a total number of 1,716 double-labelled neurons, brainstem cholinergic neurons accounted for an average of  $94.5 \pm 4.19\%$ , while the basal forebrain accounted for the remaining  $5.5 \pm 4.19\%$  (Table 2). These results confirm that the majority of cholinergic inputs to region of the probe was from the brainstem cholinergic group.

## DISCUSSION

The principal finding of this study is that ACh release in the thalamus is high both during REM sleep and wake, and significantly lower during SWS. Thalamic ACh release was sensitive to TTX and to calcium-free perfusate, which indicates that the release was due to action potential generation as well as calcium-dependent synaptic transmission. Furthermore, retrograde tracing with FG combined with ChAT immunohistochemistry verified that the majority of cholinergic inputs in the vicinity of the microdialysis probe arose from the mesopontine tegmentum. These results demonstrate unequivocally that mesopontine cholinergic neurons are active during wake.

Figure 8. Combined ChAT immunohistochemistry and retrograde labelling from the thalamus with Fluoro-Gold. A, C. Photomicrograph of ChAT-positive neurons in the LDT/PPT and in the basal forebrain, respectively. B, D. Same field as in A and C, except under ultra-violet fluorescence showing retrograde labelled neurons. Of the 114 cholinergic neurons counted in A, 73 were found to be double-labelled in B (4 examples shown by arrowheads). In contrast, 112 cholinergic neurons were counted in C, but only 2 were found to be double-labelled in D (arrows). Scale bar = 50  $\mu$ m.



Rat #	Basal Forebrain			LDT/PPT		
	FG+ChAT	ChAT+	(%)	FG+ChAT	ChAT+	(%)
10	21	579	(14)	124	243	(86)
15	21	894	(7)	264	481	(93)
18	2	721	(1)	228	584	(99)
26	6	682	(3)	221	331	(97)
31	3	818	(4)	325	503	(96)
32	21	985	(4)	470	642	(96)
Total	84	4679	(5)	1632	2784	(95)

Table 2. Distribution of double-labelled cholinergic neurons. For each rat, the number of double-labelled cells (FG+ChAT) versus the total number of cholinergic neurons (ChAT+) is shown in the left half of each column for the basal forebrain and the brainstem (LDT/PPT). Numbers in parentheses show the percentage of all cholinergic neurons projecting to the thalamus. These results show that the majority of inputs to the thalamic probe originated from brainstem cholinergic neurons.

Based upon the state-dependent activity of serotonergic and noradrenergic neurons (Hobson et al., 1975; McGinty and Harper, 1976; Trulson and Jacobs, 1979; Aston-Jones and Bloom, 1981) and the observation that NA and 5-HT inhibit identified LDT cholinergic neurons *in vitro* (Chapter II; Williams and Reiner, 1993; Luebke et al., 1992; Leonard and Llinás, 1994) we hypothesized that mesopontine cholinergic neurons would be profoundly inhibited during wake (Kamondi et al., 1992; Chapter II; Williams and Reiner, 1993). One prediction of this hypothesis is that thalamic ACh release by mesopontine cholinergic neurons would be much higher during REM sleep than during wake. The present results clearly show that this prediction is in error: thalamic ACh release during wake and REM sleep are essentially the same. Such a conclusion is in concordance with the results of electrophysiological studies which suggested that mesopontine cholinergic neurons are active during EEG desynchronized states (Steriade et al., 1990b; El Mansari et al., 1989).

The *in vitro* recovery rates through the dialysis probes indicated that detection of changes in ACh concentration within short time periods ranging from 30 sec to 2 min was 57-89% accurate. This result suggests that the rate of ACh release measured during REM sleep may have been underestimated, as these samples may have been contaminated with dialysate from SWS periods. If this were the case, then it may be possible that ACh release is higher during REM sleep than wake. This is supported by the observation that while many LDT/PPT neurons were tonically active both during wake and REM sleep, their firing rates were significantly higher in REM sleep as compared to wake (Steriade et al., 1990b). On the other hand, the *in vitro* sampling error may reflect a time constraint on the rate at which the probes could detect sudden changes in ACh concentration. Behavioral state changes were much more gradual, and as stated earlier, transition periods were discarded. Therefore, it is also possible that the sampling

procedure was more accurate *in vivo* than the *in vitro* data suggest. In any case, whether extracellular ACh concentrations during REM sleep were underestimated or not, it is clear that mesopontine cholinergic neurons are active during wake as well as REM sleep. This finding is consistent with previous reports showing the state-dependence of ACh release in the cortex (Kanai and Szerb, 1965; Jasper and Tessier, 1971; Szerb, 1967; Marrosu et al., 1995) and in the hippocampus (Kametani and Kawamura, 1990; Marrosu et al., 1995), suggesting that this behavioral profile is the same for both mesopontine and basal forebrain cholinergic neurons.

The results of the present study contrast with the current view that ACh release in the mPRF is one mechanism for the induction of REM sleep. This hypothesis is based on the observation that microinjection of the cholinergic agonist, carbachol into the mPRF induces a state that is indistinguishable from REM sleep (Amatruda et al., 1975; Baxter et al., 1969; George et al., 1964; Mitler and Dement, 1974; Gnadt and Pegram, 1986; Quattrochi et al., 1989; Bourgin et al., 1995). *In vivo* microdialysis of the cat mPRF has shown that extracellular ACh concentrations in the mPRF are very high in both REM sleep (Kodama et al., 1990; Leonard and Lydic, 1995) and in carbachol-induced REM sleep (Lydic et al., 1991) as compared to wake. Using retrograde tracing combined with ChAT immunohistochemistry, Semba et al. (1990) found that 45-88% of mesopontine cholinergic neurons projecting to the mPRF also projected to the thalamus. Taken together with the results of the present study, these data suggest that brainstem cholinergic neurons may release ACh differentially with respect to region, ACh being released in the thalamus at equal rates during both wake and REM sleep, but at significantly higher rates in the mPRF during REM sleep than during wake or SWS. There are several possible mechanisms for this type of ACh release.

First, Semba et al. (1990) also reported distinct populations of brainstem cholinergic neurons that had inputs exclusively to either the thalamus or to the mPRF. This raises the possibility that cholinergic neurons projecting only to the mPRF are selectively active during REM sleep. However, this scenario is unlikely for two reasons. First, Semba et al. (1990) reported that the majority of cholinergic neurons projecting to the mPRF also projected to the thalamus. Second, electrophysiological recordings *in vivo* have demonstrated that the majority of LDT/PPT neurons are tonically active during wake and REM sleep (Steriade et al., 1990b; El Mansari et al., 1989; Kayama et al., 1992). Steriade et al. (1990b) did observe a minority of cells that were selectively active during REM sleep, but it is not clear whether these were cholinergic.

A second possible mechanism for differential ACh release is inhibitory axoaxonic inputs onto presynaptic cholinergic terminals in the mPRF, which would suppress ACh release during wake. Anatomical studies have shown that there are neurons in the pontine reticular formation that are immunoreactive for GABA (Brodal et al., 1988) or for glycine (Fort et al., 1993). Microinjection of the GABA<sub>A</sub> agonist, muscimol, into the rat PRF increases the latency to SWS and to REM sleep as well as the total amount of time spent in wake. Likewise, the GABAergic antagonist, bicuculline, reduces the latency to SWS and to REM sleep (Camacho-Arroyo et al., 1991). These findings indicate that GABAergic neurotransmission may be involved in the sleep-wake cycle, and presynaptic inhibition of cholinergic terminals in the mPRF may be one aspect of this involvement.

In addition, the rat mPRF receives both serotonergic and (to a lesser extent) noradrenergic inputs arising from the dorsal raphe and locus ceruleus, respectively (Semba, 1993). Semba (1993) hypothesized that waking may be "maintained" in part by the convergence of ACh, NA, and 5-HT onto mPRF neurons, and that REM sleep may be induced upon depolarization of mPRF

neurons by ACh in the absence of noradrenergic and serotonergic input (Ito and McCarley, 1984). Further studies directed at the morphology and cytochemistry of synaptic inputs to the mPRF are needed to address these issues.

In conclusion, the present study has demonstrated that ACh is released in the thalamus at equal rates during wake and REM sleep and much more slowly during SWS. This finding supports the hypothesis that mesopontine cholinergic neurons play an important role in EEG desynchronized states (Steriade et al., 1990b). These data have important implications for the role of ascending as well as descending projections of mesopontine cholinergic neurons in behavioral state control.



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

##### **INTRODUCTION**

Following 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 (Guevara-Guzman et al., 1994; Strasser et al., 1994; Silva et al., 1995; Lonart et al., 1992; Lorrain and Hull, 1993), regulation of local cerebral blood flow (Adachi et al., 1992; Northington et al., 1992), synaptic plasticity (Schuman and Madison, 1991; O'Dell et al., 1991; Shibuki and Okada, 1991), resetting of the circadian clock (Ding et al., 1994; Weber et al., 1995; Amir 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). To date, few studies have addressed the role of neurotransmitters which co-localize 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<sup>ω</sup>-nitro-monomethyl-L-arginine (L-NMMA) reduced wakefulness in rats. In contrast, administration of L-NAME (i.c.v.; Kapás et al., 1994) or 7-nitroindazole (i.p.; Dzoljic et al., 1996) reduced sleep in rats. Consistent with the modulatory role of NO in neurotransmission, local infusion of the NOS inhibitor, N<sup>ω</sup>-nitro-L-arginine (N-ARG), into the mPRF reduced both ACh release (Leonard and Lydic, 1995), and REM sleep (Leonard and Lydic, 1996).

Based upon the evidence for the state-dependent activity of mesopontine cholinergic neurons (Chapter III; 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 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 upon the conversion of the ferrous form of hemoglobin (oxyhemoglobin, or HbO<sub>2</sub>) into the ferric form, methemoglobin (metHb) by NO (Murphy and Noack, 1994) was utilized for the detection of NO. Others have shown previously a linear relationship between NO production and the conversion of HbO<sub>2</sub> 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<sub>2</sub> was prepared from bovine, double crystallized hemoglobin (Sigma) as follows. A Sephadex G-25 column (Pharmacia) 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 one ml NaPO<sub>4</sub> buffer) followed by 1 mM hemoglobin (17 mg in one ml NaPO<sub>4</sub> buffer). The conversion of metHb to HbO<sub>2</sub> 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<sub>4</sub> buffer (Dixon and McIntosh 1967). In order to protect HbO<sub>2</sub> from auto-oxidation, superoxide dismutase (500 units/ml) and catalase (2000 units/ml) were added immediately to the eluent.

Ten µl of the HbO<sub>2</sub> stock was diluted in 3 mls of the NaPO<sub>4</sub> buffer, and the concentration determined by measuring the absorbance at 577 nm using either a Bausch and Lomb Spectronic

21 or a Pharmacia Biotech Ultrospec 3000 spectrophotometer. According to the Lambert-Beer Law, the absorbance density ( $D$ ) of a substance is proportional to both the concentration ( $c$ ) and the thickness (defined by the optical pathlength of the sample-containing cuvette) of the sample ( $l$ ). Thus the concentration of the  $\text{HbO}_2$  was determined from the equation:

$$D = \epsilon * c * l, \text{ or } c = D/(\epsilon * l)$$

The extinction coefficient,  $\epsilon$  (the optical density of a sample at 1 mmol/L, measured with a pathlength of 1 cm), of  $\text{HbO}_2$  at 577 nm is  $15.37 \text{ mM}^{-1} \text{ cm}^{-1}$  (Van Assendelft, 1970). The diluted solutions approximated 2  $\mu\text{M}$ . This calculation was used as the basis for preparing a series of  $\text{HbO}_2$  solutions ranging in concentration from 0.5 to 5  $\mu\text{M}$  to construct the calibration curve for the determination of NO concentration in the dialysate samples.

The conversion of  $\text{HbO}_2$  to metHb was measured at 577 and 591 nm with a Biorad diode-array spectrophotometer containing a flow-through cell. A Waters HPLC pump was used to deliver the 100  $\mu\text{l}$  samples at a rate of 1.0 ml per minute. The mobile phase consisted of 50 mM  $\text{NaPO}_4$  buffer, pH 7.4, degassed and filtered. Ideally, the mobile phase should consist of the exact same constituents as the dialysate (described below), as it is used as the reference, however, no change in absorbance was detected when the dialysate without the hemoglobin was measured against the buffer. The buffer was therefore used as the mobile phase since it was less corrosive to the HPLC system. Since  $\text{HbO}_2$  exhibits an absorbance maximum at 577 nm, and is isosbestic with metHb at 591 nm (where the absorbance does not change as  $\text{HbO}_2$  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  $\text{HbO}_2$  to metHb (0.5-5  $\mu\text{M}$ ) by aqueous NO (Figure 9).

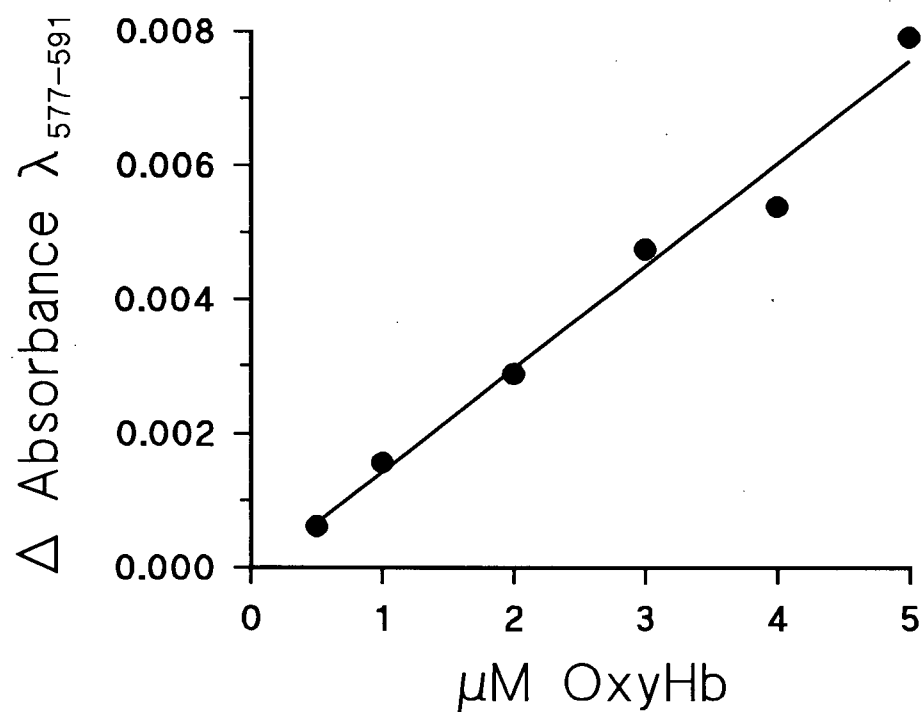


Figure 9. Calibration curve obtained by quantitative oxidation of increasing concentrations of HbO<sub>2</sub> to metHb (0.5-5 μM) by aqueous NO. Values along the y-axis are the absorbance differences between 577 and 591 nm obtained for metHb subtracted from that obtained from corresponding concentrations of HbO<sub>2</sub>. NO concentrations were estimated using the slope of the line.

Aqueous NO solutions were prepared as described previously by Feelisch (1991). Briefly, 20 mls of the 50 mM sodium phosphate buffer were saturated with argon for 40 minutes in an air-tight flask equipped with an exhaust outlet. Afterwards, the solution was bubbled with NO for up to ten minutes. The concentration of a saturated NO solution at room temperature is approximately 2 mM (Feelisch, 1991). In order to determine the detection limit of the assay, known concentrations of aqueous NO were transferred using a gas-tight syringe to buffer solutions containing 1  $\mu$ M HbO<sub>2</sub>, and the absorbance measured at 577 and 591 nm, described above. The detection limit of the assay was 10 pmols per 100  $\mu$ l sample (Figure 10).

### *Surgery*

Surgical procedures for implanting EEG electrodes and transverse dialysis probes in the thalamus were identical to those described in the previous chapter. However, cellulose ester membranes (C-DAK 4000) were used for the dialysis probes instead of the Hospal membrane for several reasons. First, the molecular weight cut-off the cellulose ester was 10 kDa, making it less likely for HbO<sub>2</sub>, a 64 kDa protein, to penetrate the membrane. Second, the cellulose ester membrane was able to withstand the greater back pressure imposed by the addition of the fused silica tubing (see below) as the outlet from the probe. Hospal membranes expanded both *in vitro* and *in vivo* under such conditions, allowing much of the HbO<sub>2</sub> contained in the sample to leak out of the probe. As described above, a decrease in absorbance at 577 nm should be indicative of an oxidation reaction, rather than leakage of the HbO<sub>2</sub> from the solution.

### *Microdialysis*

Microdialysis and sample collection procedures were similar to those described in the previous chapter, with the exception of a few modifications. The dialysis probes were perfused with ACSF containing (in mM) 147 NaCl, 3 KCl, 1.3 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, 1.0 NaPO<sub>4</sub> buffer, pH

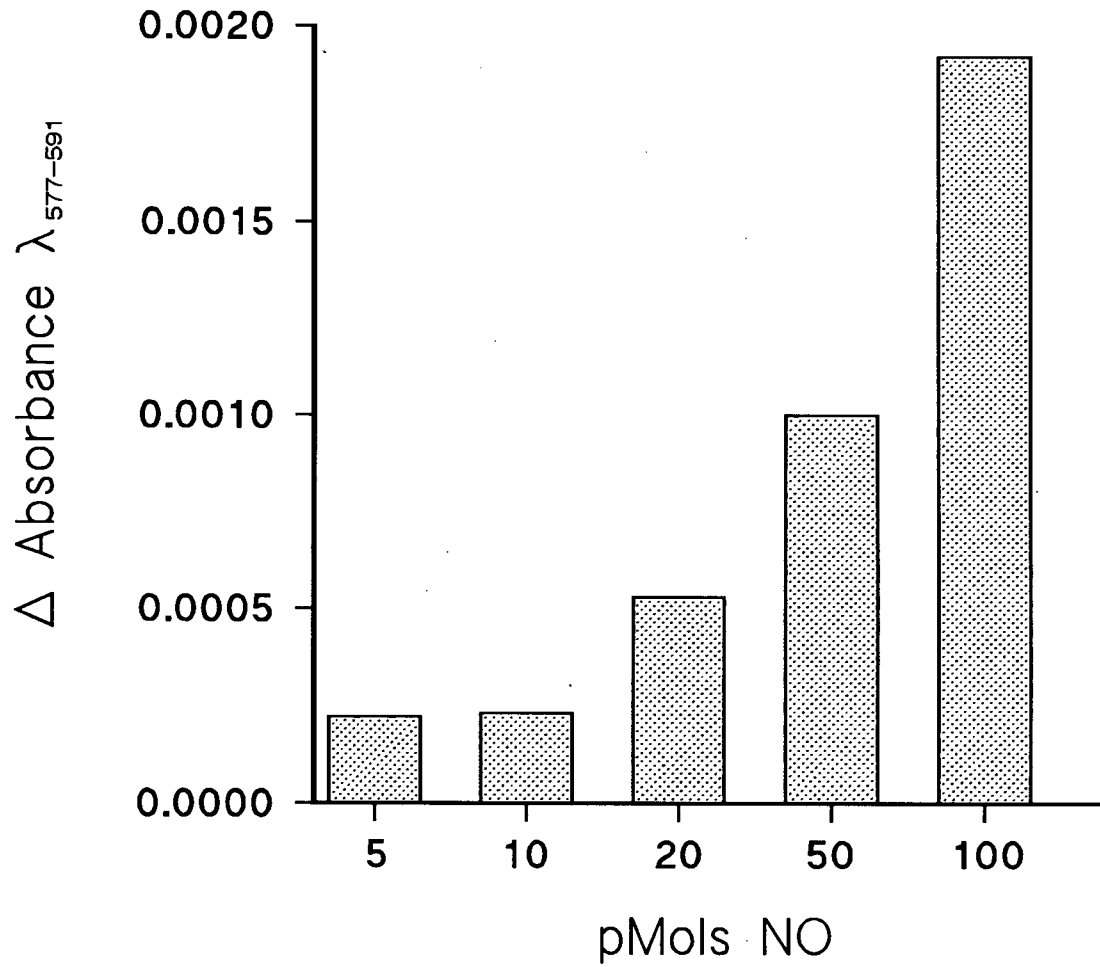


Figure 10. Detection limit of the NO assay. Absorbance differences between HbO<sub>2</sub> solutions containing known concentrations of NO and control solutions are plotted above. The larger values correspond to a greater amount of HbO<sub>2</sub> oxidation, and therefore to larger concentrations of NO. The lower detection limit was 5-10 picomoles per 100  $\mu$ l sample.

7.4, and 1  $\mu\text{M}$   $\text{HbO}_2$  for the assay of NO. The perfusion rate was 5  $\mu\text{l}$  per minute, controlled by a syringe pump (Harvard Apparatus), and samples were collected in volumes of 100  $\mu\text{l}$ , or 20-minute 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. In order to ensure the stability of  $\text{HbO}_2$ , the dialysate was replaced each hour. Both the probe inlet and outlet consisted of polyethylene tubing as used in the previous experiment (chapter III), except each contained an inner fused-silica tubing (100  $\mu\text{m}$  i.d., 200  $\mu\text{m}$  o.d.; Polymicro Technologies, Inc.) to maintain an air-tight environment. Prior to sample collection, animals were dialyzed for 60 minutes in order 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" as before, except they 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 two days prior to the experiment. On the day of the experiment, animals were anesthetized with urethane (1.5-2.0 g/kg, i.p.), 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 nucleus (LDT) 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) controlled by a rectal probe (Yellow Springs Instrument Co., Ohio).

Upon completion of experiments, animals were killed with an overdose of sodium pentobarbital, and probe placement verified using standard techniques (Figure 11A). Placement

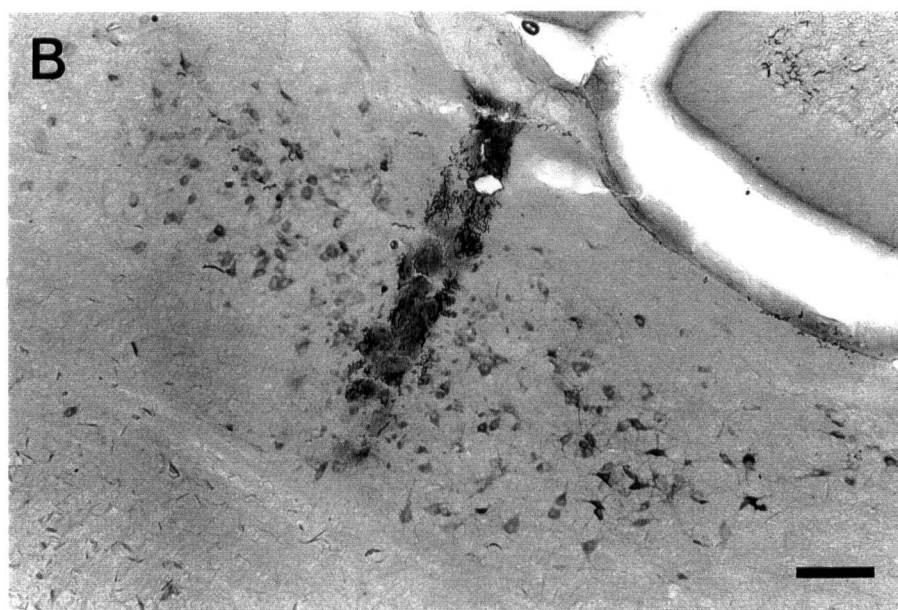
of stimulation electrodes were verified by using NADPH-diaphorase histochemistry, a reliable marker of the mesopontine NOS-positive cholinergic neurons (Vincent et al., 1983). Briefly, fixed brains were trimmed to a block containing the pontine brainstem, sliced into 40  $\mu$ m sagittal sections on a microtome and mounted onto chrome-alum coated slides. Slides were incubated at 37° in a solution containing 1 mg/ml NADPH, 0.1 mg/ml nitro blue tetrazolium in a 0.3% Triton-x-100/0.1M phosphate buffered saline for 45-60 minutes. Slides were observed under a light microscope for determining whether electrode tips were in or near the diaphorase-positive neurons in the mesopontine tegmentum (Figure 11B).

### *Statistical Analyses*

A one-way analysis of variance (ANOVA) with repeated measures was used to analyze the effects of various treatments on NO production, including behavioral state, electrical stimulation of laterodorsal tegmental nucleus (LDT) and cerebellum, applications of perfusates containing either high potassium, BAPTA (1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid [tetrapotassium salt]; Molecular Probes), or TTX (tetrodotoxin; Sigma), and systemic injections of NOS inhibitors. Scheffe's test was used for the comparison of NO production 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 production is reported as means  $\pm$  SEM in picomoles per minute (pmol/min).



Figure 11. Histochemical verification of placement of microdialysis probes and stimulating electrodes. A. Cresyl violet-stained coronal section showing horizontal probe track through the thalamus. Scale bar = 500  $\mu\text{m}$ . B. NADPH-diaphorase-stained sagittal section showing the site of the stimulating electrode tip among NOS-containing neurons in the laterodorsal tegmental nucleus. Scale bar = 100  $\mu\text{m}$ .



## RESULTS

### *Pharmacological Properties of Thalamic NO Release*

The first set of experiments was designed to determine whether the HbO<sub>2</sub> 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<sup>ω</sup>-nitro-L-arginine (N-ARG) baseline NO production in awake, freely moving animals was significantly reduced from  $1.31 \pm .07$  to  $0.86 \pm .06$  pmols/min ( $p < .001$ ,  $n=4$ ; Figure 12A).

While these data suggest that our technique is indeed capable of measuring NO *in vivo*, they do not address the question of the source of NO. Because we hypothesized that thalamic NO might derive from neuronal activity, 7-nitroindazole (7-NI), a specific inhibitor of neuronal NOS (Babbege et al., 1993) was administered to urethane-anesthetized rats. While 7-NI slightly reduced the baseline as well as blocked a stimulation-induced increase (see below), the vehicle, DMSO, produced the same effect. It is possible that this is due to DMSO's action as a free-radical scavenger (Kharasch and Thyagarajan, 1983; Arroyo et al., 1992). We therefore abandoned the pharmacological approach and resorted to other means to determine whether the NO signal was derived from neuronal sources.

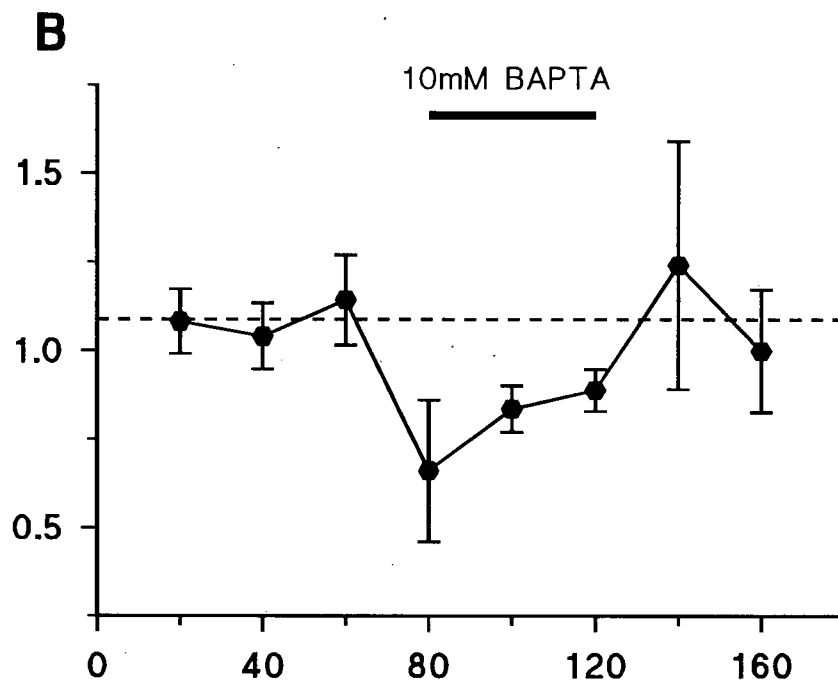
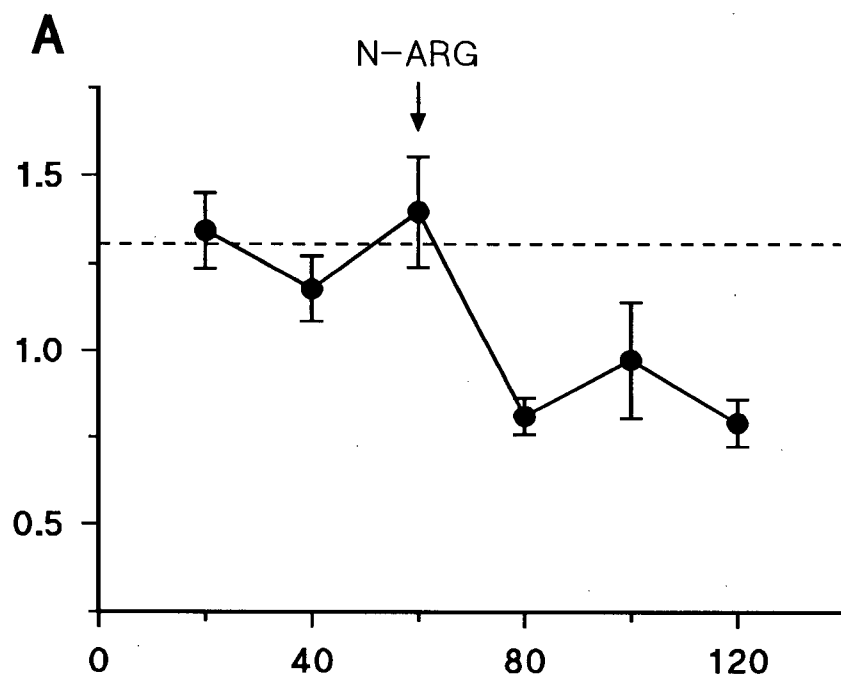
We next sought to determine if the changes in measured NO production were due to action-potential-dependent activity. In order to address this question, 1  $\mu$ M TTX, a compound which blocks voltage-dependent sodium channels, was added to the dialysate. Surprisingly, there was no change in NO output 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.

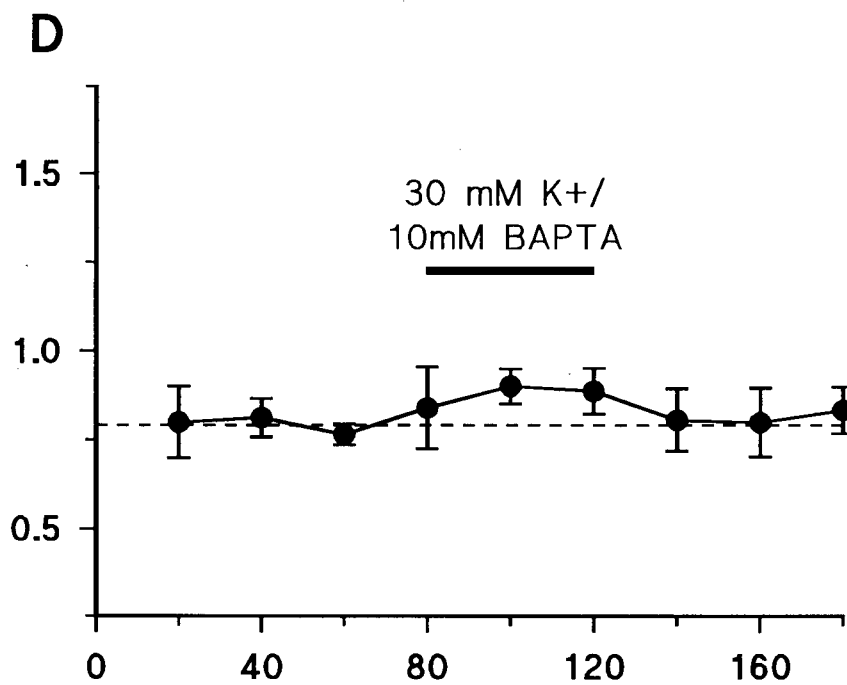
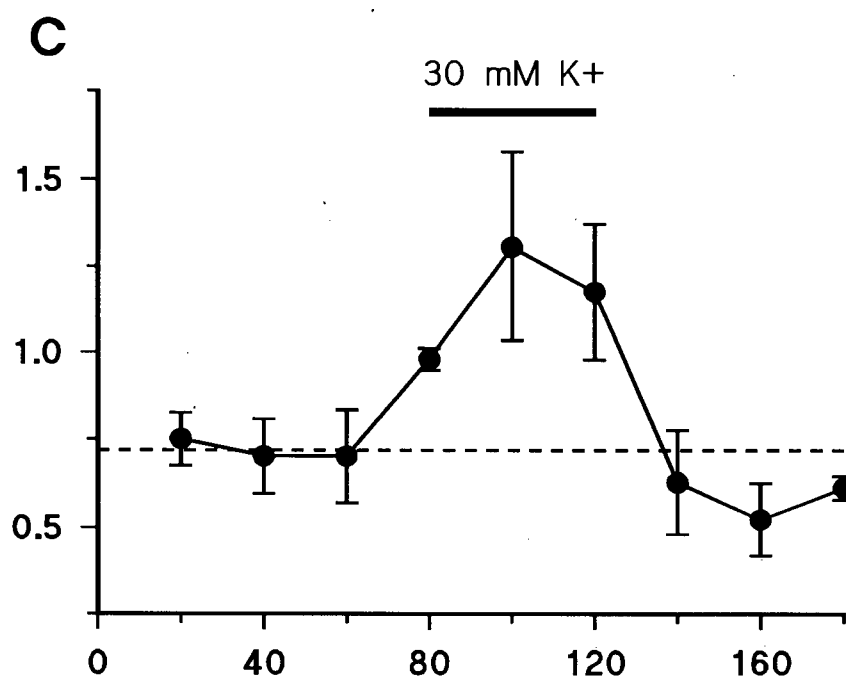
We therefore tested to see if other manipulations of neuronal activity could affect NO release. We focused upon calcium since NOS is a calcium-dependent enzyme (Bredt and Snyder, 1990). Perfusion with a nominal  $\text{Ca}^{2+}$ -free dialysis solution did not affect thalamic NO release ( $n=2$ ); however, when 10 mM of the  $\text{Ca}^{2+}$  chelator BAPTA was added, baseline production was significantly reduced in awake animals from  $1.09 \pm .05$  to  $0.80 \pm .07$  pmols/min ( $p<.01$ ,  $n=3$ ; Figure 12B). 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 in order to maintain osmolarity) significantly increased NO output in urethane-anesthetized animals from a baseline of  $0.72 \pm .06$  to  $1.15 \pm .11$  pmols/min ( $p<.001$ ,  $n=4$ ; Figure 12C). Finally, to determine whether the potassium-induced increase was  $\text{Ca}^{2+}$ -dependent, 30 mM potassium was added to a 10 mM BAPTA/ $\text{Ca}^{2+}$ -free solution. The BAPTA/ $\text{Ca}^{2+}$ -free solution prevented the effect of potassium ( $n=3$ ; Figure 12D). Taken together, these 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  $\mu\text{l}$  dialysis samples collected from each behavioral state was consistent across the seven animals tested. After a 5-6 hour 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  $101.77 \pm 10.95$  seconds across the seven animals tested; durations of active wake or SWS periods ranged from 40 seconds to a full 20 minutes. Values obtained from the SWS samples were averaged for each animal for the

Figure 12. On-line experiments demonstrating that the HbO<sub>2</sub> oxidation measured in the samples was due to NO synthase (NOS) and Ca<sup>2+</sup>-dependent activity. In each figure, the mean  $\pm$  SEM of extracellular NO in pmols/minute are shown versus time in minutes. Dashed lines represent the baseline average. A. Intraperitoneal injection of the NOS inhibitor, N-ARG, significantly reduced baseline NO production in awake animals ( $p < .001$ ;  $n=4$ ). B. Application of a Ca<sup>2+</sup>-chelator, BAPTA (10 mM) in a Ca<sup>2+</sup>-free dialysate solution significantly and reversibly reduced baseline production in awake animals ( $p < .01$ ;  $n=3$ ). C. Application of a solution containing 30 mM K<sup>+</sup> significantly increased NO release in urethane-anesthetized rats ( $p < .001$ ;  $n=4$ ). D. Addition of 10 mM BAPTA to the high-K<sup>+</sup> solution blocked the potassium-induced increase ( $n=3$ ), suggesting that the increase was due to Ca<sup>2+</sup>-dependent mechanisms. Although baselines varied slightly between 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 ( $p < .001$ ).





statistical analyses. The mean NO output during wake was  $1.34 \pm .07$  pmol/min; during SWS,  $0.97 \pm .03$  pmol/min; and during REM,  $1.42 \pm .12$  pmol/min (Figure 13). A repeated measures ANOVA showed that NO production varied significantly across state ( $p < .04$  with the Greenhouse-Geisser correction). Scheffe's post-hoc analysis showed that NO production did not vary significantly between wake and REM but was significantly less during SWS ( $p < .01$ ).

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, an electrode was placed in the LDT of urethane-anesthetized rats (1.5-2.0 g/kg), and thalamic NO release was measured in response to electrical stimulation. After collecting 3 baseline samples (one hour), the LDT was stimulated for a 20-minute period at 60 Hz (bipolar, sine-wave pulse) for 10s with 30s 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 10s 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 30s interstimulus interval, the EEG exhibited high amplitude, slow-wave activity, similar to that observed during natural SWS (Figure 14A). After the 20 minute stimulation period, the cortical EEG gradually reverted back to the burst-discharge state within 5 or 10 minutes. The 20 minute sample collected during the stimulation period was compared against the baseline average. LDT stimulation significantly increased thalamic NO release in 10 out of 12 rats from  $1.09 \pm .05$  to  $1.62 \pm .15$  pmols/min ( $p < .001$ ,  $n=12$ ; Figure 14B).



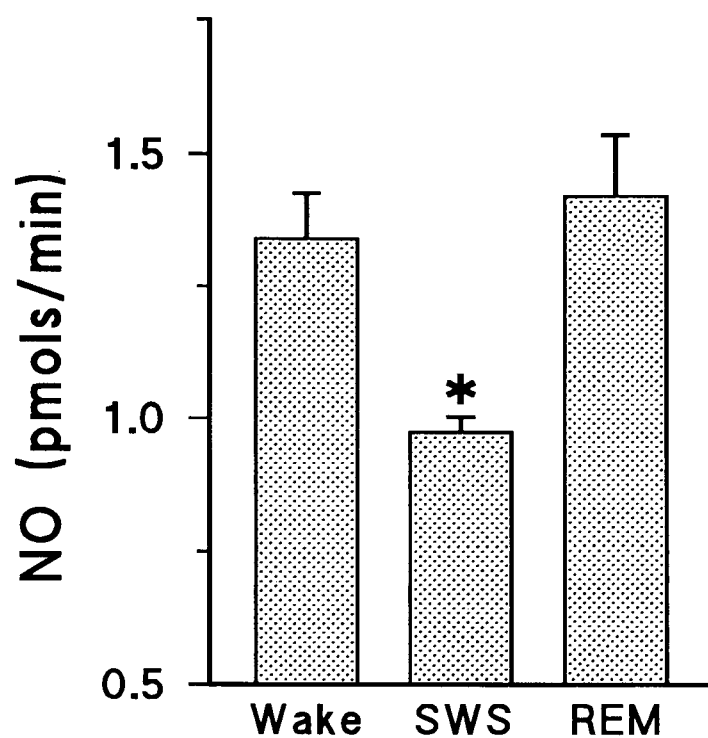
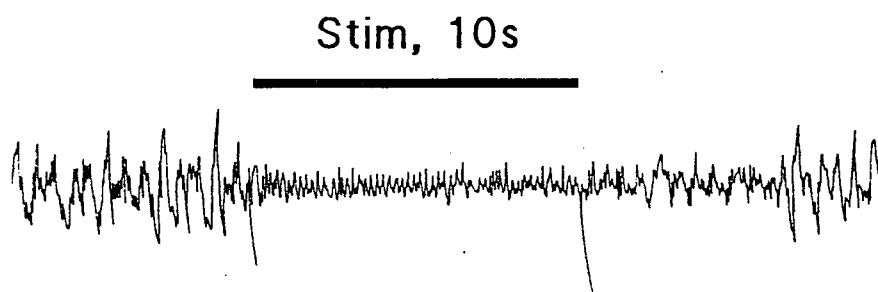
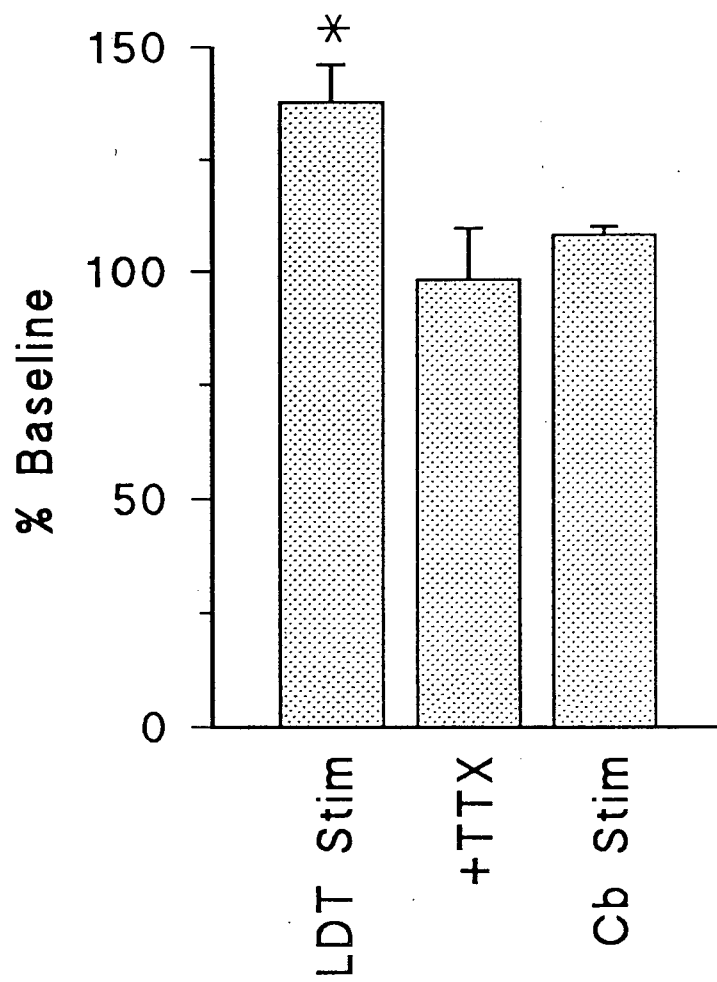


Figure 13. Extracellular NO across behavioral state in the rat thalamus. Mean NO production ( $\pm$  SEM) is reported for wake, slow-wave sleep (SWS), and REM sleep ( $n=7$ ). NO production did not differ between wake and REM, but was significantly lower during SWS ( $p<.01$ ).

Figure 14. Effects of electrical stimulation of the laterodorsal tegmental nucleus (LDT) on cortical EEG and NO production in the thalamus of anesthetized rats. A. EEG recording during a 10-second stimulation pulse exhibits low-amplitude waves (6-8 Hz) as compared to the high amplitude slow-wave activity during the interstimulus intervals (see results). B. LDT stimulation significantly increased NO output ("LDT Stim";  $n=12$ ;  $p<.001$ ). This effect was blocked by addition of 1  $\mu$ M TTX to the ringer's solution ("+TTX";  $n=3$ ). No change was observed when the stimulating electrode was placed in the cerebellum ("Cb Stim";  $n=3$ ).

**A****B**

In order to determine whether the stimulation-induced increase was due to action-potential-dependent activity, 1  $\mu$ M TTX was added to the dialysate. Consistent with the observations in awake animals, addition of TTX had no significant effect upon baseline NO production in anesthetized rats, however, the LDT-stimulated increase in NO production was completely abolished ( $n=3$ ; Figure 14B).

Increases in thalamic NO output in response to stimulation occurred when the electrode tip was in or near (within 600  $\mu$ m in the rostral direction) the LDT. However, when the electrode was placed in the cerebellum, stimulation had no effect upon the cortical EEG or on thalamic NO release (Figure 14B). 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 cyclic guanosine 3',5'-monophosphate (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). 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 voltammetry to

detect NO in the cortex, Cespuglio et al. (1996) showed that NO concentrations were highest during wake as compared to SWS and REM.

The origin of NO in the thalamus may arise from several brain regions including the hypothalamus, tectum, dorsal raphe, LDT/PPT, as well as from the cell bodies which stain for NOS in the ventral lateral geniculate nucleus of the rat (Vincent and Kimura, 1992; Gabbot and Bacon, 1994; J.A. Williams, unpublished observations). Based upon 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, as it was blocked by TTX, as well as site-specific, as no change in production 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 voltammetry. Taken together with our previous findings which showed that thalamic ACh release from mesopontine cholinergic terminals exhibited a similar behavioral profile (Chapter III; Williams et al., 1994), these data support the hypothesis that the state-dependence of thalamic NO release is largely derived from the corresponding activity of the NOS-containing mesopontine cholinergic neurons.

#### *NOS-dependent Signalling*

The second series of experiments verified the extent to which the assay for NO was sensitive to NOS-dependent activity. HbO<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> was controlled to some extent by addition of superoxide dismutase and catalase to the HbO<sub>2</sub> stock solution (Murphy and

Noack, 1994; see 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), the oxidation of HbO<sub>2</sub> by CO is unlikely for several reasons. First, the affinity of HbO<sub>2</sub> for NO is one-thousand-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<sub>2</sub> oxidation in the dialysate was indeed sensitive to NOS activity, as peripheral injections of the NOS inhibitor, N-ARG significantly reduced the amount of HbO<sub>2</sub> oxidation in the samples, reflecting a decreased concentration of NO. In addition, HbO<sub>2</sub> 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<sup>2+</sup> channels, thereby facilitating NOS activity. Indeed, the potassium-induced increase in NO was abolished by addition of a Ca<sup>2+</sup> chelator, BAPTA, indicating the Ca<sup>2+</sup>-dependence of NO production. Baseline production was also Ca<sup>2+</sup>-sensitive, as a BAPTA/Ca<sup>2+</sup>-free solution caused a significant decrease. Taken together, these observations suggest that the changes in HbO<sub>2</sub> 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 due to neuronal-dependent activity. However, to our surprise application of 1 μM TTX did not affect baseline production in awake animals. We have previously found that this concentration of TTX effectively reduced baseline concentrations of thalamic ACh (Chapter III). 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 basal concentrations of other neurotransmitters, such as glutamate or GABA, are also unaffected (or even increased) by TTX (Hashimoto et al., 1995; Abarca et al., 1995; Bradford et al., 1987; Tanganelli et al., 1994; 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, where animals were continuously active for the duration of the application. Any number of sequelae of this hyperaroused state may have increased thalamic NO production from 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  $\text{Ca}^{2+}$  dependent, while iNOS is not (Nathan and Xie, 1994; Förstermann and Kleinert, 1995). Because we were able to detect  $\text{Ca}^{2+}$ -sensitive activity, the majority of the NO signal was most likely due to either eNOS or neuronal NOS activity. NO was initially recognized to be an endothelial 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 highest levels occurring during wake and REM, and at progressively slower rates with each successive stage of SWS in humans (Hoshi et al., 1994; Hajak 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 may also 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 may also 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 upon sensory responses or upon the modulation of  $I_h$  remains to be determined.

## V. GENERAL DISCUSSION

Four major conclusions were drawn from the experiments presented in the previous chapters. First, the experiments presented in Chapter II revealed that mesopontine cholinergic neurons express both voltage-dependent and receptor-coupled ion channels which may be important determinants of their firing activity across behavioral state. The majority of type III neurons, which express both the transient outward potassium current,  $I_A$ , and the transient inward calcium current,  $I_T$ , were cholinergic. Type III neurons also accounted for the majority of the cholinergic population, as smaller numbers were found to be either type I (expressing only  $I_T$ ) or type II (expressing only  $I_A$ ). It is possible that the distribution of these types of cholinergic neurons reflect subpopulations of cells which may also express one or more of the neuropeptides known to exist in the LDT (Vincent et al., 1983a, Vincent et al., 1986; Sutin and Jacobowitz, 1988; Standaert et al., 1986; Saper et al., 1989). The observation that these cell types are expressed by LDT neurons are consistent with the findings of Leonard and Llinás (1990) who recorded from guinea pig brain slices. However, they found that the majority of cholinergic neurons in this preparation were type II neurons. Whether this discrepancy was due simply to differences in sampling, recording techniques, or species has yet to be determined. Another possibility is that in some instances  $I_A$  is more readily visible in current clamp than  $I_T$ . Kamondi et al. (1992) observed that when neurons which appeared to be type II were studied in voltage clamp or in the presence of a potassium channel inhibitor, 4-aminopyridine, they expressed  $I_T$ , which suggested that  $I_A$  might be modulated in such a way as to mask  $I_T$ . The second important finding described in Chapter II was that NA hyperpolarizes cholinergic neurons by activating the G-protein coupled inwardly rectifying potassium current,  $I_{KG}$  via an  $\alpha_2$ -adrenergic receptor. Other studies have shown that 5-HT and ACh also inhibit LDT cholinergic neurons by activation of  $I_{KG}$ .

(Luebke et al., 1992; 1993; Leonard and Llinás, 1994). Application of one or more of these transmitters did not have additive effects, which suggests that the various receptors through which these neurotransmitters act may all couple to the same ion channel (Leonard and Llinás, 1994). Based upon the extracellular recordings of raphe and LC neurons *in vivo* which showed that these cells exhibited highest firing rates during wake, we had hypothesized that brainstem cholinergic neurons would be profoundly inhibited during wake. However, as described in Chapter III, using *in vivo* microdialysis to measure ACh release in the thalamus, we found that this was not the case. ACh was released at high rates during wake and REM sleep, and at significantly slower rates during SWS. Retrograde tracing with fluorogold combined with ChAT immunohistochemistry revealed that the majority of cholinergic inputs to the vicinity of the dialysis probe arose from the mesopontine tegmentum. Finally, *in vivo* microdialysis coupled with the hemoglobin-trapping technique revealed that thalamic NO output likely from mesopontine cholinergic terminals was also high during EEG desynchronized states. Taken together, these findings raise a paradox: mesopontine cholinergic/nitergic neurons maintain a high activity rate during wake despite the presumed inhibitory aminergic input. They also raise the question of the function of this inhibitory input, which will be addressed below.

*Firing Activity of Mesopontine Cholinergic/Nitergic Neurons in vivo.*

The work in this thesis established that the majority of cholinergic neurons express the intrinsic voltage-dependent currents,  $I_A$  and  $I_T$ , as well as a G-protein coupled inhibitory current,  $I_{KG}$ . A key question is to what extent can these intrinsic currents predict the behavior of these cells *in vivo*? Using extracellular recording techniques *in vivo*, Steriade and co-workers observed that thalamically projecting LDT neurons exhibited both state-dependent firing as well as activity relating to PGO waves (Steriade et al., 1990b, 1990c). At least six varieties of firing patterns

were described in relation to PGO waves. These included either burst-firing or tonic activity immediately preceeding or following PGO waves recorded in the lateral geniculate nucleus of the thalamus. The burst-type neurons included one with a slow intraburst frequency, or “sluggish-bursts” which followed a hyperpolarization, and a second type which exhibited very high intraburst frequencies ( $>500$  Hz) over a background firing of 10-40 Hz. The “sluggish burst” neurons most likely represent the type III cholinergic neurons, as expression of  $I_A$  in addition to  $I_T$ , which underlies the low-threshold burst, would function to slow the intraburst firing (compare Figure 2A with 2C, page 20). The high-frequency bursting cells may reflect type I neurons, and finally, the cells exhibiting only the tonic activity may be type II neurons. While identification of some of the membrane properties of cholinergic neurons has allowed us to predict their firing patterns *in vivo*, the significance of the regulation of firing activity at the level of behavior remains unclear.

Both  $I_A$  and  $I_T$  require a hyperpolarization for removal of inactivation (Hille, 1992a). Based upon the observations that cholinergic neurons are hyperpolarized by NA, 5-HT, and ACh (Chapter II; Luebke et al., 1991; 1993; Leonard and Llinás, 1994) via activation of  $I_{KG}$ , one hypothesis is that this hyperpolarization functions to remove inactivation of  $I_A$  or  $I_T$ .  $I_T$  is known to require a minimum of 80 ms for de-inactivation (Kamondi et al., 1992; Hille, 1992a).

Activation of  $I_{KG}$  via a G-protein-coupled receptor agonist can produce a hyperpolarization lasting hundreds of milliseconds (North, 1989), which is sufficient for this prerequisite. Therefore, inhibition of cholinergic neurons by the amines or by autoregulation may be important for mediating burst-firing activity in conjunction with PGO waves, or possibly in regulating the rate of tonic firing activity during wake, as described below.

Of the LDT single units that were studied across the sleep-wake cycle in cats (Steriade et al., 1990b), the majority of thalamically projecting neurons fired at an average rate of 21 Hz during wake, but exhibited an oscillatory discharge pattern (0.8-1 Hz), with frequencies reaching as high as 100 Hz during the accelerated spike trains. In contrast, this acceleration-deceleration discharge pattern persisted during SWS, but with a lower average rate of 13 Hz. Finally, during REM sleep, these neurons fired at steady, high frequency rates averaging 30 Hz. This state-dependent firing pattern is consistent with the profile of thalamic ACh output (Chapter III), which suggests that these cells were cholinergic. The following scenario may account for this firing activity *in vivo*. First, several findings have suggested that mesopontine cholinergic neurons receive excitatory glutamatergic inputs. Local stimulation or application of glutamate receptor agonists in an *in vitro* slice preparation containing the LDT elicited excitatory post-synaptic potentials in and depolarization of, respectively identified cholinergic/nitrergic neurons (Sanchez and Leonard, 1994). Ionotropic glutamate receptor subunits have been reported to be expressed in mesopontine cholinergic/nitrergic neurons as identified by immunohistochemistry combined with NADPH-diaphorase staining (Inglis and Semba, 1996). Glutamatergic inputs may originate from a number of possible sources, such as the mPRF (Jones, 1995; Lai et al., 1993; Semba and Fibiger, 1992), the medial prefrontal cortex (Semba and Fibiger, 1992; Cornwall et al., 1990), and from some of the cholinergic/nitrergic neurons themselves which contain glutamate (Clements and Grant, 1990; Clements et al., 1991). Glutamatergic input from these sources may therefore provide a tonic excitatory tone upon cholinergic neurons, accounting for their high activity rate during wake or during REM sleep. Second, unit recordings *in vivo* have shown that both raphe and LC neurons exhibit firing rates of 2.5-4 Hz during wake (Aston-Jones and Bloom, 1981; Hobson et al., 1975; Harper and McGinty, 1976; Trulson and Jacobs, 1979). Stimulation of the

LC *in vitro* elicited inhibitory post-synaptic potentials (IPSP's) in LDT neurons which were greater than 400 ms in duration (data not shown). Although these cells were not histochemically identified as cholinergic, nor was it determined whether the IPSP was mediated by NA, a prediction based upon the data obtained from the recordings *in vitro* (Chapter II) is that NA would indeed exert this effect upon cholinergic neurons in the natural setting. Therefore, periodic inhibition of the mesopontine cholinergic/nitroergic neurons by NA or 5-HT may account for the oscillatory firing patterns observed during wake. Subsequent activation of  $I_{KG}$  would antagonize the short-lasting EPSP's elicited by glutamate, as the decreased resistivity of the cell would not allow small calcium conductances to evoke any significant change in the membrane potential. As discussed below, these oscillatory discharge patterns may influence thalamic processing of sensory and motor afferents.

#### *Implications for Ascending Projections*

The experiments in Chapters III and IV demonstrated that both ACh and NO are released in the thalamus at high rates during EEG desynchronized states as compared to SWS. These data are consistent with the excitatory role that has been suggested for both ACh and NO in the thalamus based on both *in vitro* and *in vivo* experiments. As discussed in the previous chapter, thalamic NO production may play a role in the modulation of firing activity (Pape and Mager, 1992), facilitation of sensory responses (Do et al., 1994; Cudiero et al., 1996), and in the control of blood flow (Koyama et al., 1994; Miyazaki et al., 1996). Similar functions have been suggested for ACh, although through different mechanisms. For example, NO is thought to suppress burst-firing activity in the thalamus by shifting the voltage-dependence of  $I_H$  via a cGMP-dependent pathway (Pape and Mager, 1992). Suppression of burst-firing activity is thought to be the underlying mechanism of EEG desynchronization (Steriade et al., 1990a; McCormick, 1992a).

ACh, on the other hand, exerts a variety of responses upon thalamic relay neurons in which the prominent overall effect is excitatory (i.e. suppression of burst-firing; reviewed in McCormick, 1992a). In particular, the cholinergic effects upon thalamic relay neurons include a rapid depolarization mediated by a nicotinic receptor, followed by a muscarinic-receptor-mediated slow depolarization resulting from a decrease in a resting potassium conductance (McCormick and Prince, 1987; McCormick, 1991). An inhibitory response to ACh has also been reported. However, this hyperpolarization is often immediately followed by the long-lasting excitatory effect (McCormick, 1992b).

NA and 5-HT also exert excitatory responses upon thalamic relay neurons in a manner similar to that of NO, by enhancing  $I_H$  (Pape and McCormick, 1989; McCormick and Pape, 1990). In addition, NA induces a slow depolarization upon thalamic relay neurons by decreasing a resting potassium conductance; an effect which was non-additive to that imposed by ACh, suggesting that these systems converge upon the same intracellular messengers or effectors (McCormick, 1992b). Finally, observations *in vivo* have suggested that NA and ACh are also involved in the facilitation of sensory responses (reviewed in McCormick, 1992a). Taken together, these data suggest that the brainstem monoaminergic and cholinergic/nitrgergic systems serve parallel functions in the thalamus important for controlling cortical EEG and arousal. Although the monoaminergic and cholinergic/nitrgergic systems appear to converge upon similar excitatory mechanisms, one hypothesis is that the monoamines may control thalamic processing of sensory and motor afferents by inhibiting mesopontine cholinergic/nitrgergic neurons, thereby reducing the frequency of cholinergic/nitrgergic excitatory input.

### *Implications for Descending Projections*

As discussed at length in Chapter III, the findings obtained by *in vivo* microdialysis have important implications not only for the ascending, but also for the descending cholinergic/nitroergic projections. The observation that NA and 5-HT hyperpolarize mesopontine cholinergic/nitroergic neurons is consistent with the hypothesis that monoamines play an inhibitory role in the generation of REM sleep. To reiterate, a widely accepted hypothesis is that ACh release from LDT/PPT terminals in the mPRF is the trigger for REM sleep. One possibility outlined in Chapter III is that NA and 5-HT may inhibit ACh release in the mPRF during wake via presynaptic receptors. In support this hypothesis, microinjection of the  $\alpha_2$  adrenergic antagonist, idazoxan, into the mPRF in cats significantly enhanced REM sleep (Bier and McCarley, 1994). This effect may be mediated by receptors on cholinergic terminals, based upon the evidence for an inhibitory role of NA in REM sleep (Karczmar et al., 1970; Tononi et al., 1991). In addition, microdialysis of the mPRF in cats has shown that ACh is released at highest rates in REM as compared to wake and SWS (Kodama et al., 1990; Leonard and Lydic, 1995). However, to date, no anatomical data are available to support these proposed interactions.

The experiments presented in Chapter IV suggest that NO is co-released with ACh in the thalamus; a similar finding may be predicted for the mPRF. Only recently have studies begun to address the role of NO output from cholinergic terminals in this region (Leonard and Lydic, 1995; 1996). Consistent with the potential excitatory role of NO in the thalamus, the studies by Leonard and Lydic (1995; 1996) suggest that NO may also have an excitatory role in the mPRF, as local application of NOS inhibitors had an inhibitory effect upon REM sleep. Further investigation of this issue is needed to determine the potential role of NO in the generation of REM sleep.



The effects of ACh upon mPRF neurons have been characterized in the brain slice preparation *in vitro* (Greene et al., 1989; Gerber et al., 1991; Stevens et al., 1993). The majority of mPRF neurons exhibited depolarizing responses upon application of cholinergic agonists, which were accompanied by a decrease in potassium conductance. These findings are consistent with an earlier study which showed that microiontophoretic application of ACh in the brainstem increased firing activity *in vivo* (Bradley and Dray, 1972), as well as with the observations that mPRF neurons increase firing rates during the onset of REM sleep (Ito and McCarley, 1984) and during carbachol-induced REM sleep (Shiromani and McGinty, 1986). However, cholinergic receptors were also found to mediate hyperpolarizing responses in separate populations of mPRF neurons (Gerber et al., 1991). Identification of the neurochemical phenotypes affected by ACh is essential for delineating the precise mechanism for triggering REM sleep. In addition, NA and 5-HT were found to evoke similar responses upon mPRF cells recorded *in vitro* (Gerber et al., 1990; Stevens et al., 1992; 1994). Whether the monoamines affect the same populations of mPRF neurons as ACh has yet to be determined.

As reviewed in the general introduction, the brainstem reticular formation is known to receive collaterals from sensory and motor pathways as well as to give rise to diffuse projections throughout the brain (Jones, 1995), and is therefore thought to have an important function in coordinating sensory and motor responses. While many of the studies described above address the role of the mPRF in triggering REM sleep, perhaps a more important function of this structure is in the "unconscious" processing of sensory information during wake. It is possible that the monoaminergic and cholinergic/nitrgic neurons converge upon the same populations of mPRF cells (Semba, 1993). Therefore, similar to the situation in the thalamus, monoamines may have an important function in modulating the processing of sensory and motor signals in the brainstem by

controlling the frequency of excitatory cholinergic input (the effects of NO upon mPRF neurons have not been studied) either via inhibition at the soma, or via actions upon the terminals.

### *Conclusions*

During the past twenty years, the actions of neurotransmitters contained the ARAS have been characterized to a large extent in the thalamus, cortex, and upon descending projection sites. As discussed above, the brainstem aminergic and cholinergic/nitroergic systems converge in at least two regions important for processing sensory and motor information: the thalamus and the mPRF. The former center is most likely involved in the "conscious" and selective processing of inputs from a variety of modalities, while the latter may be for the "unconscious" processing of similar input perhaps important for adaptation to sensory signals or for coordinating motor and sensory signalling. Pharmacological data derived from work in this thesis as well as others (Luebke et al., 1992; Koyama and Kayama, 1993; Leonard and Llinás, 1994) suggest that the amines may play an important modulatory role during wakefulness in the regulation of the tonic excitation mediated by cholinergic/nitroergic input to both regions.

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