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Cholinergic Mechanisms Of Rem Sleep (Brainstem, Rats, Rapid Eye Movement).

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CHOLINERGIC MECHANISMS OF REM SLEEP

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CHOLINERGIC MECHANISMS OF REM SLEEP

by

JAMES W. GNADT

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology and Biophysics in the Graduate School, The University of Alabama at Birmingham

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ABSTRACT OF DISSERTATION
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Title Cholinergic Mechanisms of REM Sleep

Several lines of evidence have implicated cholinergic mechanisms in the control of the sleep-wake cycle, and in the control of the rapid eye movement stage of sleep (REM sleep) in particular.

Theoretically, cholinesterase inhibitors can be used to potentiate physiological cholinergic activity in vivo. When administered acutely in rats, the "irreversible" cholinesterase inhibitor, di-isopropyl-fluorophosphate (DFP), was found to produce toxic effects which were incompatible with the occurrence of sleep and which disrupted sleep non-specifically. These toxic side effects, however, subside when the drug is administered chronically.

Following chronic administration of DFP, the amount of REM sleep was increased relative to controls due to an increased number of REM sleep periods and not an increased duration. This finding suggests that cholinergic mechanisms are involved in the initiation of REM sleep and not necessarily in its maintenance.

Historically, the pons has been implicated as the location of a REM "sleep center." Cannula microinjection techniques were used to deliver the cholinergic agonist carbachol into multiple brainstem sites in rats. These studies demonstrated two sites within the brainstem which produced increased REM sleep when stimulated by carbachol. One site lay within the pontine reticular formation medial to the trigeminal motor nucleus. The other site was in the caudal midbrain dorsal to the brachium conjunctivum. These data demonstrate that more than one location within the brainstem is involved in the generation of REM sleep.

In parallel with the effects of DFP, the pontine injections of carbachol increased the number and not the duration of the REM sleep periods. The finding is compatible with the hypothesis that cholinergic mechanisms are involved in the initiation of REM sleep.

Co-injection of the specific muscarinic receptor antagonist atropine blocked the carbachol-induced increase in REM sleep. This finding indicates that carbachol is mediating its effect via muscarinic cholinergic receptors.

Abstract Approved by: Committee Chairman G Vernon Pegram

Program Director GR Hageman

Date 5/31/85 Dean of Graduate School D Rozen

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LIST OF ABBREVIATIONS

AChE	acetylcholinesterase
Ci	Curie
c.i.	confidence interval
cm	centimeter, 10^{-2} meter
CSF	cerebrospinal fluid
DFP	di-isopropyl-fluorophosphate
EEG	electroencephalogram
EMG	electromyogram
Fig.	Figure
FTG	gigantocellular tegmental field
g	gram
ga.	gauge
hrs.	hours
Hz	hertz, cycles per second
I.D.	internal diameter
i.m.	intramuscular
in.	inch
Kg	kilogram, 10^{3} gram
LD_{50}	lethal dose, 50%
mg	miligram, 10^{-3} gram
min.	minute
ml	mililiter, 10^{-3} liter
mm	milimeter, 10^{-3} meter
NREM sleep	non-REM sleep
PGO waves	pontine-geniculo-occipital cortex waves
QNB	quinuclidinyl benzilate
REM sleep	rapid eye movement sleep
sec.	second
SEM	standard error of the mean
SWS	slow wave sleep
µCi	microcurie, 10^{-6} Curie
µg	microgram, 10^{-6} gram
µl	microliter, 10^{-6} liter
µm	micrometer, 10^{-6} meter
µV	microvolt, 10^{-6} volt

I. INTRODUCTION

Sleep is an actively generated behavioral and physiological state that has been studied extensively in mammals. Sleep is defined by the occurrence of several physiological and behavioral activities which occur in concert. Because the orchestration of these activities occurs so predictably, sleep generally is considered to be a phenomenon representing one or more consistent behavioral states. However, under certain conditions variations and disassociations of these various aspects do occur. These disassociations are indications that as a state phenomenon sleep is actually several processes occurring in parallel. Recent reviews of the various physiological aspects of sleep are provided by Orem and Barnes (1980), McGinty and Drucker-Colin (1982) and Parmeggiani *et al.* (1985). The pervasiveness of the phenomenon of sleep upon mammalian physiology is attested by the fact that all but a very few behavioral, somatic, and autonomic functions are modulated by transitions into and out of the states of sleep.

Early history of modern sleep research

The first systematic studies of normal sleep using electrophysiological techniques, specifically the electroencephalogram (EEG), were undertaken in the mid 1930s in both humans (Loomis *et al.*, 1935; Gibbs *et al.*, 1935; Blake and Gerard, 1937; Davis *et al.*, 1938) and in lower animals (Derbyshire *et al.*, 1936; Jasper, 1936; Rheinberger and Jasper, 1937). The later contributions of Clark and Ward (1945) and Hess *et al.* (1953) on sleep in the cat under natural conditions are also noteworthy.

Early studies utilizing experimental approaches to elucidate the controlling mechanisms mediating sleep can be traced to the now classic studies of Frederic Bremer. Bremer found that when the brain axis in cats was transected at the level of rostral mesencephalon, a preparation which he called the "cerveau isole" (Bremer, 1935), a synchronized EEG pattern was produced; a pattern not unlike that seen in normal sleeping cats. More caudal transections at the junction of the medulla and spinal cord, a preparation Bremer termed the "encephale isole" (Bremer, 1936), produced cats which exhibited both desynchronized EEG patterns typical of wakefulness as well as the synchronized EEG typical of sleep. Bremer attributed the sleep-like EEG of the cerveau isole to the loss of sensory afferents (all but those from the first and second cranial nerves), and attributed the waking EEG of the encephale isole to the additional sensory afferentation

of the other cranial nerves (Bremer, 1935; Bremer, 1936; Bremer, 1937).

On this basis, sleep was considered for many years a passive process due to the withdrawal of sensory stimulation. Interestingly, sleep was considered but one of many "disturbances of consciousness" (Gibbs et al., 1935) on a continuum of vigilance with such unnatural conditions as coma and barbiturate anesthesia.

Further evidence implicating sleep as a passive lack of wakefulness came in the late 1940s from the classic studies of Moruzzi and Magoun, and Lindsley and colleagues (Moruzzi and Magoun, 1949; Lindsley et al., 1949; Lindsley et al., 1950). Moruzzi and Magoun (1949) described a diffusely organized system within the brainstem reticular formation which mediated an EEG desynchronizing effect. This system was termed the "ascending reticular activating system" and was postulated to maintain the state of wakefulness and its concomitant desychronized EEG. Lesions of this system produced synchronized EEG patterns similar to that seen in the sleeping animal (Lindsley et al., 1949). Additionally, Lindsley et al. (1950) proposed that the synchronizing effect produced by brain transections was due not to deafferentation per se, as proposed by Bremer, but due to the loss of excitatory influence produced by sensory stimulation upon the reticular activating system of Moruzzi and Magoun (1949).

Despite some early evidence to the contrary (Hess, 1929; Nauta, 1946; Hess, 1947), this notion of sleep as a passive

process prevailed through much of the 1950s. As early as 1929, Hess had suggested that sleep was "the consequence of a state of excitation of certain portions of the central nervous system" (Hess, 1929). The idea that sleep was actively produced by particular "sleep centers" gained further support from the studies of Nauta (1946), who found that lesions involving the preoptic region of the hypothalamus in rats could produce complete or partial insomnia. As discussed by Nauta, insomnia following lesion of a specific brain region implicates that region as a sleep center. Extending his earlier studies, Hess later was to describe a medial thalamic "somnogeneous zone" using electrical stimulation techniques in cats (Hess, 1947). Also using electrical stimulation techniques in cats, Sterman and Clemente (1962) described a basal forebrain area just rostral to the optic chiasm which could produce EEG synchronization and behavioral sleep. Unique to this study was the finding that both low and high frequency stimulation was effective. Also noteworthy of the early studies to implicate specific regions of the brain as sleep centers is the report of Batini et al. (1958), who suggested that the caudal pons may exert a "sleep-inducing" influence upon the forebrain. Further discussion of the pons as a sleep center is continued below (Pontine mechanisms of sleep). For a comprehensive review of the early years of modern sleep research (circa 1930-1965), the extensive review by Moruzzi (1972) is suggested.

A summary of the state of the field in the late 1960s is provided by Moruzzi (1972, p. 114):

> To summarize the results of both lesion and stimulation experiments we may draw the following conclusions: i) the ascending reticular system and a group of neurons lying in the posterior hypothalamus are endowed with a tonic activating influence. They are probably concerned with the maintenance of wakefulness. ii) The lower brainstem and the basal forebrain area contains structures with an opposing function, which exert a tonic deactivating influence and lead ultimately to sleep.

Multiple states of sleep

The description of a state of sleep in which much of the brain and peripheral physiology becomes highly activated (Aserinsky and Kleitman, 1953) did much to dispel the notion of sleep as a simple, passive, and quiescent process. Ironically, this activated state of sleep had been reported previously in two of the earliest electrophysiological studies of sleep in both humans (Loomis *et al.*, 1935) and cats (Derbyshire *et al.*, 1936). These early researchers, however, had failed to recognize the implications of these findings with respect to a separate, active state of sleep. Nevertheless, since the classic description of Aserinsky and Kleitman (1953), mammalian sleep has generally been divided into two major states (see Parmeggiani *et al.*, 1985 for an overview): 1) A state characterized by relative behavioral and physiological quiescence, by generalized synchrony of the EEG (high-voltage, low-frequency activity and/or "sleep spindles"), by slow rolling eye movements, by maintained

though reduced somatomotor tone, by slow, regular heart and respiratory rates, and by increased parasympathetic activity combined with a slight attenuation of sympathetic activity. Homeostatic mechanisms are maintained under conditions favoring anabolic processes (Parmeggiani, 1985). Traditionally, this stage of sleep is called Slow Wave Sleep (SWS) by association with its EEG characteristics. Additional terminology includes synchronized sleep, quiet sleep, and non-REM (NREM) sleep (see below). 2) A second major stage of mammalian sleep is characterized by desynchronized EEG (low-voltage, high frequency activity) from neocortex, by homogeneous theta (7-10 Hz) EEG from the hippocampi, by characteristic rapid eye movements, by postural atonia with phasic myoclonic twitches, by irregular heart and respiratory rates, by phasic ascending volleys of activity from brainstem to forebrain (pontine-geniculo-occipital cortex or PGO waves), and by tonically decreased sympathetic activity with phasic excitatory bursts and phasic decreases in parasympathetic activity. Homeostasis, in at least some systems (thermoregulation), is suspended (Parmeggiani, 1985; Szymusiak and Satinoff, 1985). This stage of sleep has been called REM sleep (for Rapid Eye Movements), paradoxical sleep (Jouvet, 1965), desynchronized sleep, active sleep, and dream sleep (Dement and Kleitman, 1957a, 1957b). The term REM sleep will be used throughout this manuscript.

Pontine mechanisms of sleep

There is considerable evidence that some mechanism involved in the generation of sleep lies within the brainstem. The pons, in particular, has been implicated in the generation of REM sleep. Much of the early evidence concerning the existence of these mechanisms was provided by Michel Jouvet and colleagues during the 1960s and early 1970s (see Jouvet, 1972 for review). In a now classic study, Jouvet (1962) performed a series of brain transections in cats at multiple rostro-caudal levels; a summary of these findings can be found in Jouvet's 1972 review. In cats with caudal pontine transections, no signs of REM sleep phenomena were evident caudal to the transection (e.g., periodic postural atonia). Rostral to the transections, characteristics of both SWS and waking (EEG, eye movements, etc.) were evident, as well as a "stade d'interpretation difficile" which may have represented a REM sleep-like phenomenon. Recent confirmation of this finding (Siegel et al., 1984) suggests that these rostral symptoms probably were REM sleep. In cats with rostal pontine transections, there was a periodic atonia coupled with unusual lateral eye movements which were interpreted to represent REM sleep-like phenomena caudal to the transection. No REM sleep-like phenomena were evident rostral to the lesion. In cats with mesencephalic transections and rostral transections similar to the cerveau isole, motor and ocular symptoms of all three states of vigilance

(awake, SWS, and REM sleep) were evident caudal to the transection. Similar findings were later reported by Hobson (1965) and Villablanca (1966) and can be summarized as follows: 1) Total brain sections always uncouple descending and ascending aspects of sleep. 2) The interconnecting pathways are diffuse (determined by partial transections; Hobson, 1965). 3) Motor, ocular, and PGO aspects of REM sleep are retained in caudal indices of REM sleep when transections are made rostral to the pons. 4) The caudal pons is necessary and sufficient for periodic atonia. 5) The pons is the generator of PGO waves.

While most data concerning pontine mechanisms of REM sleep control come from the cat species, a recent report by Lavie et al. (1984) suggests that similar mechanisms may be present in humans. REM sleep was nearly totally absent in a patient with a brain lesion which apparently was localized to the pons.

Since these initial, if crude, demonstrations of the importance of the brainstem in the generation of REM sleep, many studies have focused on investigating what structures within the brainstem are responsible for generating REM sleep.

The monoaminergic theory of sleep

Restricted lesions within the brainstem initially suggested that the monoaminergic systems of the brainstem were

primarily involved in the generation of sleep, and in REM sleep in particular (Jouvet, 1972). Lesions of the raphe nuclei were found to produce an insomnia which was proportional to both the extent of the lesion and the reduction of forebrain serotonin (Jouvet and Renault, 1966; Pujol et al., 1971). In addition, lesions of the locus coeruleus (Jouvet and Delorme, 1965; Roussel et al., 1967) or projections from the locus coeruleus (Jones et al., 1973) were found to disrupt various aspects of REM sleep and waking. Lesions of the caudal locus coeruleus region abolishes the motor paralysis normally associated with REM sleep (Jouvet and Delorme, 1965; Roussel et al., 1967), while lesions involving the rostral locus coeruleus and ascending norarenergic projections through the midbrain produced a decrease in EEG patterns of waking (Jones et al., 1973).

Pharmacological evidence also suggested the involvement of monoaminergic systems in the regulation of sleep (Jouvet, 1972). Inhibition of serotonin synthesis by para-chlorophenylalanine (a tryptophan hydroxylase inhibitor) was reported to decrease sleep and was reversed by administration of 5-hydroxytryptophan (Koella, 1968; Pujol et al., 1971). Theoretically, 5-hydroxytryptophan restored normal sleep by restoring serotonin (5-hydroxytryptamine) synthesis (Koella, 1968). The 5-hydroxytryptophan is the precursor to the final enzymatic step of serotonin synthesis by 5-hydroxytryptophan decarboxylase. However, other mechanisms may have been involved (Pujol et al., 1971). The

pharmacological evidence implicating noradrenergic systems was more tenuous (Jouvet, 1972; Jacobs and Jones, 1978; Ramm, 1979), and is not reviewed here.

Based on these data and others (see Jouvet, 1972 for review), Jouvet proposed a theory of the mechanisms responsible for the generation of the sleep-wake cycle: A Monoaminergic Theory of the Sleep-Waking Cycle. According to Jouvet, the brainstem raphe neurons mediated behavioral and EEG aspects of SWS through their neurotransmitter serotonin. The caudal raphe (raphe pontis, magnus, pallidas, and obscurus) were involved in "priming mechanisms" for REM sleep, while the rostral raphe (raphe centralis superior, and dorsalis) were involved in EEG synchronization. Jouvet called the caudal 2/3 of the noradrenergic locus coeruleus complex the "executive neurons" of REM sleep, specifically mediating postural atonia, PGO waves and the rapid eye movements. The rostral 1/3 of the locus coeruleus, via the dorsal noradrenergic bundle, and the mesencephalic noradrenergic neurons were thought to mediate behavioral and EEG arousal.

During the following five to ten years, further studies failed to confirm the monoaminergic theory. Inconsistent results between species (Rechtschaffen et al., 1969) and between laboratories (Jacobs and Jones, 1978; Ramm, 1979) cast serious doubt on the validity of the original interpretation of the pharmacological and lesions studies concerning serotonin and the raphe. Additionally, unit recording studies of the mesencephalic raphe nuclei (raphe dorsalis and

centralis superior) in freely behaving cats has since demonstrated that these serotonergic raphe neurons exhibit a steadily decreasing amount of activity from wakefulness, through SWS, with virtual quiescence during REM sleep (McGinty and Harper, 1976; Trulson and Jacobs, 1979; Trulson et al., 1984; Rasmussen et al., 1984). The medullary raphe nucleus, raphe pallidus, which projects caudally rather than rostrally like the mesencephalic raphe, also exhibits very low activity during REM sleep and some decrease during SWS (Heym et al., 1982; Trulson and Trulson, 1982). Only the raphe magnus has been found to deviate from this pattern (Sheu et al., 1974; Cespuglio et al., 1981). Neurons in this nucleus were found to have their lowest activity in SWS, their highest activity during REM sleep, and their activity during wakefulness intermediate. These neurons, however, do not exhibit the autoinhibition by serotonergic agonists that other serotonergic neurons exhibit (see Rogawski and Aghajanian, 1981), and therefore may not be serotonergic (Cespuglio et al., 1981).

These findings make the proposition unlikely that the serotonergic raphe neurons are primarily involved in the generation of sleep, and SWS in particular. Furthermore, recent studies by Trulson et al. (1981) and Steinfels et al. (1983) have shown that the activity of the dorsal raphe is a concomitant of the REM sleep atonia, and not REM sleep per se.

An inhibitory role of the serotonergic system upon REM sleep has been proposed (McCarley, 1980; Jacobs and Jones, 1978), and has been convincingly demonstrated for the PGO waves in particular (Simon et al., 1973). Considering the activity profiles of the raphe during REM sleep (McGinty and Harper, 1976; Trulson and Jacobs, 1979; Heym et al., 1982; Trulson and Trulson, 1982; Trulson et al., 1984; Rasmussen et al., 1984), perhaps a more appropriate description of the role of the serotinergic system in REM sleep is to be disinhibitory. The role of this system in the control of SWS remains obscure, although an inverse relation between the mesencephalic raphe activity and sleep spindles during SWS (Trulson and Jacobs, 1979; Trulson et al., 1984; Rasmussen et al., 1984) may implicate a role for these nuclei in SWS similar to the role proposed for REM sleep.

With respect to the noradrenergic system, pharmacological studies have failed to confirm this aspect of monoaminergic theory as well. Inhibition of catecholamine synthesis by alpha-methyltyrosine (a tryosine hydroxylase inhibitor) produced no decrease in REM sleep in rats (Marantz and Rechtschaffen, 1967) and even an increase in REM sleep in cats (King and Jewett, 1971). Later lesion studies (Jones et al., 1977), in which care was taken to produce lesions specific to the region of the noradrenergic neurons of the locus coeruleus, while sparing the adjacent pontine reticular formation, showed that the locus coeruleus is not necessary for the occurrence of REM sleep. Furthermore, the

unit activity profile for locus coeruleus neurons across the sleep-wake states has been found to be similar to that of the dorsal raphe (Hobson et al., 1975; Aston-Jones and Bloom, 1981); that is, highest activity during waking, lower during SWS, and nearly absent during REM sleep. As proposed for the raphe, the noradrenergic system is now thought to be generally inhibitory upon REM sleep (Hobson et al., 1975; Jacobs and Jones, 1978) and upon PGO activity in particular (Jones et al., 1977). During REM sleep, the inhibitory activity of both the serotonergic and the noradrenergic systems appears to be withdrawn. Considering this role of the monoaminergic systems in the modulation of sleep, it is of interest that both the dorsal raphe and the locus coeruleus appear to be responsive to sensory stimuli (Jacobs et al., 1979; Trulson and Jacobs, 1979; Aston-Jones and Bloom, 1981; Trulson et al., 1984; Rasmussen et al., 1984).

The pontine reticular formation as a REM "sleep center" and the reciprocal interaction theory

In 1962, Jouvet demonstrated in cats that electrolytic lesions which involved the caudal portion of the nucleus reticularis pontis oralis and the rostral part of the nucleus reticularis pontis caudalis could suppress REM sleep relatively independent of changes in SWS or arousal (Jouvet, 1972; reviewed in Jouvet, 1967). This finding has been confirmed by Jones (1979) and Friedman and Jones (1984) using

more restricted radio frequency heat lesions. Furthermore, it appears to have been the destruction of neurons within lateral pontine reticular formation, or fibers passing through this region, which was responsible for producing this effect. Destructive lesions restricted to the lateral aspect of the reticular formation mimicked the effect of the larger regions, while lesions in the medial aspect failed to produce any major long-term disruptions in REM sleep or sleep-wake cycle (Friedman and Jones, 1984). In agreement with this finding that the medial caudal pontine reticular formation is not necessary for REM sleep are the findings of Sastre et al. (1981) and Drucker-Colin and Pedraza (1983) that kainic acid lesions of the gigantocellular tegmental field (FTG, Bermen, 1968) did not produce disruption of REM sleep. Kainic acid was used to destroy the FTG cells while sparing fibers in passage.

While those findings demonstrate that the medial pontine reticular formation is not necessary for REM sleep to occur, it does not preclude this region from being involved in the normal generation of REM sleep. Cholinergic stimulation in this area can produce REM sleep (see Cholinergic mechanisms of sleep). This response to cholinergic stimulation in the FTG is abolished by kainic acid lesions in the FTG (Drucker-Colin, 1983).

In the early 1970s, several reports from Hobson, McCarley, and colleagues (Hobson et al., 1974; McCarley and Hobson, 1975a; Hobson et al., 1975) suggested that neurons

within the gigantocellular reticular field (FTG; Berman, 1968) of the medial caudal pontine reticular formation were primarily involved in the generation of REM sleep. In an unanesthetized head restrained cat preparation, Hobson et al. (1974) reported that neurons within the FTG exhibited relatively higher rates of activity during REM sleep than during wakefulness or SWS. Their state specific activity profile and discharge pattern during REM sleep (McCarley and Hobson, 1975a) led Hobson and McCarley to postulate that the FTG neurons were "a critical part of an executive system" for the generation of REM sleep (Hobson et al., 1975). As pointed out by McCarley and Hobson (1975a), the widespread ascending, descending, and local axonal projections of the FTG giant cells were consistent with these cells serving a generalized state-related function.

Furthermore, noting a state specific decrease in activity of presumed noradrenergic locus coeruleus neurons (Hobson et al., 1975), Hobson and McCarley proposed an interaction of these two neuronal groups in the mediation of REM sleep. A reciprocal interaction between the locus coeruleus and the FTG was proposed (Hobson et al., 1975; McCarley and Hobson, 1975b). During wakefulness and SWS the activity of the locus coeruleus was thought to be inhibitory upon the FTG. As REM sleep approached, the activity of the locus coeruleus waned, thus progressively disinhibiting the FTG. At a critical point, the FTG would escape locus coeruleus inhibition and quickly recruit activity through intrapontine

self-excitatory mechanisms, thus mediating the onset of REM sleep. As FTG activity continued, the FTG would slowly recruit activity within the locus coeruleus until the inhibitory influence of the locus coeruleus would again prevail, thus turning off FTG activity and ending the REM sleep period. Based on indirect histochemical and pharmacological evidence, the FTG neurons were proposed to be both cholinoceptive and cholinergic.

Subsequent studies in other laboratories, however, have failed to confirm these original findings. In unrestrained cats, Siegel and colleagues (Siegel and McGinty, 1977; Siegel et al., 1977; Siegel and Tomaszewski, 1983a and 1983b) found that unit activity within the medial reticular formation is functionally related to motor activity. Most medial reticular formation activity was related to specific movements or groups of movements, while no activity was found which was exclusively related to REM sleep (Siegel and Tomaszewski, 1983a and 1983b). Similar findings have been reported for the rat species (Vertes, 1977 and 1979). The contradictory findings of these later studies have been attributed to the fact that the cats in the Hobson and McCarley studies had been conditioned to accept head restraint and therefore exhibited very little spontaneous movement (Siegel et al., 1977).

During REM sleep central motor systems become highly activated (Siegel et al., 1977), but behavioral expression of this activity is masked by the occurrence of a strong

postsynaptic inhibition of somatic motor neurons (Chase and Morales, 1985). Therefore, the REM sleep specific activity of FTG cells noted by Hobson and McCarley may have been a result of REM sleep and not a causal factor in REM sleep.

As reviewed above, the findings of Sastre et al. (1981) and Drucker-Colin and Pedraza (1983) that kainic acid lesions of FTG neurons do not eliminate REM sleep also speaks against the postulate that FTG cells are primarily involved in the generation of REM sleep. Furthermore, Jones et al. (1977) also have shown that the locus coeruleus is not necessary for REM sleep.

In support of the earlier findings concerning the medial pontine reticular formation, a recent report of intracellular recordings of medial pontine reticular neurons (Ito and McCarley, 1984) suggests that these neurons do exhibit a state-specific tonic depolarization and increase in excitability during REM sleep. However, whether this effect is a consequence of REM sleep or a contributing factor to producing REM sleep remains to be determined.

To date, no specific information is available on the state-related activity profiles of neurons located in the lateral pontine reticular formation, which recently has been implicated as a REM sleep center by Friedman and Jones (1984) using lesion techniques.

Cholinergic mechanisms of sleep

A large body of indirect evidence concerning the involvement of cholinergic systems in sleep has been reviewed (King, 1971; Jouvet, 1972; Jacobs and Jones, 1978) and will not be repeated in detail here. A few selected lines of evidence will be reviewed with emphasis on the involvement of cholinergic mechanisms in the generation of REM sleep, particularly those mechanisms within the brain stem.

A very early indication in the history of experimental sleep research that cholinergic mechanisms might be involved in the generation of sleep came from the report by Dikshit (1934) that introduction of acetylcholine into the lateral ventricle or hypothalmic region can produce a condition "closely resembling sleep." Later, in the early 1960s, Hernandez-Peon and colleagues (Hernandez-Peon, 1964; Hernandez-Peon et al., 1963; Velluti and Hernandez-Peon, 1963) mapped out an extensive cholinergic hypnogenic system in the brain using local application of acetylcholine and/or physostigmine (a cholinesterase inhibitor); see Hernandez-Peon, 1965 for review. This cholinergic system included several regions of cerebral cortex, medial thalmic nuclei, portions of the striatum, the limbic-midbrain circuit of Nauta (Nauta, 1958), the pontine reticular formation, and portions of cervical spinal gray. The specificity of Hernandez-Peon's methods of drug delivery (local application of "minute crystals" via indwelling cannulae) has been questioned (King,

1971); however, his data did strongly suggest that cholinergic mechanisms were involved in the mechanisms producing sleep. A later study by Hazra (1970) showed that inhibition of acetylcholine synthesis in cats by hemicholinium-3 resulted in profound and complete loss of REM sleep and a dose-dependent increase in SWS.

Another indirect approach to the investigation of cholinergic involvement in sleep involves the use of cholinesterase inhibitors. By inhibiting the degradation of synaptically released acetylcholine, theoretically one can potentiate physiologically occurring cholinergic activity. In cats, the cholinesterase inhibitor di-isopropyl-fluorophosphate (DFP) can increase REM sleep time; however, the effect appeared to be somewhat unreliable (Belenky et al., 1968). In another study (Domino et al., 1968), it was found that physostigmine in cats could produce episodes of REM sleep directly from an awake or "drowsy" state. Normally, REM sleep appears only after a requisite period of SWS. Similarly, in humans, the onset of REM sleep is advanced relative to normal by the infusion of physostigmine (Gillin et al., 1978).

Several clinical studies in humans have investigated the effects of cholinesterase inhibitors on sleep (Grob et al., 1947; Grob and Harvey, 1958; Metcalf and Holmes, 1969; Duffy et al., 1979). In studies of the effects of two different cholinesterase inhibitors, DFP (Grob et al., 1947) and sarin (isopropyl methyl phosphonofluoridate; Grob and

Harvey, 1958), it was found in both cases that a prominent symptom was subjective reports of excessive dreaming. No objective measures of sleep parameters were made. In a later report by Metcalf and Holmes (1969), objective measures of all-night sleep records were performed on a population of industrial and agricultural workers who had been exposed to cholinesterase inhibitors in industrial accidents.[1] The studies of 9 of 12 subjects were described as "narcoleptic-like" sleep records. In another study of workers having accidental exposure to sarin (Duffy et al., 1979), it was reported that the exposed population of workers had increased amount of REM sleep when compared to a matched control group.

A recent report of DFP administration in rats (Gnadt et al., 1985b) has reported increased REM sleep in that species as well. The results of this study is presented in Section II. Systemic Studies.

One other line of evidence concerning the effect of cholinesterase inhibitors on sleep is the fact that in decerebrate cats, administration of physostigmine precipitates what may be a rudimentary REM sleep-like phenomena (Matsuzaki et al., 1967; Pompeiano and Hoshino, 1976).

To investigate brainstem cholinergic mechanisms of REM sleep, a number of investigators have utilized techniques of

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[1] A species of cholinesterase inhibitors, organophosphates, are manufactured commercially as pesticides and warfare agents due to their potent biocidal activity.

delivering cholinergically active agents directly into the brainstem of cats (George et al., 1964; Baxter, 1969; Mitler and Dement, 1974; Amatruda et al., 1975; Van Dongen et al., 1978; Silberman et al., 1980; Masserano and King, 1982; Hobson et al., 1983; Baghdoyan et al., 1984a, 1984b). The laboratory of Hobson (Amatruda et al., 1975; Silberman et al., 1980; Hobson et al., 1983) and most recently Baghdoyan of the Hobson group (Baghdoyan et al., 1984a, 1984b) have pursued this approach most vigorously in pharmacological tests of the reciprocal interaction theory of Hobson and McCarley (Hobson et al., 1975). Recently, similar studies have been undertaken in the rat species (Gnadt, 1985a, 1985b, 1985c).

Although studies using electrical stimulation techniques have met with some success in producing REM sleep from stimulation within the brainstem (Monti, 1970), it must be remembered that many ascending and descending fiber systems course through the brainstem. Selective stimulation of neurons endogenous to the stimulation site, without also stimulating fibers in passage, is impossible with electrical stimulation techniques. Indeed, many of the brainstem nuclei proposed to be involved in the mediation of sleep lie very near or even among fiber systems that would be postulated to be disruptive to sleep if stimulated. Moreover, the pontine reticular formation, which is implicated in the mediation of REM sleep, overlaps extensively with the ascending reticular activating system of Moruzzi and Magoun (Moruzzi and Magoun, 1949).

Theoretically, chemical stimulation techniques should provide a means of selectively stimulating receptors of neurons endogenous to the stimulation site, as well as providing information on the neurochemical coding of the mechanisms in question. The major drawback of the chemical stimulation techniques lies in the difficulty in limiting and controlling for the diffusion of the agent to remote sites. Despite this technical deficiency, chemical stimulation techniques have provided valuable information concerning the cholinergically mediated mechanisms of REM sleep.

The beginning of investigations of cholinergic mechanisms within the brainstem which are specifically related to REM sleep can be traced to the study of George et al. (1964). These investigators found that injections of the cholinergic agonist carbachol (carbamylcholine chloride, an amide resistant to hydrolysis by acetylcholinesterase) or the specific muscarinic agonist oxotremorine into the nucleus reticularis pontis caudalis or oralis could produce a "behavioral and electroencephalographic picture indistinguishable from [REM sleep]." In some cases, however, the cats appeared to be awake, but to have a complete absence of muscle tone. This atonia during wakefulness is now thought to be a disassociation of REM sleep atonia into the awake state, and can be seen to occur spontaneously in humans afflicted with the sleep disorder narcolepsy (Roth, 1980). The common term for the phenomenon in humans is cataplexy.

This drug-induced REM sleep-like phenomenon in cats persisted sometimes for as long as 50 min, and could be reversed by local injection of atropine (a selective muscarinic antagonist) into the same injection site, or by atropine given systemically. Atropine alone, epinephrine, norepinephrin, serotonin, dopamine, nicotine and isotonic potassium chloride injected into the same sites failed to produce noticeable behavioral or EEG effects. In this study, drugs were delivered either unilaterally or bilaterally in saline vehicle at 1 to 2 μl volumes.

In 1969, Baxter reported what he called "a novel form of REM sleep" produced by administering carbachol to the midbrain gray near the cerebral aqueduct (Baxter, 1969). Following a 9 to 14 min period of generalized excitement and "emotional behavior," the cats lapsed into a REM sleep-like state directly from wakefulness. An unusual feature of this phenomenon was that the cats were completely unresponsive to any sensory stimulation, including surgical incision of the skin. Clearly, then, this phenomenon was not truly REM sleep even though virtually all of the signs of REM sleep were present. In addition, the specificity of the carbachol stimulation in this study was questionable. The carbachol was applied to the stimulation site as crystalline powder. The proximity of the stimulation site to the cerebral aqueduct makes fairly likely the possibility that the carbachol entered the ventricular system. In fact, Baxter proposed

that the effects were due, at least in part, to diffusion of the drug into the ventricular system.

Two other reports of carbachol-induced atonia following pontine injections have been reported (Mitler and Dement, 1974; Van Dongen et al., 1978). In an attempt to model human narcolepsy-cataplexy, Mitler and Dement (1974) injected carbachol into the mediodorsal aspect of the rostral pontine reticular formation, just ventral to the central gray in cats. These injections precipitated prolonged periods of atonia, sometimes accompanied by PGO waves. However, the cats would track visual targets and blink to threat and, therefore, were awake. Focal injection of atropine reversed the atonia within 2 to 5 min. Injections were bilateral in 1 µl volumes per side, delivered over 45 to 60 sec. It was concluded by the authors that the carbachol-induced atonia did not resemble the narcolepsy-cataplexy syndrome closely enough to be utilized as an animal model for the disorder.

A more extensive study by Van Dongen et al. (1978) found similar results to those found by Mitler and Dement. Injections of carbachol in the pontine reticular formation near the locus coeruleus produced a state of generalized atonia. As found by George et al. (1964), unilateral injections could produce bilateral, generalized atonia. All injections were 0.5 µl. Atropine injections could block or reverse the effect, but mecamylamine (a nicotinic antagonist) did not. The effect was not reproduced by injection of physostigmine, norepinephrine, serotonin, alpha adrenergic

agonists (clonidine and oxymetazoline), a beta adrenergic agonist (isoprenaline), an alpha adrenergic antagonist (piperoxane), or a beta adrenergic antagonist (propanolol). Also, the carbachol-induced atonia was not affected by systemic administration of the alpha adrenergic blocker, phenoxybenzamine, or by the beta adrenergic blocker, propranolol. Carbachol injections into the fourth ventricle did not produce atonia.

These data suggested that the atonic effect was produced specifically by muscarinic mechanisms and did not involve adrenergic mechanisms. The authors also suggested that the effect was not produced by vascular changes due to cholinergic stimulation since adrenergic agents, which are known to affect cerebral vasculature, did not mimic the effect. The negative finding regarding physostigmine is surprising, but another cholinesterase inhibitor (neostigmine) has been found to be effective in a similar paradigm (Baghdoyan et al., 1984b; see below). The most effective site for producing the atonia was a location medioventral to the locus coeruleus. Injections into more ventral portions of the pontine ventricular formation produced the additional symptom of relaxed nictitating membrane (usually present only during sleep); however, the authors imply that the cats remained awake.

In order to test predictions regarding the cholinergic nature of the reciprocal interaction model of REM sleep control (Hobson et al., 1975), Hobson and colleagues have

published a series of papers using cholinergic stimulation in the brainstem (Amatruda et al., 1975; Silberman et al., 1980; Hobson et al., 1983; Baghdoyan et al., 1984a, 1984b). Initially, large volume unilateral injections (3 µl) into the FTG of the pons were found to produce a REM sleep-like state (Amatruda et al., 1975). The phenomenon was variable both qualitatively and quantitatively. Disassociations sometimes were noted and autonomic phenomena (i.e., panting and salivation) were sometimes present. Despite this, the phenomenon was reported to occur at a rate of as much as 3.5 times baseline values of REM sleep. The state was increased both in number and length compared to baseline REM sleep during the first two hours post-injection. The effect appeared to be dose-dependent and was blocked by atropine in one trial. The most effective site for producing the effect was the FTG. Injections of carbachol into the locus coeruleus reduced the amounts of REM sleep, but so did the injection vehicle (an artificial CSF solution).

In a later study (Silberman et al., 1980), a less variable and more natural state was achieved using smaller injection volumes (1 µl and 0.25 µl) in a saline vehicle. As in the original study, injection sites were centered in the FTG. Behaviorally and electrophysiologically, the cats appeared to be in normal REM sleep. They could be aroused by sensory stimulation. At times the drug-induced state occurred directly from wakefulness. Quantitatively, the REM-like state occurred at a rate of as high as 12 times

baseline values of REM sleep. The increase was due to a shorter latency and longer duration of episodes. The saline vehicle did not produce measurable effects upon sleep parameters. These studies were interpreted as providing evidence for the cholinergic aspects of the reciprocal interaction theory, at least with respect to the FTG. The findings concerning the locus coeruleus were inconclusive, but a later study by Masserano and King (1982) confirmed that cholinergic stimulation in the locus coeruleus could selectively decrease REM sleep compared to vehicle controls.

The findings concerning the pontine reticular formation were confirmed later (Hobson et al., 1983) using a selective muscarinic agonist, bethanachol (beta-methyl carbamylcholine chloride). The 0.5 or 1.0 µl injections again induced a state indistinguishable from normal REM sleep. The effects were most prominent for the first hour postinjection, but could persist for as long as 30 hours. The effect was dose-dependent and restricted to the pons. Injections into the midbrain or medullary reticular formation either reduced REM sleep or had no effect. This study, therefore, reinforces the suggestion that cholinergic stimulation in the pontine reticular formation can produce REM sleep through muscarinic mechanisms.

The site-dependency of this cholinergic stimulation was later confirmed by Baghdoyan et al. (1984b), who showed that injections (0.5 µl) of carbachol into midbrain and medullary reticular formation decreased amounts of REM sleep, while

pontine injections enhanced the REM sleep-like state. Unlike bethanechol (Hobson et al., 1983), the carbachol effect in the pons appeared to be most pronounced in the second, third, and fourth hours postinjection. An area in the anteromedial pons appeared to be the optimal site for producing the REM sleep-like state in its most natural expression.

If cholinergic mechanisms within the pontine reticular formation are involved in the generation of REM sleep, then theoretically it should be possible to enhance the occurrence of REM sleep by potentiating physiological cholinergic activity by means of cholinesterase inhibition. In a recent study, Baghdoyan et al. (1984a) placed injections of the cholinesterase inhibitor, neostigmine, into the pontine reticular formation. Injections were .25 µl and were located in the pontine reticular formation rostral to the genu of the facial nerve. Neostigmine was found to increase REM sleep in a dose-dependent manner, due to a decreased latency and to increased number and duration of episodes. This effect was blocked by concomitant injection of atropine.

Presumably, this study would seem to indicate that potentiation of cholinergic activity in the pontine reticular formation by slowing the degradation of the neurotransmitter acetylcholine can augment REM sleep. However, the failure of physostigmine to produce the same effect (Van Dongen et al., 1978; Baghdoyan et al., 1984a) casts some doubt on that interpretation. Baghdoyan et al. (1984a) suggests that

neostigmine, in part, may be stimulating the acetylcholine receptors directly. This inconsistency awaits further investigation.

All of the studies described here, in which cholinergic stimulation was applied directly to brainstem structures, have been performed in the cat species. The cat is considered by many to represent an atypical species for the study of mammalian, and particularly human, sleep. The cat is an excessively somnolent species with very weak entrainment to circadian cues. To date, only one study using another species for detailed investigation of the effects of small volume injections of cholinergically active agents has been performed (Gnadt, 1985a, 1985b, 1985c). The results of this study are presented in Section III. Microinjection Studies.

Summary

In summary, there is a large body of evidence to implicate cholinergic mechanisms in the generation of REM sleep. The brainstem reticular formation, and in particular the pontine reticular formation, seems to be the locus of a cholinoceptive system which, at the least, is facilitory upon REM sleep, and perhaps is executive in its generation.

There is a cholinoceptive mechanism located ventromedial to the locus coeruleus which is involved in the mediation of REM sleep atonia (Mitler and Dement, 1974; Van

Dongen et al., 1978). Lesion studies (Henley and Morrison, 1974), unit recording studies (Sakai, 1980), and anatomical studies (Sakai et al., 1979) also implicate this area in the mediation of REM sleep atonia (see Sakai, 1985 for review). Cholinergic stimulation in the more ventral portions of the pontine reticular formation (George et al., 1964; Amatruda et al., 1975; Silberman et al., 1980; Hobson et al., 1983; Baghdoyan et al. 1984a, 1984b; Gnadt, 1985a) implicates this structure as being involved in the generation of the entire concordant REM sleep state. Autoradiographic localization of muscarinic receptors using the specific high affinity ligand quinuclidinyl benzilate (QNB) in the rat brainstem has shown that the pontine reticular formation has a low but finite amount of muscarinic receptors (Wamsley et al., 1981).

Baghdoyan et al. (1984b) suggest that the optimal site for cholinergic stimulation of REM sleep is in the medial pontine reticular formation just rostral to the level of the genu of the facial nerve. However, lesion studies (Sastre et al., 1981; Drucker-Colin and Pedraza, 1983; Friedman and Jones, 1984) have shown this area of the brainstem not to be necessary for REM sleep. Extracellular unit recording studies (Siegel and McGinty, 1977; Siegel et al., 1977; Vertes, 1977, 1979; Siegel and Tomaszewski, 1983a, 1983b) also cast doubt upon the selectivity of activity in the medial pontine reticular formation for REM sleep. Ito and McCarley (1984), on the other hand, have used intracellular recording in

medial pontine neurons to demonstrate a state-dependent depolarization in medial pontine reticular neurons.

The reciprocal interaction theory as originally proposed by Hobson and McCarley (Hobson et al., 1985) seems to have been largely discredited by more recent anatomical and neurophysiological studies. The cellular aspects of the theory in particular, have been called into question. However, pharmacological evidence in the cat, at least, has been generally compatible with the neurochemical predictions of the theory. The field of sleep research, it would seem, is in need of new or redesigned theories of the cellular control of sleep based on the current empirical evidence available.

Experimental goals

The experiments described below were undertaken with the intent to investigate cholinergic involvement in the generation of REM sleep. The series of experiments involved two different experimental approaches and two sets of experimental goals. These two sets of experiments will be considered separately. The first is a series of experiments utilizing systemic administration of cholinesterase inhibitors in rats (Section II). The second series of experiments involves the use of microinjection techniques to investigate cholinergic mechanisms within the brainstem (Section III).

The term as used in this manuscript requires some definition. In its strictest use, the term most appropriately describes injection techniques which can apply drugs or solutions to one or a few neurons. This specificity of action can be achieved only by use of iontophoretic or micropressure injection techniques. The term microinjection as used here is a relative term to describe techniques of localized injection in the tenths of microliter range, which can apply solutions to localized regions of brain or to specific brain nuclei. The specificity of this technique is sufficient for inference concerning the generalized function of a brain nucleus or of aggregates of neurons.

Systemic studies. In consideration of the findings concerning cholinesterase inhibitors in humans, a series of experiments were undertaken to determine the effects of these agents in rats. It was hoped that a time and cost effective model of excessive somnolence would be developed. The effects of cholinesterase inhibitors in humans had been described as narcoleptic-like. Data from both humans and cats had shown that cholinesterase inhibitors could produce effects upon REM sleep, and much experimental data implicated cholinergic mechanisms in the generation of REM sleep. The goal of these studies was to develop an experimental model which might mimic the human sleep disorder narcolepsy. Such a model would be valuable in investigating possible mechanisms producing this disorder, and perhaps in the mechanisms generating REM sleep itself.

Microinjection studies. Once it could be shown that probable cholinergic mechanisms were involved in the generation of REM sleep in rats by using the systemic studies described above, this effect would need to be investigated more thoroughly. At what locus was the cholinesterase inhibitors having their effect? The pontine reticular formation would be a likely candidate. What was the pharmacologic nature of their effect? Muscarinic mechanisms had been implicated previously in the brainstem control of REM sleep in cats.

Additionally, virtually all experimental data to date concerning cholinergic mechanisms of REM sleep were gleaned from the cat species. Similarities and differences in cross-species studies would likely be illuminating.

Finally, microinjection studies of cholinergic mechanisms in the brainstem would serve as independent tests of the neurochemical predictions of the reciprocal interaction theory of REM sleep control. While the cellular aspects of the reciprocal interaction theory have come into question in recent years, the neurochemical predictions concerning cholinergic mechanisms of the theory still seem to be valid.

II. SYSTEMIC STUDIES

The following studies were undertaken to investigate possible cholinergic mechanisms involved in the control of sleep. Several lines of evidence have suggested a cholinergic involvement in the control of REM sleep in particular. These data have been reviewed above.

Theoretically, it should be possible to potentiate cholinergic function *in vivo* by use of the cholinesterase inhibitors. By slowing the enzymatic degradation of acetylcholine at synaptic sites, the post-synaptic activity of physiologically released acetylcholine should be enhanced. Thus the cholinesterase inhibitors represent a powerful pharmacological tool with which to study cholinergic involvement in the control of sleep. The organophosphate cholinesterase inhibitors are particularly well suited for this use due to their long term activity. The organophosphates bind covalently to the esterase enzyme at the active site and hence have been termed "irreversible" cholinesterase inhibitors. *De novo* synthesis of new enzyme is required for return of the esterase activity. An overview of the chemistry of the organophosphates is provided by Taylor (1980).

As noted above, the effects of several organophosphates upon sleep in humans have been reported (Grob et al., 1947; Grob and Harvey, 1958; Metcalf and Holmes, 1969; Duffy et al., 1979). Grob et al. (1947) and Grob and Harvey (1958) have reported excessive dreaming in subjects given the organophosphates DFP and sarin, respectively. Metcalf and Holmes (1969) and Duffy et al. (1979) have used objective measures of sleep to document increased amounts of REM sleep in humans exposed to a variety of organophosphates. Furthermore, Metcalf and Holmes (1969) described the sleep in their subjects as "narcoleptic-like."

The following studies were undertaken in an attempt to develop a time and cost effective model of excessive sleepiness in a rodent species using an organophosphate cholinesterase inhibitor. It was hoped that the model would have particular relevence to the clinical disorder narcolepsy.

The rat was chosen as the experimental species for a number of reasons:

1. The rat represents a particularly cost effective experimental species.
2. The sleep-wake cycle of the rat is short and concentrated into daylight hours, making it a time effective experimental model for the study of circadian and ultradian cycles.
3. The inbred nature of the laboratory rat makes it an excellent candidate for experimental study, particularly with respect to biochemical studies.

4. The central biochemical effects of organophosphates are well documented in the rat species.
5. Established techniques for chronic unit recording in the rat make correlations of pharmacological, biochemical, behavioral and neurophysiological variables possible.
6. The rat is resistant to the "delayed neurotoxic effects" of organophosphates seen in many other species.

The following two manuscripts document the findings concerning the administration of the organophosphate cholinesterase inhibitor, DFP, on sleep in rats. The first manuscript (Gnadt et al., 1985b) reports the effects of chronic administration of the drug on sleep. The second manuscript (Gnadt et al., 1985a) discusses the acute effects of the drug. The findings concerning acute administration of another organophosphate, soman (Meighen et al., 1985), also are discussed briefly.

The acetylcholinesterase inhibitor di-isopropyl-fluorophosphate increases REM sleep in rats

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ABSTRACT

In this experiment, rats were treated chronically with moderate doses of the acetylcholinesterase inhibitor di-isopropyl-fluorophosphate (DFP). After an initial injection of 1.0 mg/kg DFP, the rats received a 0.5 mg/kg injection every third day thereafter for a total of 5 injections (13 days). Following the treatment regimen, the rats were found to have increased amounts of REM sleep compared to vehicle control rats. The time spent awake and in slow wave sleep was relatively unaffected. The increase in REM sleep appears to be due to increased numbers of REM sleep episodes and not an increase in the average length of the REM sleep episodes. Furthermore, the increased REM sleep does not appear to be due to REM rebound or to disruptions of circadian rhythm.

Key Words: acetylcholinesterase inhibitor, REM sleep, DFP, di-isopropyl-fluorophosphate, rats, organophosphate.

INTRODUCTION

Cholinergic mechanisms are known to be involved in the control of sleep and its various physiological parameters. By manipulating cholinergic systems pharmacologically, it is possible to affect the sleep-wake cycle or individual physiological responses that would normally occur in concert in the normal sleep-wake cycle. As several reviews are available,[17,19,20,23] we do not attempt to review the vast literature here.

Pharmacological agents which have been used to manipulate cholinergic systems in relation to sleep include the ACh synthesis inhibitor, hemicholinium-3,[5,13] and the muscarinic antagonist, atropine,[5,16,20] as well as direct application of acetylcholine or cholinomimetics into the the brain.[1,3,7,14,21,25,30,32]

Other agents which act indirectly upon cholinergic systems are the acetylcholinesterase (AChE) inhibitors. AChE is the enzyme which degrades ACh at synaptic sites. It has been shown that the AChE inhibitor, physostigmine, can produce episodes of REM sleep directly from wakefulness in cats.[5] Normally, REM sleep appears only after a requisite period of non-REM sleep. Additionally, physostigmine can induce a REM sleep-like phenomenon in animals pretreated with the monoamine depletor, reserpine,[18] and can induce

postural atonia (a REM sleep phenomenon) in decerebrate cats.[28]

Sitaram et al.[31] have shown in humans that physostigmine can induce wakefulness when given during REM sleep, but when administered during non-REM sleep, physostigmine shortens the latency to the next REM sleep period. A later study by the same group[8] has shown that the effect of physostigmine is to promote the induction of REM sleep, but not to alter the length of REM sleep periods.

Another class of AChE inhibitors of interest to the study of sleep are the "irreversible" AChE inhibitors. These organophosphate compounds bind covalently to AChE at the active site of the enzyme, thereby inactivating the enzyme permanently. De novo synthesis of the enzyme is required for return of the esterase activity. Studies of humans exposed to organophosphates have demonstrated an increase in REM sleep.[6,24] Additionally, there are reports of "narcoleptic-like symptoms"[24] and excessive dreaming and nightmares[11,12] in humans exposed to organophosphates. Belenky and colleagues[4] report that organophosphates in cats may produce increased REM sleep.

To our knowledge, there have been no reports to date on the effects of organophosphates on sleep in the rat species. This series of experiments was performed to document the effects of di-isopropyl-fluorophosphate (DFP), an organophosphate, on sleep in the rat. Since the sleep stages cycle about every 15 minutes in the rat, this species should

be an excellent model to examine the detailed changes in sleep-wake cycles as well as circadian rhythm changes due to organophosphates.

We have found that acute doses of DFP in the rat produce the well known dose-dependent generalized cholinergic crisis, which indirectly produces effects upon the sleep-wake cycle.[2] These effects, however, are not likely due to direct effects upon sleep mechanisms. They are more likely reactions to the discomforts and illness of overstimulation of the peripheral muscarinic and nicotinic systems and the associated symptoms. Using acute administration of DFP, we have observed some effects upon REM sleep such as narcoleptic-like intrusion of REM sleep into wakefulness and disruption of sleep architecture. Domino et al.[5] have reported a similar finding in cats treated with physostigmine. However, we found these effects unreliable and difficult to quantify.

On the other hand, chronic administration of DFP does produce effects upon sleep that are more reliable and quantifiable, and do occur in the absence of the overt illness associated with large, acute doses. It is these results that we report here. A portion of these data has been presented previously.[10]

METHODS

Twenty-two adult (250-350 mg) Sprague-Dawley laboratory rats were randomly assigned to a control group (n=10) or an

experimental group (n=12). All rats were female. Each rat was surgically implanted with electrodes for recording EEG, and in some cases EMG. EEG was recorded from neocortex and dorsal hippocampus. Bipolar EMG was recorded from dorsal neck musculature.

Standard surgical methods were used to implant the recording electrodes under halothane anesthesia (1.5-2.0%). EEG electrodes were made of stainless steel wire (.25 mm) soldered to male gold connectors (Amphenol). EMG electrodes were made by soldering short lengths (<2.5 cm) of Teflon insulated 38 gauge, braided stainless steel wire (Cooner Sales Co.) to male gold connectors (Amphenol). Cortical electrodes were placed bilaterally over frontal and occipital cortex. Two subcortical electrodes were placed bilaterally into the dorsal hippocampus at 3.5 mm caudal to bregma and 3.8 mm from midline; these electrodes were 3 mm in length, thus lying approximately 2 mm below the pial surface. The free ends of the EMG wires were led subdermally to the dorsal aspect of the neck and placed in the belly of the muscle. The electrodes were held in place by a mound of dental acrylic and reinforced with two screws secured into the cranium.

After at least 4 days recovery from surgery, the animals were recorded for 7 1/2 undisturbed hrs (0830-1600) to obtain baseline measures of their sleep-wake cycle. The recordings were performed in a ventilated, sound attenuated chamber following 4 days habituation to the chamber and to a

0600/1800 light/dark schedule. Groups of four rats were recorded simultaneously. Each rat was individually housed in a 10 cm x 30 cm opaque plastic cage of 37 cm height. Rats were allowed food and water ad libitum. Recording leads were attached to the recording electrodes on the rats' heads and led out of the isolation chamber to polygraph amplifiers.

A neocortical EEG (frontal to contralateral occipital), a hippocampal EEG (dorsal hippocampus to contralateral neocortex), and either EMG or a movement trace was recorded from each rat. The movement trace was produced by referencing a free, ungrounded lead near the rat's head to frontal cortex and amplifying the artifact produced by the movement of the free lead in space. The three recording channels for each rat were recorded on a 16 channel polygraph. The day following the baseline recording, we began a drug administration regimen of di-isopropyl-fluorophosphate (DFP) as developed by Glow et al.[9] This protocol is reported to reduce brain cholinesterase activity to approximately 30% of normal and to maintain the activity at that level for as long as the injections are maintained.[9,29] DFP in arachis oil (20 mg/ml) was injected i.m. at 1.0 mg/kg initially and 0.5 mg/kg every third day thereafter for a total of 5 injections (13 days). All injections were made sometime between 1300 and 1600. Control rats received injections of arachis oil only.

The second day following the last injection (day 15) the rats were recorded as in the baseline to determine the

effects of DFP on the sleep-wake cycle. The recordings were not performed before the second day after the injection to avoid the possibility of acute effects.

The record scoring was done by visual analysis and scored according to 50 sec epochs (1 pg at 6 mm/sec). Each epoch was designated as the stage which predominated within that epoch. The stages were scored according to standard criteria into awake (mixed frequency, low voltage EEG), slow wave sleep (SWS; low frequency, high voltage EEG, and sleep spindles), and REM sleep (mixed frequency, low voltage EEG from neocortex, homogeneous 7-8 Hz theta EEG from hippocampus, absent tonic EMG). In cases where a REM period was 25 sec or more, but was distributed across two epochs in which it did not predominate within either epoch, one of the epochs was designated as REM.

The scores by epoch were entered into a Nova computer (Data General) and the data compiled into a form amenable to graphical and statistical analyses. For each record the sleep record was analyzed in terms of percent, and number of each stage. Additionally, the average length of the REM sleep episodes for a recording day was calculated from the sum of the time in seconds of each REM sleep period more than 10 sec in length divided by the total number of episodes more than 10 sec in length.

A second, longitudinal study was then undertaken to examine the possibility of REM sleep deprivation occurring during the chronic dosing regimen and to evaluate the effect

of chronic DFP upon circadian sleep-wake rhythm. This second study utilized an additional eight rats. These eight rats were evaluated separately from the initial 22 rats, as described below. These rats received a baseline record followed by the same drug regimen as the previous groups. These rats were recorded on day 8 and day 10 of the dosing schedule, as well as a 32 hour recording starting the second day after the last dose (days 15-16). Please note that the recording on day 8 follows the day after a DFP injection, whereas, day 10 falls upon an injection day. This was done to look for acute deprivation or rebound phenomena on an injection day or the following day. The recording on day 10 had to be disturbed for about 30 min to perform these injections and then extended by that amount of time at the end of the day to keep total recording time constant. During the 32 hour record on days 15 and 16, the recording was interrupted for the last 30 min of each 8 hrs to allow for technical maintenance of recording apparatus and housekeeping purposes.

RESULTS

Following the initial injection of DFP (1.0 mg/kg), rats exhibited signs of DFP exposure within 10 to 30 min. The observed effects varied from mild to severe, with most cases being mild to moderate. Effects included salivation, diarrhea, piloerection, muscle fasciculations, tremor and

respiratory distress. In no cases did we see cataplectic-like weakness, exopthalmia, or death which is sometimes seen with larger doses. By the next day, most rats did not exhibit overt signs of DFP exposure. Subsequent doses of DFP (0.5 mg/kg) rarely produced observable effects. The potency of the DFP mixtures was tested periodically by giving 3.0 mg/kg doses to spare laboratory rats. In all cases the test rats exhibited severe cholinergic crisis and respiratory distress within 10 to 30 min and were sacrificed by barbiturate overdose.

With chronic administration of DFP, we never observed any evidence of disassociations of EEG and behavioral indices of sleep. It was not possible to choose between records of control versus DFP treated rats by visual inspection of EEGs. The DFP treated rats exhibited the same three state related EEG patterns as the vehicle control rats.

In the initial between-groups study, 30% (13/44) of the rat-days of recording were lost due to rats destroying their recording leads and related technical problems. Therefore, the statistical analyses presented here were based on pooled data from 14 (of 22) rats for baseline and 17 (of 22) rats for post-injection. Because the distributions of all the variables appeared near normal, the two-tailed t-test was used to compare these means.

For the baseline recordings, no statistical differences between the control and DFP groups were found for any of the measured variables. Because no differences between the

control and DFP groups were found for the baseline records, it is likely that any differences between groups for the post-injection records were due to DFP.

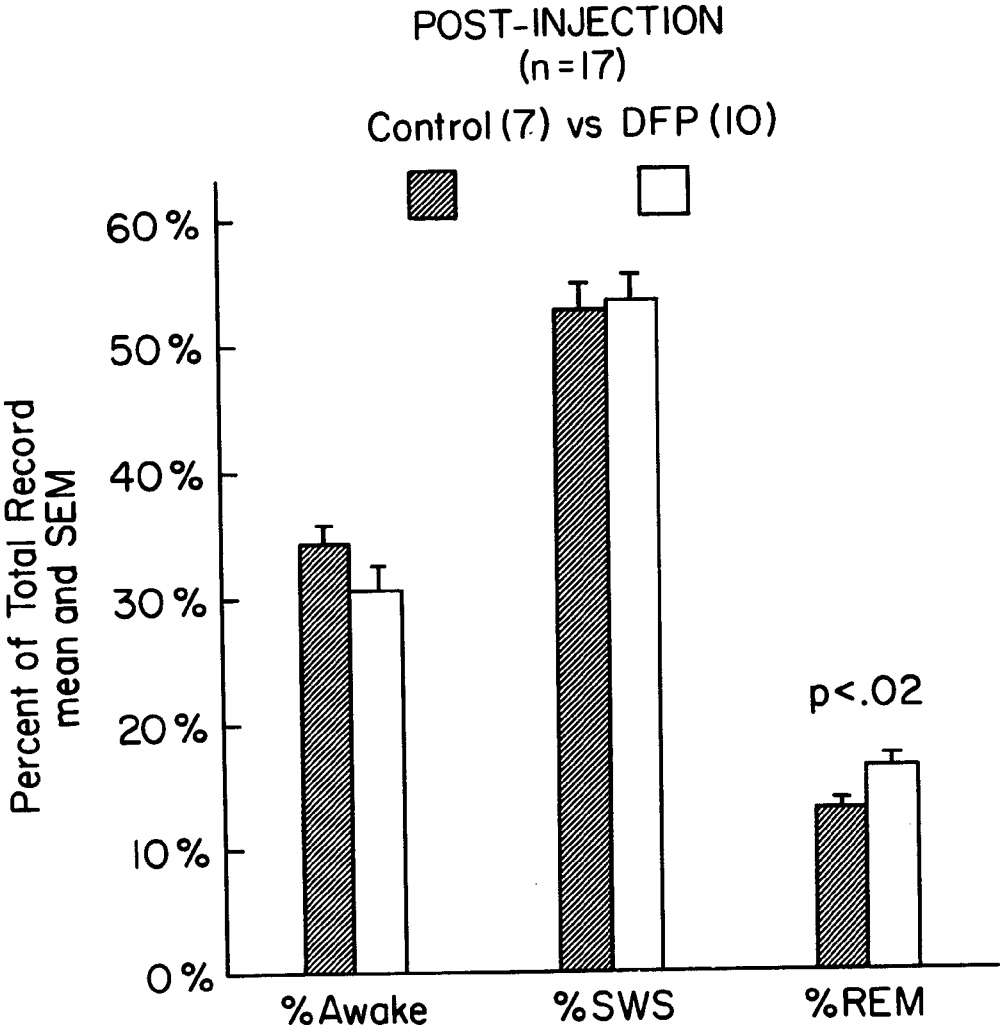
The results for the post-injection records are shown in Fig. 1. The mean of the percent of REM sleep for the DFP rats (16.3 $\pm$ 0.9, $\pm$ SEM) was significantly ($p<.02$) greater than the mean for the control rats (13.0 $\pm$ 0.7). There was no statistical difference between groups for percent awake or percent SWS.

The means for the average REM sleep length and number of REM sleep episodes per recording are shown in Fig. 2. While there was no difference between groups for average REM sleep length, the DFP treated rats had significantly ($p<.05$) more REM sleep episodes (44 $\pm$ 4.5) than the controls (31 $\pm$ 2.6).

The analysis of the percent REM sleep for the 8 rats from the second, longitudinal study is shown in Fig. 3. As in the between-groups study above, not all rats successfully completed every recording day. For this reason, it was not possible to use the more appropriate paired-difference t-test to analyze these data statistically. Instead, the t-test to compare means was used. This analysis represents a more conservative test in this case. Additionally, inspection of the data from the individual rats which did successfully complete each recording day does not appear to contradict the finding reported here. On days 15 and 16 the rats had significantly more REM sleep than at baseline. The rats

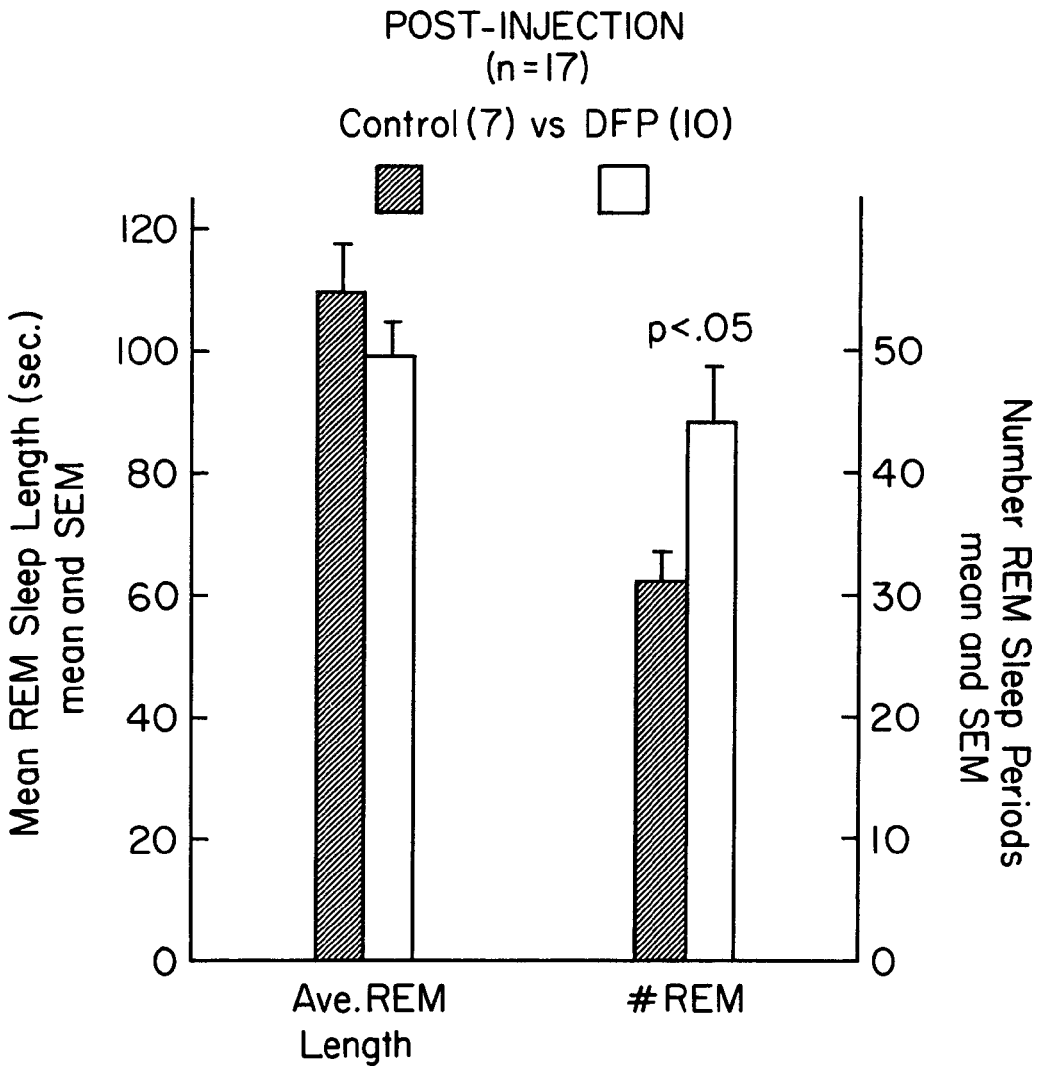
LEGEND FIGURE 1

DFP EFFECTS UPON THE SLEEP-WAKE CYCLE. Following chronic treatment with DFP, the percent of REM sleep time from the 7 1/2 hour day time records (day 15) is increased in DFP treated rats compared to vehicle control rats (two-tailed t-tests). Time awake, Slow Wave Sleep (SWS), and REM sleep (REM) are plotted as a percentage of total recording time.


POST-INJECTION
(n=17)
Control (7) vs DFP (10)
60%
50%
40%
30%
20%
10%
0%
Percent of Total Record
mean and SEM
p<.02
%Awake
%SWS
%REM

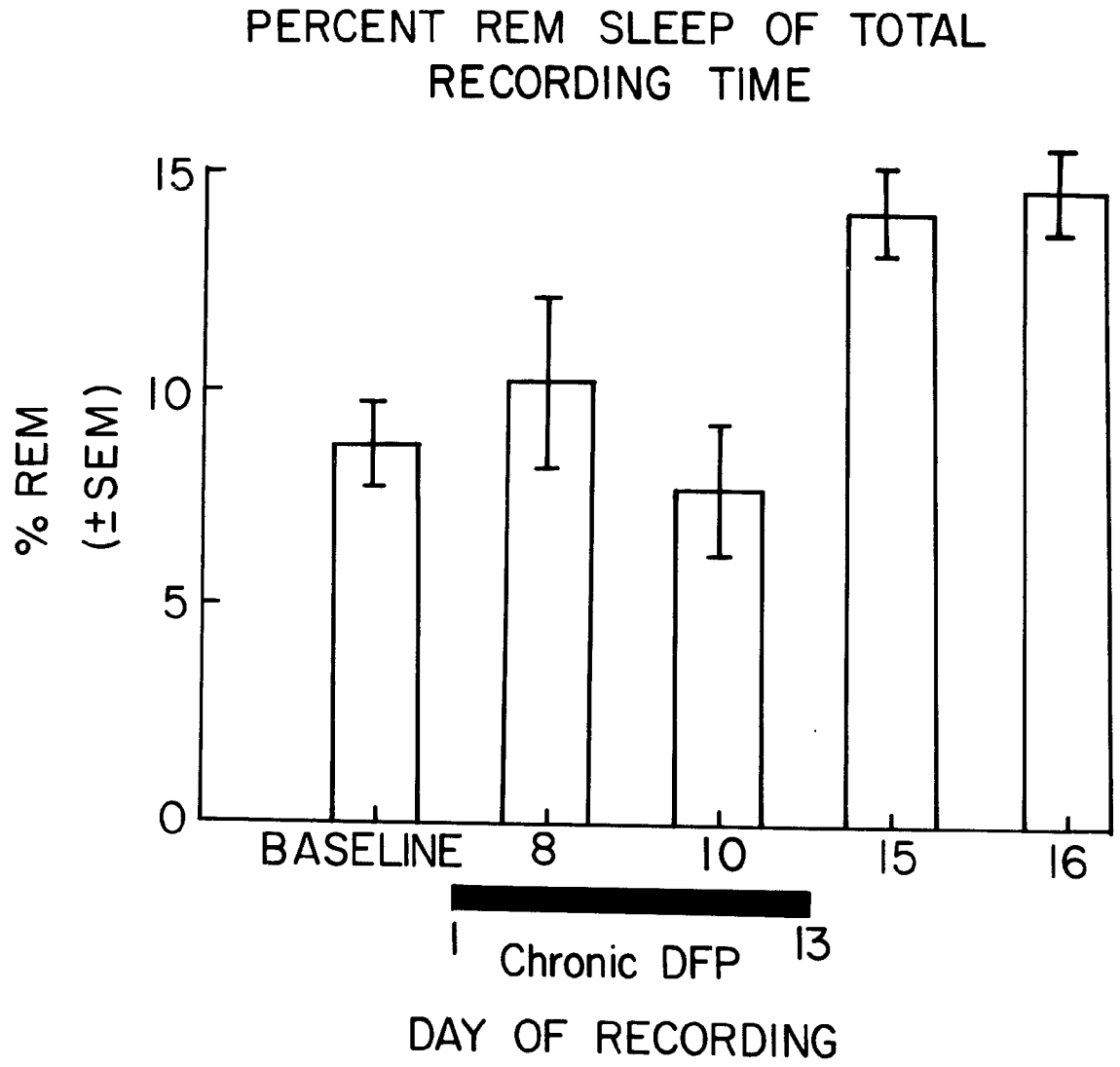
LEGEND FIGURE 2

DFP EFFECTS UPON REM SLEEP PERIODS. Following chronic treatment with DFP (day 15), the number of REM sleep episodes is increased (two-tailed t-test), while the average length of REM sleep periods appears to remain unchanged.


POST-INJECTION
(n=17)
Control (7) vs DFP (10)
Mean REM Sleep Length (sec.)
mean and SEM
120
100
80
60
40
20
0
p<.05
50
40
30
20
10
0
Number REM Sleep Periods
mean and SEM
Ave. REM
Length
REM

LEGEND FIGURE 3

CHANGES IN REM SLEEP TIME, LONGITUDINAL STUDY. DFP was administered chronically for 13 days following baseline. Rats do not appear to be REM sleep deprived during the dosing period. Note that the record on day 10 was disturbed to administer DFP injections. Percent REM for days 15 and 16 are significantly greater than baseline (two-tailed t-test).


PERCENT REM SLEEP OF TOTAL RECORDING TIME
% REM (±SEM)
15
10
5
0
BASELINE
8
10
15
16
1
Chronic DFP
13
DAY OF RECORDING

do not differ significantly from baseline on days 8 and 10. As noted above, the amount of sleep on day 10 is lower than might have been otherwise due to disturbing the rats during the recording to give an injection.

The results of the post-DFP 32 hr record from the second study is shown in Fig. 4A. Please note that the first 7 1/2 hrs of this record is analogous to the post-DFP record from the between-groups study and that these data are the same as shown for days 15 and 16 of Fig. 3. This figure shows the amount of time spent in each stage of the sleep-wake cycle as a function of time of day. For comparison, Fig. 4B shows a typical sleep-wake record from a normal rat from a previous study (unpublished results). As for a normal condition, the DFP-treated rats spent relatively more time awake and less time asleep during the dark hours of the day than during the light hours. This figure demonstrates that the circadian rhythm of the sleep-wake cycle is not seriously disrupted in these DFP-treated rats.

DISCUSSION

The results of this study demonstrate that chronic treatment of rats with the AChE inhibitor DFP produces increased REM sleep.

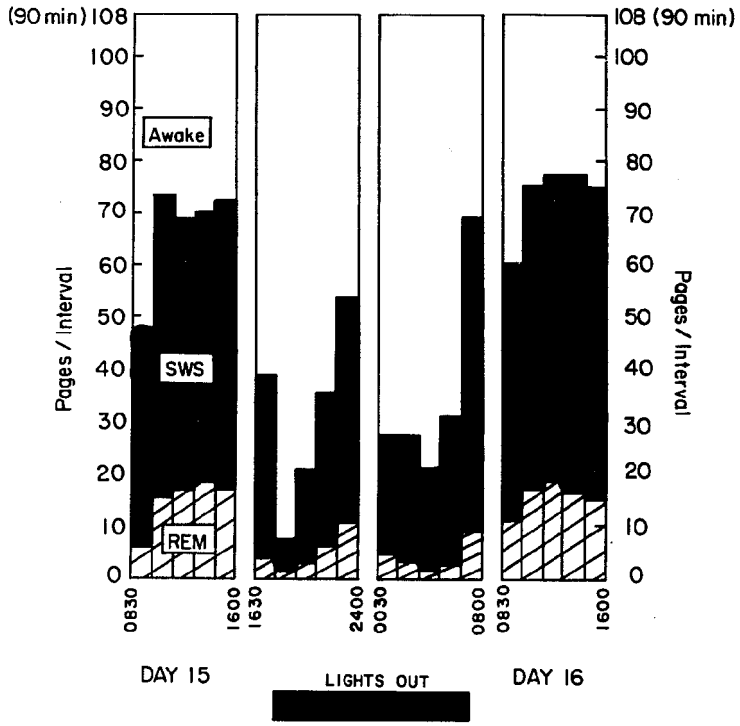
While there was an increase in the percent REM sleep for the DFP treated rats, there was no significant difference between the DFP and control groups for the percent of

LEGEND FIGURE 4

CIRCADIAN SLEEP-WAKE CYCLE AFTER CHRONIC DFP, LONGITUDINAL STUDY. Sleep-wake stages are shown as a function of time of day. Rats were on a 0600-1800 light-dark cycle. A. A composite 32-hr record of sleep-wake stages for 8 rats from the longitudinal study. Recording started at 0830 on day 15 and continued until 1600 on day 16. Rats received DFP chronically for days 1-13. B. For comparison, a typical record for a normal rat is shown. The circadian sleep-wake rhythm of the DFP-treated rats does not appear to be seriously disrupted.

(A)

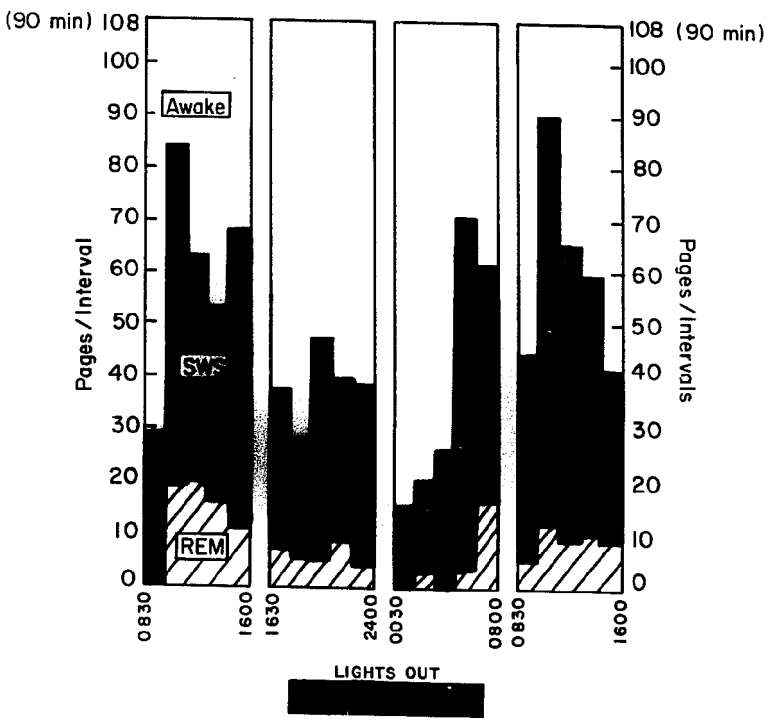
32 HOUR SLEEP WAKE RECORD FOLLOWING
CHRONIC DFP (Days 1-13) Averages of 8 Rats



Time of Day by 90 min. Intervals

(B)

TYPICAL 32 HOUR SLEEP WAKE RECORD
OF NORMAL RAT



Time of Day by 90 min. Intervals

the recording time spent awake or in SWS (Fig. 2). This suggests that these latter two stages of the sleep-wake cycle are relatively unaffected by the DFP treatment.

The increased time spent in REM sleep could be due to either more frequent REM sleep episodes, longer REM sleep episodes, or a combination of these. The increased REM sleep time does not appear to be due to longer REM sleep episodes for there is no significant difference between the DFP and control groups for the postinjection measures of average length of REM sleep episodes (Fig. 3). Conversely, the DFP treated rats do have a significantly larger number of REM sleep episodes (Fig. 2). This indicates that most of the increase in the REM sleep time is due to increased number of REM sleep episodes.

Furthermore, the increase in REM sleep following chronic treatment with DFP does not appear to be due to REM rebound following chronic sleep deprivation during the dosing regimen (Fig. 3). That the increased REM sleep persists for at least the second and third days following the last injection is another indication that this effect is a persistent effect of the drug and not a transient effect such as REM rebound. Our experience through years of working with mice and rats with a variety of psychoactive agents and with sleep deprivation has always shown rebound (if any) to occur within 24 hours.[27] The quickness with which the rat species consolidates REM rebound has been noted by others.[23]

Neither does this effect appear to be due to disruption of circadian sleep cycle (Fig. 4).

It is unfortunate that 30% of our rat-days of recording were lost due to rats destroying or tampering with their recording leads. The inquisitive nature of the rat makes long-term undisturbed recording of unrestrained rats a non-trivial problem. All of our attempts to decrease either the availability or the desirability of the recording leads also decreased either rat mobility or recording lead flexibility. Any recording methodology which significantly impedes the rat's mobility or impairs the rat's ability to choose preferred sleeping postures leads to disrupted sleep patterns. The simplest procedure which we have found to alleviate this problem is simply to repair damaged leads at the time the problem occurs. However, considering the modest magnitude of the DFP effect in this paradigm, we felt that these recordings should be performed undisturbed despite the loss of valuable data. It is unlikely that this loss of data systematically biased our findings. Empirically, the fraction of records lost from DFP treated and control groups are roughly equivalent: 2/12 and 3/10, respectively.

These data are consistent with the view that cholinergic mechanisms are involved in the control of REM sleep. These data complement the findings of other investigators who have studied the effects of AChE inhibitors on sleep.[4,5,8,18] In animals pretreated with reserpine (a monoamine depletor), Karczmar et al.[18] report being able to

produce dramatic REM sleep-like episodes with acute doses of physostigmine. We have shown here that, even in the absence of reserpine, it is possible to produce REM sleep with chronic administration of a long-acting AChE inhibitor, albeit less dramatically. Despite the modest size of the DFP effect shown here, we do not find this contradictory to our hypothesis of the mechanism of action, as discussed below. The fact that the direction of the apparent DFP effect is to increase REM sleep is important. REM sleep is a rather delicate state; many manipulations appear to nonspecifically decrease REM sleep. Very few things have been found to increase REM sleep. In fact, DFP, when administered acutely in larger doses, decreases REM sleep,[2] probably through nonspecific side effects. Additionally, the finding that the increased REM sleep appears to be due to an increased number of REM sleep episodes would seem to parallel the findings of Gillin et al.,[8] that AChE inhibitors seem to facilitate REM sleep initiation, but not alter its episode length. It should be noted that the REM sleep effects we have described here appear to be an increase in true physiological REM sleep and not the occurrence of REM sleep-like disassociative phenomena that can occur in reserpine treated rats.

In this study, the between-groups comparison of DFP treated rats to the vehicle treated rats establishes that chronic administration of DFP can produce increased REM sleep. However, the possibility that this increase in REM sleep could be due to a REM rebound phenomena must be

considered. The longitudinal study of the eight additional DFP treated rats shows that the rats do not appear to be REM sleep deprived during the injection regimen, even when injection day and postinjection day effects are considered. One must be cautious, however, in the interpretation of the data from the longitudinal study. These data should not be interpreted as representing a curve describing the development of the DFP effect across time. First, the DFP effect here is confounded by a repeated recording effect in which there is a tendency for rats to exhibit increased REM sleep following repeated recording periods. The original between-groups design was used to avoid this difficulty initially. Secondly, in order to control for acute effects, the recordings made on days 8 and 11 were staggered with respect to day of injection and, therefore, not entirely analogous with the recordings on days 15 and 16.

The mechanism of action of DFP is to potentiate cholinergic activity by inhibiting the hydrolysis, and thus deactivation, of acetylcholine at synaptic sites. It is possible that DFP is acting to bias the balance between cholinergic systems and other chemically distinct neurotransmitter systems which are controlling the initiation of REM sleep.

The findings of Karczmar *et al.*[18] suggest that there is an interaction between monoaminergic and cholinergic systems which are involved in producing REM sleep. Hobson and McCarley[15,22] have proposed a model of REM sleep control in

which the balance between cholinergic and monoaminergic systems is critical. How the results of this study may relate to the details of their model is not known; however, we find these results compatible with the pharmacological predictions of such a model. Assuming that REM sleep is initiated by a cholinergic trigger mechanism, which is critically balanced against an inhibitory mechanism, DFP would bias such a trigger towards threshold thus making the initiation of REM sleep a more likely event.

ACKNOWLEDGMENTS

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Cholinesterase inhibitors, acute toxicity, and sleep. Di-isopropyl-fluorophosphate (DFP)

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ABSTRACT

Acute administration of the organophosphate di-isopropyl-fluorophosphate (DFP) produces behavioral and physiological symptoms indicative of excessive cholinergic stimulation. This behavioral toxicity was found to be incompatible with the occurrence of sleep, despite the fact that chronic administration of DFP has been shown to increase the rapid eye movement stage of sleep.

DFP was found to decrease all stages of sleep and to increase wakefulness in a dose-dependent manner. The behavioral toxicity also was noted to be dose-dependent by qualitative assessment. Atropine sulfate, at doses of 3.0 mg/kg, was ineffective in blocking the DFP effects upon sleep.

Running Head: DFP, toxicity, and sleep

Key Words: cholinesterase inhibitor, sleep toxicity, DFP, organophosphate

INTRODUCTION

Cholinergic mechanisms have long been known to be involved in the control of sleep (King, 1971; Jouvet, 1972; Jacobs and Jones, 1978). Cholinergic mechanisms particularly are implicated in the control of the rapid eye movement stage of sleep (Hobson, 1982). This stage of sleep, commonly called REM sleep, is the phase of sleep in which nightly dreaming occurs (Dement and Kleitman, 1957).

Cholinesterase inhibitors can be used to manipulate cholinergic function in vivo, and therefore represent a useful tool in the study of cholinergic mechanisms in sleep. In humans, the organophosphate cholinesterase inhibitors have been reported to produce excessive dreaming (Grob et al., 1947; Grob and Harvey, 1958). Later studies by Metcalf and Holmes (1969) and Duffy et al. (1979) have shown that humans exposed to organophosphates exhibit increased amounts of REM sleep. The effects of organophosphates in humans have been described as "narcolepticlike" (Metcalf and Holmes, 1969), in reference to the disorder of excessive sleepiness which occurs in humans (Roth, 1980) and in canines (Baker et al., 1982).

In the interest of developing an animal model of organophosphate-induced excessive sleepiness, our laboratory has investigated the effect of organophosphates in the rat species. We have reported that chronic administration of the organophosphate, di-isopropyl-fluorophosphate (DFP) in rats produces an increase in REM sleep (Gnadt et al., 1985).

We report here the effects of acute administration of DFP upon sleep in rats. Preliminary results of these data have been presented (Atwood et al., 1984).

The toxic effects of systemic administration of organophosphates is well documented (Grob et al., 1947; Jovic, 1974; Baron, 1981; Gordon et al., 1983; Lim et al., 1983; Soliman, 1983). Although the rat species is resistent to the reported "delayed neurotoxic effects" (Baron, 1981; Soliman, 1983; Gordon et al., 1983), the acute muscarinic and acute neuromuscular effects of the drug are present in this species (Jovic, 1974; Lim et al., 1983). Muscarinic effects include excessive salivation, lacrimation, and defecation. Neuromuscular effects include fibrillation, fasciculation, tremor, and generalized motor weakness. Generalized and behavioral effects include hyper-reactivity to stimuli, anorexia and weight loss, adipsia and dehydration, and respiratory distress. In the most severe forms the symptoms lead to death.

Unlike the chronic administration of DFP, in which there is an increase in REM sleep, toxic effects of the drug predominate when DFP is administered acutely. The effects upon sleep which are produced by this toxicity are described here. A similar finding concerning this toxicity for the organophosphate soman (0,1,2,2-trimethylpropylmethylphosphonofluoridate) is reported in a companion paper (Meighen et al., 1985).

A secondary goal of this study was to determine if the muscarine antagonist, atropine, could block the DFP effects upon sleep.

METHODS

Surgical and electrophysiological techniques.

Thirty-three female Sprague-Dawley rats (Charles River), 200-300 g, were used in this study of the effects of DFP on sleep. Under halothane anesthesia, each rat was surgically implanted with electrodes for recording of the electroencephalogram (EEG). Some rats also received electrodes for recording of the electromyogram (EMG) from posterior muscles of the neck. These procedures have been described elsewhere (Gnadt *et al.*, 1985). The EEG, and either EMG or body movement, were used to determine stages of the sleep-wake cycle. Following the surgery, each rat was housed individually in a 0600-1800 light-dark cycle, and provided food and water *ad libitum*. The rats were allowed at least four days recovery from surgery before being used for sleep studies.

Recordings of the sleep-wake cycle were performed using standard polygraphic methods. Four rats were recorded at a time, individually housed within a sound attenuated, ventilated isolation chamber. All recordings were performed for the 7 1/2 hours from 0830 to 1600. Recordings were made on the day before the injection day (baseline), the injection day, and postinjection days 1 and 3.

According to standard EEG and EMG or body movement criteria, each 50 sec of the 7 1/2 hour record was designated as awake, Slow Wave Sleep (SWS), or REM sleep. From those data the percentage of the total recording time for each stage of the sleep-wake cycle was determined. A more detailed description of polygraphic and recording methods can be found in Gnadt et al. (1985).

Drugs doses and injections

All injections were performed at 0815 to 0830, immediately prior to recording on the injection day. The 33 rats were assigned randomly to one of three groups: a control group (n=12), a low dose group (n=10), or a high dose group (n=11). The low dose group received injections of 1.25 mg/kg of DFP in a 20% ethanol-saline vehicle. The high dose group received injections of 2.5 mg/kg (n=9) or 2.0 mg/kg (n=2) of DFP. The control group received injections of the injection vehicle alone. All injections were intraperitoneal. The DFP was provided graciously by Dr. Steve Barker, manufacturer. In addition, one-half of the rats in each DFP group were given 3.0 mg/kg atropine sulfate (Sigma Chem. Co.), subcutaneously, 30 min prior to receiving the DFP injection. This dose of atropine was chosen to avoid the behavior-EEG disassociations which occur at higher doses (Longo, 1966).

Body weight as an index of toxicity

It has been suggested for soman that body weight loss serves as a general index of toxicity (J.H. McDonough, personal communication). Because daily values for body weights of the above animals were not available, the following additional experiment was performed. Thirteen additional rats received sham surgeries and were divided into three groups: a control group (n=4), a low dose group (n=4), and a high dose group (n=5). After one week recovery from surgery, a baseline body weight was determined for each rat. The following day the low dose group received a 1.25 mg/kg injection of DFP and the high dose group received a 2.5 mg/kg injection of DFP; the control group received injection of the drug vehicle alone. Five hours later, the body weight was determined for each surviving rat. Subsequent body weights were determined for the first, third, and fifth postinjection days.

Statistical analysis

Values for the baseline day were analyzed using a one-way analysis of variance for each sleep parameter. Analysis of the changes produced by the DFP injections was performed using the changes from baseline for each stage of the sleep-wake cycle for each subsequent recording day. The two-tailed t-test of difference scores was used to determine levels of significance. The effects of atropine were

tested using the Mann-Whitney U to compare the rats which received atropine, for each of the DFP doses.

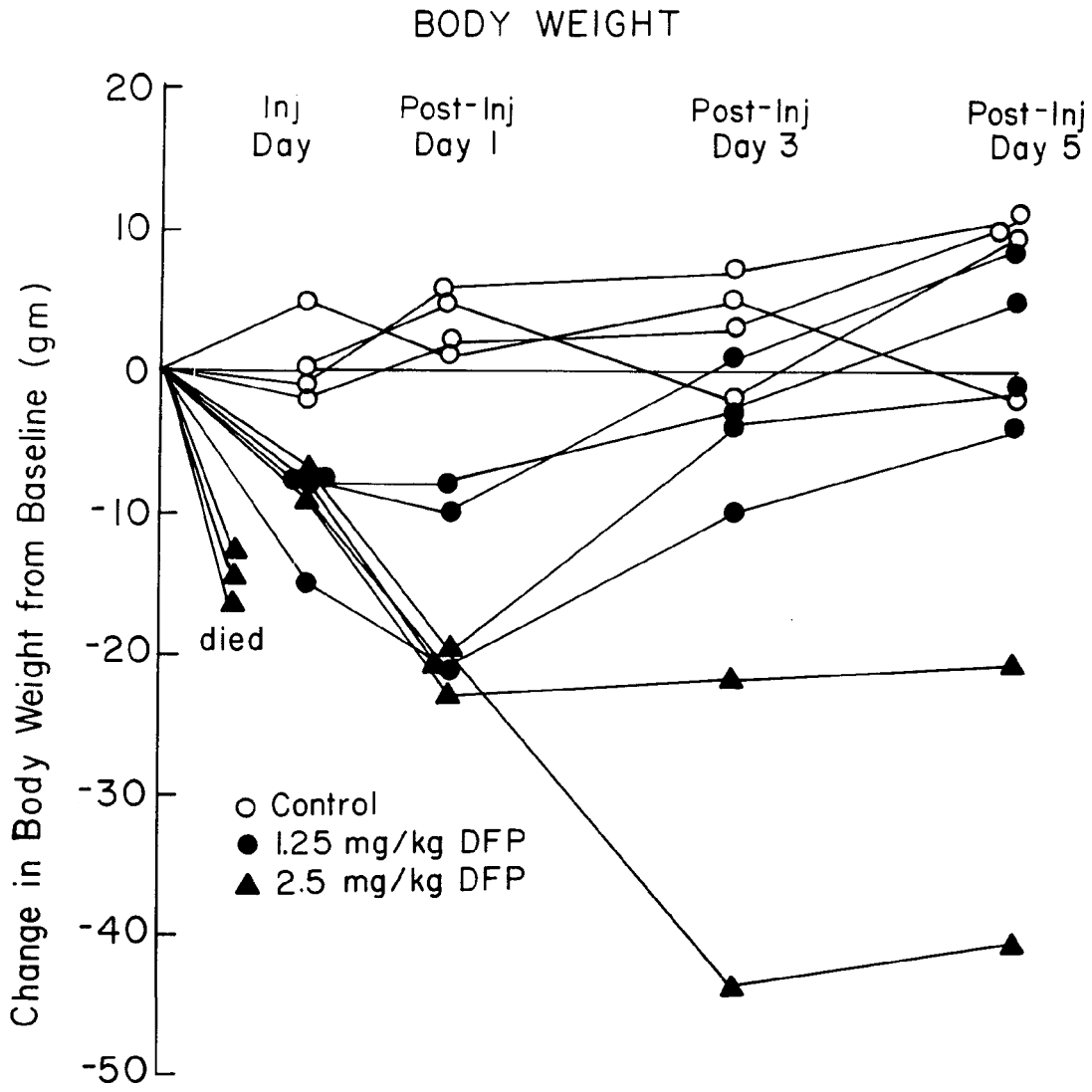
RESULTS

Behavioral effects. The behavioral effects described here have been observed in the 34 DFP-treated rats in this study, as well as other rats from pilot studies in which behavioral observation was not restricted by the nature of the experiment. For obvious reasons, once the rats described in this study were placed into isolation, only remote observation through one-way mirrors was possible. Occasionally, the recordings were disturbed briefly during the first 2 hours of recording to assess the rats' condition more directly. The effects described here are grouped according to general categories and are listed in roughly increasing order of severity. General behavior: cessation of spontaneous activity, exaggerated startle to sudden sensory stimuli (i.e., snapping fingers, clapping hands or gentle touch), motor incoordination, tremor with motor effort, periodic spontaneous tremor, exaggerated respiratory effort, generalized weakness with prostrate posture, and desperate respiratory effort. Autonomic systems: excessive mouth movements and licking apparently due to increased salivation, defecation, piloerection, lacrimation, excessive salivation, excessive secretion of upper respiratory tract (nasal discharge and rales during breathing), and unusual postures suggestive of severe abdominal cramps. Neuromuscular

system: isolated fasciculations, isolated twitches involving ears and proximal muscles of the limbs, motor incoordination and tremor as listed above, severe (almost flaccid) generalized weakness, and exopthalmia perhaps due to spasm of the extraocular muscles. During recovery over a period of two to three days, some animals exhibited an unusual periodic, cataplectic-like, weakness which could be overcome with voluntary effort. This effect was refactory to atropine sulfate and cognetin (benztropine mesylate) and, therefore, probably due to peripheral neuromuscular factors and not to central muscarinic mechanisms (unpublished observations). Food and water intake: anorexia and adipsia were common, with an associated weight loss and dehydration. The latency to recovery of these functions, and the associated recovery of body weight was roughly correlated with severity of toxic symptoms.

No attempt was made to quantify these subjective assessments of behavioral toxicity. Individual rats from both DFP doses exhibited these effects to varying degrees. It can be stated that as a general rule the rats receiving the high dose of DFP exhibited more of the symptoms to a more severe degree than did the group receiving the low dose. The body weight data, shown in Fig. 1, support this qualitative assessment. Note that the rats which received 2.5 mg/kg of DFP exhibited a more severe and prolonged weight loss than the rats which received 1.25 mg/kg.

Fig. 1 Body Weight as a function of DFP dose and time postinjection. Body weight is expressed as the change from baseline values. Three rats which received 2.5 mg/kg DFP died soon after injection.


BODY WEIGHT
Inj Day
Post-Inj Day 1
Post-Inj Day 3
Post-Inj Day 5
20
10
0
-10
-20
-30
-40
-50
Change in Body Weight from Baseline (gm)
died
Control
1.25 mg/kg DFP
2.5 mg/kg DFP

An additional index of severity is the mortality rate. Table I shows mortality as a function of dose of DFP and of latency from injection for the 33 rats used for the sleep studies. There were no mortalities for the low dose of DFP. Only 2 rats received a 2.0 mg/kg dose, but neither of these rats died. Five of 9 rats receiving 2.5 mg/kg died. One rat died soon after injection and, therefore, could not be included in the subsequent analysis of sleep parameters. This rat died of apparent asphyxiation due to severe respiratory compromise. Four more rats died over the course of the next 3 days, probably secondary to compromise of multiple physiological systems and severe dehydration.

Effects upon sleep. For the analysis of sleep parameters, 5 rat-days of recording were precluded from use due to technical difficulties related to the rats tampering with their recording leads. These 5 records were distributed among the several recording days and between the several groups so that it is unlikely that this loss of data produced any systematic bias in the analyses.

The baseline values of the sleep parameters for each of the stages of the sleep-wake cycle for each group of animals are shown in Table II. There were no significant differences between groups for any of the sleep parameters: awake, $F(2,29)=2.25$; SWS, $F(2,29)=2.94$; REM sleep, $F(2,29)=0.22$.

Table I Mortality from DFP is shown as a function of dose and latency from injection. The proportion of deaths to total N is given for each dose of DFP.

MORTALITY

DFP Group	DFP Dose	Total N	Day of Death				Proportion
			Injection Day	Post-Inj. Day 1	Post-Inj. Day 2	Post-Inj. Day 3	
Low dose	1.25mg/kg	10	-	-	-	-	0/10
High Dose	2.0 mg/kg	2	-	-	-	-	0/2
	2.5 mg/kg	9	1	2	1	1	5/9

Table II Baseline sleep parameters. The means for each stage of the sleep-wake cycle is shown for each group. Values are the percent of the recording time spent in each of the three stages.

BASELINE SLEEP PARAMETERS

	Control (n=12)			DFP, low dose (n=10)			DFP, high dose (n=11)		
	% Awake	% SWS	% REM	% Awake	% SWS	% REM	% Awake	% SWS	% REM
Mean	40.8	48.7	10.5	45.5	45.0	9.5	35.7	54.4	9.9
Standard Deviation	14.4	11.8	4.1	5.7	6.7	3.8	8.1	5.8	3.5

The DFP effects on the sleep parameters were analyzed as changes from baseline for the subsequent days of recording. In consideration of the small numbers of animals per group, this type of analysis minimizes individual differences among the different rats.

The changes in sleep parameters relative to baseline for the injection day, the postinjection day 1 and postinjection day 3 are shown in Figs. 2, 3 and 4. On the injection day, both the low dose and high dose DFP groups spent more time awake ($p<.001$, Fig. 4), and less time in SWS ($p<.001$, Fig. 3) and REM sleep ($p<.001$, Fig. 4). By the postinjection day 1, the low dose group had recovered to baseline levels for all three stages of the sleep-wake cycle. On the first postinjection day, the high dose group continued to have elevated levels of wakefulness ($p<.02$, Fig. 2) with reduced levels of SWS ($p<.01$, Fig. 3), while the percent of recording time for REM sleep had returned to baseline levels. By the postinjection day 3, the high dose group continued to have diminished amounts of SWS ($p<.01$, Fig. 3), while the other two parameters had returned to preinjection values. The non-significant increase in REM sleep seen for all three groups for the third postinjection day (Fig. 4) is a typical finding when rats are recorded repeatedly. This may be related to habituation to handling and the recording apparatus.

Fig. 2 Effects of DFP on wakefulness. Changes in the percent of the recording time awake are shown for the injection day and postinjection days 1 and 3. All values are differences from baseline values. Levels of significance are designated as: *, $p<.02$; +, $p<.01$; ++, $p<.001$ (two-tailed t-test of difference scores).

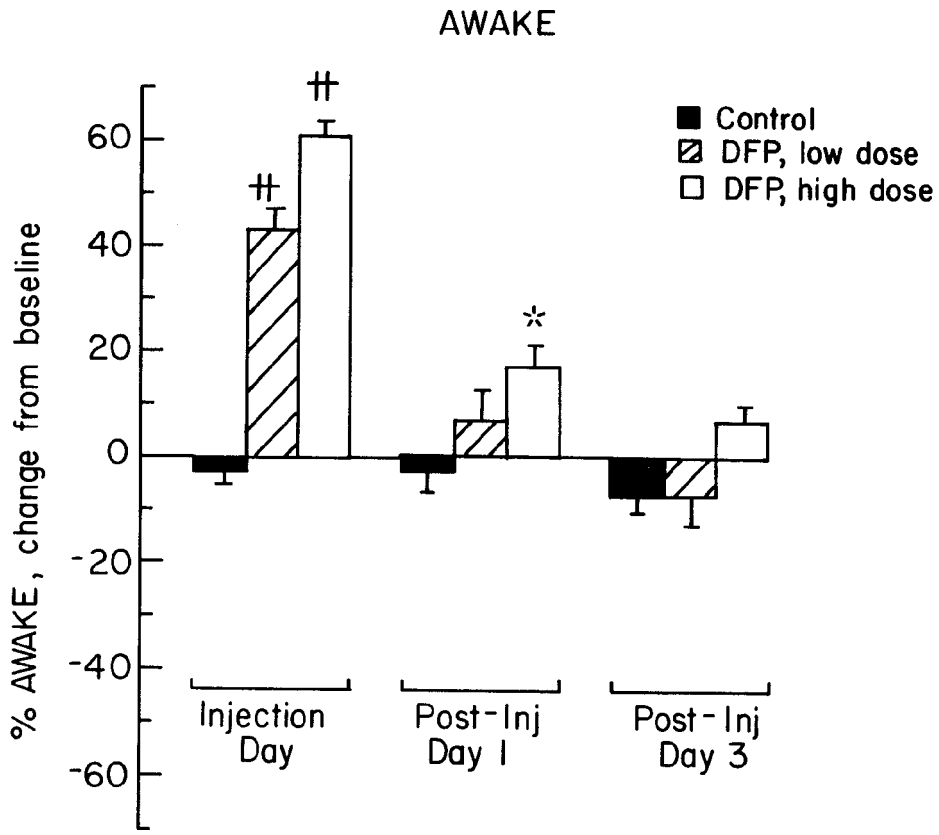

AWAKE
Control
DFP, low dose
DFP, high dose
% AWAKE, change from baseline
60
40
20
0
-20
-40
-60
Injection Day
Post-Inj Day 1
Post-Inj Day 3

Fig. 3 Effects of DFP on SWS. Changes in the percent of the recording time in SWS. Values are differences from baseline values. Symbols same as Fig. 4.

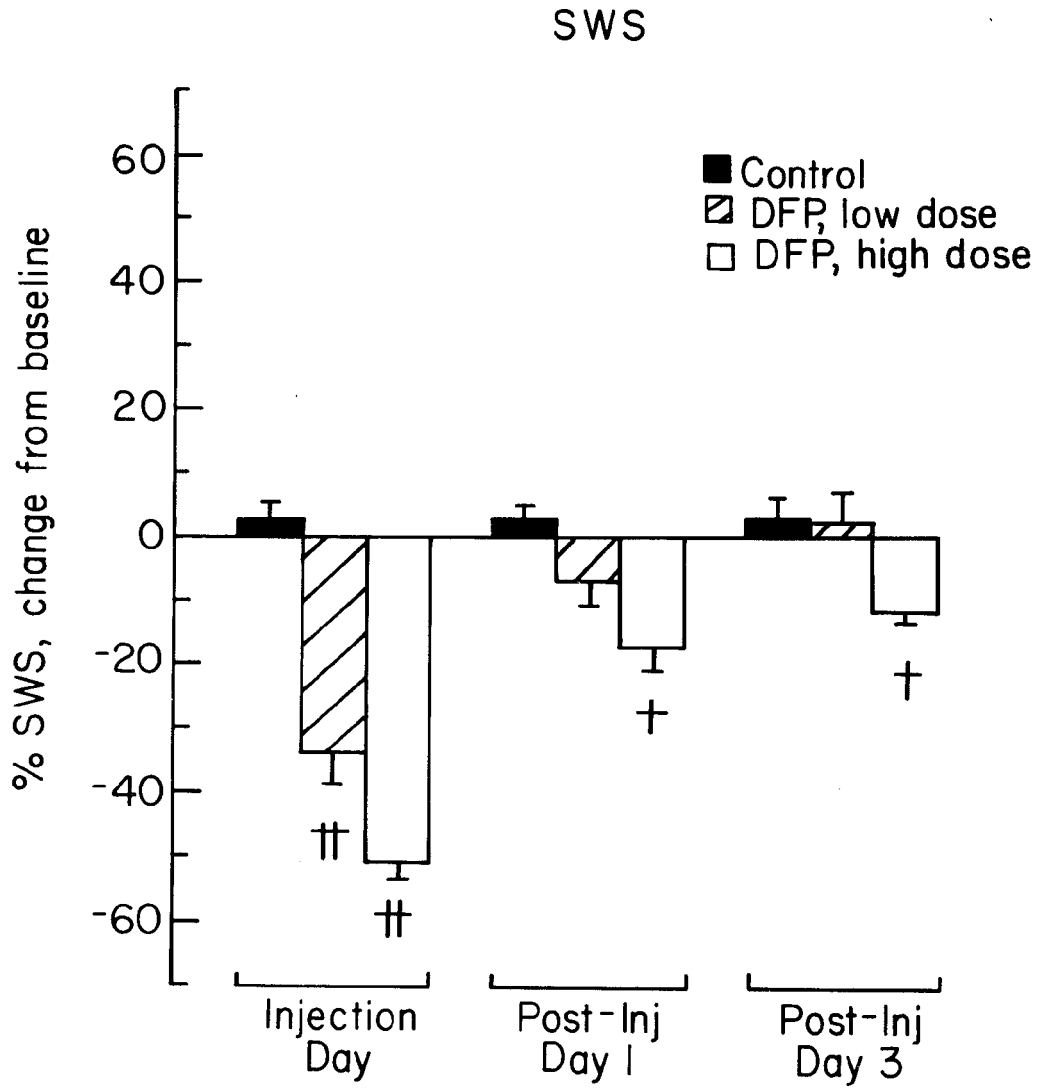
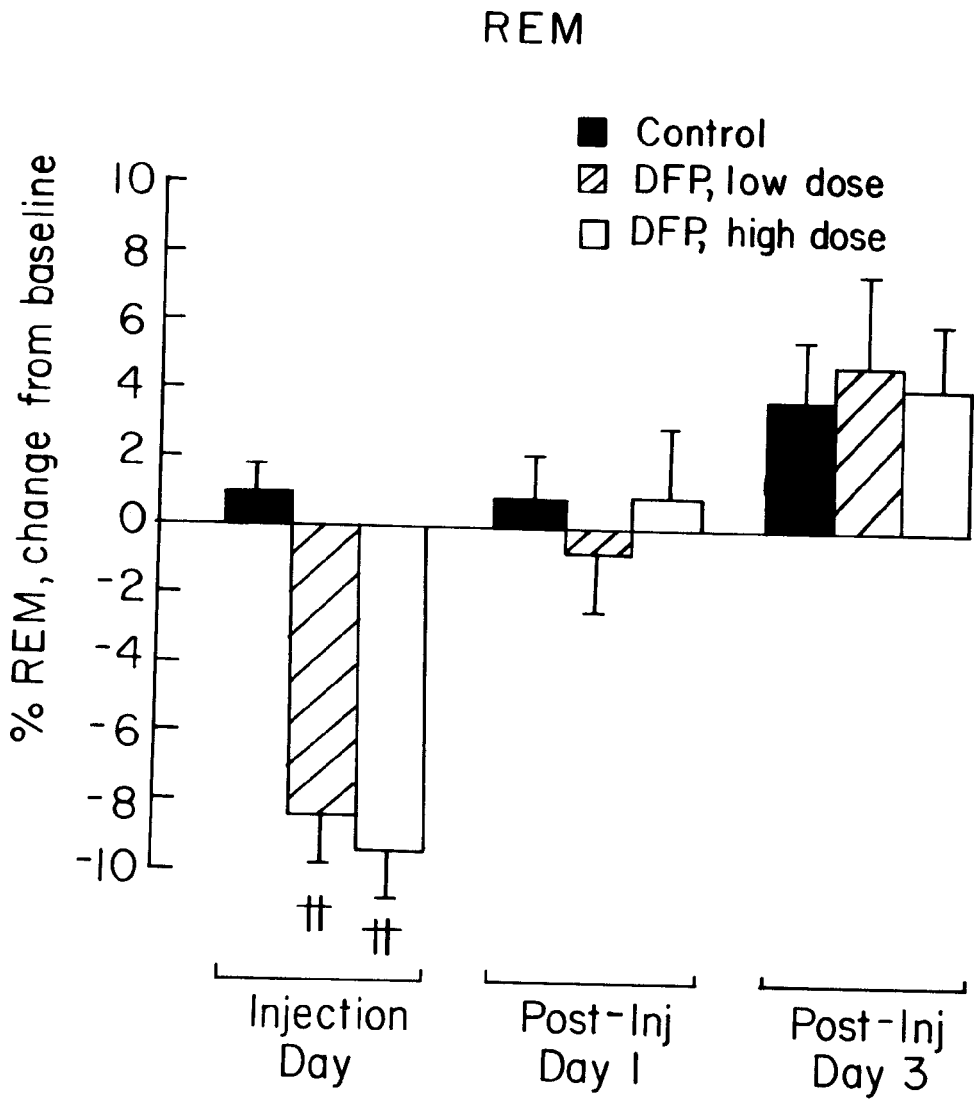

SWS
Control
DFP, low dose
DFP, high dose
% SWS, change from baseline
60
40
20
0
-20
-40
-60
Injection Day
Post-Inj Day 1
Post-Inj Day 3

Fig. 4 Effects of DFP on REM sleep. Changes in the percent of the recording time in REM sleep. Values are differences from baseline values. Symbols same as Fig. 4.


REM
Control
DFP, low dose
DFP, high dose
% REM, change from baseline
10
8
6
4
2
0
-2
-4
-6
-8
-10
Injection Day
Post-Inj Day 1
Post-Inj Day 3

Comparison of the rats which received atropine prior to DFP injection to those which did not receive the atropine is shown in Table III. There are no significant differences between these groups (Mann-Whitney U) for any of the sleep parameters. Furthermore, qualitative assessment of the toxic symptoms did not reveal any substantial relief of the toxic symptoms by atropine.

DISCUSSION

The behavioral symptoms of DFP toxicity described here are similar to those described by Jovic (1974) for the organophosphate cholinesterase inhibitor soman. The correlation of body weight loss and severity of generalized symptoms, also, has been noted by others (McDonough et al., 1983; Churchill et al., 1985; Lim et al., 1983). The presence of these symptoms of toxicity was found to be incompatible with the occurrence of sleep, despite the fact that under certain conditions DFP promotes increased amounts of REM sleep (Gnadt et al., 1985) and that these studies were performed at the time of day when sleep propensity is greatest (the rat is a nocturnal species).

In response to acute administration of DFP, there was a dose-dependent decrease in both stages of sleep and an inversely related increase in wakefulness. The higher dose of DFP produced decreased amounts of sleep, larger in magnitude and longer in duration than the lower dose of DFP. For the low dose of DFP, values of the sleep parameters had returned

Table III Effects of atropine on DFP effects. Median values for each stage of the sleep-wake cycle is given for the rats grouped by whether or not they received atropine, for both DFP doses. There were no significant differences between the rats which received atropine and those which did not (Mann-Whitney U).

EFFECTS OF ATROPINE

	DFP, low dose				DFP, high dose		
	% Awake	% SWS	% REM		% Awake	% SWS	% REM
without Atropine	99.1	0.9	0	Median	100	0	0
	74.1-100	0-25.9	0-0	Range	97.4-100	0-1.9	0-0.7
with Atropine	81.1	18.9	0	Median	100	0	0
	59.1-100	0-32.4	0-8.5	Range	66.5-100	0-32.0	0-1.5

to baseline levels by the first postinjection day. For the high dose of DFP, recovery to baseline values was nearly complete by the third postinjection day; only a decreased amount of SWS persisted at that time.

The pattern of decreased sleep for the DFP-treated rats roughly paralleled the pattern of body weight loss. Body weight served as a general index of severity of toxicity.

Pretreatment of the rats with 3 mg/kg atropine sulfate did not block the effects of DFP on sleep.

In summary, the toxicity of acutely administered DFP secondarily results in decreased amounts of sleep. The recovery of sleep parameters parallels the recovery of behavioral toxic symptoms. Atropine sulfate, at doses of 3 mg/kg, failed to block the toxic symptoms of either dose of DFP, and also failed to block the effects of DFP on sleep.

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In addition to the studies described above, our laboratory also has been involved in the study of the effects of another organophosphate, soman, on sleep in rats (Meighen et al., 1985). Both soman and DFP are "irreversible" inhibitors of acetylcholinesterase. Soman is a more potent agent, having a toxicity (as measured by LD_{50}) of approximately 1 1/2 log units greater than that of DFP. The occurrence of seizures as a prominent symptom of toxicity for soman suggests that the ratio of central to peripheral activity of this drug is higher than that for DFP.

In relation to its effects upon sleep, similar findings concerning acute toxicity have been found for soman as that found for DFP. On the day of injection of soman there was an orderly dose-dependent increase in time spent awake and a corresponding decrease in time spent in SWS. The doses of soman tested were 40, 50, and 60 mg/kg. There was a small decrease in REM sleep on the injection day seen only for the highest dose of soman. Pilot studies had shown that interpretation of sleep data from higher doses of soman were complicated by the occurrence of prolonged seizures that usually ended in death. By the day following the injection, the sleep parameters for all rats had returned to baseline values.

Studies concerning chronic administration of soman are currently underway. It is anticipated that these studies will confirm our findings concerning chronic administration of DFP.

Summary of systemic studies

The findings of the chronic DFP studies described above are compatible with hypotheses postulating that cholinergic mechanisms are involved in the generation of REM sleep. They suggest that cholinergic systems may be particularly important in the mechanisms which initiate REM sleep.

The effects of DFP on sleep in rats, however, can not be termed as narcoleptic-like. Two hallmarks of the human sleep disorder are the occurrence of REM sleep directly from wakefulness without intervening non-REM sleep (REM onset sleep), and the occurrence of an associated cataplexy (Roth, 1980). Neither of these phenomena were seen with chronic administration of DFP. Rarely, REM onset sleep was noted in rats given DFP acutely; however, the effect usually occurred after one or more hours of DFP-induced insomnia. It would be imprudent to attribute this effect to direct action of DFP on sleep mechanisms. The cataplectic-like effect seen in rats recovering from acute injections of DFP were suggestive of a similarity to clinical cataplexy. Despite this, several factors make it unlikely that this effect was truly analogous to the clinical syndrome: 1) The effect was not

relieved by atropine and, therefore, probably not mediated by central muscarinic mechanisms. The cataplexy of the canine narcolepsy (Delashaw et al., 1979), is thought to involve central muscarinic mechanisms. 2) The periodic weakness observed in the DFP treated rats often was associated with probable neuromuscular symptoms such as tremor with motor effort. 3) The weakness in rats could be overcome by voluntary effort. Cataplectic attacks in humans and canines are incapacitating.

On the other hand, the finding that the cholinesterase inhibitor did seem to promote the initiation of REM sleep could suggest that a similar mechanism may be involved in the two forms of hypersomnolence. It has been suggested (Gnadt et al., 1985b) that the mechanism of action for the effects of the cholinesterase inhibitor in REM sleep may be to bias cholinergic mechanisms towards a critical threshold for initiating REM sleep. Narcolepsy appears to be an uncontrolled interruption of REM sleep, or REM sleep phenomena, into whatever state of vigilance is present at that time. The difference between the two syndromes of excessive REM sleep could be a matter only of degree. Indeed, administration of physostigmine exacerbates cataplexy in narcoleptic dogs (Delashaw et al., 1979). The effects of physostigmine on the REM sleep of the narcoleptic dogs were not assessed in that study.

The differences in the effects upon sleep between chronic and acute administration of DFP can be attributed to

the toxicity associated with acute administration of the drug (Gnadt et al., 1985a). The behavioral tolerance to organophosphates when administered chronically is a well documented phenomenon (Russell et al., 1969; Overstreet et al., 1974; Russell et al., 1975). Several laboratories have shown that the probable mechanism of this tolerance is, at least in part, a down regulation in the number of muscarinic receptors in the brain (Schiller, 1979; Ehlert et al., 1980; Costa et al., 1982; Yamada et al., 1983; Churchill et al., 1984a and 1984b). It is interesting to note, however, that muscarinic receptor sites in the pontine reticular formation do not exhibit this down regulation (Yamada et al., 1983; Churchill et al.; 1984a and 1984b). This finding has interesting implications concerning the effects of chronic administration of DFP on sleep with respect to brainstem mechanisms. This could explain why the sleep effects persist in the chronic paradigm after the acute toxic effects have abated.

III. MICROINJECTION STUDIES

Considerable evidence, which has been reviewed in the Introduction, has suggested that pontine mechanisms are involved in the generation of REM sleep. The pontine reticular formation, in particular, seems to play an important role in the generation of REM sleep. In the mid-1970s Hobson, McCarley and Wyzinski (1974) proposed a theory which formalized one hypothesis concerning the role of the pontine reticular formation in the generation of REM sleep. Their theory also made several predictions concerning the neurochemical mechanisms that might be involved. Their theory suggested that cholinergic stimulation of neurons within the pontine reticular formation was responsible for the initiation of REM sleep, whereas cholinergic stimulation of monoaminergic neurons in the locus coeruleus was responsible for the termination of REM sleep. Implicit in this model was the hypothesis that cholinergic stimulation in the pontine reticular formation, coupled with diminished monoaminergic activity, was necessary for REM sleep to occur.

Pharmacological studies in the cat have suggested that the pontine reticular formation does contain cholinergic mechanisms which are involved in the generation of REM sleep

(Hobson, 1982). This effect appeared to be restricted to the pontine reticular formation (Baghdoyan et al., 1984), and the robustness of the effect (e.g., Silberman et al., 1980; Hobson et al., 1983) suggested that the cholinergic mechanisms which are involved are of primary importance. Despite this, several lines of evidence (see Introduction) have suggested that while cholinergic mechanisms within the pontine reticular formation may be facilitatory upon REM sleep, it may not be necessary.

In the studies described below, microinjection techniques were used to investigate possible cholinergic mechanisms in the brainstem of the rat species. These studies serve to investigate more thoroughly the presumed cholinergic effect upon REM sleep produced by systemic administration of cholinesterase inhibitors in rats, and to address some of the issues concerning brainstem cholinergic mechanisms of REM sleep. Furthermore, these studies allow cross-species comparisons to similar studies performed in the cat species.

Cholinergic brainstem mechanisms of REM sleep in the rat

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Abstract

Injection of carbachol into the brainstem of rats produced an increase in REM sleep which was site- and dose-dependent. Effective locations for carbachol to stimulate REM sleep included the pontine reticular formation at the level of the trigeminal motor nucleus and the dorsal parabrachial area in the caudal midbrain. The carbachol effect in the caudal pons was dose-dependent. Additionally, this effect was blocked by concomitant administration of the muscarinic antagonist atropine. Control experiments suggested that the drug-induced phenomenon appeared to be an increase in normal physiological REM sleep.

Key words: REM, sleep, carbachol, brainstem, reticular formation, rats

INTRODUCTION

Cholinergic mechanisms are involved in the control of sleep, and in the generation of REM sleep in particular. Inhibition of acetylcholine synthesis completely and specifically eliminates REM sleep.[18] Systemic administration of cholinesterase inhibitors, which presumably facilitate physiologic cholinergic activity, can produce an increased REM sleep in humans,[11,25] in rats,[17] and perhaps in cats.[6] Studies using the cholinesterase inhibitor physostigmine in cats[10] and in humans[38] have suggested that this agent promotes the initiation of REM sleep, but not necessarily its maintenance.[15] A similar finding has been noted in the rat using the "irreversible" cholinesterase inhibitor, DFP.[17]

Lesion and transection studies in the 1960s implicated the pons as a locus for REM sleep generating mechanisms.[21] More recent lesion studies by Friedman and Jones[13] have suggested that the pontine reticular formation may contain the critical elements for the generation of REM sleep.

Another line of evidence using chemical stimulation techniques has implicated cholinergic mechanisms within the brainstem in the generation of REM sleep.[1-3,5,14,19,37] Other laboratories have described a cataplectic phenomenon in response to cholinergic stimulation in the brain stem[26,39] This phenomenon probably represents a dissociation of REM sleep atonia into wakefulness, a condition which occurs spontaneously in humans or canines afflicted with the

disorder narcolepsy.[4,30] Baghdoyan et al.[3] have found in cats that the most effective site within the brainstem for the cholinergic stimulation of REM sleep is in the anteromedial pontine reticular formation. Furthermore, these effects appear to involve muscarinic mechanisms.[2,14,19,39]

Despite these findings, lesion studies using kainic acid have suggested that the medial pontine reticular formation may not be necessary for REM sleep.[9,32] Additionally, extracellular unit recording studies in cats[33-36] and in rats[40,41] have failed to identify neurons within the pontine reticular formation with activity profiles specific for REM sleep.

With the exception of the investigations of Vertes,[40,41] all of the studies described above concerning pontine mechanisms of REM sleep utilized the cat as the experimental species. To date, detailed investigation of brainstem cholinergic mechanisms in other species has not been reported. This report describes cholinergic mechanisms of REM sleep in the brainstem of the rat species using localized injection techniques. These studies serve to investigate the presumably cholinergic mechanisms of REM sleep recently reported in the rat,[17] and to provide independent cross-species comparison to studies in the cat.

METHODS

Surgeries. Sixty-seven Sprague-Dawley rats (Charles River) were surgically implanted under halothane anesthesia with chronic electrodes for the recording of electroencephalogram (EEG) and electromyogram (EMG). All rats were adult males (240-310g). The construction of the stainless steel electrodes with gold contacts has been described previously.[17] The EEG electrodes were implanted supradurally in holes drilled into the calvarium. Bipolar EMG was recorded from muscles of the dorsal aspect of the neck.

Each rat received 4 EEG electrodes: Two electrodes were placed bilaterally over the frontal cortices (2 mm anterior to bregma and 2 mm lateral), one electrode was placed over the left hippocampus (4 mm posterior to bregma and 3.8 mm lateral) and the fourth electrode was placed 3-4 mm posterior to the right frontal electrode and was used to ground the animal. Each rat received two EMG electrodes.

In addition to the recording electrodes, each rat received a 26 ga. stainless steel guide cannula (Small Parts, Inc. or Plastic Products). The guide cannula was implanted stereotaxically through a hole drilled in the calvarium with the tip lying 5-6 mm below the dura, and was aimed unilaterally at various brainstem targets using the Pellegrino et al.[29] atlas as a reference. The incisor bar of the stereotaxic instrument (Kompf) was set at 5 mm above interaural zero and targets within the pons were approached from a 15°

lateral to medial angle to avoid the fourth ventricle and the locus coeruleus.

The bases of the electrodes and of the guide cannula were buried in a mound of dental acrylic along with a stainless steel screw secured into the cranium. Patency of the cannula was maintained by a removable stylus.

After surgery the rats were given 30,000 units penicillin and were allowed 5 or more days recovery. During recovery, rats were housed individually in a ventilated, sound-attenuated isolation chamber. Before and after surgery lights were maintained on a 0600/1800 light/dark cycle.

Injections. Nineteen hours before the experiments, the rats were habituated to a 10 cm x 30 cm opaque plastic recording cage of 37 cm height. Throughout the experiment food and water was available ad libitum. The injections were made at 1500 hours in hand restrained, unanesthetized subjects.

Each injection was delivered using a 33 ga. injection cannula (Plastic Products) which passed 2-3 mm beyond the tip of the guide cannula. The injection cannula was connected to a micrometer-driven (Sherr-Tumico, Inc.) 0.5 µl Hamilton syringe by 25 cm of flexible plastic tubing (0.0075 in. I.D., Autoanalyzer pump tubing, Technicon Corp.).

All injections were 0.1 µl delivered over 2-2 1/2 min and the injection cannula was left in place for an additional 60 sec before being withdrawn. Solutions were drawn into the apparatus by backfilling several microliters into

the needle and tubing. The reliability of the apparatus was found to be ± 0.01 µl by injecting a radiolabeled solution (^{3}H choline, 7.7 µCi/µl) into scintillation fluid and counting the radioactivity ejected.

The vehicle for all drug injections was normal saline (pH=7.0) with 0.5% alcian blue (ingrain blue 1, Fischer Scientific). Alcian blue is a histological dye used to help locate the injection sites. The drug injections consisted of 0.1, 0.5, 1.0, or 5.0 µg of carmabamylcholine chloride (carbachol, Sigma Chem. Co.), 0.41 µg atropine sulfate (Sigma), or a combination of the two. This dose of atropine was chosen to effectively produce 100% inhibition of binding of a 1.0 µg dose of carbachol, as calculated from affinity constants for these two agents for high affinity muscarinic binding sites in rat brain.[7,20] Four of the control rats received injection of saline only; all others received the drug vehicle solution. Three of these 4 rats were used later for injections of carbachol. All other rats received only one injection.

Recording. Immediately following the injection, the rats were connected to polygraph amplifiers by flexible cables and returned to their recording cages. The cables were suspended from above by a spring-loaded apparatus which allowed complete freedom of movement with minimal distraction. Injections and recordings were performed on 4 subjects at a time. Care was taken to yoke different drug or dose conditions within each group of 4.

Behavioral notes were made during the 15-30 min required to inject and to prepare the rats for recording. Visual observation continued during the following 30-60 min, and when unusual behavior was noted, the isolation chamber was entered for more direct assessment of the behavior. Disturbance of the animals was kept to a minimum whenever possible. Periodic behavioral observation continued for the first 2-3 hours of the record and occasionally thereafter. Early studies had shown that if anomalies were produced by the injections they would be evident at the start of the recording. In a few cases the recording had to be disturbed briefly for repair of recording leads and equipment.

Standard polygraphic methodologies were used to record two EEGs and a bipolar EMG on each rat. The EEGs were recorded by referencing the electrode over the hippocamus to the ipsilateral and to the contralateral frontal electrodes. Using standard criteria, the 6 hour record was scored visually into awake (mixed frequency, low voltage EEG), Slow Wave Sleep (SWS, low frequency, high voltage EEG and sleep spindles), and REM sleep (homogeneous 7-8 Hz EEG from the hippocampal electrode, absent tonic EMG) according to 60 sec epochs (1 page at 5 mm/sec). Each epoch was designated as the stage which predominated within that epoch. In cases where a REM sleep period was 30 sec or more, but was distributed across two epochs in which it did not predominate within either one of the epochs was designated as REM sleep. The records were analyzed in terms of the percent of the

recording time spent in each stage of the sleep-wake cycle, by 90 min intervals. In some cases the number and average duration of the stages were analyzed as well. For REM sleep, the number and average episode duration were calculated using all REM sleep episodes more than 10 sec in length. Latency of the first REM sleep period from start of the record also was analyzed for some rats.

Histology. Twenty to 48 hours after the injections, each rat was sacrificed for histological confirmation of the injection site. The rats were deeply anesthetized with pentobarbital and perfused through the heart with saline followed by 10% buffered formalin phosphate. The brains were removed and soaked in the fixative for 2-24 hours then frozen on dry ice. The brains were blocked and mounted for cutting of coronal sections at 25 m in a cryostat. Every fourth section through the brainstem was mounted on gelatin treated slides and stored overnight in 70% ethanol. Sections were stained using cresyl violet or the Kluver-Berrera method with luxol blue (solvent blue 38, Sigma) and safranin O (Sigma) then dehydrated with ethanol and xylenes and coverslipped. For each rat, the section containing the center of the injection site was drawn and the representative anterior-posterior level of the brain with respect to the Pellegrino et al. (1979) atlas was determined. For demonstration purposes, composite drawings of the injection sites were made by projecting the injection site centers from

the individual drawings onto drawings adapted from the brain atlas.

Spectral analysis. As a control experiment the EEG of one rat was quantified by spectral analysis before and after injection of carbachol. Three representative 10 sec epochs of EEG from each of the three stages of the sleep-wake cycle were chosen from the record of a control (vehicle injected) rat. These EEG samples were analyzed by digitizing the signal at 100 times per second (Nova Computer, Data General) and determining the spectral power from 0-20 Hz at 1/4 Hz increments by Fast-Fourier analysis. Three days later the same rat received a 1.0 µg dose of carbachol into the same injection site. The record following this injection was analyzed in the same manner as for the control condition. The spectral analysis for each of the stages of the sleep-wake cycle was compared between the two recordings.

Autoradiography. As another control experiment, 6 rats were injected with a radiolabeled solution to estimate the spread of a 0.1 µl injection into brain tissue. Because a commercially available source of radiolabeled carbachol was unavailable, ^{3}H choline (78 Ci/mmol, Amersham) was used. The ^{3}H choline was concentrated to 7.7 µCi/µl (10^{-4} Molar) in 0.9% saline. Carbachol was added to this to give a .055 Molar solution of carbachol (1.0 µg/.1 µl). The rats were lightly anesthetized with halothane and given injections of 0.1 µl of the radiolabeled carbachol/choline solution. Three of the rats were killed by decapitation immediately

following the injection (under deep anesthesia). The other 3 rats were allowed one hour survival times before being reanesthetized and killed by decapitation. After decapitation, the brains were removed quickly, rinsed in saline, and quick-frozen by immersion in 2-methyl butane cooled with dry ice. The brains then were sectioned in a cryostat at 40 μm. Every third section was thaw mounted onto coverslips on a hot plate. Adjacent sections were mounted onto gelatin treated slides for staining by cresyl violet. The cover slips were opposed to the emulsion of LKB Ultrafilm ^{3}H and mounted into x-ray cassettes. One each of rats having the 0 and 1 hour survival times were exposed for 2 weeks. The sections from the other 4 rats were exposed for 3 weeks. The films were developed for 5 min in GBX developer (Kodak) and fixed in Kodak x-ray fixer.

Standards were made by making serial dilutions of the injection solution and drying 1 μl droplets of the dilutions onto a coverslip. Dilutions were made such that the total radioactivity in each droplet was less than the total radioactivity injected into the rat by factors of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}.

The diameter of the spot of silver grains on the film produced by the injections was measured at the center of the injection site and for the two sections before and after the center. The spots were measured along their longest axis. Irregular halos of trace radioactivity ($<10^{-10}$ Ci) around the central, densely exposed spot were not included. The

mean of these diameters estimated a cross section of the volume of tissue exposed to the solution with a sensitivity to more than a 10^{-4} dilution of the injected material. The radius estimated the approximate distance from the injection site center through which the injection solution spread radially before diluting substantially.

Statistical methods. Site-dependency was analyzed by comparing values of individual carbachol injections to the distribution of the values for the control injections. Values lying outside of the 95% confidence interval for the controls were considered to be statistically significant.

The dose-dependency of the injections was analyzed with consideration for time from injection. For statistical analysis, the 6 hour recording time was divided into four 90 min intervals. The F-test was used to determine the effects of carbachol dose and its interaction with time. Since the four time intervals represent a repeated measure, analyses including this variable were performed using the multivariate profile analysis of repeated measures.[27] Analyses which do not include this interaction effect were performed by simple one-way analysis of variance.

The t-test was used to compare the differences between individual group means unless the distributions of the samples were decidedly non-Gausian. In such cases, the nonparametric Mann-Whitney U was used to compare the sample distributions.

The effects of atropine were analyzed using the one-way analysis of variance, with planned comparisons to the control group and between the carbachol and the carbachol-atropine group.

RESULTS

Behavioral effects. Except as noted below, the behavior of most of the rats was unremarkable. During wakefulness, the rats would move about their cage, eat, groom, or lie quietly. During SWS, the rats would lie recumbent, usually in a curled position, with maintained posture. During REM sleep, the rats would lose their postural tone and lie slackly on the floor of the cage with phasic twitches of the limbs, ears, and vibrisse. Occasionally the rats would awaken spontaneously from REM sleep with a sudden jerk.

Common behaviors during the injection included spontaneous mouth movement or chattering of the teeth, twitching of the vibrisse, sometimes more vigorously ipsilateral to the injection, and occasional sudden body jerks. Many rats did not exhibit any unusual symptoms. Mouth movements and twitching of the vibrisse were the most common symptoms noted, and could occur with injection of any of the drug or control conditions. These symptoms were most prominent for injections which were localized in the caudal pons, and for the larger doses of carbachol.

Injections of carbachol into the pons or rostral medulla sometimes produced an ipsilateral contraction of the axial musculature, particularly in the neck and thorax. In its mildest form, this appeared as an ipsiversive circling behavior. In its most severe form, the contracture completely drew the rat into an incapacitating ipsilateral flexion of the body. This symptom was produced by injection doses of 1.0, 5.0 and 10.0 µg of carbachol, with increasing severity. With 1.0 µg of carbachol, this effect was rare and never severe; the symptom abated within 15-30 min. With 5.0 µg carbachol, the contracture sometimes lasted as long as 60-90 min, diminishing steadily during that time period. With 10.0 µg carbachol, the symptom was very prominent and could disturb the animal for several hours. Due to this disturbance and other behavioral anomolies, data from rats receiving 10.0 µg of carbachol have not been included in analysis in this paper, other than their behavioral description here.

One rat exhibited excessive lacrimation of the ipsilateral eye, in addition to the ipsilateral turning behavior. Both symptoms abated in about 30 min. The injection site for this rat lay in the reticular formation medio-rostral to the facial nucleus.

Five rats exhibited a generalized flacid-type weakness. This symptom was substantial in three of the rats and mild in the other two. The weakness was never a complete paralysis and could be overcome with sufficient effort when the

rat was prodded manually. This weakness was accompanied by a diminished EMG. The symptom did not persist more than 30-40 min, although sleep onset during this time made further investigation difficult. In two cases, this hypotonia was associated with an elevated REM sleep as reported below.

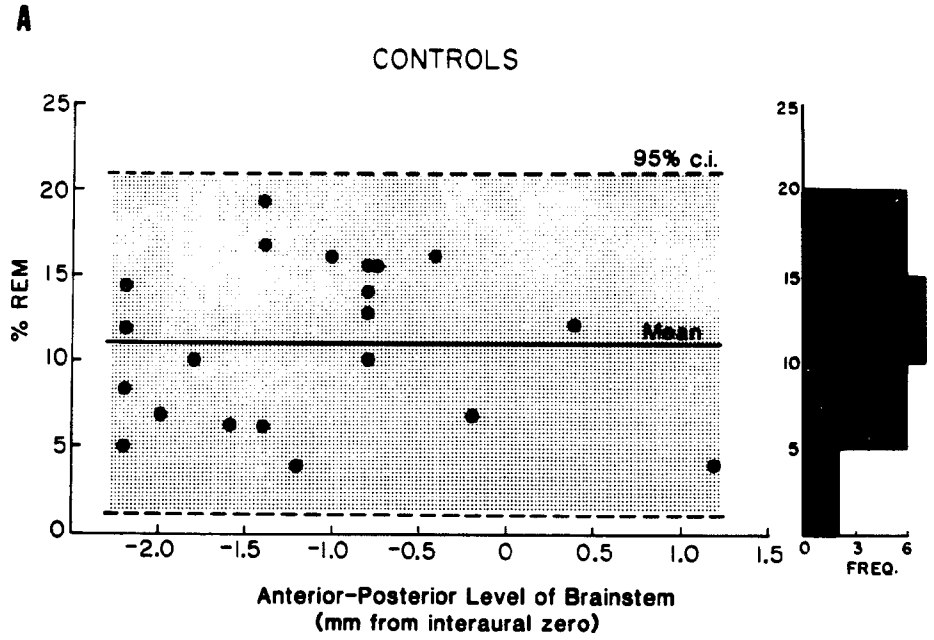
Sleep effects. The effect of carbachol injections into the brainstem was found to be site- and dose-dependent. Fig. 1 shows this effect as the percent of the recording time spent in REM sleep for the first 3 hours of the record. These values are plotted as a function of the anterior-posterior level of the brain. The level of the site of each injection is plotted relative to interaural zero according to the atlas of Pellegrino et al. (1979). Fig. 1A shows the data for the 21 control injections. Also plotted is the mean and a 95% confidence interval for the sample. A frequency histogram is shown to the right.

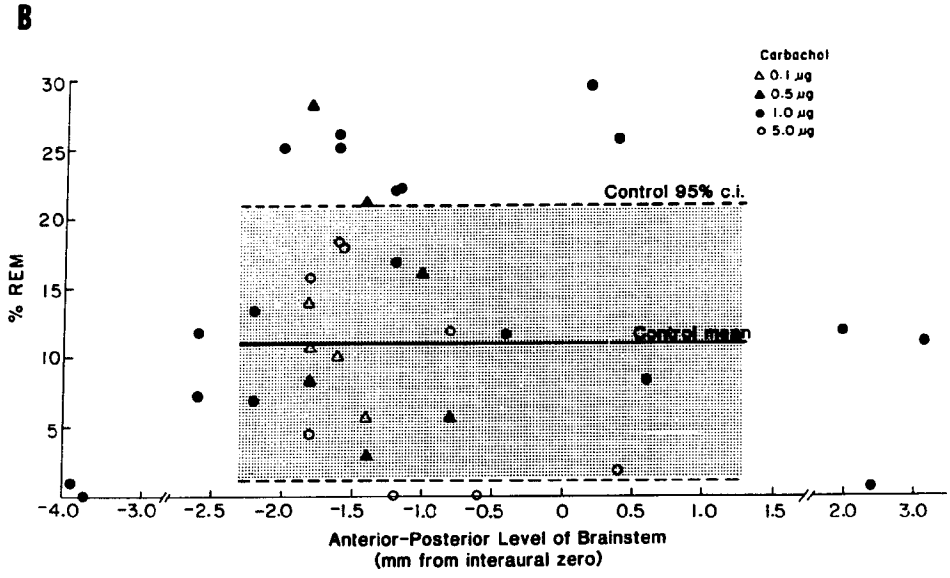
Fig. 1B is a similar plot for all 37 of the carbachol injections, grouped as follows: 0.1 µg, n=4; 0.5 µg, n=6; 1.0 µg, n=19; 5.0 µg, n=8. Superimposed upon this graph is the mean and 95% confidence interval for the control group. Note that injections produced an increase in the amount of REM sleep above control values only when they lie between 2.0 to 1.2 mm posterior to zero, and when they lie at approximately 0.4 mm anterior to zero. All other injection sites produce either a decrease or no effect on REM sleep. The level between 2.0 and 1.2 mm posterior to zero corresponds to the caudal pons. The level at 0.4 mm anterior to

Fig. 1. The percent of the recording time in REM sleep as a function of anterior-posterior level of the injection site in the brainstem. The data are from the first 3 hours postinjection. The scale of the abscissa is millimeters posterior (negative) or anterior (positive) to interaural zero.

A. Values for the control animals with their mean and a 95% confidence interval.

B. Values for the carbachol injections superimposed upon the mean and 95% confidence interval for the controls.


A
CONTROLS
95% c.i.
Mean
% REM
25
20
15
10
5
0
-2.0
-1.5
-1.0
-0.5
0
0.5
1.0
1.5
0
3
6
FREQ.
Anterior-Posterior Level of Brainstem
(mm from interaural zero)


B
Carbachol
Δ 0.1 μg
▲ 0.5 μg
● 1.0 μg
○ 5.0 μg
Control 95% c.i.
Control mean
% REM
30
25
20
15
10
5
-4.0
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0
0.5
1.0
1.5
2.0
3.0
Anterior-Posterior Level of Brainstem
(mm from interaural zero)

zero corresponds approximately to the junction of the pons and midbrain. Note also that only the 0.5 and 1.0 μg doses are effective in increasing REM sleep, even within the caudal pons.

The injection sites from 2.6 mm to 0.4 posterior to zero for the 0.5 and 1.0 μg doses are shown in coronal sections in Fig. 2. The most effective location for the injections to produce increased REM sleep appears to lie in the pontine reticular formation just medial and slightly ventral to the trigeminal motor nucleus, near the junction of the nucleus reticularis pontis caudalis and oralis.

The more rostral level where injections also produced increased REM sleep is shown in Fig. 3. The two effective injection sites lie in the midbrain just dorsal to the brachium conjunctivum. Another injection ventral to the brachium failed to increase REM sleep.

The dose-dependency of this effect was determined for the caudal pons. Fig. 4 shows the percent of the recording time spent in REM sleep as a function of time from injection and dose of carbachol. These data are from the 26 rats whose injection sites lie between 2.0 to 1.2 mm posterior to interaural zero. The rats are grouped according to dose as follows: Control, n=7; 0.1 μg, n=4; 0.5 μg, n=4; 1.0 μg, n=6; 5.0 μg, n=5. The 6 hour recording time has been analyzed as four 90 min time intervals. There is a significant effect of dose to increase REM sleep, which is dependent upon time from injection $F(12,50) = 3.778$, $p<.001$.

Fig. 2. Injection sites from the rostral medulla to the rostral pons for 0.5 and 1.0 µg doses of carbachol. All injection sites lie within 0.2 mm of the representative sections shown. Filled symbols (● ■) represent injections which elevated REM sleep above control values, unfilled symbols (○ □) represent injections which failed to elevate REM sleep. Squares (■ □) are 0.5 µg, circles (● ○) are 1.0 µg. The half-filled circle represents an injection in which only the percent REM within total sleep time was elevated. The scale bar is 1 mm. A = cerebral aqueduct, BC = brachium conjunctivum, CG = central grey, CT = central tegmental nucleus, IC = inferior colliculus, LC = locus coeruleus, P = pontine nuclei, PT = pyramidal tract, RGC = nucleus reticularis gigantocellularis, RPC = nucleus reticularis pontis caudalis, RPO = nucleus reticularis pontis oralis, SC = superior colliculus, 3 = oculomotor nucleus, 4V = fourth ventricle, 5n = trigeminal nerve, 7 = facial nucleus, 7n = facial nerve.

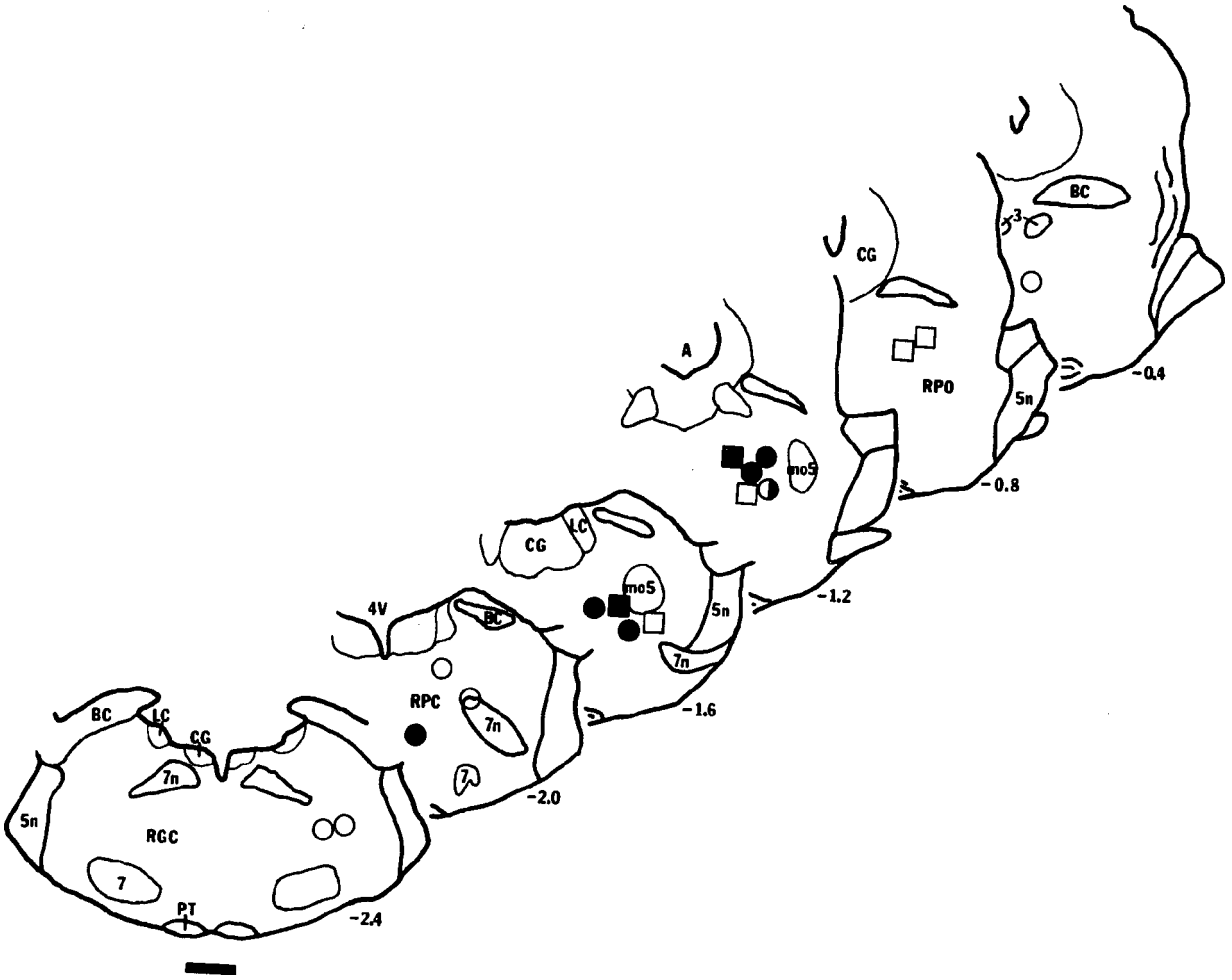
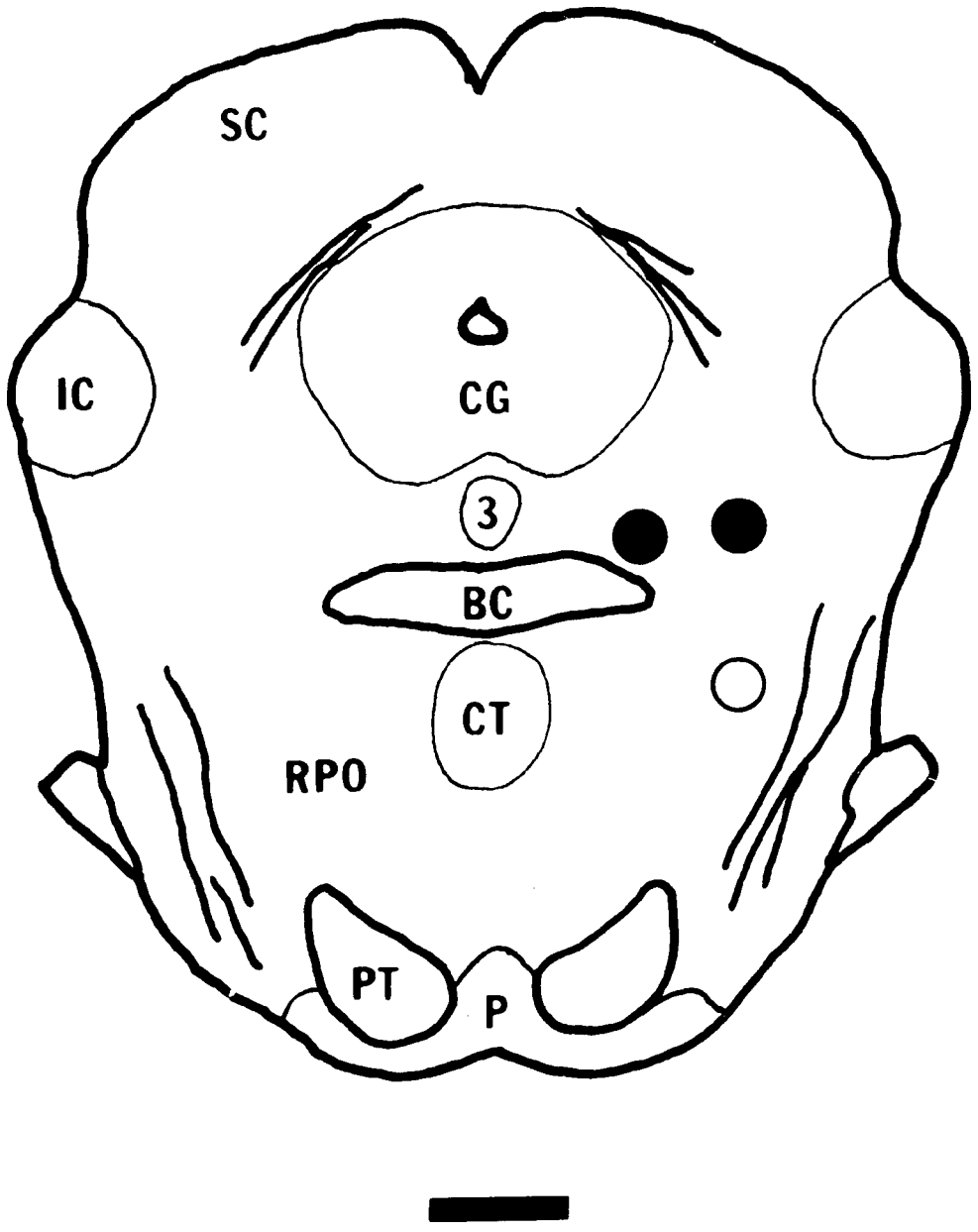

BC
CG
RPO
5n
-0.4
-0.8
A
mo5
LC
CG
-1.2
4V
BC
mo5
5n
7n
RPC
7n
-1.6
BC
LC
CG
7n
7
-2.0
5n
RGC
7
PT
-2.4

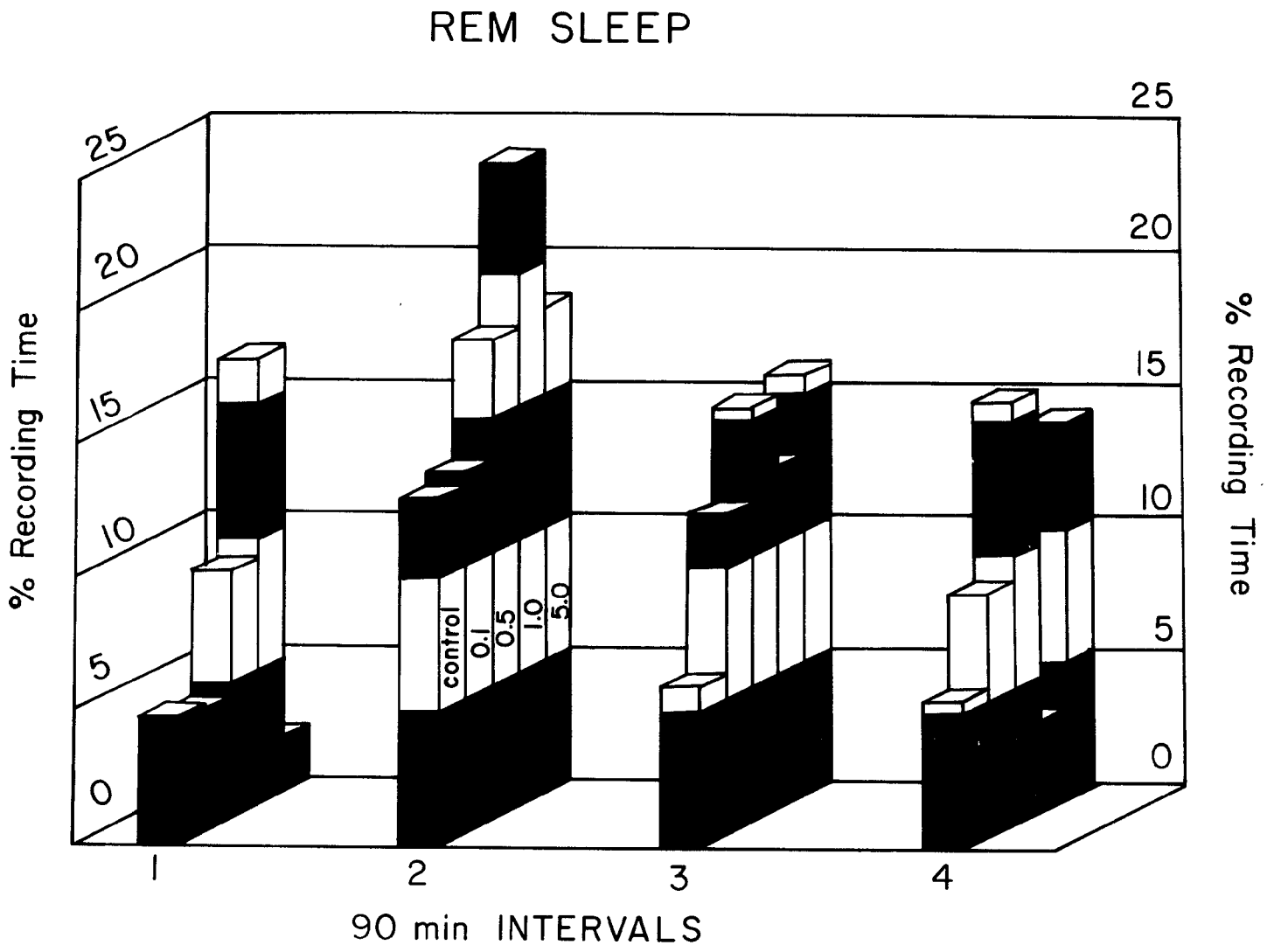
Fig. 3. Injection sites near the junction of the pons and midbrain. All injections lie within 0.2 mm of the representative section shown. All symbols as in Fig. 2.


SC
IC
CG
3
BC
CT
RPO
PT
P

During the first 3 hours of the record (intervals 1 and 2), there is a significant effect of dose upon REM sleep, $F(4,21)=3.627$ $p<.02$. The mean percentage of recording time in REM sleep for the 1.0 µg dose of carbachol is significantly higher than the corresponding mean for the control group, $F(1,21)=11.351$ $p<.005$. The lower percentage of REM sleep during the first 90 min. for the 5.0 µg group, relative to the 1.0 µg dose, is probably due to the motor disturbances which were prevalent for this highest dose of carbachol.

An increased percent of REM sleep could be produced by an increase in the number of REM sleep periods, an increase in the duration of the REM sleep periods, or a combination of the two. The number of REM sleep periods and the average duration of the REM sleep periods for the first 3 hours postinjection was determined for the 7 rats with injections in the caudal pons which had an increase in the REM sleep time. The mean number of REM sleep periods for these carbachol-treated rats (20.0 ± 1.6, mean $\pm$ standard error) was significantly higher ($p<.001$, one-tailed t-test) than the corresponding mean for the control rats from the caudal pons (8.9 ± 1.0). There were no statistical differences for the values of the average duration of the REM sleep periods between the two groups, despite a tendency for the carbachol-treated rats to have longer REM sleep periods. These data were analyzed by non-parametric methods (Mann-Whitney U) due

Fig. 4. The percent of the recording time in REM sleep as a function of dose of carbachol and time from injection. All injections lie within the caudal pons (-2.0 to -1.2 mm, Fig. 1B). Along the abscissa, the 6 hours of recording has been divided into four 90 min intervals. Increasing doses of carbachol from control values through 5.0 µg are plotted into the depth of the page. The alternation of shaded and unshaded sections of the vertical bars indicates increments of 5% as a visual aid to estimate the bar heights. See text for statistical analyses.


REM SLEEP
% Recording Time
25
20
15
10
5
0
control
0.1
0.5
1.0
5.0
1
2
3
4
90 min INTERVALS

to an apparent outlier for the control group: control median = 85 sec vs. carbachol median = 122 sec.

Another index of the propensity for REM sleep is the latency from injection to the first REM sleep period. The REM sleep latency for the drug-treated rats is reduced when compared to control values (two-tailed Mann-Whitney U, $p<.05$). Again, the non-parametric analysis was used due to differences in variance between the two groups: control median = 69 min vs. carbachol median = 39 min.

The dose-dependent effects of the carbachol injections into the caudal pons on the percent SWS is shown in Fig. 5. There is a dose-dependent decrease in SWS, $F(4,21)=2.938$ $p<.05$, which is not time dependent, $F(12,50)=.884$. The mean percentage recording time in SWS for the 5.0 μg dose is significantly less than for the control group, $F(1,21)=10.120$ $p<.005$. This decrease was due to a decrease in the average duration of the SWS periods (one-tailed t-test, $p<.001$): control mean 4.2 min $\pm$ 0.22 vs. carbachol mean 2.2 min $\pm$ 0.46. The number of SWS periods was not statistically different between the two groups (Mann-Whitney U): control median = 32 vs. carbachol median = 22.

For completeness, the effects of carbachol injections into the caudal pons on the percent awake is shown in Fig. 6. These values are not statistically significant, $F(4,21)=1.303$.

Fig. 5. The percent of the recording time in SWS as a function of dose of carbachol and time from injection. See. Fig. 4 for explanation.

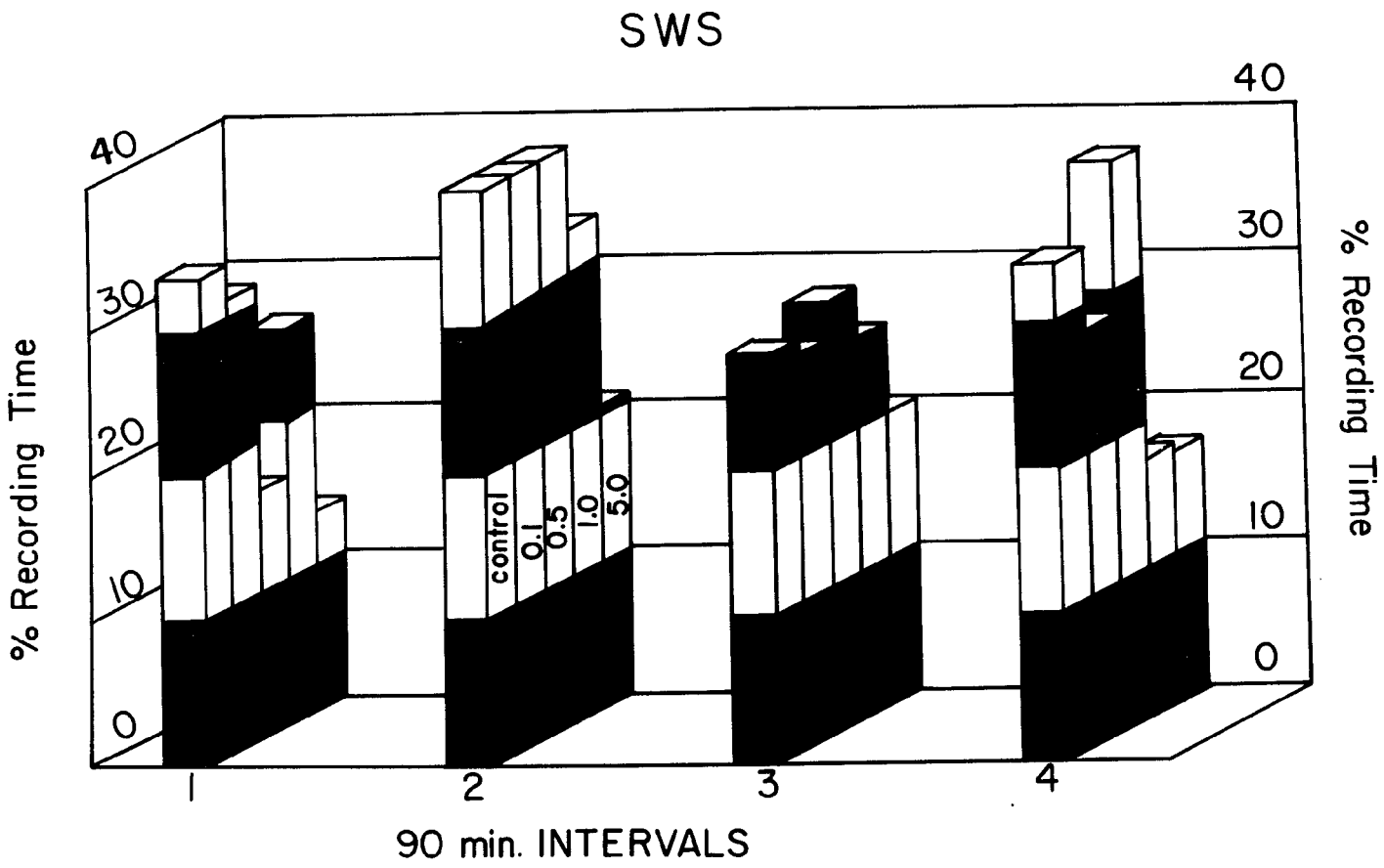
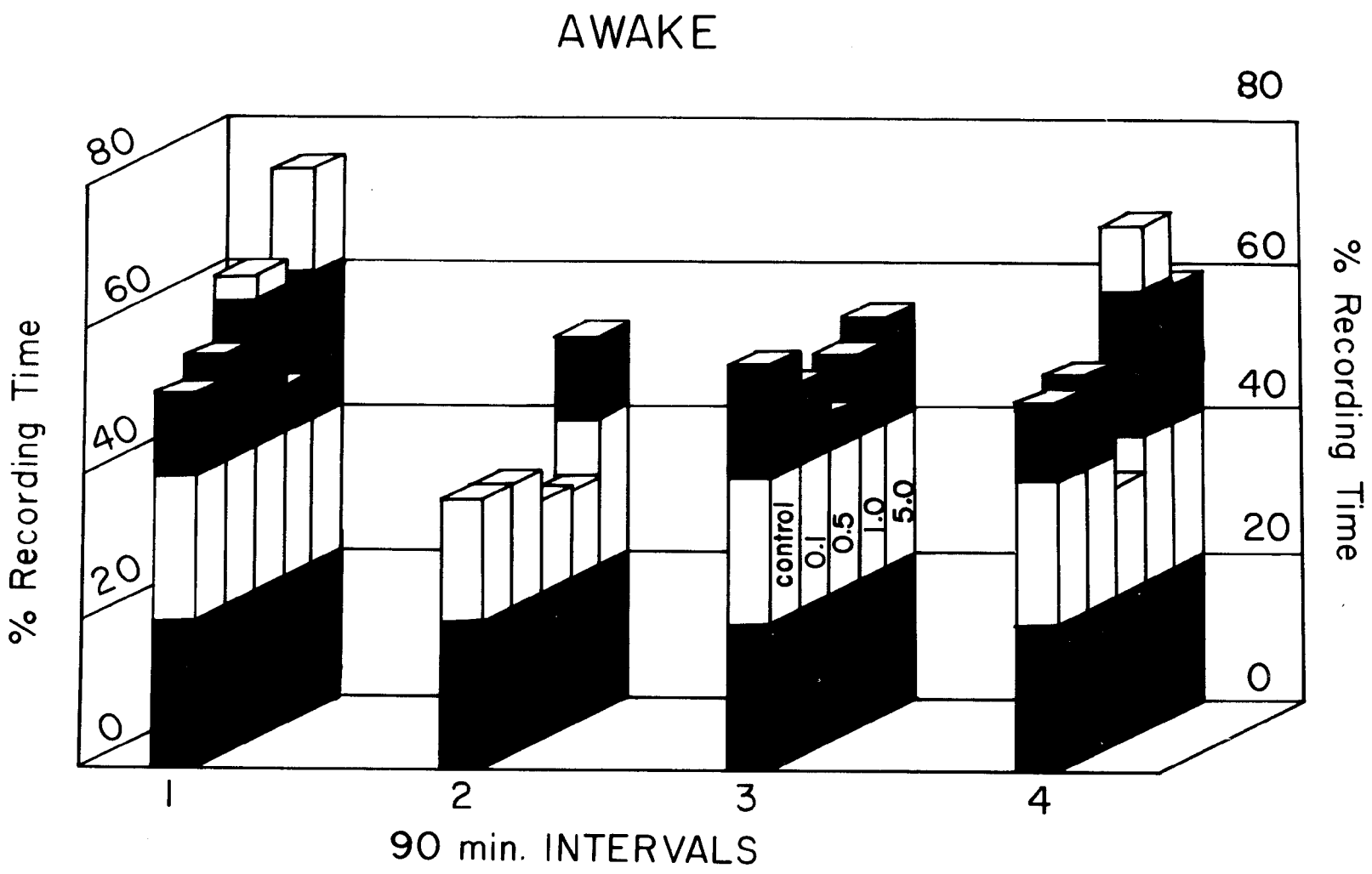

SWS
40
30
20
10
0
% Recording Time
control
0.1
0.5
1.0
5.0
1
2
3
4
90 min. INTERVALS

Fig. 6. The percent of the recording time awake as a function of dose of carbachol and time from injection. See Fig. 4 for explanation.


AWAKE
% Recording Time
80
60
40
20
0
1
2
3
4
90 min. INTERVALS
control
0.1
0.5
1.0
5.0

Effects of atropine. The effects of atropine are shown in Fig. 7 for the 6 hour recording time of 4 groups of rats. All of the rats represented here received injections into the caudal pons. The control group and the 1.0 μg carbachol group have been described above. The carbachol-atropine group represents 7 additional rats which received injections containing 1.0 μg carbachol and 0.41 μg atropine sulfate. The atropine group represents 5 additional rats which received 0.41 μg atropine sulfate. One-way analysis of variance indicates that there is a significant effect of these injections only on the REM sleep variable, $F(3,21)=7.238$ $p<.002$, and that only the carbachol group differs from controls, $F(1,21)=12.711$ $p<.002$. The atropine was found to block the effect of carbachol, when compared to the group receiving carbachol alone, $F(1,21)=19.782$ $p<.001$.

Spectral analysis. The spectral analysis of the EEGs from a control rat and the same rat later receiving 1.0 μg carbachol is shown in Fig. 8. Comparison of the EEGs before and after the drug treatment for each stage of the sleep-wake cycle does not reveal noticeable differences, despite the fact that the carbachol injection produced an increased amount of REM sleep (38.2% of the recording time during 3 hours postinjection).

Autoradiography. The autoradiographs of the four rats which had 3 week exposure times allowed detection of amounts of radioactivity to more than 4 log units more dilute than the original concentration of radioactivity. The diameters

Fig. 7. The effects of atropine. The percent of the recording time in REM sleep for 6 hours postinjection for 4 groups of rats. The control group received injections of the drug vehicle. The carbachol group received 1.0 µg carbachol. The carbachol-atropine group received 1.0 µg carbachol and 0.41 µg atropine sulfate concomitantly. The atropine group received 0.41 µg atropine sulfate. All injections were made in the caudal pons. See text for statistical analysis. Error bars are the standard error of the mean.

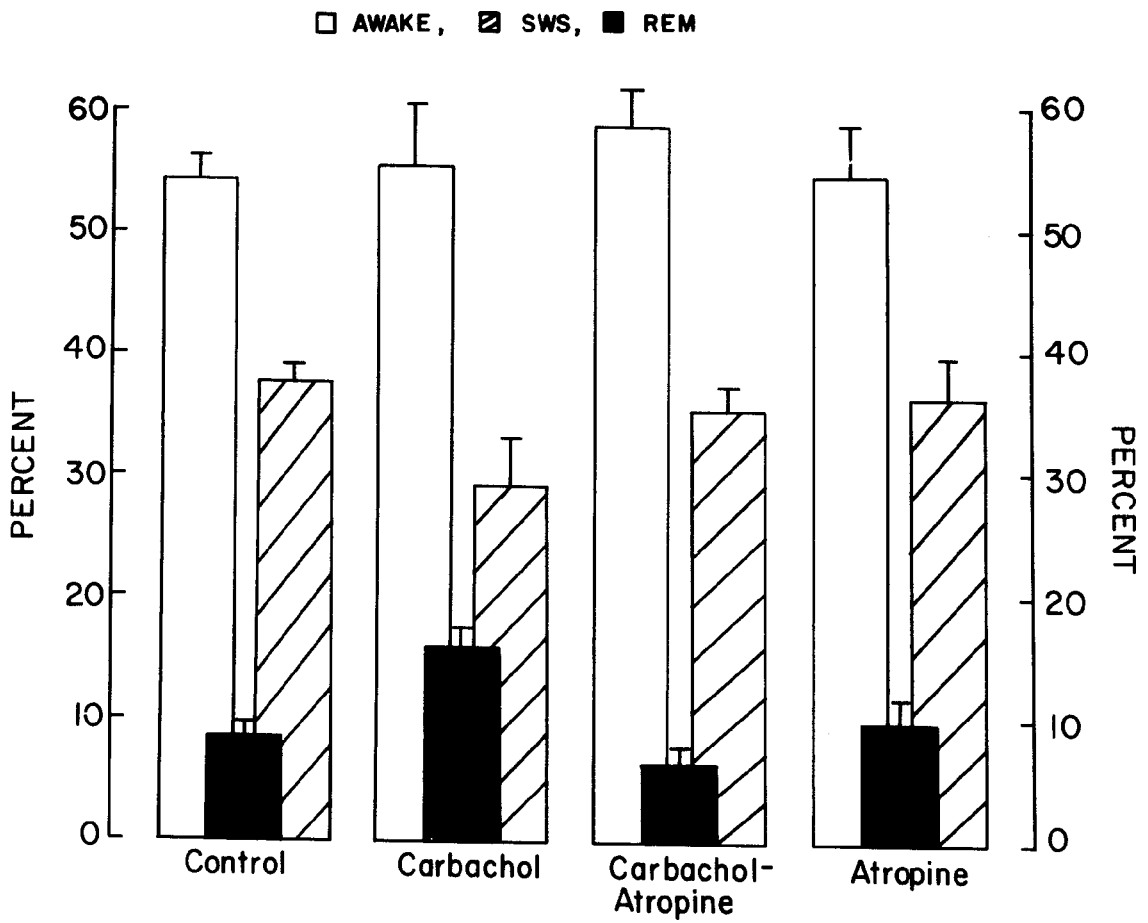
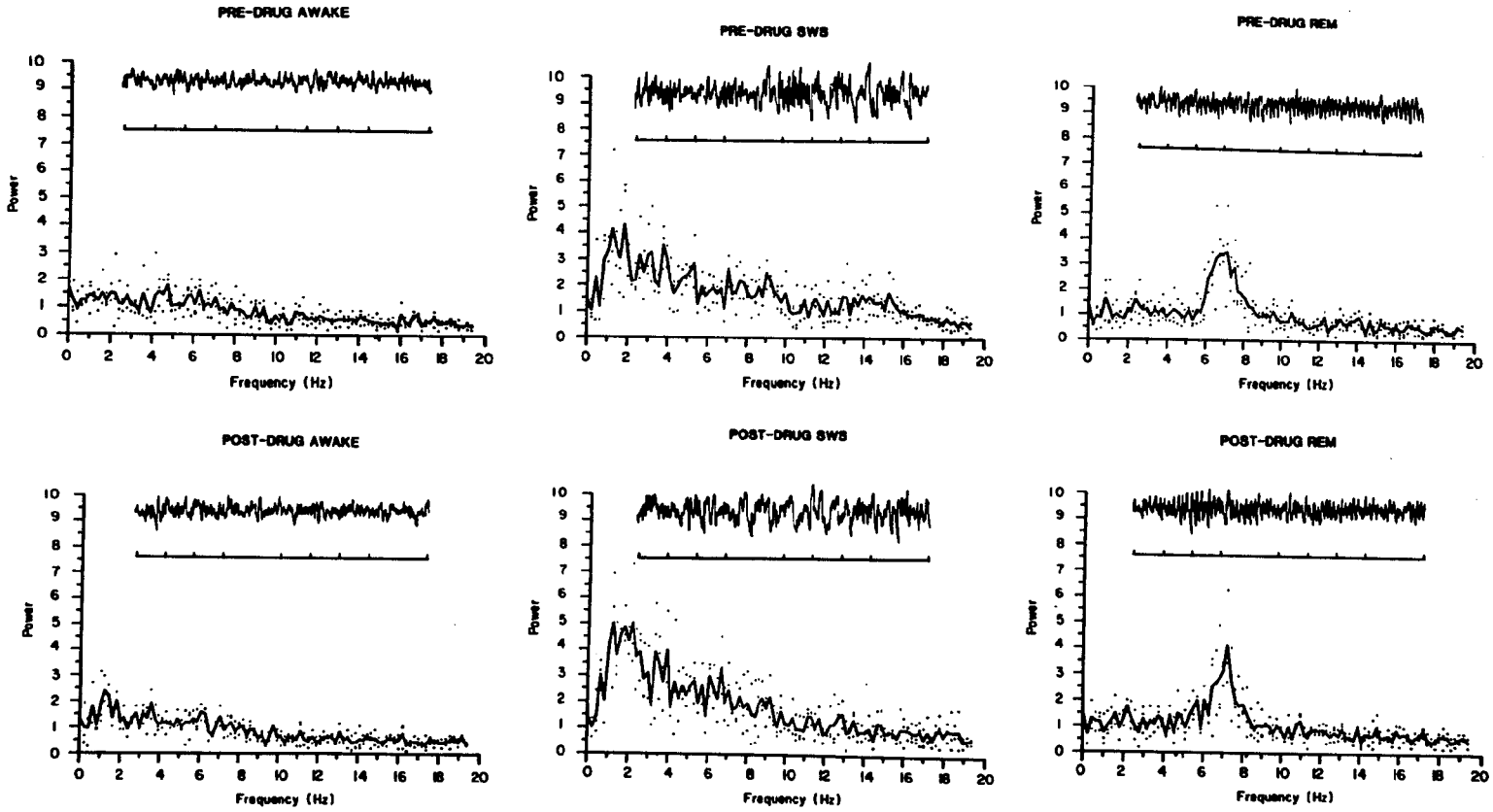

AWAKE, SWS, REM
PERCENT
60
50
40
30
20
10
0
Control
Carbachol
Carbachol-
Atropine
Atropine

Fig. 8. Spectral analysis of the EEG. The data are from one rat which received a vehicle injection (pre-drug) and later received a 1.0 µg dose of carbachol (post-drug). Each panel represents analysis of three 10 sec samples of each stage of the sleep-wake cycle (dots) and the mean values for the three samples (line). One of the three samples is shown in each panel along with a 10 sec time code.


PRE-DRUG AWAKE
PRE-DRUG SWS
PRE-DRUG REM
POST-DRUG AWAKE
POST-DRUG SWS
POST-DRUG REM
Power
Frequency (Hz)

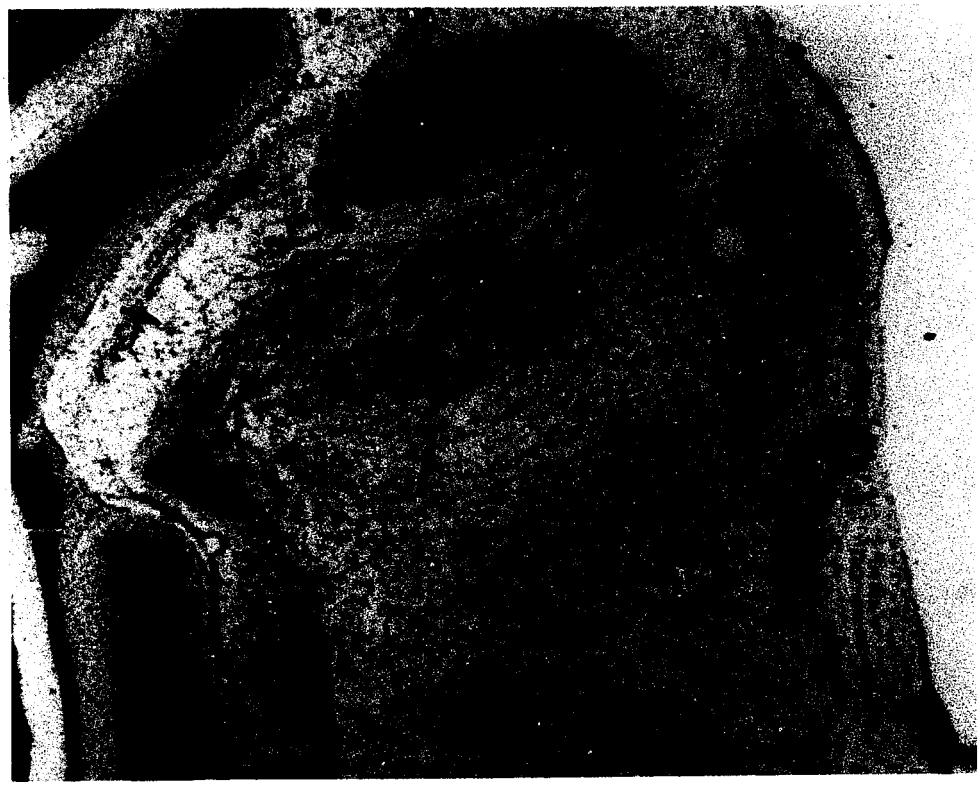
of the spots on the films were 1.7 and 2.4 mm for the 2 rats which had been sacrificed immediately after injection. The 2 rats which had 1 hour survival times had spot diameters of 2.0 and 2.3 mm. The spots were roughly spherical with a tendency for traces of the radioactivity to trail up the path of the injection needle to the tip of the guide cannula. An example of the autoradiogram from one of these rats is shown in Fig. 9, along with an adjacent section stained with cresyl violet.

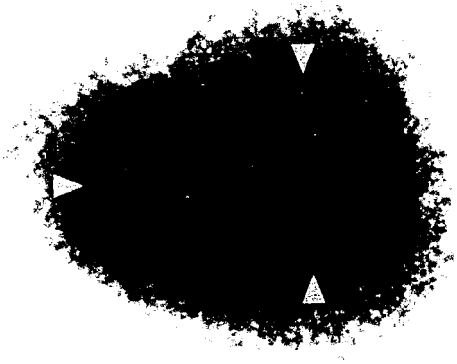
The autoradiographs of the 2 rats which had 2 weeks exposure times showed similar results at lower levels of sensitivity. The diameters of the spots for these rats was about 1 mm.

DISCUSSION

The autoradiographic findings concerning the volume of tissue exposed to the 0.1 µl injections used in this study provide a rough estimate of the distance from the injection site that effects of the material might be expected to be manifest. Dilutions of the agents to less than 10^{-4} of the original concentration are unlikely to be important relative to the effects produced by the higher concentrations of the agent near the injection center. From inspection of the autoradiogram shown in Fig. 9, one can appreciate that there is a continuous diminution of radioactivity from the injection site center to its periphery. The smaller size spot

Fig. 9. The autoradiogram from the center of an injection site and an adjacent section stained with cresyl violet. Arrowheads designate equivalent sites on the two photographs. The long arrow points to a small lesion produced by the tip of the injection cannula. The short arrow shows the direction from which the injection cannula approached the injection site. The distance between the two horizontal arrowheads is 1 mm.





seen with the shorter, and therefore less sensitive, exposure times also demonstrates this phenomenon. This finding is typical of injections which have been made sufficiently slowly.[12] The distance from the injection site center at which the drug concentrations become inconsequential cannot be determined with certainty, but these findings suggest that distances more than one millimeter from the center probably are not affected substantially. Relative to the effects produced by the concentration at the center of the injection site, this effective radius may be less. The diameter of the injection spot produced by this volume is within the typical range seen with both autoradiographic[12] and horseradish peroxidase assay techniques.[42]

The large volume of tissue exposed to the drug solutions when using this technique prevents the determination of the specific neurons or structures which mediate the effects produced by the injections. However, the technique is useful for making inference concerning the generalized function of groups of neurons and in determining the approximate location of particular functional activities.

With the exception of the limited motor and behavioral side effects produced by large doses of carbachol and the few rats which exhibited the brief postural hypotonia, the general behavior and the sleep behavior of the drug treated rats was indistinguishable from the control rats. Excluding the brief periods of hypotonia, the EMG patterns typical for normal sleep-wake patterns were present in both the drug

treated rats and the controls. Additionally, quantitative analysis of the EEG also failed to reveal differences between the control and carbachol treated conditions. On these bases, the increased REM sleep noted in the drug treated rats is considered to be an increase in physiologically normal REM sleep.

The hypotonia noted in 5 rats would seem to be analogous to the atonia which can occur in cats when cholinergic agonists are injected near the locus coeruleus.[26,39] The injection sites in these 5 rats were located in the same general location in which injections produced increased REM sleep. The effective doses of carbachol ranged from 0.1 to 5.0 μg. It is not known why these trials produced the hypotonia while other injections having similar doses into the same region of the reticular formation did not produce the effect. These findings have been presented in detail elsewhere.[16]

This study represents the first report of detailed investigation of cholinergic mechanisms of REM sleep in the brainstem of the rat species. There are both similarities and differences in the findings in this species and findings from similar studies in the cat species.[1-3,5,14,19,26,37,39]

Using cats as the experimental species, Baghdoyan et al.[3] have reported that injections of carbachol into the pontine, but not the medullary or the midbrain, reticular formation can produce a state indistinguishable from REM sleep. These investigators reported that the most effective

location for this effect was in the anteromedial pontine reticular formation.

Injection of carbachol in rats also showed site differentiation in its effects upon REM sleep. Injections into the caudal pontine reticular formation produced an increase in apparent REM sleep. The most effective location within the caudal pons was near the junction of the nucleus reticularis caudalis and oralis, just medial to the trigeminal motor nucleus. This site corresponds approximately to the location in which Friedman and Jones[13] have shown in cats that lesions prevent the occurrence of REM sleep. The placement of injection sites in this study did not allow sufficient differentiation between medial versus lateral placement.

Unlike the cat, however, another site near the junction of the pons and midbrain also is effective in increasing REM sleep. This site is near the brachium conjunctivum at the level where these fibers decussate. The two injections in this region which produced increased amounts of REM sleep lay in the midbrain dorsal to the brachium and rostral to the dorsal parabrachial nucleus. This location lies near a region which has been implicated in the generation of PGO waves in cat.[28,31]

Injections at levels of the brainstem other than those described above produced either a decrease or no effect on REM sleep.

The increased REM sleep produced by injections into the caudal pons were due primarily to an increase in the number

of REM sleep periods during the first 3 hours postinjection. This finding is in contradiction to that described in the cat in which prolonged REM sleep periods were typical.[1-3,14,19,37] This finding parallels a similar effect of systemically administered cholinesterase inhibitor in rats,[17] in which there was an increase in the number and not duration of REM sleep periods. This finding supports the hypothesis that cholinergic mechanisms may serve as an excitatory input onto a REM sleep trigger.[17] The same conclusion was reached by Gillin et al.[15] in their studies of the effects of a cholinesterase inhibitor in humans. Furthermore, this study suggests that the integration of this cholinergic input may occur in the brainstem reticular formation.

The finding of two anatomically distinct sites in which carbachol can induce increased REM sleep can have several different interpretations. One site could contain the integrating mechanisms of a REM sleep trigger, while the neurons from the other site serve as a cholinergically mediated relay onto the first site. Another possibility is that both sites could contain independent REM sleep triggers. Alternatively, both sites (and perhaps others, as well) could jointly contain the integrating mechanisms of the trigger, such that activity within one site would be sufficient to recruit activity within the other. The results of this study cannot determine conclusively which, if any, of these interpretations are valid. Several investigators recently have suggested that the mechanisms which produce REM sleep

may be widely distributed within the brainstem.[8,22-24,31] The findings of this study are compatible with that hypothesis. With respect to the issue of recruitment of activity, the fact that these unilateral injections were able to produce an apparently ordered, non-disruptive change in a phenomenon which affects virtually every neuronal and somatic system of the body suggests that the mechanisms which generate REM sleep are able to affect the activity of very many or very divergent neuronal systems.

The dose-dependence of the carbachol effect suggests that the carbachol is producing its effects through a receptor mediated mechanism, either by recruiting the response of more receptors or by recruiting more response per receptor. The ability of atropine to block the effect of carbachol on sleep suggests that the effect is mediated by muscarinic cholinergic receptors. It can not be determined from these studies whether the receptors in question are postsynaptic or presynaptic, but the involvement of fibers in passage would seem to be ruled out.

Van Dongen et al.[39] have shown previously that local injection of adrenergic agents near the locus coeruleus in cats did not mimic the effect of carbachol injections to produce atonia. Because adrenergic agents are known to produce vascular changes, but did not mimic the effect of carbachol, it is unlikely that changes in cerebrovascular perfusion were responsible for the carbachol-induced effects. The autoregulatory nature of cerebrovascular blood flow also

argues against the possibility that vascular changes would be responsible for such long term effects as produced by the carbachol. Additionally, necrosis due to ischemia would not be expected to increase REM sleep, since destructive lesions in this area of the pons eliminate REM sleep rather than facilitate it.[13]

The finding that atropine had no effect on REM sleep, despite being injected into the same location in which carbachol produced substantial effects, was an unexpected finding. The 0.41 µg dose of atropine sulfate (0.012 molar atropine base) was sufficient to block the effects of a 1.0 µg dose of carbachol (.055 molar carbamylcholine) and would be expected to be in high enough concentration to inhibit binding of endogenous acetylcholine. If cholinergic stimulation at this site were a necessary condition for REM sleep to occur, one would predict that atropine would reduce the amount of REM sleep. Interestingly, two other laboratories also have reported a failure of atropine to produce the opposite effects of carbachol.[14,34]

This finding suggests that while cholinergic stimulation in the caudal pons may be facilitory upon REM sleep, it may not be necessary. Drucker-Colin and Pedrazza[9] found in cats that kainic acid lesions of the medial pontine reticular formation abolishes the ability of cholinergic stimulation at that site to produce REM sleep, but does not eliminate the REM sleep state. Further investigation of this hypothesis will be necessary to establish its validity.

This hypothesis, however, is compatible with the postulate that cholinergic mechanisms may play an integral role in generating REM sleep. It merely implies that other non-cholinergic mechanisms also are involved in the triggering of REM sleep. This also could explain why carbachol-injected rats continued to have relatively long latencies to REM sleep, even though their latencies were decreased compared to controls. Assuming many chemically coded inputs onto a REM sleep trigger, some excitatory and some inhibitory, cholinergic stimulation need not be necessary nor sufficient for the occurrence of REM sleep; yet it could still bias the trigger towards more or less frequent occurrence.

The prolonged effect of carbachol on REM sleep suggests that non-classical synaptic mechanisms are responsible for the effect. The time constants of the traditional ligand-induced ion fluxes are too short to account for increased REM sleep several hours after the injection of carbachol.

In summary, injections of carbachol into the brainstem of rats produced a site- and dose-dependent effect upon REM sleep. Two sites were found to be effective in stimulating increased REM sleep. One site was located in the pontine reticular formation at the level of the trigeminal motor nucleus. The second site was located in the dorsal parabrachial region in the caudal midbrain. The effect within the caudal pons was dose-dependent and blocked by the muscarinic receptor antagonist atropine. The increased REM sleep was due to increased numbers of REM sleep periods without

substantial increase in the duration of the REM sleep periods. By qualitative analysis and by quantitative analysis of the EEG, the sleep-wake states in the drug treated animals were indistinguishable from those of the control animals.

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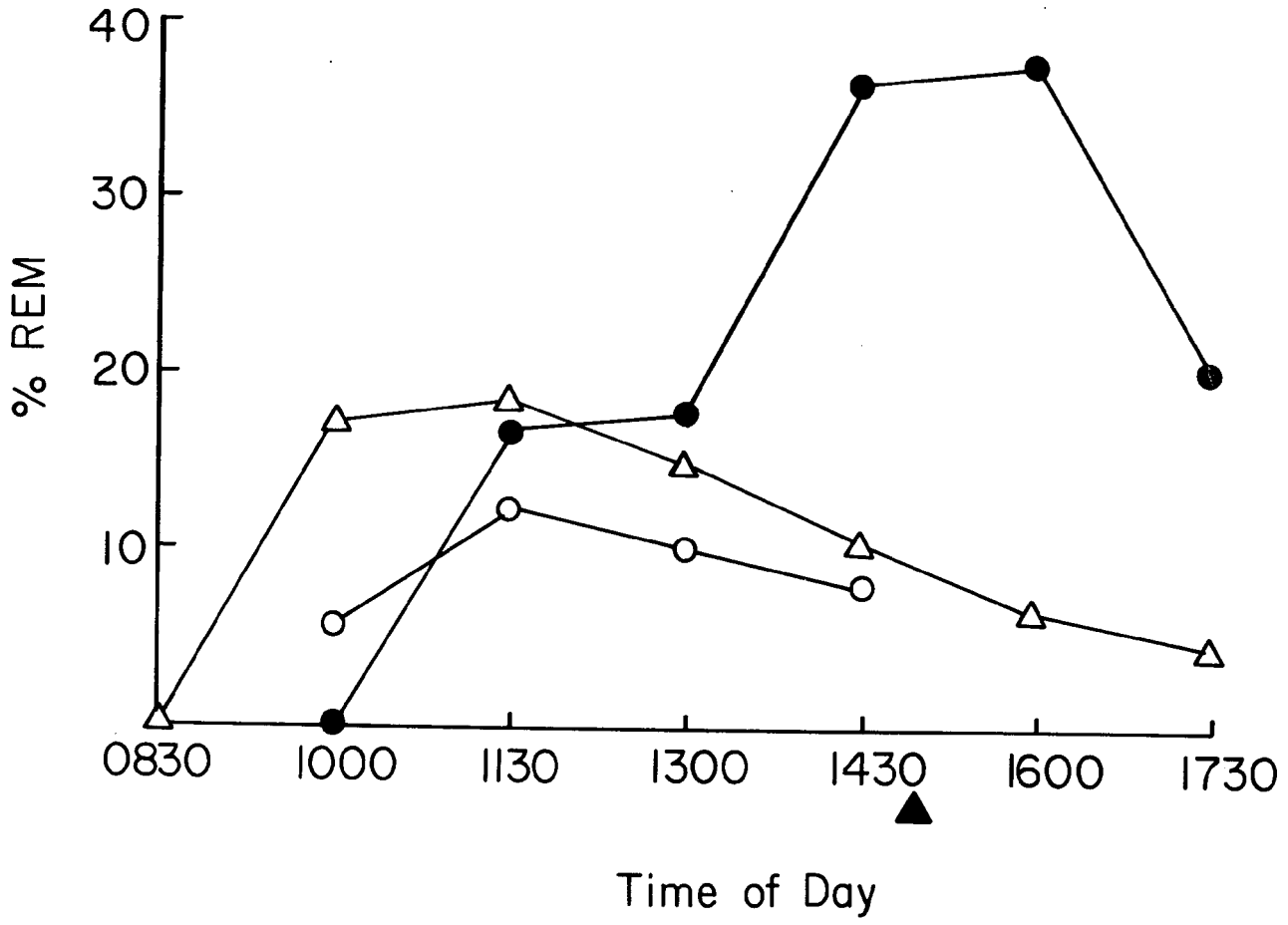
Circadian cycle and the effect of carbachol on REM sleep

An additional finding of interest concerning the effects of brainstem injections of carbachol in rats is one which involves circadian phase. Pilot studies of more than 15 trials of injections given at 1000 rather than 1500 hours suggested that carbachol fails to produce increased sleep at earlier phases of the circadian cycle. An example of this finding is illustrated in Fig. 10. The percent of the recording time in REM sleep is shown as a function of time of day for two different rats.

The data represented by circles were collected from one rat on two different occasions. The unfilled circles represent data from an injection of saline into the caudal pontine reticular formation given at 1000. One week later this same rat received an injection of 5.0 µg carbachol into the same location. Note that the carbachol injection does not produce a substantial effect upon REM sleep until 4 1/2 hours later at about 1430. The percent REM sleep then appears to remain elevated during the next 4 1/2 hours of recording. For comparison, typical data from an uninjected control animal from a previous study (unpublished results) has been plotted as unfilled triangles.

These findings and similar findings from more than 20 trials of microinjections of the cholinesterase inhibitor, DFP, suggested that cholinergic stimulation in the caudal

Fig. 10. The percent of the recording time in REM sleep as a function of time of day. Each point on the graph represents the data for 90 min beginning at the time shown. The circles (● O) represent data from the different trials from one rat. The filled circles (●) are data from an injection of carbachol. The unfilled circles (O) are data from a control injection. The triangles (△) are data from an uninjected normal rat. The arrowhead shows the time of day at which injections were made for the previously described studies. See text for explanations.


40
30
20
10
% REM
0830
1000
1130
1300
1430
1600
1730
Time of Day

pons fails to produce increased REM sleep when given earlier during the light phase than approximately 1430. Coincidentally, the highest propensity for REM sleep in the rat species occurs during the late afternoon up until the onset of the dark phase of the circadian cycle (1800 in these rats).

These data represent one more line of evidence that, for the rat species, cholinergic stimulation is not the only mechanism involved in the induction of REM sleep. Cholinergic stimulation in the caudal pontine reticular formation is neither sufficient nor necessary for REM sleep to occur.

In some respects, the studies described here appear to confirm some of the neurochemical predictions of the reciprocal interaction theory of Hobson and McCarley (Hobson, 1982). Presumed cholinergic stimulation in rats by cholinesterase inhibition was found to facilitate the occurrence of REM sleep (Gnadt et al., 1985b). Cholinergic stimulation in the caudal pontine reticular formation was found to mimic this effect and was found to produce a more robust effect than seen with systemic administration of the cholinesterase inhibitor (Gnadt and Pegram, 1985). Furthermore, the stimulatory effect was due to a facilitation of the initiation of REM sleep, but not its maintenance. It is of interest to note here that the finding in cats of prolonged REM sleep periods in response to cholinergic stimulation actually are in contradiction to predictions of the model.

Other findings in the rat species, however, do not support the theory proposed by Hobson and McCarley. Locations other than the caudal pontine reticular formation are sensitive to cholinergic stimulation of REM sleep. Additionally, cholinergic stimulation in the caudal pons appears to be neither sufficient nor necessary for REM sleep to occur.

This author favors other hypotheses that the trigger mechanisms for the initiation of REM sleep are distributed widely throughout the brainstem. Several other investigators recently have made this suggestion as well (McGinty and Drucker-Colin, 1982; McGinty, 1985; Sakai, 1985; McCarley and Ito, 1985; Corner, 1985).

Currently, there is accumulating evidence that the outputs for REM sleep phenomena from the brainstem may be anatomically separate (Sakai, 1985; Vertes, 1984; Corner, 1985; Chase, 1983), and that they may be independently stimulated (e.g., Mitler and Dement, 1974; Van Dongen et al., 1978; Vertes, 1982) or independently eliminated (e.g., Hendricks et al., 1982; Morrison and Reiner, 1985).

Using intracellular recording within the medial pontine reticular formation, McCarley and Ito (1985) may have identified a mechanism which could mediate a distributed integrating mechanism within the brainstem. These investigators suggested that a tonic membrane depolarization during REM sleep throughout a widely distributed neuronal pool may mediate the mechanisms which integrate the various outputs during REM sleep. However, the source of this tonic

membrane depolarization remains to be identified. It is unclear at this time how this mechanism may be related to the integration of inputs onto a REM sleep trigger.

IV. SUMMARY

The modern history of sleep research has been reviewed with particular emphasis on brainstem mechanisms of REM sleep. The evidence concerning the cholinergic nature of these mechanisms was discussed. The experimental results reported here have demonstrated that cholinergic mechanisms are involved in the generation of REM sleep in the rat species.

Acute administration of the "irreversible" cholinesterase inhibitors to rats was found to disrupt all stages of sleep due to behavioral toxicity associated with this form of administration. These toxic effects, however, subsided when the drug was administered chronically.

After chronic administration, the cholinesterase inhibitor, DFP, produced an increased amount of REM sleep compared to controls. The mode of action was to facilitate the initiation of REM sleep without substantially prolonging the occurrence of individual episodes.

Microinjection techniques were used to apply a cholinergic agonist (carbachol) to localized sites within the brainstem of rats. Two sites within the brainstem were found to produce increased REM sleep when stimulated with

carbachol. One site was located in the pontine reticular formation, a location which has been implicated in the control of REM sleep by chemical stimulation and by lesions in the cat species. A second site was found in the caudal midbrain dorsal to the brachium conjunctivum. This site has been implicated in the generation of PGO waves (a REM sleep phenomenon) in cats.

The effect within the caudal pons was found to be dose-dependent and blocked by the muscarinic receptor antagonist atropine. Atropine injections into the caudal pons had no effect on REM sleep. Control experiments were performed which suggested that the carbachol-induced increase in REM sleep was an increase in physiologically normal REM sleep.

It was suggested that while cholinergic stimulation in the caudal pontine reticular formation may be facilitory upon REM sleep, it is neither necessary nor sufficient.

The implications of these findings on theories concerning the mechanisms which trigger REM sleep were discussed. In particular, these findings were compared to predictions of the model of REM sleep control postulated by Hobson and McCarley.

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APPENDICES

APPENDIX I

Postural hyotonia following pontine injections of carbachol

Legend Fig. Ia.

A sample record from a rat exhibiting hypotonia. This continuous sample was taken 5 min after injection. Note the waxing and waning of the EMG and spontaneous bursts of EMG during attempted movements. Behavioral assessment of this rat revealed a substantial flacid weakness which could be overcome by sufficient effort. EMG = electromyogram from dorsal aspect of neck, EEGi = electroencephalogram from over left hippocampus to ipsilateral frontal cortex. EEGc = electroencephalogram from over left hippocampus to contralateral frontal cortex. Vertical scale bars: EMG = 25 μV, EEG = 100 μV. Horizontal scale bar = 2 sec.

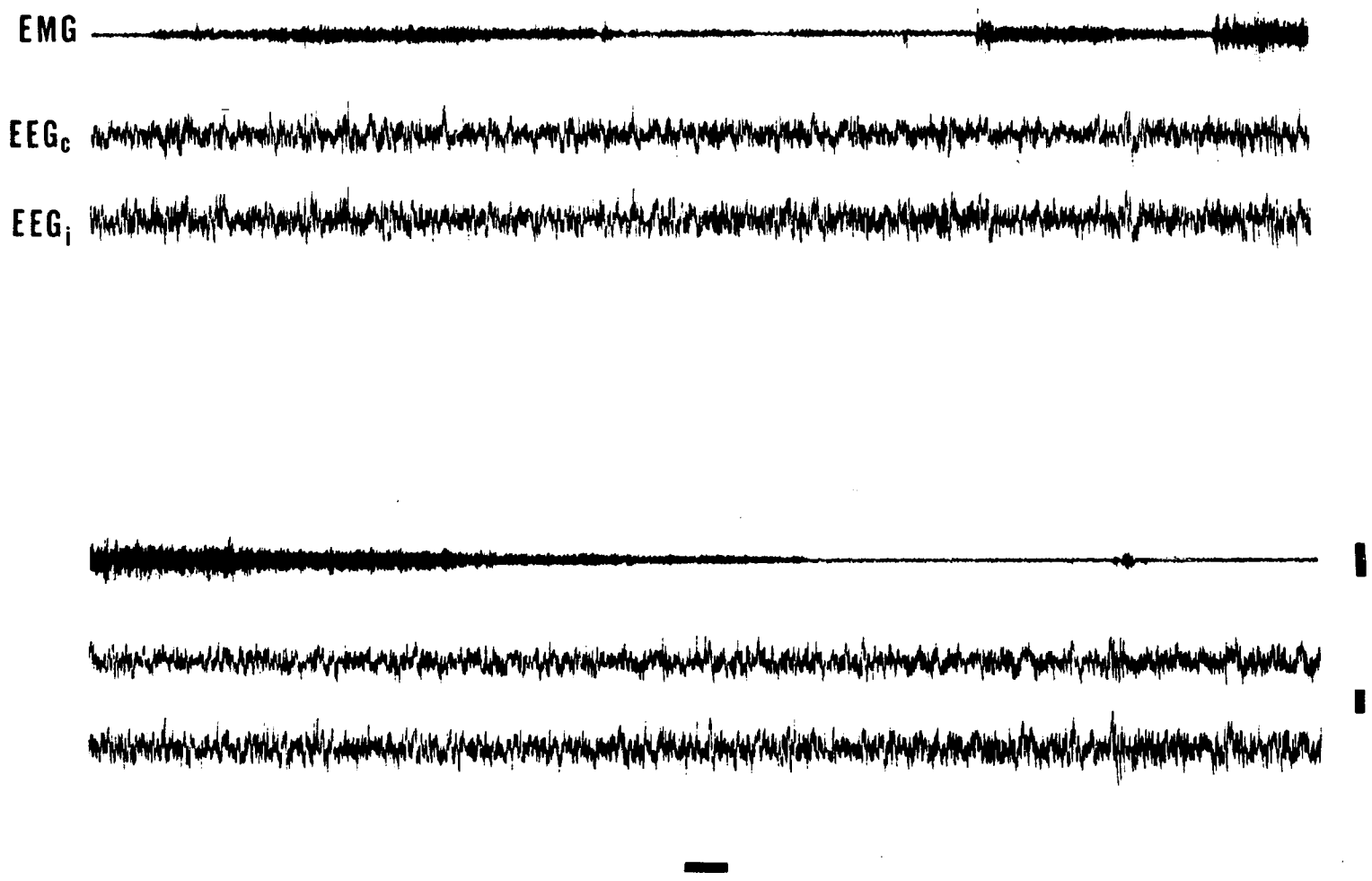

EMG
EEG$_c$
EEG$_i$

Fig. Ib.

A sample record from a rat exhibiting hypotonia. This sample was taken 15 min after injection. Note the very diminished EMG activity even at this higher sensitivity. The arrows designate a brief REM sleep period directly from wakefulness. All symbols and scale bars as in Fig. Ia.

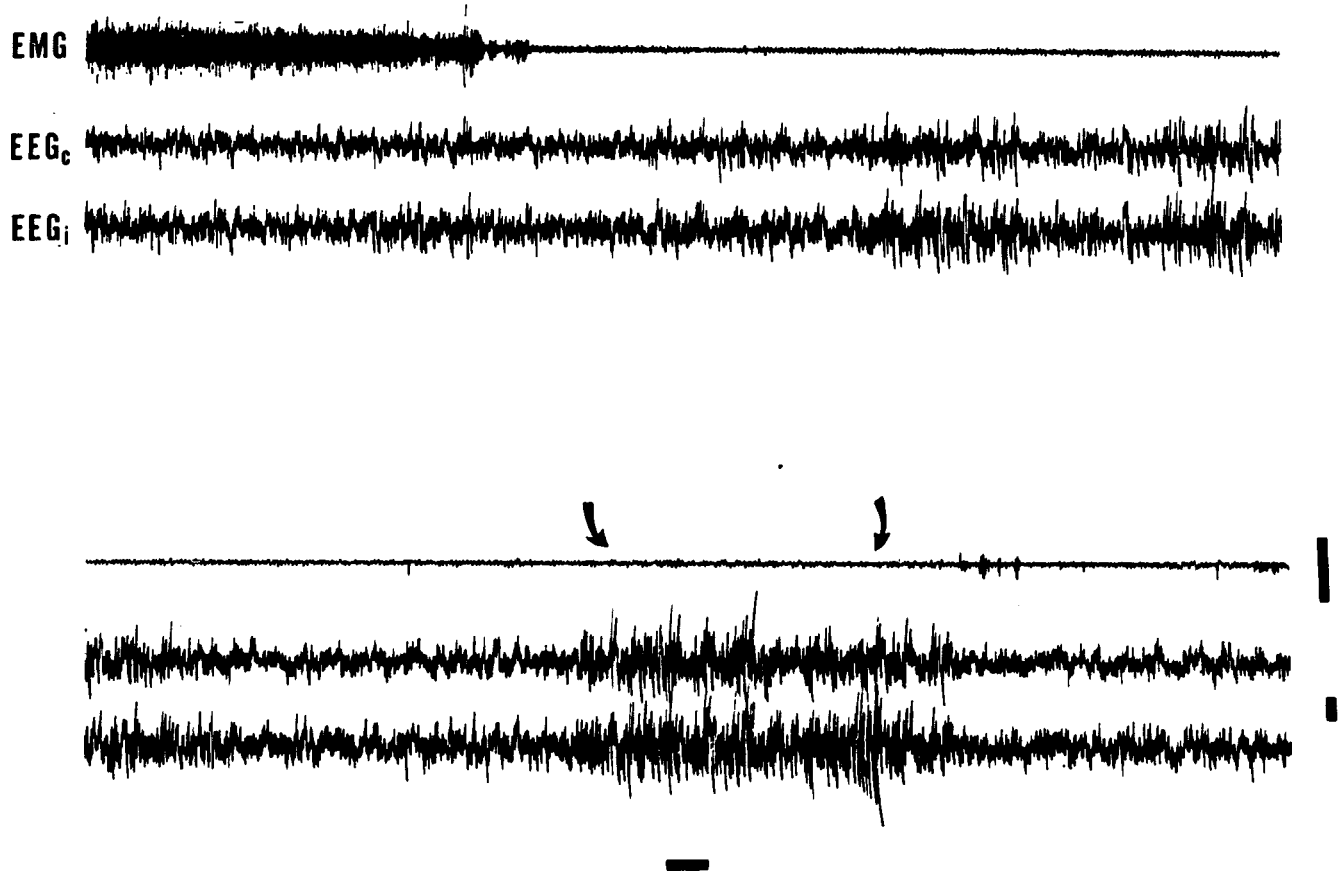

EMG
EEG$_c$
EEG$_i$

Fig. Ic.

A sample record from a rat exhibiting hypotonia. This sample was taken 1 hour after injection when recovery from the hypotonia appeared to be complete. This record shows a transition from REM sleep to awake (arrow). All symbols and scale bars as in Fig. Ia.

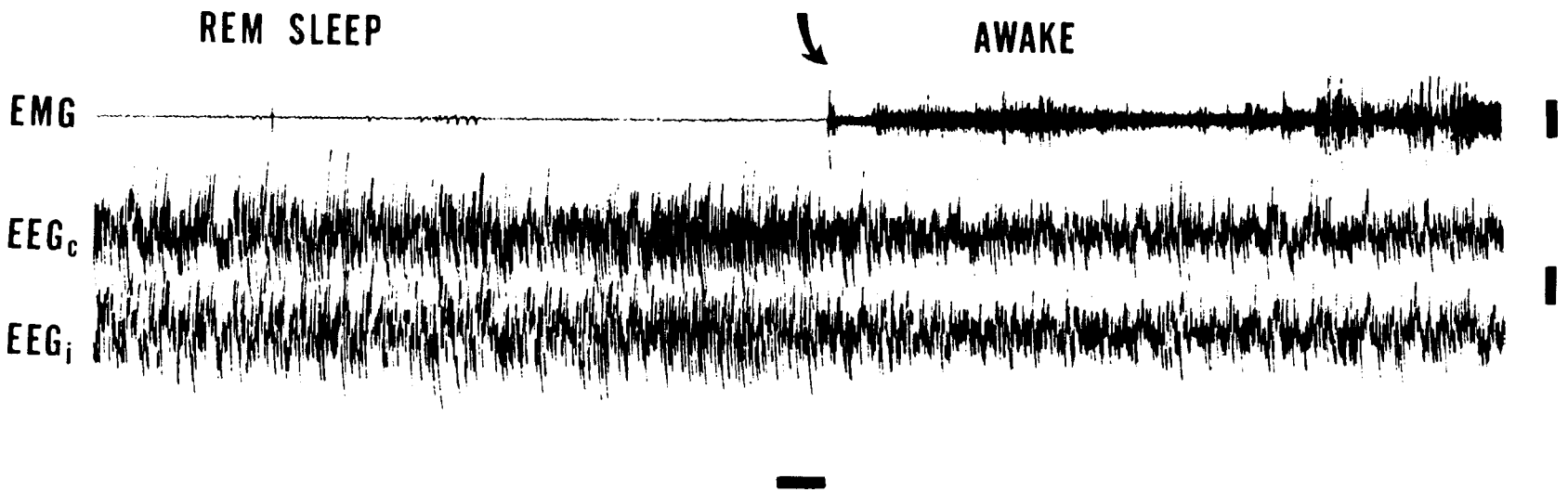
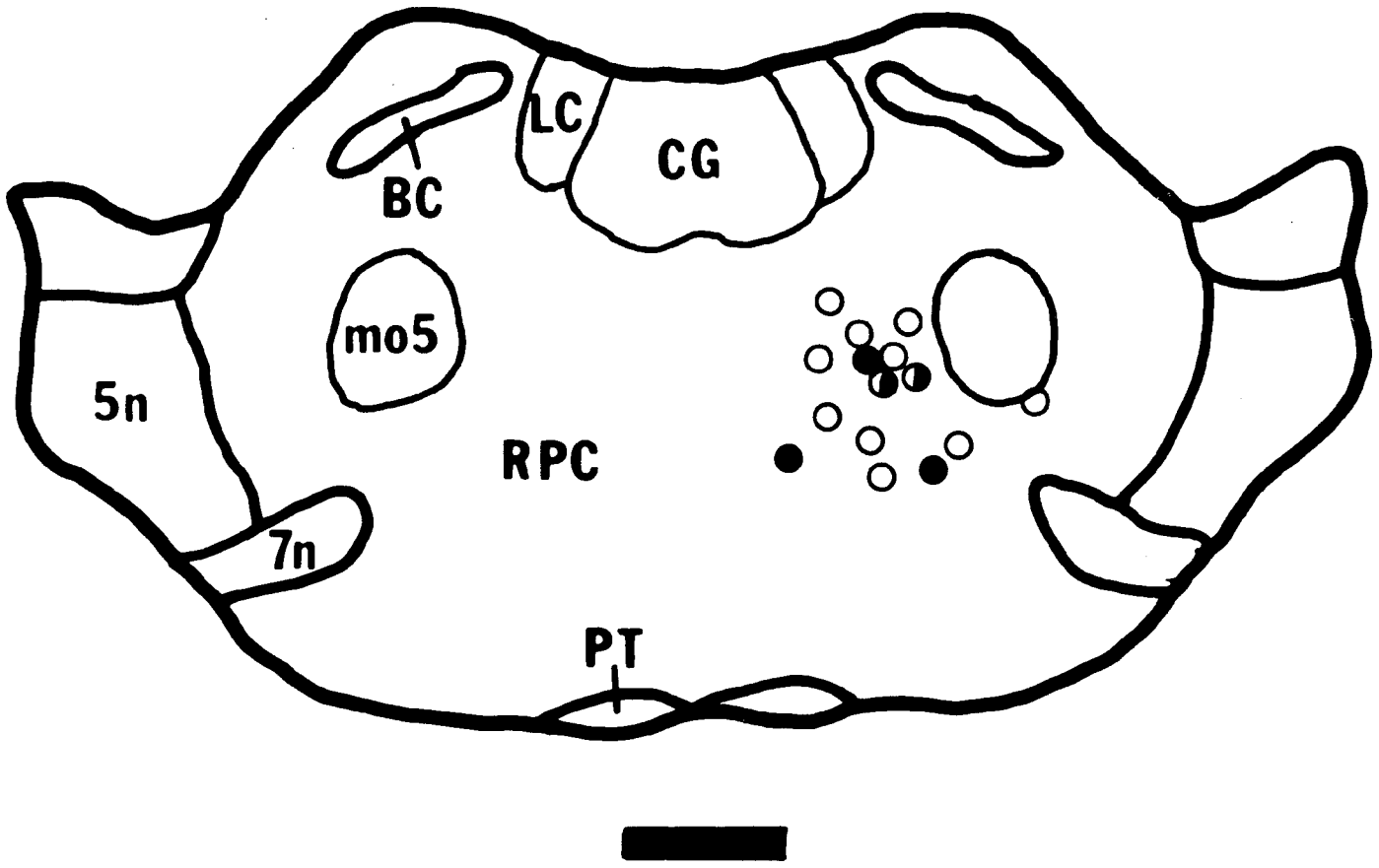

REM SLEEP
AWAKE
EMG
EEG$_c$
EEG$_i$

Fig. 1d.

Coronal section of the pons showing sites of carbachol injection within the caudal pons which produced, and which failed to produce, hypotonia. Doses of carbachol range from 0.1 to 5.0 µg. Filled circles represent injections which produced substantial hypotonia. Partially filled circles represent injections which produced mild symptoms. Unfilled circles represent injections which did not produce hypotonia. All injections lie within 0.2 mm of the representative section shown here, except for the most medio-ventral site which lies 0.4 mm caudal. BC = brachium conjunctivum, CG = central gray, mo5 = trigeminal motor nucleus, RPC = nucleus reticularis pontis caudalis, PT = pyramidal tract, 4V = fourth ventricle, 5n = trigeminal nerve, 7n = facial nerve. Scale bar = 1 mm.


LC
CG
BC
mo5
5n
RPC
7n
PT

APPENDIX II

Injection locations for atropine and carbachol-atropine studies

Fig. IIa.

Coronal section of the pons showing sites of injection for the five rats which received 0.41 µg atropine sulfate. All injections lie within 0.22 mm of the representative section shown. Abbreviations and symbols same as Fig. Id.

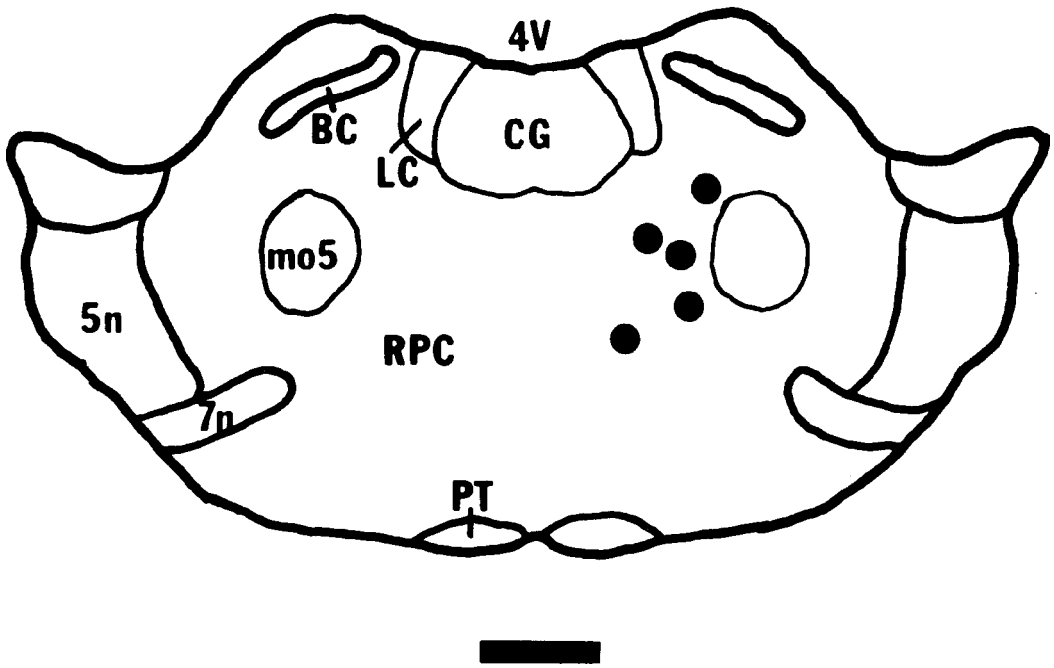
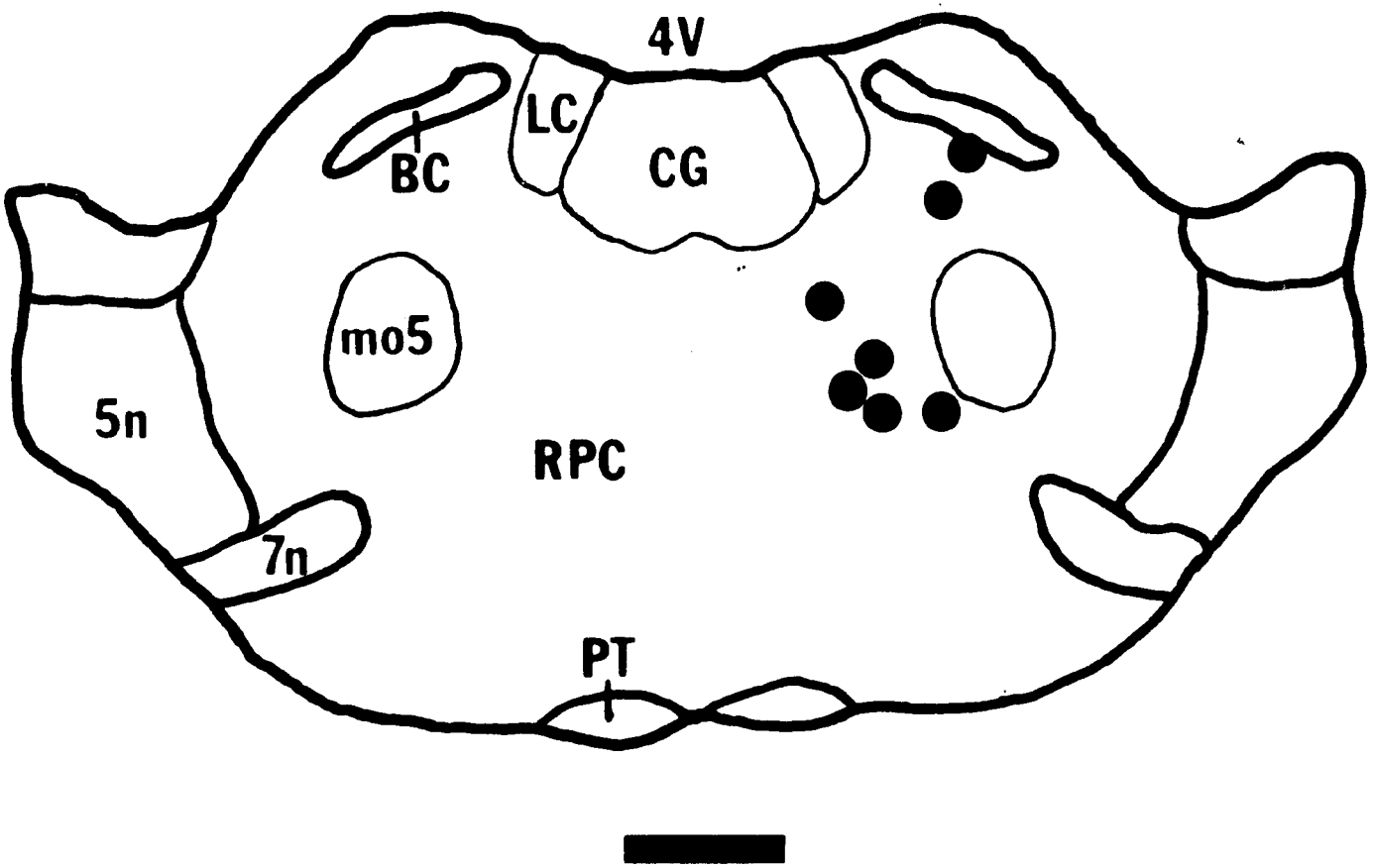

4V
BC
LC
CG
mo5
5n
RPC
7n
PT

Fig. IIb.

Coronal section of the pons showing sites of injection for the seven rats which received 1.0 µg carbachol and 0.41 µg atropine sulfate. All injections lie within 0.2 mm of the representative section shown. Abbreviations and symbols same as Fig. Id.


4V
LC
BC
CG
mo5
5n
RPC
7n
PT

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