The Role of Cholinergic, Monoaminergic, and GABAergic Neurons of the Brainstem in Paradoxical Sleep

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Abstract

Multiple lines of evidence indicate that neurons within the brainstem pontomesencephalic tegmentum (PMT) are critically involved in the initiation of paradoxical sleep (PS). Evidence suggests that unidentified but "possibly" cholinergic neurons discharge at higher rates during PS than during slow wave sleep or even Waking and would thus play an active role, whereas "presumed" monoaminergic neurons cease firing during PS and would thus play a permissive role in PS generation. Efferent projections from these PMT neurons have been thought to stimulate PS through modulation of 1) adjacent neurons in the oral pontine reticular formation, 2) more caudally distributed neurons of the medullary reticular formation, and 3) more rostrally distributed neurons in the ventral mesencephalic tegmentum, as well as thalamic and extrathalamic relay systems implicated in limbic and cortical activation.

In the present study, dual-immunostaining for c-Fos, as a marker of cellular activation, and choline acetyltransferase (ChAT), serotonin (Ser), tyrosine hydroxylase (TH), or glutamic acid decarboxylase (GAD) permitted immunohistochemical identification of active neurons during PS rebound as compared with PS deprivation and PS control conditions. In the PMT, there was an increase in ChAT+/c-Fos+ cells, a decrease in Ser+/c-Fos+ and TH+/c-Fos+ cells, and an increase in GAD+/c-Fos+ cells during PS rebound. These results support the hypothesis that cholinergic neurons are active, whereas monoaminergic neurons are inactive during PS and possibly inhibited by surrounding GABAergic neurons. In the reticular formation, there was an increase in c-Fos+ cells and a decrease in GAD+/c-Fos+ cells during PS rebound in the oral pontine reticular nucleus, suggesting a disinhibition of these reticular neurons. In contrast, in the caudal pontine and medullary reticular nuclei, there was an increase in GAD+/c-Fos+ cells during PS rebound, and covariaton with EMG in a manner to suggest an inhibitory role of these GABAergic neurons in muscle atonia. In the midbrain, there was an increase in TH+/c-Fos+ cells in the ventral tegmental area during PS rebound. The results suggest that DA neurons may increase activity during PS and therein, via connections with limbic and cortical structures, contribute to the unique physiological and cognitive aspects of that state, including dreaming.

Résumé

Plusieurs études démontrent que les neurones localisés au niveau du tegmentum pontomésencephalique (TPM) jouent un rôle dans l'initiation du sommeil paradoxal (SP). Il a été suggeré que les neurones présumés cholinergiques du TPM soient plus actifs durant le SP que durant le sommeil lent (SL) ou même l'état d'éveil (E). Ces neurones cholinergiques auraient donc un rôle actif dans la régulation du SP, tandis que les neurones présumés monoaminergiques seraient moins actifs et joueraient un rôle passif durant le SP. On pense que les efférences de ces neurones du TPM induisent le SP par la modulation 1) des neurones adjacents dans la formation réticulée pars pontis oralis, 2) plus caudallement, des neurones dans la formation réticulée pontis caudalis et medullarie et 3) plus rostrallement, des neurones dopaminergiques distribués au niveau du tegmentum mésencephalique ventral ainsi que des relais thalamiques et extrathalamiques impliqués dans l'activation limbique et corticale. Afin de caractériser la nature des neurones impliqués dans la régulation du sommeil, nous avons comparé l'activité de ces neurones durant la déprivation du SP, durant le rebond de SP suite à une déprivation ou durant des conditions du SP contrôlés. Pour ce faire, nous avons effectué des expériences de double marquages immunohistochimiques pour c-Fos, un marqueur d'activité neuronale, et l'un des marqueurs suivants: la choline acétyltransférase (ChAT), la sérotonine (Ser), la tyrosine hydroxylase (TH), ou la glutamique acide décarboxylase (GAD). Dans le TPM, nous avons noté une augmentation du nombre de neurones ChAT+/c-Fos+, une diminution du nombre de neurones Ser+/c-Fos+ et TH+/c-Fos+, alors qu'il y avait une augmentation du nombre de neurones GAD+/c-Fos+ durant le rebond du SP. Ces résultats sont en accord avec l'hypothèse selon laquelle les neurones cholinergiques sont actifs durant le SP, tandis que les neurones monoaminergiques sont inactifs, vraissemblament inhibés par les neurones GABAergiques. Dans le noyau réticulé pontique pars oralis, nous avons détecté une augmentation du nombre de neurones c-Fos+ durant le rebond et une diminution du nombre de neurones GAD+/c-Fos+, ce qui suggère que ces neurones réticulés sont désinhibés. Par opposition, nous avons observé une augmentation du nombre de neurones GAD+/c-Fos+ dans les régions caudales du pons et de la medulla durant le rebond du SP. Cette augmentation co-varie avec le profil d'activité électromyographique d'une façon qui laissent suggérer que les

neurones GABAergiques de cette région jouent un rôle dans la régulation du tonus musculaire. Finalement, nous avons noté une augmentation du nombre de neurones TH+/c-Fos+ dans l'aire tegmentaire ventrale durant le rebond du SP. Ces résultats suggèrent que les neurones dopaminergiques modulent leur activité dans le SP et de ce fait pourraient jouer un rôle dans la régulation de l'aspect physiologique et cognitif de ce stade du sommeil.

Statement of Originality

The work contained in this thesis is part of an ongoing research program concerning the neurochemical and neuroanatomical substrates of sleep in the laboratory of Dr. Barbara Jones. The work involved the central participation and supervision of Dr. Jones. The thesis is comprised of three experimental chapters based on three major manuscripts that have either been published or have been submitted for publication in the Journal of Neuroscience.

The surgery on rats for the implantation of chronically indwelling electrodes and execution of the experimental, PS deprivation, protocol was done by myself. The scoring of sleep-wake states, analysis and parsing of the data was also performed by myself. This aspect of my research confirmed the success of the flower pot technique for the selective deprivation of PS and also demonstrated the established PS rebound effect after such deprivation.

I learned to perform some of the immunohistochemistry associated with such experiments, although the immunohistochemical staining for c-Fos and neurotransmitters or their enzymes for these studies was done by Lynda Mainville.

I was responsible for the handling and processing of all the data generated by these experiments. This included analyzing and compiling the raw EEG data, and the mapping or plotting of the distribution of single- and dual-immunostained cells through the brainstem using a computer based image analysis system equipped with a computer based atlas previously developed by Dr. Jones. I performed all the statistical analysis using Systat software.

I composed the manuscripts that were later edited with Dr. Jones into a condensed manner suitable for publication. Production of the figures was done by myself, and included developing the format for the presentation, layout of the data, and producing and editing the photomicrographs.

Finally, this body of work represents a unique contribution to the field of sleep research. By utilizing this dual immunohistochemical procedure for c-Fos (as a marker of cellular activity) and neurotransmitters or their enzymes, these experiments provide

visualization of the activity in neurochemically defined neuronal populations during PS. At the initiation of these experiments it had not been possible to identify the neurochemical type of cells recorded in single unit recording studies, and it is still impossible to evaluate the activity in a population of neurochemically defined neurons by other means. Hence, these experiments offered a unique insight into the activity of neurochemically defined neuronal populations during PS. In the first experiment, evaluating cellular activity in the pontomesencephalic tegmentum (PMT), the results support the hypothesis that cholinergic neurons are active, whereas monoaminergic neurons are inactive during PS. These results confirm single unit recording studies that have only been able to hypothesize as to the chemical identity of recording units. The results of this first experiment also provide a unique insight into the possible mechanisms that may modulate cellular activity in this region during PS by demonstrating an increase in activity of GABAergic neurons co-distributed there within. The second experiment extended these findings into regions innervated by the cholinergic neurons and provided evidence for a disinhibition of reticular neurons by local GABAergic neurons in the pontine reticular nucleus, pars oralis (PnO). Of all the regions studied in these experiments, only this region, the PnO, exhibited a decrease in GABAergic activity during PS. In contrast, through the caudal pons and medulla, there was an increase in GABAergic activity during PS rebound, and this activity covaried with EMG in a manner to suggest a GABAergic role in muscle atonia. This experiment provided novel visualization of the pattern of activation in GABAergic subgroups through the pons and medulla during PS, and provided clear localization in the rat of a unique area involved in PS induction that had been somewhat controversial. The third experiment, evaluates activity in dopaminergic and GABAergic neurons of the ventral mesencephalic tegmentum (VMT), whose role in PS has been of some debate. The findings demonstrate increased GABAergic activation in the VMT and increased dopaminergic activity in the ventral tegmental area during PS. The results suggest that GABAergic neurons of the substantia nigra may contribute to dampening motor activity during sleep, and that dopamine neurons of the ventral tegmental area may change their pattern of activity in PS and therein contribute, via projections to limbic and cortical structures, to the unique physiological and cognitive aspects of that state. This experiment provides evidence that

dopaminergic activity change over the sleep-waking cycle; a point that had been difficult to ascertain previously. Altogether, these studies offer a unique insight into the activity of neuronal populations through the brainstem during PS, as has not before been possible.

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Dr. Barbara Jones, my supervisor, has provided me with a scientific education. Beyond the scope of what we have studied together, I have learned from her a genuine respect for scientific inquiry, etiquette and protocol. I will revisit the discipline she shared with me throughout my life as one of the highest values I have encountered. I thank her for being such an exceptional example.

I would like to thank Dr. Jean Gotman and Dr. Edith Hamel for agreeing to be on my thesis advisory committee. Though I did not utilize their support as much as I could have, it was comforting to know that it was there.

I owe special thanks to other teachers: Dr. R. Melzak, for broadening my imagination and lighting my beacon to McGill University; the late Dr. J. Macnamara, for his philosophical and historical context of science; and Dr. J. Ramsay, for his understanding of mathematic mischievousness and providing me with a statistical puzzle (the bell curve as an artifact of counting).

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This thesis is dedicated to my parents, though I extend important and personal thanks to all members of my family. Their support has sustained me and my efforts, and made this thesis possible.

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"It is the glory of God to conceal a matter,
to search out a matter is the glory of kings."

(Proverbs 25, 2)

Chapter One

Introduction

Introduction

1.1 Literature Review

1.1.1 The States of the Sleep-Waking Cycle

Over the mammalian sleep-wake continuum, the changing pattern of behavioural characteristics, physiological events and electroencephalogram (EEG) parameters have led researchers to the differentiation of three major states. The term Slow Wave Sleep (SWS) is applied when the organism is behaviourally asleep, the threshold for response to environmental stimuli is decreased, and the EEG is distinguished by the presence of high amplitude, irregular slow, including delta (1.0-4.0 Hz), waves. Prior to and during SWS, the EEG also manifests high amplitude spindle (9.0-14.0 Hz) waves. High amplitude in the EEG is indicative of a large population of neurons operating in assemblies, activated in a synchronized fashion, and hence SWS has traditionally been referred to as 'synchronized' sleep. The term Wakefulness is applied when the organism is behaviourally responsive, and the EEG manifests low voltage, fast activity, which has been described as 'cortical arousal or activation'. The third major state of the sleep-waking cycle has been termed Paradoxical Sleep (PS) because of the paradoxical association of behavioural sleep and an EEG pattern resembling wakefulness or cortical arousal. Also called rapid eye movement (REM) sleep, because of the occurrence of eye movements during behavioural sleep, PS (or REM sleep) is characterized by a unique array of phasic and tonic physiological events. During PS, the organism is relatively immobile, though there are twitching movements that may be observed in limb extremities and facial muscles. Furthermore, while the posture may not change from SWS, there is a complete loss of muscle tonus as recorded in the EMG (electromyogram). The EEG of Waking and PS, has traditionally been referred to as 'desynchronized', because of the presence of low amplitude, high frequency waves. However, the description of 'desynchronization' in reference to these states is misleading on two accounts. Firstly, during the entire period of PS and in waking states associated with a high degree of arousal or behavioral activity, an almost sinusoidal wave in the theta (4.5-8.5 Hz) band appears on which the high frequencies of 'desynchronization' tend to ride

(Parmeggiani and Zanocco, 1963). Secondly, in the low amplitude, high frequency EEG that defines 'desynchronization', regular oscillatory activity in the gamma range (30-60 Hz) has been recorded (Maloney and Jones, 1997). It therefore appears that while amplitude of EEG fluctuation is indicative of the number of neurons engaged in synchronized oscillatory activity, this activity (especially at higher frequencies) can also occur in smaller neuronal assemblies at lower amplitudes. In sum, there are the three major states of the sleep-waking cycle, including Wake and PS that, while behaviourally different, manifest similar electrophysiological characteristics with synchronized oscillatory activity in the theta and gamma frequency bands.

Considerable research has been dedicated to developing an understanding of brain mechanisms responsible for the generation of these different behavioral states. Initial studies, which demonstrated the importance of the brainstem for their generation, relied on lesion techniques and brain stimulation. Later research utilized the knowledge of neurochemistry and immunohistochemistry to elaborate on these early findings, superimposing upon them the neurotransmitters involved and the neurochemical pathways active during these differentially 'defined' states. Single cell recording provided another angle of approach, demonstrating that certain cells in certain areas display varying firing patterns over the sleep-wake cycle. Furthermore, these neuronal firing characteristics could be neurochemically modulated, indicating the possibility that neuronal populations could be differentially regulated across the sleep-waking cycle by input from specific neurotransmitter systems. However, the results from single cell recordings cannot be generalized to neuronal populations, and it has not until very recently been possible to identify the neurochemical type of cells recorded *in vivo* (Manns et al., 2000). Hence, though single cell recording studies may suggest the chemical specificity of recorded neurons, these findings have been limited and generalizations to neuronal populations have been prevented.

1.1.2 Classical Lesion Studies and the "Ascending Reticular Activating System"

Historically, Wakefulness was viewed as a state of sensory processing and hence the processing of sensory stimuli was believed to be the arousing factor that resulted in the state of Wakefulness. In 1935, Bremer attempted to prove this principle by transecting the brainstem at various levels to interrupt sensory input to the cortex (Bremer, 1935). The first transection he made was below the medulla, separating the brainstem from the cerebrum. This transection did not alter the cyclical pattern of cortical EEG activation and inactivation associated with the states of the sleep-waking cycle. Bremer reasoned that although the ascending sensory system from the body had been interrupted, the sensory input from the head had not and these stimuli were adequate to preserve the state of Wakefulness. A second transection through the rostral midbrain, removing sensory input from the face, head and ears as well as the body, eliminated wakefulness, as evidenced by a lack of cortical activation. However, Bremer erred in his interpretation of these results as was later demonstrated by Moruzzi and Magoun in 1949 (Moruzzi and Magoun, 1949). They were to show that the loss of cortical activation following the rostral midbrain transection was not due to elimination of ascending sensory stimuli but to the elimination of the ascending influence from the reticular formation upon the cerebral cortex.

The reticular formation, a large network of neurons and fibers located in the central core of the brainstem was found to be particularly important in cortical arousal and hence, states that exhibit such activity, Wake and PS. Electrical stimulation of the reticular formation produced cortical activation while lesioning resulted in its elimination (Moruzzi and Magoun, 1949). The reticular formation receives collateral axons from the ascending sensory pathways and hence, it was hypothesized that sensory information activated the reticular formation, which in turn activated the cerebral cortex. If this were true, then lesioning of the sensory pathways rostral to the reticular formation should not interfere with the arousing effects of sensory stimuli, even though theoretically the animals could not 'feel' the stimuli. This was shown to be the case (Lindsley et al., 1950). Moreover, sensory stimuli produced only a mild and brief arousal when the reticular formation was lesioned (Lindsley et al., 1950). The reticular formation became

known as the primary generator of cortical arousal and EEG activation and a potential generator of Wake and PS.

The means by which the reticular formation exerts its influence to implement the EEG and behavioural characteristics associated with sleep-wake states, and particularly Wake and PS, involves both ascending and descending systems. Ascending fibers from the reticular formation were revealed to course along two major ascending routes, dorsally, into the thalamus, and ventrally, into the hypothalamus and basal forebrain (Nauta, 1958; Scheibel and Scheibel, 1958). Stimulation of either of these terminal sites, the thalamus or basal forebrain, elicited the cortical activation associated with states of Wake and PS (Starzl and Magoun, 1951; Casamenti et al., 1986). In the initiation and generation of the specific state of PS, studies revealed that efferent projections from the pons was the most critical. Lesions caudal to the pons eliminated muscle atonia associated with PS but not the manifestation of phasic and tonic characteristics of PS in rostral structures, while lesions rostral to the pons eliminated PS characteristics in structures that would receive ascending inputs but not the peripheral characteristic of muscle atonia (Jouvet, 1962). These lesion studies, and studies demonstrating that stimulation of the pontomedullary reticular formation produced a generalized motor inhibition (Magoun and Rhines, 1946; Chase et al., 1986), suggested that the reticular formation could also provide the descending inhibitory influence to the spinal motor neurons that result in the induction of the behavioural characteristic of PS, muscle atonia. Hence, these ascending and descending fibers of the reticular formation became known to be important in the initiation and generation of PS, and their potential neurochemical modulation by the neighboring cholinergic, monoaminergic systems located within the brainstem as well as neurochemical modulation in areas of their projections became of interest in the study of PS.

1.1.3 Components of the Activating System

Acetylcholine

The role that acetylcholine (ACh) plays in cortical activation and in sleep-wake states was first recognized in early pharmacological studies. Intraventricular

administration of hemicholinium-3, which blocks the uptake of choline, and thereby prevents enzymatic conversion to ACh by the synthetic enzyme choline acetyltransferase (ChAT), resulted in a decrease of Wakefulness and an elimination of PS (Hazra, 1970). Physostigmine, which inhibits the catabolic enzyme acetyl cholinesterase (AChE) and thus prolongs the postsynaptic activation of ACh, was shown to first enhance cortical activation during Wakefulness and secondly to shorten the latency to and prolong the duration of PS (Domino et al., 1968). When physostigmine was administered during SWS, it produced PS (Sitaram et al., 1976). Pilocarpine, a cholinergic muscarinic receptor agonist, was found to enhance cortical activation associated with wakefulness and also shorten the latency to PS following arousal (Hinman and Szeto, 1988). Atropine, a cholinergic muscarinic receptor antagonist, produced a decrease in vigilance and PS (Matsuzaki, 1968). Injections of carbachol, a cholinergic receptor agonist, into the reticular formation resulted in either cortical activation and muscle atonia associated with PS or, in some animals, eye movements associated with alert Wakefulness (George et al., 1964). In fact, carbachol injections into the pons had the capacity to elicit many components of PS including cortical activation and muscle atonia (Katayama et al., 1984; Yamamoto et al., 1990). These carbachol effects were antagonized by the co-administration of atropine (Ashby, 1960). Furthermore, studies demonstrated that ACh release in the cerebral cortex is highest in association with cortical activation, as during the natural states of Wakefulness and PS, or as induced by electrical stimulation of the reticular formation (Celesia and Jasper, 1966; Jasper and Tessier, 1971). Hence, ACh appeared to contribute to the mechanisms underlying the processes of cortical activation as recorded in the EEG and/or the generation of the states, Waking and PS, associated with such activity.

Research also utilized the knowledge of neurochemistry and immunohistochemistry to elaborate on the early findings of the ascending reticular formation and superimpose upon it a cholinergic element. AChE staining was employed as a marker for cholinergic fibers and staining indicated the presence of an ascending cholinergic system arising from the reticular formation (Shute and Lewis, 1963). This presumed cholinergic system was relayed to the cortex via two routes, one dorsally through the thalamus and one ventrally up to the basal forebrain (Shute and Lewis, 1963).

Also described was a second major, presumed cholinergic, fiber system originating in the basal forebrain to reach the cerebral cortex (Krnjevic and Silver, 1965). However, AChE is not exclusive to cholinergic neurons and it wasn't until an antibody for the synthetic enzyme, choline acetyltransferase (ChAT), was discovered that pathways were reliably evaluated for their ACh content.

Within the pontomesencephalic tegmentum (PMT), cholinergic cells were more restricted in their location than first indicated by AChE histochemistry and found predominately within the laterodorsal and pedunculopontine tegmental nuclei (LDT and PPT) (Mesulam et al., 1984; Jones and Beaudet, 1987). The dorsal projection to the thalamus was confirmed, and a rich cholinergic innervation to the nuclei of the thalamocortical projection system was visualized as well as a collateral projection to the thalamic reticular neurons (Jones and Webster, 1988; Steriade et al., 1988). The cholinergic neurons of the PMT were also found to project locally into the surrounding reticular formation, caudally to the pons and medulla (Jones, 1990), and to the VMT in rostrally ascending projections coursing into the hypothalamus (Beninato and Spencer, 1987). On the other hand, the cholinergic contingent of the ventral, extrathalamic, route to the cortex, did not appear to be as important as first thought by AChE staining (Jones and Webster, 1988; Jones and Cuello, 1989). Within the basal forebrain, the fiber system innervating the cortex was confirmed as cholinergic by ChAT immunostaining (Mesulam and Van Hoesen, 1976).

Stimulation of the PMT results in cortical activation and increased cortical ACh release, though the direct influence of ACh in the cortex was found to derive from the cholinergic neurons in the basal forebrain (Jasper and Tessier, 1971). *In vivo* recordings of the presumed cholinergic neurons in the basal forebrain found that their average firing rate was roughly five times higher during states of cortical activation, Wake and PS, than during SWS (Detari et al., 1984; Szymusiak and McGinty, 1986). Direct stimulation of the basal forebrain can produce a sevenfold increase in ACh release in its major target area, the cerebral cortex (Kurosawa et al., 1989), and increased fast EEG activity has long been known to be associated with ACh release in this area (Kanai and Szerb, 1965). Chemical lesions in the basal forebrain area by kainic acid or ibotenic acid result in a significant slowing of the cortical EEG (Stewart et al., 1984; Buzsaki et al., 1988; Ray

and Jackson, 1991), as would be expected if the basal forebrain's cholinergic innervation contributed to cortical activation. The cholinergic neurons of the PMT would thus evoke cortical activation with enhanced ACh release in the cortex by stimulating basal forebrain neurons directly or more likely, given their limited direct projections, indirectly by exciting other neurons of the reticular formation or VMT.

After immunohistochemical localization of the cholinergic neurons in the PMT, it was demonstrated that neurotoxic lesions of these neurons did not diminish waking but did result in the loss of PS (Jones and Webster, 1988). *In vivo* recordings of presumed cholinergic neurons in the PMT found that their tonic firing rate was increased during states of cortical activation, Wake and PS, in comparison to SWS. Some presumed cholinergic neurons fired at even higher rates during PS than during Waking (Sakai, 1985; ElMansari et al., 1989; Kayama et al., 1992). Furthermore, ACh release in the pontine and medullary reticular formation was reported to be double during PS as compared to Wake (Kodama et al., 1990), thus indicating that ACh may modulate surrounding cholinoceptive reticular formation neurons which may in turn contribute to generation of PS characteristics such as cortical activation via the ventral extrathalamic ascending relay and also influence descending systems associated with muscle atonia. ACh release in the thalamus as measured by *in vivo* microdialysis showed that extracellular ACh concentrations were highest during both Wake and PS (Williams et al., 1994), and ACh release in the thalamus has been shown to promote single spike mode of firing (Steriade et al., 1988). EEG activity has been shown to be tightly coupled to the type of activity generated by the thalamocortical relay neurons, so that when thalamic neurons exhibit burst activity, the EEG is synchronized (as in SWS), while when the thalamocortical neurons exhibit single spike activity, the EEG is desynchronized (as in Wake or PS) (Steriade and Deschenes, 1984; Steriade and Llinas, 1988). Hence, it would appear that PMT projections to the surrounding reticular formation and medulla as well as to the thalamus could modulate the behavioural and EEG changes seen during states of Wake and PS.

These results demonstrate that the activity of presumed cholinergic neurons in the PMT is associated with cortical arousal, and the states of Waking and PS. Through projections to the surrounding reticular formation, as well as to the caudally located

medulla, or the rostrally located the thalamus or VMT, and by their cholinergic influence in these regions, the neurons of the PMT are in a position to generate these states of cortical arousal. However, since these neurons are active during both Waking and PS, and these states differ behaviourally, other neurons located within the brainstem may be in a position to differentially modulate certain cells in the reticular formation and other areas of PMT projection. Monoaminergic neurons of the locus coeruleus and raphe nuclei, whose activity has been hypothesized to be highest in association with Wake and virtually cease during PS, as well as GABAergic neurons co-distributed within the PMT, might provide this differentially modulation which would distinguish the states of Wake and PS in the reticular formation and in other areas of PMT projection.

Monoamines

Monoamines include the catecholamines (CAs), dopamine (DA) and noradrenaline (NA), and the indolamine—serotonin (5-HT). Early pharmacological manipulation of the synthetic/catabolic pathway of the CAs, demonstrated a role for CAs in arousal, alertness and Wakefulness. AMPT, which blocks the enzymatic conversion of tyrosine to DOPA by inhibiting tyrosine hydroxylase (TH), results in a reduction of CAs and is associated with an attenuation of behavioural arousal and a decrease in electrocortical activation (Keane et al., 1976). Reserpine, which reduces CAs by interfering with their vesicular storage, results in a tranquilizing effect and was used to calm psychotic patients in the 1940's and 50's (Keane et al., 1976). Administration of the dopamine precursor L-DOPA as occurs in the treatment of Parkinson patients, results in an increase in CAs and behavioral arousal, cortical activation and a decrease in PS (Jones, 1972; Keane et al., 1976; Hernandez-Lopez et al., 1996). Amphetamine, which stimulates CA release, results in heightened alertness and a reduction of sleep and was frequently given to soldiers and pilots during WWII. Cocaine, which increases the effect of CAs by blocking re-uptake, results in euphoria and insomnia (Johanson et al., 1999). Interestingly, withdrawal from cocaine in chronic users has also been shown to increase PS in a manner similar to PS rebound (Kowatch et al., 1992). Hence, CAs appear to contribute to the mechanisms associated with behavioral arousal and cortical activation, and appear to antagonize mechanisms associated with sleep induction including PS.

The distribution of monoaminergic neurons in the central nervous system was initially studied using classic histofluorescent techniques that did not distinguish between the various CAs (Dahlstrom and Fuxe, 1964). Data obtained in these earlier studies did however reveal that CA perikarya were located predominantly in the brainstem regions; regions known to be important for Wakefulness and PS (see above). These findings were re-examined in light of newer immunohistochemical techniques with antibodies against tyrosine hydroxylase (TH), serotonin and dopamine, and in combination with retrograde labeling, for an accurate picture of their organization within the brainstem and their projections.

Noradrenaline

Neurons that synthesize noradrenaline (NA) are located in the pontine and medullary brainstem and clustered within the pontine nucleus, the locus coeruleus (LC), from where they give rise to diffuse projections through the central nervous system. Neurons of the LC project to the thalamus, the hypothalamus, the basal forebrain and the entire cortex, as well as caudally to the spinal cord (Jones and Moore, 1977).

Early pharmacological studies, which showed that CAs could induce Wakefulness and arousal (see above), together with lesion studies and *in vivo* electrophysiological recording studies over the sleep-waking cycle, suggested that noradrenergic neurons were inactive during PS (see for review, (Jones, 1991)). Pharmacological studies, resulted in a proposal that noradrenaline mediated arousal (or at least the suppression of sleep). Electrolytic and 6-OHDA lesions of the midbrain tegmentum, where ascending noradrenaline pathways course, produced a decrease in Wakefulness and associated EEG activation (Lidbrink, 1974; Jones et al., 1973). Reversible cooling of the LC was found to produce sleep in the waking animal, while electrical stimulation produced arousal in the sleeping animal (Cespuglio et al., 1982). LC lesions were also associated with a loss of high frequency EEG activity in response to sensory stimuli (Delagrange et al., 1989). On the other hand, localized thermolytic lesions of the LC neurons did not result in a loss of Wakefulness or alter cortical activation (Jones et al., 1977; Jacobs and Jones, 1978), indicating that noradrenergic activity was not necessary for Wakefulness. Single-unit recording studies demonstrated that LC neurons change their firing rate over the sleep-

waking cycle. During quiet waking, LC neurons demonstrate a regular, slow rate of spontaneous activity, which reduces in SWS and virtually ceases in PS (Steriade and Hobson, 1976). Noradrenaline neurons were also shown to increase their rate of firing approximately 3 seconds before the onset of waking (Aston-Jones and Bloom, 1981), and transiently in association with responses to sensory stimuli (Aston-Jones and Bloom, 1981; Rasmussen et al., 1986). This pattern of LC neuronal activity indicates that noradrenergic activity is associated with certain waking behaviors and is absent during PS. This inactivity during PS is compatible with a popular hypothesis of sleep generation, which suggested noradrenergic cells play a permissive role in PS induction and are part of a PS gating mechanism (Hobson et al., 1975).

The LC noradrenergic neurons may inhibit the induction of PS directly though connections with the cholinergic nuclei within the PMT. Pharmacological evidence suggests that noradrenaline inhibits these cholinergic tegmental neurons known to be instrumental in PS generation (Hobson et al., 1975; Luebke et al., 1992; Williams and Reiner, 1993; Leonard and Llinas, 1994). Simultaneous unit recording of assumed cholinergic PS-on and monoaminergic PS-off cells reveal a mirror image in the changes in their discharge as to suggest mutual inhibit (Sakai, 1988). Because ACh is excitatory to monoaminergic neurons (Egan and North, 1986; Li et al., 1998), these cholinergic effects are likely mediated though inhibitory interneurons.

In summary, noradrenergic activity is increased with waking in comparison to PS. While not necessary for waking, noradrenergic activity appears to increase during specific behavioural conditions and, as mentioned (see above), its pharmacological augmentation enhances and prolongs cortical activation associated with waking. Through diffuse projections, noradrenergic activity may modulate activity in areas in a manner to antagonize the sleep process, which when removed may allow the induction of PS through cholinergic modulation of these regions. The inhibition of noradrenergic activity during PS may be induced in part by surrounding GABAergic interneurons.

Serotonin

Early neurochemical and pharmacological studies demonstrated that serotonin (5-HT) varied in a circadian fashion and was involved in sleep generation. Evidence suggested that there were cyclic fluctuations in levels of tryptophan, the primary precursor of serotonin, over the 24-hour day (Wurtman et al., 1974). In the pineal glands, serotonin levels demonstrated circadian variations and were shown to double in the rat after the onset of light (Johanson et al., 1999). Tryptophan is converted to 5-hydroxtryptophan (5-HTP), in the synthesis of serotonin, by the enzyme tryptophane hydroxylase. The drug, PCPA, as the preferred substrate for tryptophan hydroxylase, inhibits serotonin synthesis and causes 90% depletion in brain serotonin levels. Injection of PCPA causes an abrupt decrease in sleep and an almost complete insomnia (Jouvet, 1972). Whereas injection of 5-HTP, the end product of tryptophan hydoxylase in the synthetic serotonergic pathway, restored sleep (Jouvet, 1972). Sleep recovery was shown to parallel the return of serotonin levels (Jouvet, 1972). Hence, serotonin was believed to be involved in sleep induction.

Serotonin was further shown to be a "sleep promoting substance/factor". Serotonin is directly released into cerebrospinal fluid (CSF) during neural activity for dissemination over the ventricular surface (Holman et al., 1977). In 1913, Henri Pieron siphoned CSF from dogs forced to stay awake into recipient dogs with the result of causing sleep in the recipient animals. Continued research led to the discovery of "sleep promoting factors" in the CSF of which serotonin was included. Interestingly, PS deprivation causes an increase in serotonin synthesis (Hery et al., 1970), an increased release of serotonin in the suprachiasmatic nucleus (the biological clock of the brain) (Grossman et al., 2000), and an increase in serotonin levels in the CSF (Radulovacki and Buckingham, 1975). Total sleep deprivation also causes an increase in serotonin levels (Cramer et al., 1973). These studies indicate that while possibly sleep promoting; the serotonergic system also appeared to be active during Wake.

Most serotonergic neurons are localized in nuclei of the raphe (Dahlstrom and Fuxe, 1964). The rostral serotonergic pontine and mesencephalic groups, located in the dorsal raphe (DR) and medianus raphe (MR), send ascending projections to innervate the thalamus (Moore et al., 1978), the hippocampus, the basal forebrain (Moore, 1978), and

through highly branched fibers to reach the entire cerebral cortex (Lidov et al., 1980). The caudal serotonergic medullary groups, located in the raphe magnus and raphe pallidus obscurus, send descending projections to the dorsal and ventral horns of the spinal cord, respectively (Basbaum et al., 1978; Jacobowitz and MacLean, 1978). Interestingly, stimulation of this bulbospinal serotonergic pathway provides both inhibition to the transmission of noxious stimuli and excitation to motor neurons (Wessendorf et al., 1981). Lesions involving 80-90% of these raphe nuclei result in an insomnia (Jouvet and Pujol, 1974). On the other hand, single cell recording studies of presumed serotonergic neurons in the DR nucleus demonstrate a reduction in firing during sleep from Wake and a virtual cessation during PS (McGinty and Harper, 1976; Trulson and Jacobs, 1979; Heym et al., 1982), and serotonergic release in the DR is lowest in association with sleep and PS (Portas and McCarley, 1994). These different results indicate that although serotonin may facilitate the onset of sleep, serotonergic neuronal activity does not directly promote sleep and in fact may even prevent PS.

The projections of the rostral serotonin groups, the dorsal raphe (DR) and the median raphe (MR), ascend and innervate structures important in the generation of PS and PS associated characteristics. Early studies suggested the importance of serotonergic influences within the basal forebrain for the generation of SWS (Denoyer et al., 1989). *In vitro* studies showed that serotonin post-synaptically hyperpolarizes cholinergic basal forebrain neurons (Khateb et al., 1993). Interestingly, serotonin in the basal forebrain has been shown to reduce gamma activity (Cape and Jones, 1998), which appears with theta during active Wake and PS (Maloney et al., 1997). Electrical stimulation of the DR and MR also resulted in a reduction of theta (Assaf and Miller, 1978), whereas lesioning of the MR produces an almost continual theta in the rat (Maru et al., 1979). Suppression of MR by injection of an autoreceptor agonist resulted in an increase of hippocampal theta (Vertes et al., 1994). Some DR neurons also exhibit theta rhythmic firing in freely moving rats (Kocsis and Vertes, 1992), which later research demonstrated to be non-serotonergic neurons (Kocsis and Vertes, 1996). Hence it would appear that serotonin inhibits theta, though some authors have argued that serotonin serves to inhibit one type of theta (type 2 theta) and induce another type (type 1 theta) (Vanderwolf et al., 1989). Hence, whether serotonin elicits Wake or sleep modes of neuronal activity may depend

on the region, however it would appear to dampen cholinergic activation of cortical gamma and hippocampal theta associated with active Wake and PS.

The medial medullary reticular formation, where the raphe magnus and raphe pallidus obscurus lie, have been implicated in inducing muscle atonia during PS. Chemical and electrical stimulation of this area elicits postural atonia (Lai and Siegel, 1988). Since serotonin increases motorneuron excitation (McCall and Aghajanian, 1979; White and Fung, 1989), and serotonergic cell firing in the raphe magnus has been shown to increase in association with motor tasks (Siegel et al., 1979; Veasey et al., 1995), it is believed that serotonin would be implicated as antagonizing the occurrence of muscle atonia during PS. Supporting this notion, carbachol-induced PS is associated with a decrease in serotonin levels in the hypoglossal nucleus along with depression in motorneuron activity, which is removed by serotonin application (Kubin et al., 1993).

In summary, the research on serotonin and sleep provide some conflicting findings that may not easily be resolved with simple models of sleep induction. On one hand, serotonin appears to be important for the induction of sleep and alters activity in some neurons in a manner consistent with SWS, and on the other hand, serotonin has been shown to be involved in motor activity and single cell recording studies show a decrease in presumed serotonergic neuronal activity during sleep and PS similar to noradrenergic neurons. An important permissive role in the induction of PS has been hypothesized for serotonergic activity (Hobson et al., 1975). Finally, if serotonergic activity is inhibited during PS, one possible mechanism for this inhibitory induction could be the surrounding and co-distributed GABAergic neurons.

Dopamine

The ventral mesencephalic tegmental (VMT) dopamine-containing neurons have been described as forming three cell groups---A8, A9 or the substantia nigra (SN), and A10 or the ventral tegmental area (VTA)---though the boundaries between these groups are relatively indistinct. However, a mediolateral gradient within the VMT is recognizable, with the SN located more laterally and the VTA more medial. The projections from the lateral, SN, dopamine cell, region course forward through the lateral

hypothalamus and internal capsule into the neostriatum, where they provide a dense innervation (Bjorklund and Lindvall, 1984). This nigrostriatal pathway is believed to play a role in motor expression (Carli et al., 1985), and degeneration of this system is associated with the motor akinesia of Parkinson's disease. The projections from the medial, VTA, dopamine, neurons extend through and innervate the lateral hypothalamus, basal forebrain, limbic (amygdala) and cortical areas (frontal, cingulate, entorhinal and visual cortices) (Hokfelt et al., 1974; Lindvall and Bjorklund, 1974; Bjorklund and Lindvall, 1984). This mesolimbocortical pathway is believed to play a role in reinforcement or incentive motivational processes (Carr and White, 1986), and excess activation of this system is believed to be associated with hallucinations and psychotic behaviours. Evolutionarily speaking, the close anatomical residence and mediolateral overlay of areas involved in incentive motivational functions in those involved with behavioral substrates makes sense in terms of learning and the execution of goal-directed behavior.

Studies analyzing the effect of lesions in these dopaminergic regions have produced varied findings. Electrolytic and 6-hydroxydopamine (6-OHDA) lesions of the VMT decreased behavioural arousal (Ungerstedt, 1971; Jones et al., 1973), while neurotoxic lesions of the dopaminergic VMT neurons has been found to decrease PS (Lai et al., 1999). Such differences could be explained in part by the different roles the SN versus the VTA dopamine neurons play. Indeed, in contrast to the hypoactivity produced by 6-OHDA lesions of the SN, hyperactivity was reported to follow lesions of the VTA (Galey et al., 1977). However, the different lesion techniques employed, the consequential different adaptive mechanisms resulting from them, as well as an appreciation of the delicate equilibrium dopaminergic activity may play in sleep-wake states, should also be considered in explaining these different results.

In vivo recordings of neurons with the VMT have shown that, unlike other monoaminergic neurons which decrease there firing rate during SWS and PS, presumed dopamine neurons did not change their average discharge rate across sleep-wake states (Gage et al., 1983; Miller et al., 1983; Steinfels et al., 1983; Trulson and Preussler, 1984). Dopaminergic neurons, however, are known to change their firing pattern from single spike mode to bursts of spikes during active or stimulated waking conditions (Freeman et

al., 1985), or during behaviorally significant and rewarding situations (Schultz, 1986; Mirenowicz and Schultz, 1996; Overton and Clark, 1997). A study of the variance in interspike interval showed significant differences across sleep-wake states, in a manner to suggest that there is an increase in bursting activity during PS (Miller et al., 1983). Interestingly, in this regard, the non-dopaminergic neurons and presumed GABAergic neurons in this region, known to exert an influence on adjacent dopaminergic neurons (Tepper et al., 1995), increase their firing during PS in comparison to SWS or Waking (Miller et al., 1983; Steinfels et al., 1983), and hence may be involved in the pacing of burst activity as has been suggested in other regions.

The dopaminergic system may also be involved in various EEG changes that occur during schizophrenia and sleep deprivation. Schizophrenia is frequently associated with EEG abnormalities (Matsuura et al., 1994), including a decrease in power in the delta frequency band (Keshavan et al., 1998). Dopamine D2 receptor blockers, which continue to be implicated in the therapeutic effects of antipsychotic drugs (Sokoloff, 1983), produce an increase in slow EEG activity (Sebban et al., 1999). Interestingly, selective PS deprivation has the opposite effect on EEG activity. In both humans and rats, selective PS deprivation results in a reduction in delta activity (Beersma et al., 1990)(Benington et al., 1994; Corsi-Cabrera et al., 1994). These results suggest that like schizophrenia, PS deprivation, causes an associated decrease in slow EEG activity, while antipsychotic drugs or D2 receptor blockers cause an increase in slow EEG activity. These results suggest that there may be an underlying dopaminergic component to EEG changes associated with schizophrenia and sleep deprivation. In this regard, it is interesting to note that sleep deprivation can lead to psychosis and hallucinations (Dement et al., 1970), and a similarity has often been made between hallucinogenic states and the cognitive correlate of PS, dreaming.

EEG studies in the preceding paragraph rarely monitored EEG frequencies above 30Hz, though dopamine VTA neurons have been implicated in the production of a behavioural position of focused attention and a corresponding EEG rhythm on the cortex in the gamma range (35-45 Hz) (Bouyer et al., 1981). VTA lesions resulted in loss of focused attention and the associated increased gamma activity (Montaron et al., 1982). Apomorphine, a dopamine agonist, restored the appearance of the gamma activity to

some degree in VTA lesioned animals (Yim and Mogenson, 1980), while haloperidol was been shown to suppress the appearance of this gamma rhythm in unlesioned animals (Ahveninen et al., 2000). Gamma activity has been shown to occur during active Wake and PS (Maloney et al., 1997), and hence if increased by activation of the dopaminergic system, its appearance may reflect an underlying activity in the dopaminergic systems during these states.

The PMT cholinergic nuclei are known to be critically involved in the generation of PS (see above) and send projections to the VMT (Beninato and Spencer, 1987). Stimulation of the PMT produces an increase in burst firing in the dopaminergic neurons (Lokwan et al., 1999) and an increase in dopamine turnover in their terminal locations (Niijima and Yoshida, 1988). Muscarinic receptor activation in the VMT and nicotinic receptor activation in the VTA is known to enhance the burst firing of dopamine neurons and dopamine release in their terminal locations (Grenhoff et al., 1986; Blaha et al., 1996; Gronier and Rasmussen, 1998). Activation of nicotine receptors has been shown to mediate direct excitation of midbrain dopamine neurons *in vitro* (Calabresi et al., 1989), while activation of muscarine receptors on VMT GABAergic neurons, which impinge on dopamine neurons, depress GABAergic transmission (Grillner et al., 2000). Bursting of dopamine neurons does not occur *in vitro* (Grace, 1988), and hence is not likely generated by intrinsic properties but rather by the interplay of either afferent inputs and/or the alteration of local GABAergic influences. The cholinergic PMT projection to the VMT may supply this afferent input and/or this alteration in GABAergic activity. Hence, while dopaminergic activity is associated with waking and sleep mechanisms the exact method of its contribution to the sleep-waking states, as well as whether there is a change in firing pattern during PS, still remains ambiguous. Therefore, unlike other monoaminergic systems, the mean discharge rate of presumed dopaminergic neurons reportedly does not change over the sleep-waking cycle, though the dopaminergic system may underlie various EEG patterns associated with the states of cortical arousal, Wake and PS.

GABA

Gamma-aminobutyric acid (GABA) has long been considered to be the most important inhibitory substance in the central nervous system. GABA is derived from glutamate after the removal of a carboxyl group by the enzyme glutamic acid decarboxylase (GAD). Techniques for localizing GABAergic neurons have used immunohistochemical techniques with antibodies directed against GAD, and revealed that GABA is mostly synthesized and released by local interneurons, though a number of projection systems have also been acknowledged both from the cholinergic pontomesencephalic tegmental (PMT) nuclei (Ford et al., 1995), and from the basal forebrain (Gritti et al., 1997). Areas with a dense distribution of GABA neurons include the pontine brainstem where the laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT), dorsal raphe (DR) and locus coeruleus (LC) nuclei lie, and the substantia nigra of the VMT (Fonnum et al., 1978). In general, GABAergic activity has long been thought to have a role in sleep, especially in view of the sedative effects of GABA agonists (Mendelson, 1985). More specifically, GABAergic activity has been suggested to play an intermediary role between cholinergic and monoaminergic neurons to affect the inhibition of the serotonergic and noradrenergic neurons during PS (Jones, 1991). However, the activity of GABAergic neurons in the various regions of the reticular formation across the sleep-waking cycle is not known.

1.2 Conclusion

Paradoxical sleep (PS) is so named because of the paradoxical occurrence of behavioural sleep with an EEG pattern similar to that of Wake. Lesions and transection studies demonstrated the importance of the pontine reticular formation in the generation of this state, and along with other approaches, revealed the ascending reticular activating system that is critical for cortical activation. However, given the pharmacological influences, the anatomical residence, and/or the effect of neurotoxic lesions, the cholinergic and monoaminergic impact on this ascending activating system must also play a critical role in PS induction and generation. From single cell recording studies it has been hypothesized that the activity in "presumed" cholinergic neurons is increased, and activity in "presumed" monoaminergic neurons is decreased during PS. However, the neurochemical identity can only be presumed by such studies and activity in single cells limits generalizations to neural populations. Suggestions have been made that descending projections from the pontine reticular formation and neighboring cholinergic nuclei initiate, through medullary relays, the characteristic of muscle atonia, while monoaminergic influences antagonize such an occurrence. While the pons and medulla where thought to relay the PS characteristic of muscle atonia from the pontomesencephalic region to the spinal cord motorneurons, the location within the brainstem, along with the neurochemical identity of such a relay, remain obscure. The cholinergic neurons within the PMTalso send ascending fibers to the VMT where dopaminergic neurons are located. Lesion and pharmacological studies have indicated that dopaminergic neurons may regulate behavioural arousal and sleep-wake states, yet recording studies have reported that there are no changes in average discharge rate across the sleep-wake cycle. To clarify these questions and examine hypothetical models of PS induction and generation, c-Fos, the early immediate gene product stimulated by Ca+ entry into the cell and hence activity (Dragunow and Faull, 1989) was used. The following studies use dual immunostaining for c-Fos protein and neurotransmitters or their enzymes to allow for immunochemical visualization of activity during PS in cholinergic, monoaminergic, and GABAergic neurons of the 1) pontomesencephalic

tegmentum (PMT), 2) the pontomedullary reticular formation, and the 3) ventral mesencephalic tegmentum (VMT).

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Chapter Two

Pontomesencephalic Tegmentum

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2. Pontomesencephalic Tegmentum

2.1 Introduction

To visualize activity in a population of neurochemically identifiable neurons during paradoxical sleep (PS), the following studies utilized a dual-immunohistochemical staining procedure. c-Fos expression, as a marker of cellular activity, was stained for visualization in neurons already identified for neurochemical type by previous staining for neurotransmitters or their enzymes. The findings of these first study in the pontomesencephalic tegmentum provide support to the hypothetical reciprocal activity between the cholinergic and monoaminergic neurons during PS that have led to the suggestion that cholinergic neurons are PS-on neurons and monoaminergic neurons are PS-off. The findings confirm the neurochemical identity of single-unit recording studies that have suggested that 'presumed' cholinergic neurons decrease their rate of firing during slow wave sleep (SWS) in comparison to Wake and PS, and fire at their highest rate during PS, and that 'presumed' monoaminergic neurons decrease their rate of firing from Wake to SWS and virtually cease firing during PS. The study also provides evidence that a possible mechanism by which monoaminergic neurons are inhibited during PS is by local GABAergic neurons.

2.2 *Differential c-Fos expression in cholinergic, monoaminergic and GABAergic cells groups of the pontomesencephalic tegmentum after paradoxical sleep deprivation and recovery*

2.2.1 Abstract

Multiple lines of evidence indicate that neurons within the pontomesencephalic tegmentum are critically involved in the generation of paradoxical sleep (PS). From single-unit recording studies, evidence suggests that unidentified but "possibly" cholinergic tegmental neurons discharge at higher rates during PS than during slow wave sleep or even waking and would thus play an active role, whereas "presumed" monoaminergic neurons cease firing during PS and would thus play a permissive role in PS generation. In the present study performed on rats, c-Fos immunostaining was used as a reflection of neuronal activity and combined with immunostaining for choline acetyltransferase (ChAT), serotonin (Ser), tyrosine hydroxylase (TH), or glutamic acid decarboxylase (GAD) for immunohistochemical identification of active neurons during PS recovery (~28% of recording time) as compared with PS deprivation (0%) and PS control (~15%) conditions. With PS recovery, there was a significant increase in ChAT+/c-Fos+ cells, a significant decrease in Ser+/c-Fos+ and TH+/c-Fos+ cells, and a significant increase in GAD+/c-Fos+ cells. Across conditions, the percent PS was correlated positively with tegmental cholinergic c-Fos+ cells, negatively with raphe serotonergic and locus coeruleus noradrenergic c-Fos+ cells, and positively with codistributed and neighboring GABAergic c-Fos+ cells. These results support the hypothesis that cholinergic neurons are active, whereas monoaminergic neurons are inactive during PS. They moreover indicate that GABAergic neurons are active during PS and could thus be responsible for inhibiting neighboring monoaminergic neurons that may be essential in the generation of PS.

2.2.2 Introduction

Since early studies using transections and lesions, the pontomesencephalic tegmentum has been known to be critical for the generation of paradoxical sleep [(PS), or rapid eye movement (REM) sleep] (Jouvet, 1962, 1972). Pharmacological evidence had indicated that acetylcholine (ACh) was important for the appearance of PS (Domino et al., 1968), and injections of the cholinergic agonist carbachol into the pontomesencephalic tegmentum was shown to elicit a state similar to natural PS (George et al., 1964). ACh was also known to be important for wakefulness, and enhancing ACh levels with acetylcholinesterase (AChE) inhibitors elicited a waking state (Domino et al., 1968) unless monoamines were first depleted with reserpine, in which case, it elicited PS (Karczmar et al., 1970). These early pharmacological studies thus suggested that cholinergic systems, particularly within the pontomesencephalic tegmentum, were involved in the generation of PS but were also involved in waking and could only elicit PS when monoaminergic systems were inactivated.

After immunohistochemical localization of the cholinergic neurons in the pontomesencephalic tegmentum (Mesulam et al., 1983; Jones and Beaudet, 1987), it was demonstrated that neurotoxic lesions of these neurons resulted in the loss of PS (Jones and Webster, 1988; Webster and Jones, 1988; Jones, 1991b). Single-unit recording within the cholinergic cell area, including the laterodorsal and pedunculopontine tegmental nuclei, found cells that discharged at higher rates during PS than during slow wave sleep (SWS) and some that fired at even higher rates during PS than during waking (El Mansari et al., 1989; Steriade et al., 1990a; Kayama et al., 1992). In contrast, presumed serotonergic raphe neurons and presumed noradrenergic locus coeruleus neurons were found to fire at their lowest rates or cease firing altogether during PS (Hobson et al., 1975; McCarley and Hobson, 1975; McGinty and Harper, 1976). These electrophysiological results supported the hypothesis that PS is generated by an active involvement of cholinergic neurons, considered to be "PS-on" cells, and a permissive role of monoaminergic neurons, considered to be "PS-off" cells (McCarley and Hobson, 1975;

Sakai, 1988; McCarley et al., 1995). The possibility was also raised that the cessation of firing by the monoaminergic neurons could be caused by active inhibition by local GABAergic neurons, which are distributed within these cell groups and neighboring areas (Jones, 1991a,b,c, 1993; Ford et al., 1995). The validation of these hypotheses depends, however, on the chemical identification of the recorded units, which has not yet been possible in naturally sleeping-waking animals.

Another method of studying neuronal activity, which permits immunohistochemical identification of the active cells, is by examination of c-Fos, the product of the immediate early gene that is expressed in association with neuronal discharge and entry of Na^+ and Ca^{2+} ions (Morgan and Curran, 1986; Dragunow and Faull, 1989). c-Fos expression combined with immunohistochemical staining for cholinergic and monoaminergic neurons has been used in cats in the study of neurons active during carbachol-induced PS (Shiromani et al., 1992; Yamuy et al., 1995, 1998), yet this state may fundamentally differ from naturally generated PS. In the present study performed in rats, we sought to examine c-Fos expression in association with naturally enhanced PS during rebound from PS deprivation (Mendelson, 1974). Dual immunostaining for c-Fos protein and neurotransmitters or their enzymes was used for relative assessment of activity in cholinergic, serotonergic, noradrenergic, and GABAergic neurons in the pontomesencephalic tegmentum of animals under conditions of PS recovery, deprivation, and control (Maloney and Jones, 1997).

2.2.3 Methods and Materials

Animals and surgery. Sixteen male Wistar rats (Charles River, Montreal, Quebec, Canada), weighing ~225 gm, were operated under barbiturate anesthesia (Somnotol; 67 mg/kg, i.p.) for the implantation of chronically indwelling electrodes. For the electroencephalogram (EEG), stainless steel screws were threaded into holes drilled in the skull so that the screws were in gentle contact with the dura. They were placed over the left and right retrosplenial, anterior frontal, parietal, and occipital cortices, as described previously (Maloney et al., 1997). One electrode was placed in the frontal bone rostral to the frontal lobes to be used as a reference for monopolar recording from each cortical lead and one in the occipital bone over the cerebellum to be used as a ground electrode. For the electromyogram (EMG), two stainless steel loops were inserted into the muscles of the neck. All leads were connected to a miniature (12 lead) plug that was cemented to the skull. Animals were allowed 2 or 3 d recovery from surgery in the animal room before being placed in recording chambers for the duration of the experiment.

Recording and experimental procedures. For recording and experimentation, each rat was placed in a Plexiglas box that was contained within a larger electrically shielded recording chamber. The rat was connected to a cable that was attached to a commutator and suspended with a balanced boom to allow free movement of the animal within the box. During the baseline day and in the control condition, the floor of the box was covered with woodchips. The animal had *ad libitum* access to food and water in containers that hung within easy reach on the sides of the box. As was the case in the animal room, a 12 hr light/dark cycle was maintained in the recording room (with lights on from 7:00 A.M. to 7:00 P.M.). The rat was placed in the recording box and connected to the cable 3 d before baseline recording to allow for habituation to the recording environment.

The EEG and EMG signals were amplified using a Grass model 78D polygraph and subsequently sent to a computer (ALR 386SX) for analog-to-digital conversion,

filtering, and storage on hard disk with the aid of Stellate Systems (Montreal, Quebec, Canada) computer software, as described previously (Maloney et al., 1997).

PS deprivation was performed using the flower pot technique that has previously been shown to cause a fairly selective deprivation of PS in rats (Mendelson, 1974). It was also shown to not be associated with significant changes in adrenal gland weights (Mendelson, 1974), thus not producing a severe level of stress in the animals. Each rat was placed on an inverted flower pot that was just large enough (~6.5 cm in diameter) to hold the animal. The flower pot was surrounded by water that filled the Plexiglas box to within 1 cm of the surface of the inverted pot. In this situation, the animal could engage in SWS but not PS, because the loss of muscle tonus that occurs with PS onset causes the animal to fall into the water and awaken. Food and water containers were positioned to be easily accessible to the animal on the flower pot. Under these experimental conditions, it was determined in preliminary recording experiments (involving four rats operated for implantation of electrodes and tested in the recording and experimental paradigm) that after the first 24 hr on the flower pots during which a certain degree of habituation to the experimental situation occurred, SWS appeared in ostensibly normal amounts but PS remained suppressed, producing a relatively selective deprivation of PS in the second 24 hr deprivation period and selective rebound of PS after the deprivation. Accordingly, an ~48 hr deprivation period on the flower pots was selected for the experimental paradigm.

The experimental protocol was performed over a 4 d period in three groups of four rats (Fig. 1). Recordings were performed in the afternoon (~12:00-3:00 P.M.) for the four consecutive days. On the first day, a baseline recording was performed on all animals. On the remaining 3 d of the experiment, the "condition" was varied for the three different groups: PS control (PSC), PS deprivation (PSD), and PS recovery (PSR). (1) For the control condition, the PSC animals remained on a bed of woodchips in their recording boxes for the 4 d. During these days, they were left undisturbed, except in the morning (~10:00-10:30 or ~11:00-11:30 A.M.) when their boxes were cleaned, and food and water were replenished. At the termination of the experiment on day 4, the PSC animals were anesthetized for perfusion (at ~3:00 or 3:30 P.M.) after the afternoon recording period

(Fig. 1). (2) For the deprivation condition, the PSD animals were placed on flower pots for the second, third, and fourth days of the experiment. On these days, they were removed from their flower pots in the morning (~10:00-10:30 or ~11:00-11:30 A.M.) while their boxes were being cleaned, and they were allowed to run around the larger dry recording chamber. On day 4 after the recording period, the PSD animals were anesthetized for perfusion (at ~3:30 P.M.), having been in the deprivation condition for ~53 hr (Fig. 1). (3) For the recovery condition, the PSR animals were also placed on flower pots for the second, third, and fourth days like the PSD animals. Similarly, they were removed from their cages each morning (~10:00-10:30 A.M.) while their boxes were cleaned. However, on day 4 after cage cleaning and after ~50 hr of PS deprivation, the animals were returned to a dry bed of woodchips in their recording boxes to allow for recovery of PS. To maximize the recovery during the final recording period 3 hr before perfusion, the cage cleaning was performed at ~11:00 A.M. on day 4, and the animals were returned to the dry box at ~11:30 A.M., thus allowing ~30 min exploration and grooming before sleep onset and recording. All three groups of animals commonly tended to remain awake and active during the ~30 min period after cage cleaning and handling (from 11:00-11:30 on day 4). After the recording period, the animals were anesthetized for perfusion (at ~3:00 P.M.), having been in the PS recovery condition for ~3 hr after PS deprivation of ~50 hr (Fig. 1).

The experiments were conducted using two recording chambers and thus on two animals at one time, running pairs of PSD-PSR or PSC-PSC animals. Because the recovery condition was considered the most constrained with regard to time, the PSR animals were always anesthetized and perfused first at ~3:00 P.M., whereas the paired PSD animals were anesthetized and perfused second at ~3:30 P.M. For the PSC-PSC pairs, one PSC animal was anesthetized at ~3:00 and the second at ~3:30 P.M. The entire course of experiments involving six pairs of rats was conducted over a 3 month period during the winter season.

Pilot animals and procedures. Before the recording and experimental study described above, a pilot experiment was performed to test the effectiveness of the experimental paradigm and immunohistochemical revelation of c-Fos together with the

neurotransmitters or enzymes. A series of twelve Wistar rats (Charles River), weighing ~225 gm, were submitted to the three different experimental conditions described above, except that the pilot animals were not operated for implantation of electrodes and not attached to cables for recording. Another group of four rats were kept in the animal room, two to a cage in the manner that they are typically housed. These four groups of animals were anesthetized and perfused in pairs at ~3:00 or ~3:30 P.M. (as above).

Perfusion and fixation. The animals were killed under barbiturate anesthesia (Somnotol; ~100 mg/kg) by intraaortic perfusion of a fixative solution. The time between the barbiturate injection and initiation of the perfusion was ~10 min. One liter of 3% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer was perfused for fixation and followed by 250 ml of 10% sucrose in buffer. The brains were immersed in 30% sucrose overnight to complete cryoprotection. The brains were frozen at 50°C and stored at 80°C.

Immunohistochemistry. Coronal sections were cut at 25 µm thickness on a freezing microtome. Up to six series of adjacent sections were collected every 200 µm for immunohistochemical processing. All immunohistochemistry was performed using the peroxidase-antiperoxidase (PAP) technique (Sternberger, 1979), according to previously published procedures (Gritti et al., 1993, 1997; Ford et al., 1995). For the immunostaining of c-Fos protein, an anti-c-Fos antiserum from sheep (Cambridge Research Biochemicals, Cheshire, UK) was used at a dilution of 1:3000. For immunostaining of neurotransmitters or their enzymes, the following antibodies were used: rabbit anti-choline-acetyl transferase (ChAT) antiserum (1:3000; Chemicon International, Temecula, CA), rabbit anti-serotonin (Ser) antiserum (1:30,000; Incstar, Stillwater, MN), rabbit anti-tyrosine hydroxylase (TH) antiserum (1:15,000; Eugene Tech International, Allendale, NJ), and rabbit anti-glutamic acid decarboxylase (GAD) antiserum (1:3000; Chemicon International). Incubations with primary antibodies were performed at room temperature overnight using a Tris-saline solution (0.1 M) containing 1% normal donkey serum (NDS) and after incubation with Tris saline containing 6% NDS for blocking. For antibodies to ChAT and TH, Triton X-100 (0.2%) was added to the incubation solutions. Appropriate secondary antisera and PAP antibodies (Jackson ImmunoResearch

Laboratories, West Grove, PA) were used after the respective primary antisera. In all brains, one series of sections was immunostained for c-Fos alone using the brown, floccular reaction product, 3,3' diaminobenzidine (DAB) as chromogen. In other adjacent series processed from the pilot and experimental brains, c-Fos was immunostained in combination with a neurotransmitter or enzyme using a sequential procedure staining c-Fos in either the first or second position. When in the first position, c-Fos was revealed with DAB, and the neurotransmitter or enzyme was revealed with the blue granular reaction product, benzidine dihydrochloride (BDHC). In one set (PSD-PSR-PSC), c-Fos was revealed with DAB intensified with nickel (DAB-Ni^{2+}) in the first position, and the neurotransmitter or enzyme revealed with DAB in the second position. In the major experimental series, the neurotransmitter or enzyme was immunostained and revealed in the first position with DAB, and c-Fos was immunostained and revealed in the second position with BDHC. Controls in the absence of primary antibodies and in the presence of normal sera were routinely run with every single and dual immunostaining procedure to ensure the absence of nonspecific single or dual immunostaining in the material. Brains from sets of PSD-PSR, which were run together experimentally, were processed in the same manner for immunohistochemistry together with an accompanying PSC brain.

In assessing the effectiveness of the experimental paradigm and immunohistochemistry, qualitative examination of the material was performed by one of the experimenters (B.E.J.) who had knowledge of the experimental groups. In comparing the numbers of c-Fos immunostained cells across the different conditions used in the pilot and experimental studies, it was apparent that few to no cells were stained in brains from animals housed in pairs in the animal colony, many cells were stained in animals kept for 4 d under control conditions housed individually in the recording chambers, and many more cells were stained in animals that had been operated for implantation of electrodes and were attached to recording cables for 4 d under control conditions in the recording chambers. It was thus clear that the base level of c-Fos expression in the pilot and more so experimental animals was relatively high and could be attributed to the degree of stress associated with isolation, a new environment, surgery, and being tethered, despite allowing habituation to the recording environment and recovery from surgery. c-Fos expression has been known to be elevated in widespread areas in response to stress

(Pezzone et al., 1993; Chen and Herbert, 1995; Cullinan et al., 1995). The level of c-Fos expression caused by these factors would however be the same for the three pilot and the three experimental groups, respectively, that were being compared among themselves. Qualitative differences in c-Fos immunostaining could be detected across the three different conditions of the pilot and experimental groups, respectively, and appeared systematic across these conditions in the two groups.

In the pilot series, c-Fos was immunostained in the first position, and the neurotransmitter or enzyme was immunostained in the second position. In this series, it was clear that more TH+ cells in the locus coeruleus were c-Fos+ in the PSD group than in the PSR group. However, it was difficult to clearly discern the ChAT and GAD immunostaining in the second position and thus to appreciate changes in the number of ChAT+ and GAD+ cells expressing c-Fos across the different groups. Thus, for the major experimental series, the immunostaining procedure was changed so that the neurotransmitter or enzyme was stained in the first position (with DAB), and c-Fos was stained in the second position (with BDHC). This procedure reduced the sensitivity of the c-Fos immunostaining particularly in the TH+ cells and also in Ser+ cells, in which the TH and serotonin staining was intense. However, it greatly enhanced the immunostaining, and thus identification of cells containing ChAT and GAD, in which ChAT and GAD staining was only moderate and c-Fos staining thus less affected. Because brains were processed in pairs from PSD-PSR animals and subsequently in a like manner from a matching PSC animal, it could be determined that the relative differences across groups were the same independent of the immunostaining procedure, although the absolute numbers of c-Fos-immunostained cells were lower with the c-Fos staining in the second position.

Analysis of sleep-wake state data. The EEG was examined by off-line analysis on computer screen and scored for sleep-wake state by visual assessment of EEG and EMG activity in 20 sec epochs using Eclipse software (Stellate Systems) for each 3 hr recording session (~540 epochs). Epochs were scored as one of the three major states (Wake, SWS, or PS) or transitional (*t*) stages between states: (1) wake marked by the presence of low-voltage fast activity associated with EMG tonus, (2) transition from wake into slow wave

sleep (*t*SWS) characterized by moderate amplitude slow or mixed slow, spindle and fast activity, (3) slow wave sleep (SWS) marked by continuous high-amplitude slow activity, (4) transition from slow wave sleep into PS (*t*PS) marked by a decrease in high-amplitude slow activity and the appearance of spindles and theta waves, or (5) PS characterized by a prominence of theta waves, in addition to low-voltage fast activity, with low EMG activity.

The number of epochs scored in each state was calculated as a percent of total epochs in the 3 hr recording session for each day. An overall statistic was performed using a repeated measures ANOVA with two trial factors ("state" and "day") and one grouping factor ("condition"). Data were further analyzed per state by repeated measures ANOVA tests with one trial factor (day) and one grouping factor (condition). When a main effect of condition was significant, *post hoc* tests were performed per day across groups (PSR or PSD vs PSC; PSR vs PSD) using Fisher's pairwise comparison. In the case in which there was a significant difference between groups (condition), another test was performed to determine whether there was also a significant difference between days 2, 3, or 4 and day 1 in that group. These tests were performed per state and condition, using a repeated measures ANOVA with one trial factor (day) and *post hoc* tests performed between the experimental days (day 2, 3, or 4) and baseline day (day 1). The number of PS episodes and the mean duration of these episodes were also calculated for the baseline day 1 and recovery day 4 of each animal in the PSR condition and analyzed by *t* test paired comparisons.

Fast Fourier Transform was performed using Rhythm software (Stellate Systems) to determine power in EEG frequency bands for the 20 sec state-scored epoch data, as described previously (Maloney et al., 1997). Frequency bands were set at the following ranges: delta, 1.5-4.0 Hz; theta, 4.5-8.5 Hz; and gamma, 30.5-58.0 Hz. The ratio of theta/delta, which reflects theta rhythmicity on the EEG, was also calculated and displayed. EMG amplitude was computed for the total spectrum up to 58.0 Hz. Changes in frequency band activities across experimental conditions were examined by statistical analysis of activities from the right retrosplenial lead. Frequency band activity was normalized for each rat according to the average amplitude values per state in the baseline

day. Statistical differences in frequency band activity per state and condition were tested by repeated measures ANOVA with one trial factor (day).

Analysis of immunohistochemical data. Sections were viewed with a Leitz Orthoplan microscope equipped with an x/y movement-sensitive stage and CCD camera attached to a computer. Single- and dual-immunostained cells were mapped using a computer-based image analysis system (Biocom, Paris, France) with a resident atlas of sections through the pontomesencephalic tegmentum (Jones, 1995). The experimenter (K.J.M.) mapping the cells did not have knowledge of the experimental group (PSD, PSR, PSC) to which the individual brains belonged. She was only given this information after all the data were tabulated on computer spreadsheets, and the group condition was inserted for the statistical analysis of the completed data set. Cell counts were tabulated automatically within each nucleus or region, including those of the cholinergic, serotonergic, and noradrenergic cell groups and the adjacent central gray areas of the pontomesencephalic tegmentum. Single c-Fos-immunostained cells were mapped and counted unilaterally (in one or two sections) at one or two representative of three stereotaxic levels corresponding approximately to anterior (A) 0.5, A 0.1, and posterior (P) 0.3, depending on the specific nucleus (Paxinos and Watson, 1986; Jones, 1995). To allow a more thorough sampling of dual immunostained cells, which were less numerous than the single c-Fos-immunostained cells and represented the focus of the present study, double-labeled cells were counted bilaterally at 200 μm intervals through the full rostrocaudal extent of each cholinergic and serotonergic cell group and individual nucleus (in four to six sections between ~A 1.1 and ~P 0.5 depending on the specific nucleus) and through the rostral to midportion of the noradrenergic cell group (for two or three sections, between ~P 0.1 and ~P 0.5 depending on the specific nucleus). GAD+/c-Fos+ cells were counted on adjacent sections to ChAT+/c-Fos+, Ser+/c-Fos+, or TH+/c-Fos+ cells in the same cell groups and nuclei, except the locus coeruleus (where too few GAD+ cells are located, (Ford et al., 1995)) and additionally in the rostral and caudal central gray areas neighboring the dorsal raphe and locus coeruleus, respectively. The bilateral cell counts for dual-immunostained cells were averaged per section across the two sides. ANOVAs or ANCOVAs were performed on the cell counts across conditions in multiple sections per nucleus per cell group (cholinergic, serotonergic, noradrenergic, or central gray) per animal. Overall

statistical differences in the number of cells caused by condition were examined in each cell group by one-way ANCOVA with condition as the grouping factor and nucleus, section, and animal as covariates. Statistical differences in the number of cells in individual nuclei within each cell group were subsequently examined using a one-way ANCOVA with condition as grouping factor and section and animal as covariates. When there was a significant main effect, differences in cell counts between individual conditions were analyzed by *post hoc* analyses using Fisher's pairwise comparisons (with significance level set at $p \leq 0.05$). For tabular presentation and regression analysis, the total number of labeled cells was calculated for each nucleus and cell group by adding (absolute or averaged) unilateral counts across sections in individual animals. Correlations between total number of labeled cells counted per nucleus or cell group and the percent PS, SWS, or wake were performed by multiple linear regression analyses with animal as a covariate. All statistics were performed using Systat for Windows (Evanston, Illinois). Figures were prepared for publication using CorelDraw (Ottawa, Ontario).

2.2.4 Results

Sleep-wake states

The PS deprivation procedure, which commenced on day 2 of the experimental paradigm for animals of the deprivation and recovery conditions (Fig. 1), was effective in producing a near complete elimination of PS, as well as a significant decrease in the transitional state into PS (*t*PS), as measured during 3 hr recording periods on days 2 and 3 in the deprivation and recovery conditions (Table 1, PSD, PSR). The decrease in PS and *t*PS was associated with a significant increase in waking. Although SWS was reduced, particularly on the first deprivation day, it was not significantly less than that in the PSC group during the deprivation days (Table 1).

In comparing the sleep-wake states across the different conditions on day 4 when the recovery group was removed from the deprivation condition (Fig. 1), it was apparent that there was a relatively selective deprivation of PS in the deprivation condition (PSD) and a relatively selective recovery of PS in the recovery condition (PSR), as compared with the control condition (PSC) in the final 3 hr before anesthesia and perfusion (Table 1). Thus, after ~53 and 50 hr of deprivation, respectively, PS represented 0% in the deprivation condition and ~28% in the recovery condition, as compared with ~15% in the control condition. SWS was less in the deprivation condition than in control, but not significantly so; wake was significantly greater in the deprivation condition (Table 1). SWS was significantly greater in the recovery condition than in control or deprivation, however it was not significantly different from baseline within the same group (Table 1, as indicated by parentheses). As compared with both control and baseline conditions, therefore, PS was the one state that was commonly altered in the two experimental conditions, being significantly decreased in the deprivation and significantly increased in the recovery condition.

Although the experimental procedure was effective in producing a relatively selective deprivation and recovery of PS, it did so without causing major changes in the EEG characteristics of the extant states of wake and SWS during deprivation, as well as of PS during recovery (Fig. 2). According to visual inspection of the record and

quantitative assessment of frequency band activity, the EEG was relatively unchanged during waking and was characterized, as in baseline and control conditions, by high-frequency gamma activity and theta waves recorded from limbic cortex [Figs. 2, right retrosplenial (*RRS*), 3, 4]. Across deprivation days, though, there was a progressive increase in gamma activity during waking that was significant for the deprivation condition ($F = 6.068$; df = 3; $p < 0.05$). The EEG during SWS was relatively unaltered and characterized as in baseline and control conditions by high-amplitude delta waves during deprivation and recovery (Figs. 2-4). There were no significant differences in amplitude of delta activity in SWS. PS during recovery (day 4) appeared similar in its EEG characteristics to that during baseline (Figs. 2, 4, *Day 1*). Gamma and theta activities were not quantitatively different from those in baseline PS. The change in PS during recovery was thus measured as being only in amount (Fig. 4), which particularly reflected consistently increased duration of PS episodes (2.44 ± 0.49 vs 1.84 ± 0.35 min, mean ± SEM; $t = 12.5$; df = 3; $p < 0.05$ with paired comparison of recovery to baseline values), in addition to frequently increased numbers of PS episodes (23 ± 4.3 vs 17 ± 2.7; $t = 1.58$; df = 3, not significant).

c-Fos+ expression in cell groups of the pontomesencephalic tegmentum

The number of single-labeled c-Fos+ cells varied significantly as a function of the experimental condition in most cell groups (Table 2, c-Fos+). However, whether there was an increase or decrease in the PS recovery condition depended on the individual cell group. In the cholinergic cell group, including its four nuclear subdivisions, c-Fos+ cells were greater in the PSR condition than in the PSD or PSC conditions (Table 2, Sum). In the serotonergic cell group, including its two nuclear subdivisions, c-Fos+ cells were not consistently different in the recovery condition. In the noradrenergic cell group, including its two nuclear subdivisions, c-Fos+ cells were lower in the recovery condition than in the deprivation condition. In the central gray areas, which lie adjacent to the cholinergic and monoaminergic cell groups, c-Fos+ cells were greater in the PS recovery condition than in the deprivation and control conditions. The different variations according to condition in c-Fos+ cells across the different cell groups appeared to depend on the predominant

cell types located in those regions, as could only be fully appreciated by dual immunostaining for c-Fos and specific neurotransmitter or synthetic enzyme (Figs. 5-8).

In the cholinergic cell group, ChAT-immunostained cells expressing c-Fos (Fig. 5*A*) varied significantly as a function of condition (Table 2, ChAT+/c-Fos+). The sum of ChAT+/c-Fos+ cells was greater in the recovery condition than in the deprivation and control conditions (Table 2, Sum). The greater number of ChAT+/c-Fos+ cells in the recovery condition as compared with the deprivation condition was apparent across the cholinergic nuclei of the pontomesencephalic tegmentum (Fig. 6, *LDTg, LDTgV, PPTgM, PPTgL*). Within individual nuclei, these differences were statistically significant for the laterodorsal tegmental nucleus (LDTg), in which the number of ChAT+/c-Fos+ cells in the recovery condition was higher than in the deprivation condition, and for the pedunculopontine tegmental nucleus, medial part (PPTgM), in which the number of ChAT+/c-Fos+ cells in the recovery condition was higher than in both the deprivation and control conditions (Table 2). Within the LDTg, the number of cells in the deprivation condition was also significantly lower than in the control condition. Across conditions, the number of ChAT+/c-Fos+ cells within the LDTg was significantly positively correlated with the percent time spent in PS during the final 3 hr recording period ($r = 0.68$; Fig. 9). It was not significantly correlated with the percent SWS but was significantly negatively correlated with the percent waking ($r = 0.73$; $df = 11$; $p < .05$). With stepwise backward removal of variables in a multiple regression linear model, removal of the waking variable did not eliminate the significant correlation of PS with the number of ChAT+/c-Fos+ cells.

In the serotonergic cell group, Ser-immunostained cells expressing c-Fos (Fig. 5*C*) varied significantly as a function of condition (Table 2, Ser+/c-Fos+). The number of Ser+/c-Fos+ cells was significantly lower in the recovery condition than in the deprivation and control conditions (Table 2, Sum). The lower number of Ser+/c-Fos+ cells in the recovery condition as compared with the deprivation condition was apparent in both the dorsal and median raphe nuclei (Fig. 7, *DR, MR*). Within both nuclei, the

difference between the recovery condition and deprivation and control conditions was significant (Table 2). Across conditions, the number of Ser+/c-Fos+ cells within the DR was significantly negatively correlated with the percent time spent in PS during the final 3 hr recording period (Fig. 9; $r = 0.73$). It was not significantly correlated with either the percent SWS or waking.

In the noradrenergic cell group, TH-immunostained cells expressing c-Fos (Fig. 5*D*) varied significantly as a function of condition (Table 2, TH+/c-Fos+). The number of TH+/c-Fos+ cells was significantly lower in the recovery condition than in the deprivation condition (Table 2, Sum). The lower number of TH+/c-Fos+ cells in the recovery condition as compared with the deprivation condition was apparent in both the locus coeruleus and subcoeruleus, part (Fig. 8, *LC*, *SubCA*). Within both nuclei, the difference between the recovery condition and deprivation condition was significant (Table 2). In addition, for both the sum and LC, TH+/c-Fos+ cells were significantly greater in the deprivation condition than in the control condition (Table 2, Sum and LC). Across conditions, the number of TH+/c-Fos+ cells within the LC was significantly negatively correlated with the percent time spent in PS during the final 3 hr recording period (Fig. 9; $r = 0.61$). It was not significantly correlated with the percent SWS but was significantly positively correlated with the percent waking ($r = 0.60$; $df = 11$; $p < 0.05$). With stepwise backward removal of variables in a multiple regression linear model, removal of the waking variable did not eliminate the significant correlation of PS with the number of TH+/c-Fos+ cells.

In all cell groups, including the central gray areas adjacent to the cholinergic and monoaminergic cell groups, GAD-immunostained cells expressing c-Fos were evident (Fig. 5*B*) and varied significantly as a function of condition (Table 2, GAD+/c-Fos+). The number of GAD+/c-Fos+ cells was significantly higher in the recovery condition than in the deprivation and control conditions in all regions (Table 2, Sum). The higher number of GAD+/c-Fos+ cells in the recovery condition as compared with the deprivation condition was apparent in all cell groups and nuclei of the pontomesencephalic tegmentum where GAD+ cells are distributed (Figs. 6-8). In every one of these nuclei, the number of GAD+/c-Fos+ cells was consistently higher in the recovery condition than in

the deprivation and control conditions (Table 2). In none of the groups or nuclei were there significant differences between the deprivation and control conditions (Table 2). Across conditions, the number of GAD+/c-Fos+ cells was significantly positively correlated with the percent time spent in PS in all nuclei, as illustrated for the cells in the LDTg ($r = 0.85$), the DR ($r = 0.92$), and the caudal central gray adjacent to the LC (Fig. 9; $r = 0.70$). It was not significantly correlated with either the percent SWS or waking.

2.2.5 Discussion

The present results demonstrate that during PS rebound, the number of c-Fos-expressing cholinergic cells is increased, whereas the numbers of c-Fos-expressing monoaminergic neurons are decreased, suggesting a reciprocal change in the activity of these cell groups. Moreover, the number of GABAergic cells expressing c-Fos during rebound is increased, suggesting that they may also be active during PS and involved in suppressing the activity of surrounding monoaminergic cells.

The changes in c-Fos expression are interpreted here as reflecting changes in neuronal activity associated with the different experimental conditions. It must be mentioned, nonetheless, that such changes may also reflect changes in other cellular processes that can be stimulated by chemical messengers independent of neuronal discharge, although also dependent on changes in intracellular calcium (Morgan and Curran, 1986).

Cholinergic cell group

PS recovery resulted in increased numbers of cholinergic neurons expressing c-Fos in the laterodorsal and pedunculopontine tegmental nuclei, and across conditions the percent PS was significantly positively correlated with their numbers, supporting the hypothesis that cholinergic tegmental neurons are actively involved in PS generation. As confirmed here, previous studies examining single-labeled c-Fos+ cells reported increases within the cholinergic cell area in association with enhanced PS (Merchant-Nancy et al., 1992; Shiromani et al., 1992; Yamuy et al., 1993). Examining dual immunostaining for c-Fos and ChAT, one study also found increases in the number of cholinergic neurons expressing c-Fos during carbachol-induced PS in cats, although, as is also the case in the present study, only a small proportion of the cholinergic cells were c-Fos+ (Shiromani et al., 1996). Another more recent study failed to confirm the latter result in cats (Yamuy et al., 1998). However, because carbachol acts directly on target neurons of the cholinergic

cells (Vanni-Mercier et al., 1989; Jones, 1990), its pharmacological effect would not depend on increased activity by the cholinergic cells. *In vivo* electrophysiological studies have identified slow-spiking neurons as possibly cholinergic neurons and reported that these cells are PS-on cells, all discharging at higher rates during PS than during SWS and some higher during PS than during waking (Sakai and Jouvet, 1980; El Mansari et al., 1989; Steriade et al., 1990a,b; Kayama et al., 1992). Recently, juxtacellular labeling with biocytin combined with staining for NADPH-diaphorase has provided histochemical evidence that such slow-spiking neurons are cholinergic (Koyama et al., 1998). Electrophysiologically characterized neurons have moreover been shown to be inhibited by carbachol microinjections (as "Carb-I PS-on" neurons) *in vivo* (Sakai and Koyama, 1996), similar to the response to carbachol documented on identified cholinergic cells *in vitro* (Leonard and Llinas, 1994). Further evidence that cholinergic tegmental neurons are PS-on cells comes from biochemical studies showing that in the thalamus and the brainstem pontine and medullary reticular formation, to which the cholinergic tegmental neurons project (Jones and Webster, 1988; Pare et al., 1988; Jones, 1990), ACh release is greater during PS than during SWS and in the brainstem, also greater than during waking (Kodama et al., 1990, 1992; Becker et al., 1994; Williams et al., 1994; Leonard and Lydic, 1995).

PS rebound was also associated with an increase in the number of GABAergic neurons expressing c-Fos within the cholinergic cell area, and across conditions, the percent PS was positively correlated with their number. A significant proportion of neurons in the cholinergic cell area that show increased c-Fos expression in association with naturally occurring PS (Merchant-Nancy et al., 1992; Shiromani et al., 1992) and also with carbachol-induced PS (Yamuy et al., 1998) would thus, as the latter study also showed, be noncholinergic and as the present study shows, most likely GABAergic. GABAergic neurons are codistributed with the cholinergic neurons in the laterodorsal and pedunculopontine tegmental nuclei (Ford et al., 1995) and could correspond in part to other electrophysiologically identified PS-on cells, which display brief spikes and discharge rapidly (Sakai and Jouvet, 1980; El Mansari et al., 1989; Steriade et al., 1990a,b; Koyama et al., 1998). In contrast to the putative cholinergic, these presumed noncholinergic, PS-on cells are excited by carbachol microinjections ("Carb-E PS-on"

neurons) *in vivo* (Sakai and Koyama, 1996). According to the electrophysiological study of these presumed noncholinergic Carb-E PS-on neurons and to the chemoneuroanatomical study of the GABAergic neurons in the cholinergic cell area, some PS-on GABAergic neurons could be projection neurons and project in parallel with the cholinergic neurons into the forebrain (Ford et al., 1995). Other GABAergic cells may be locally projecting neurons and innervate cell bodies or dendrites of neighboring monoaminergic neurons (Jones, 1991a,b), which they could thus inhibit during PS. In either case, they would appear to be active in parallel and perhaps also in series with the cholinergic neurons during PS.

Monoaminergic cell groups

In the dorsal and median raphe nuclei, PS recovery resulted in a decrease in the number of serotonergic cells expressing c-Fos, supporting the claim that presumed serotonergic neurons decrease or cease firing during PS (McGinty and Harper, 1976; Tulson and Jacobs, 1979). Although a recent study with carbachol-induced PS in cats found no significant difference as compared with saline-injected controls in numbers of c-Fos-expressing serotonergic neurons, this lack of difference was also interpreted as being caused by a lack of discharge by the serotonergic neurons during PS (Yamuy et al., 1995). Support for the cessation of serotonergic neuronal discharge during PS comes from biochemical studies that have shown a marked decrease in serotonin release during PS (Portas et al., 1998). In the present study, PS recovery also resulted in an increase in the number of GABAergic cells expressing c-Fos in the raphe and central gray, revealing potentially active inhibitory interneurons that could be responsible for the suppression of serotonergic activity. These GABAergic cells could represent a proportion of dorsal raphe nonserotonergic c-Fos-expressing cells that were increased in number with carbachol-induced PS (Yamuy et al., 1995). The GABAergic c-Fos-expressing neurons may correspond to neurons that were originally considered to be inhibitory interneurons, based on their distinct discharge properties and response to stimulation, which was reciprocal to that of the dorsal raphe serotonergic neurons (Aghajanian et al., 1978). Presumed nonserotonergic neurons have been recorded across the sleep-waking cycle, and some

reported to increase their discharge during PS (Sheu et al., 1974; Shima et al., 1986; Kocsis and Vertes, 1992). GABA release measured in the dorsal raphe has been reported to be higher during PS than during SWS or waking (Nitz and Siegel, 1997a). Microperfusion with $GABA_A$ antagonists, bicuculline, or picrotoxin in the dorsal raphe has been reported to lift inhibition of serotonergic neurons during sleep (Levine and Jacobs, 1992) and also to decrease PS (Nitz and Siegel, 1997a). In summary, within the raphe, the inverse correlations of percent PS with GABAergic versus serotonergic c-Fos-expressing neurons suggests together with other evidence, that GABAergic raphe neurons are PS-on cells, which may be partly responsible for the important inhibition of codistributed serotonergic PS-off cells.

In the locus coeruleus and subcoeruleus, there was a significant decrease in the numbers of noradrenergic c-Fos-expressing cells during PS recovery as compared with deprivation and a significant negative correlation in these numbers with the percent PS across conditions. These results support the claim that noradrenergic locus coeruleus neurons cease firing during PS and may accordingly play a permissive role in PS generation (Hobson et al., 1975; McCarley and Hobson, 1975; Aston-Jones and Bloom, 1981a). With carbachol-induced PS in cats, no significant difference was documented in c-Fos-expressing noradrenergic neurons relative to controls (Yamuy et al., 1995). In the present study, PS recovery resulted in an increase in the number of c-Fos-expressing GABAergic neurons, which were located in the vicinity of the noradrenergic cells and which could thus act as local inhibitory neurons. These GABAergic cells could represent a proportion of the non-noradrenergic c-Fos-expressing cells in the locus coeruleus region that were found to be increased with carbachol-induced PS in cats (Yamuy et al., 1995). In support of increased activity during PS of GABAergic neurons innervating the locus coeruleus, biochemical studies have found greater release of GABA in the locus coeruleus during PS than during SWS or waking (Nitz and Siegel, 1997b). Moreover, the cessation of discharge by locus coeruleus neurons during this state can be reversed by microinjection of the $GABA_A$ antagonist bicuculline (Gervasoni et al., 1998), which also results in a decrease in PS (Kaur et al., 1997). In summary, the present results showing inverse correlations of PS with GABAergic versus noradrenergic c-Fos-expressing

neurons provide evidence that GABAergic neurons are active during PS and may be responsible for the important inhibition of adjacent noradrenergic neurons during PS.

Interaction between cell groups

The present results substantiate the concept that cholinergic tegmental neurons are actively involved, as PS-on cells, whereas monoaminergic neurons may be permissively involved, as PS-off cells, in PS generation (Sakai, 1988; McCarley et al., 1995). Given pharmacological evidence that serotonin and noradrenaline inhibit cholinergic tegmental neurons (Luebke et al., 1992; Williams and Reiner, 1993; Leonard and Llinas, 1994; Leonard et al., 1995), the monoaminergic neurons could tonically inhibit cholinergic neurons during the waking state, rendering them PS-on/Wake-off cells. The reciprocal increase in noradrenergic locus coeruleus c-Fos-expressing neurons and decrease in cholinergic laterodorsal tegmental c-Fos-expressing neurons during PS deprivation could reflect such an influence in the present study, as well as confirming the role of noradrenergic neurons in behavioral and cortical arousal and stress (Foote et al., 1980; Aston-Jones and Bloom, 1981a,b; Abercrombie and Jacobs, 1987; Jones, 1991c; Pezzone et al., 1993; Tononi et al., 1994). However, in view of the reported high levels of ACh release in the thalamus during waking (Williams et al., 1994), it is unlikely that all cholinergic tegmental neurons are tonically inhibited during this state. Another possibility is that a subset of cholinergic neurons having particular projections are PS-on/Wake-off cells, such as those projecting into the brainstem (Jones, 1990), where ACh release is greater during PS than during waking (Kodama et al., 1990, 1992). Electrophysiological evidence has recently been presented that there are two subsets of putative cholinergic neurons, one that is PS-on/Wake-off and inhibited by serotonin, and another that is PS-on/Wake-on and unaffected by serotonin (Thakkar et al., 1998). The present results would support the hypothesis that a subset of cholinergic neurons may be selectively active during PS and reciprocally related in their activity to monoaminergic neurons across the sleep-waking cycle.

In addition, the present results indicate that GABAergic neurons that are codistributed with cholinergic and monoaminergic neurons in the pontomesencephalic tegmentum are also active during PS and could accordingly, as PS-on cells, inhibit the monoaminergic PS-off cells. Simultaneous unit recordings of putative cholinergic PS-on and monoaminergic PS-off cells during transitions into and out of PS have revealed mirror image changes in their discharge, such as to suggest a mutual inhibitory relationship between them (Sakai, 1988). Because ACh is excitatory to monoaminergic neurons (Egan and North, 1986; Li et al., 1998), the cholinergic cells could only effect such an inhibition via local interneurons (Jones, 1991b). Reported putative noncholinergic PS-on cells that are excited by carbachol (Sakai and Koyama, 1996) could correspond in part to such GABAergic interneurons. GABAergic neurons in the pontomesencephalic tegmentum would accordingly play an integral role in the generation of PS.

2.3 Conclusions

The pontomesencephalic tegmentum has long been known to be important in the induction and generation of PS. Lesioning rostrally projecting efferents from this region result in a loss of PS characteristics in rostral brains structures, while lesioning caudally projecting efferents results in a loss of PS characteristics in caudal brain structures and the periphery (Jouvet, 1962). Within the pontomesencephalic tegmentum, c-Fos expression in cholinergic neurons was increased while c-Fos expression in monoaminergic neurons was decreased in association with PS rebound, confirming the earlier hypothesis of an active cholinergic role, and a permissive monoaminergic involvement, in PS generation. Moreover, c-Fos expression in GABAergic neurons co-distributed with cholinergic and monoaminergic neurons was increased during PS rebound. The hypothesis that GABAergic neurons are responsible for the inhibition of monoaminergic neurons during PS is presented. The efferent projections from these pontomesencephalic tegmental neurons can provide modulatory influences on ascending and descending systems that may mediate the initiation and generation of PS characteristics in other brains structures.

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2.5 Tables and Figures

Table 1.

Percentage of time spent in Wake, tSWS (transition from Wake to SWS), SWS, tPS (transition from SWS to PS), and PS per day (scored per 20 sec epoch over ~3 hour recording period) across the four experimental days for animals in each condition (mean ± SEM, n = 4). A repeated measures ANOVA was performed on the entire data set with two trial factors ("state" and "day") and one grouping factor ("condition") and revealed significant variance across state with a significant interaction of state with condition, day, and condition and day. The data were further analyzed per state by repeated measures ANOVA tests with one trial factor (day) and one grouping factor (condition). There was a significant main effect of condition for W, SWS, tPS, and PS. In these cases, post hoc tests were performed per day across conditions (PSR or PSD vs PSC; PSR vs PSD) using Fisher's pairwise comparisons. In cases where there was a significant difference between conditions on days 2, 3, or 4, another post hoc test was performed to determine whether there was a significant difference between those experimental days and day 1 (baseline) within that group. Differences with respect to PSC are indicated by * $p \leq 0.05$. Differences in PSR with respect to PSD are indicated by $p \leq 0.05$. In those cases in which there was not a significant difference with respect to baseline, the symbols are placed within parentheses.

Table 1. Sleep-Wake states across days for Paradoxical Sleep (PS) Control, Deprived (PSD) and Recovery (PSR) Groups.

		DAY 1	DAY 2	DAY 3	DAY 4
PSC	WAKE	21.82 ± 5.10	19.05 ± 5.46	13.85 ± 4.09	21.00 ± 2.00
	tSWS	27.34 ± 1.43	28.75 ± 4.40	32.00 ± 1.00	30.00 ± 5.00
	SWS	11.50 ± 3.14	15.72 ± 6.39	10.39 ± 4.38	9.00 ± 3.00
	tPS	25.75 ± 4.21	23.75 ± 4.13	28.67 ± 1.67	25.00 ± 4.00
	PS	11.61 ± 2.91	12.73 ± 1.13	10.57 ± 3.95	14.50 ± 6.50
PSD	WAKE	21.65 ± 3.59	72.89 ± 3.18*	52.89 ± 7.75(*)	56.32 ± 3.87*
	tSWS	36.92 ± 7.02	22.05 ± 3.49	33.75 ± 6.28	34.00 ± 4.74
	SWS	8.65 ± 2.46	2.25 ± 0.59	5.96 ± 1.19	3.04 ± 1.53
	tPS	23.54 ± 5.01	0.50 ± 0.29*	7.25 ± 2.18(*)	6.25 ± 1.03(*)
	PS	12.43 ± 4.57	0.0 ± 0.0*	0.3 ± 0.5*	0.0 ± 0.0*
PSR	WAKE	20.89 ± 2.88	76.35 ± 2.37 *	49.90 ± 3.23*	16.36 ± 1.28 (*)
	tSWS	21.88 ± 3.83	20.07 ± 2.59	25.18 ± 0.84	10.75 ± 2.60
	SWS	25.64 ± 1.67*†	2.95 ± 0.61	13.58 ± 1.50†	25.31 ± 1.06(*)
	tPS	18.25 ± 4.42	0.0 ± 0.0*	10.36 ± 3.21 *	19.25 ± 1.32(*)
	PS	12.83 ± 1.23	0.0 ± 0.0 *	0.0 ± 0.0 *	28.57 ± 1.86(*)

Table 2.

Cell counts were taken from five adjacent series of sections cut at 25 μm and collected at 200 μm intervals.

[a] c-Fos+ cell numbers correspond to the mean ± SEM of the total number of single-labelled c-Fos+ cells counted unilaterally per nucleus in two sections (at ~A 0.5 and ~A 0.1) for all rostral structures or in one section at (~P 0.3) for LC, SubCA, and cCG. Sum refers to the mean ± SEM of the total unilateral cell count for nuclei of the cholinergic, serotonergic, noradrenergic, or central gray cell groups.

[b] Double-labelled cell numbers correspond to the mean ± SEM of the total number of cells counted bilaterally and averaged for the two sides in sections extending through the cholinergic, serotonergic, noradrenergic, or neighboring central gray cell groups. Double-labelled cholinergic and serotonergic cells were counted through the major extent of their rostrocaudal distribution, which varies according to the specific nucleus (Ford et al., 1995). Double-labelled noradrenergic cells were counted through the rostral to middle levels of their distribution. Double-labelled GABAergic cells were counted on adjacent sections at corresponding levels in each cell group and nucleus (except the LC) and also in rostral and caudal central gray areas neighboring the DR and LC, respectively. Accordingly, counts were performed in the following number of sections for each nucleus. ChAT+/c-Fos+ cells or GAD+/c-Fos+ cells were counted: in four sections for the LDTg (between ~A 0.7 and ~A 0.1) and the PPTgM (between ~A 0.9 and ~A 0.3) and in five sections for LDTgV (between ~A 0.7 and ~P 0.1) and PPTgL (between ~A 1.1 and ~A 0.3). Ser+/c-Fos+ or GAD+/c-Fos+ cells were counted in five sections for the DR and MR (between ~A 0.7 and ~P 0.1). TH+/c-Fos+ cells were counted in three sections for the LC (between ~P 0.1 and ~P 0.5), and TH+/c-Fos+ or GAD+/c-Fos+ cells in two sections for the SubCA (at ~P 0.1 and ~P 0.3). GAD+/c-Fos+ cells were also

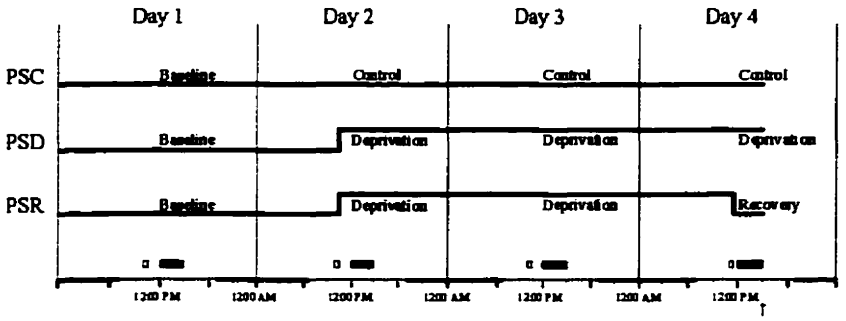
counted in five sections for the rCG (between ~A 0.9 and ~A 0.1) and in three sections for the cCG (between ~P 0.1 and ~P 0.5). Sum refers to the mean ± SEM of the total (average) unilateral cell count for corresponding nuclei of the cholinergic, serotonergic, noradrenergic, or central gray cell groups.

[c] Statistical tests of the variation in cell numbers for the sum of neurons in cholinergic, serotonergic, noradrenergic, or central gray cell groups were performed using one-way ANCOVAs with condition as grouping factor and nucleus, section, and animal as covariates. For Sum, a significant main effect for condition is indicated by $p \leq 0.05$ in the column under F. With a significant main effect for sum, individual ANCOVAs were performed for each nucleus with condition as grouping factor and section and animal as covariates. For individual nuclei, a significant main effect for condition is indicated by $p \leq 0.05$ in the column under F. For both sum and individual nuclei with significant main effects of condition, post hoc tests between individual conditions were performed using Fisher's pairwise comparisons. A significant difference with respect to PSC is indicated by * $p \leq 0.05$ and with respect to PSD by † $p \leq 0.05$.

Table 2. Number of identified single-labelled, c-Fos+, or double-labelled ,ChAT+/c_fos+ neurons, Ser+/c-Fos+ neurons, TH+/c-Fos+ neurons and GAD+/c-Fos+ neurons in the cholinergic, serotonergic, noradrengic and adjacent periventricular gray areas of the pontomesencephalic tegmentum in Paradoxical sleep (PS) Control (PSC), Deprived)PSD) and Recovery (PSR).

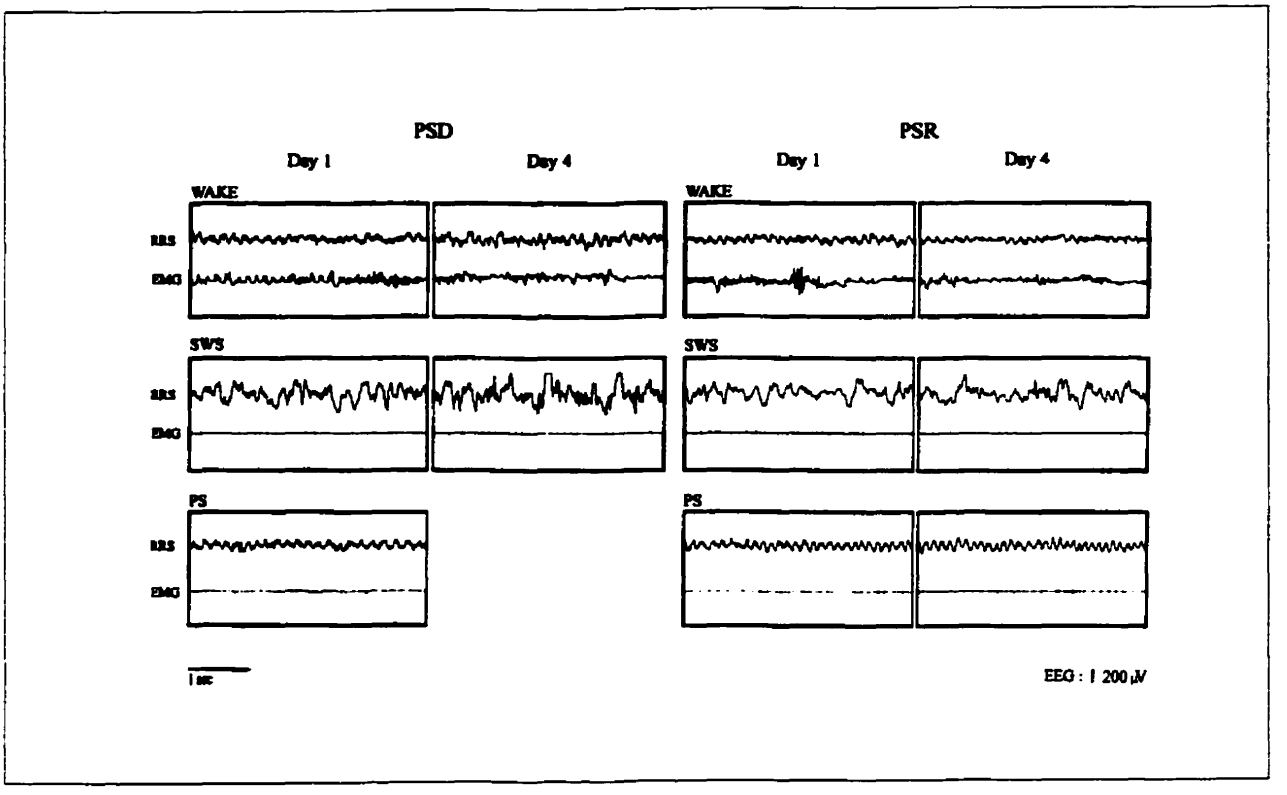
	c-Fos+[a]				ChAT+/c-Fos+[b]				Ser+/c-Fos+[b]				TH+/c-Fos+[b]				GAD+/c-Fos+[b]			
Nucleus	PSC	PSD	PSR	F	PSC	PSD	PSR	F	PSC	PSD	PSR	F	PSC	PSD	PSR	F	PSC	PSD	PSR	F
LDT_g[1]	61.25±10.08	63.25±12.94	106.38±14.89		42.13±3.53	23.33±5.93	53.83±6.57										29.50±10.77	21.00±2.78	87.33±13.37	
LDT_gV[1]	33.75±5.48	53.13±5.23	61.75±6.42		20.88±3.19	15.00±1.53	16.50±1.80										11.67±2.96	18.17±5.29	31.00±6.76	
PPT_gM[1]	113.25±34.79	83.50±7.63	96.25±7.64		12.75±3.43	9.83±0.44	20.83±4.34										26.17±11.61	19.33±2.59	44.41±9.68	
PPT_gL[1]	177.50±57.47	153.00±18.06	257.50±77.33		24.63±4.71	27.17±4.92	32.00±1.00										29.17±2.79	30.00±2.65	57.33±21.36	
[2]	305.63±89.83	352.88±22.38	527.88±102.62		100.25±12.83	75.35±11.32	123.17±12.41										96.33±17.46	88.50±4.36	220.07±46.25	
DR[1]	37.50±3.75	64.50±28.12	113.63±40.11						24.87±3.60	29.17±5.96	128.3±0.88						10.50±2.75	7.67±2.60	29.17±4.34	
MR[1]	267.75±109.45	213.33±43.91	109.33±32.09						33.75±7.62	39.75±11.25	141.7±6.93						44.67±14.17	38.00±10.61	104.83±35.55	
[2]	303.25±100.54	234.50±44.57	225.00±25.51						58.50±11.26	67.17±8.59	27.00±7.28						53.50±12.86	45.67±8.82	134.00±31.27	
LC[1]	28.24±4.50	32.75±3.43	15.00±2.38										9.00±0.74	12.13±2.59	7.67±0.60					
SubCA[1]	63.00±12.99	97.50±15.60	61.00±13.37										1.38±0.47	2.50±0.71	0.50±0.50		31.33±5.21	28.00±4.00	51.00±10.82	
[2]	91.25±16.67	130.25±17.19	76.00±14.01										10.37±0.47	14.63±2.95	8.00±0.29					
rCG[1]	159.00±43.54	142.75±11.81	238.00±30.05														47.67±11.67	24.50±6.81	122.50±12.68	
cCG[1]	124.00±27.48	120.25±16.47	161.00±19.79														47.50±22.53	43.83±8.25	74.00±9.47	
[2]	283.00±43.02	263.00±27.97	399.00±48.56														90.17±32.76	68.33±11.11	196.50±13.44	

Figure 1. Diagram illustrating time course of treatments for the three different experimental conditions: PSC, PSD, and PSR. Whereas the PSC group remained on a bed of woodchips, the PSD and PSR groups were placed on inverted flower pots surrounded by water for ~53 or ~50 hrs deprivation of PS (Days 2-4) in the recording chambers. The PSR group was returned to the woodchips to allow ~3 hr recovery of PS. Animals were anesthetized and perfused (†) at the end of the control, deprivation, or recovery periods (on day 4). EEG recording was performed each day for 3 hr in the afternoon. Animals were maintained on a 12 hr light/dark cycle (with lights on from 7:00 A.M. to 7:00 P.M., as indicated by light shading). See Materials and Methods for further details.



On Flower Pot
Cage Cleaning
Recording Periods
Light/Dark
† Anesthesia and Perfusion

Figure 2. EEG and EMG associated with wake, SWS, and PS on day 1 (baseline) and day 4 from two representative animals within the PSD (left) and PSR (right) groups. There is no apparent difference in EEG activity during wake or SWS in either group or during PS in the recovery group between days 1 and 4. The EEG was recorded from the RRS cortex.


PSD
PSR
Day 1
Day 4
Day 1
Day 4
WAKE
WAKE
IRS
EMG
SWS
SWS
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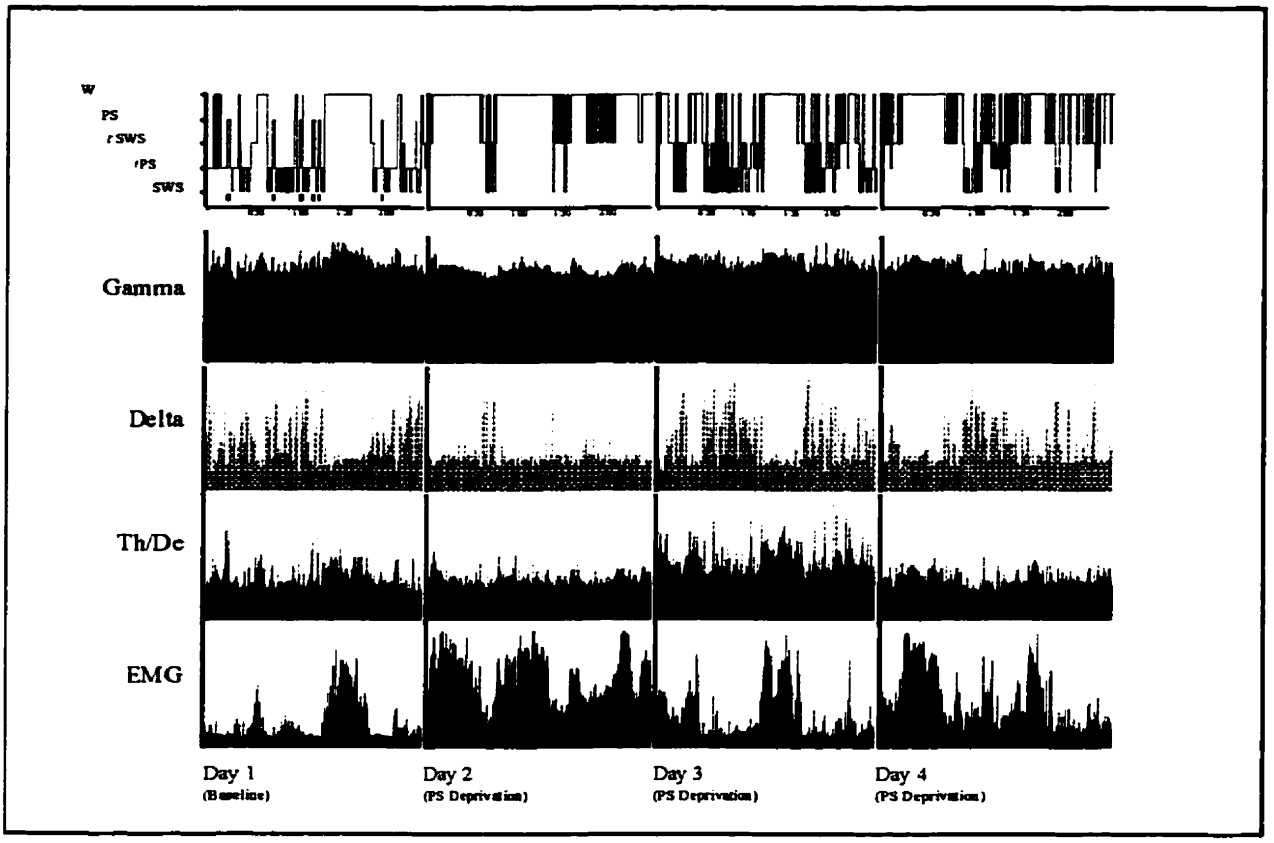
EMG
PS
PS
IRS
EMG
1 sec
EEG : 1 200 µV

Figure 3. Hypnogram showing sleep-wake states (top row, with PS also indicated by black bars underneath; scored per 20 sec epoch during the 3 hr recording period) over the four experimental days in one representative animal of the PSD condition. Activity in the gamma (30.5-58.0 Hz) and delta (1.5-4.0 Hz) frequency bands and the ratio of theta (4.5-8.5 Hz) to delta (Th/De, indicative of theta activity) are shown for EEG. Total activity (1.5-58 Hz) is shown for EMG. Parallel increases in gamma and theta reflect cortical activation during wake and PS, which is also accompanied by low EMG activity, whereas high delta activity reflects SWS. Note on days 2, 3, and 4, the persistence of SWS marked by high-amplitude delta, despite the deprivation of PS. Activity displayed as amplitude units scaled to maximum.


W
PS
SWS
PS
SWS
Gamma
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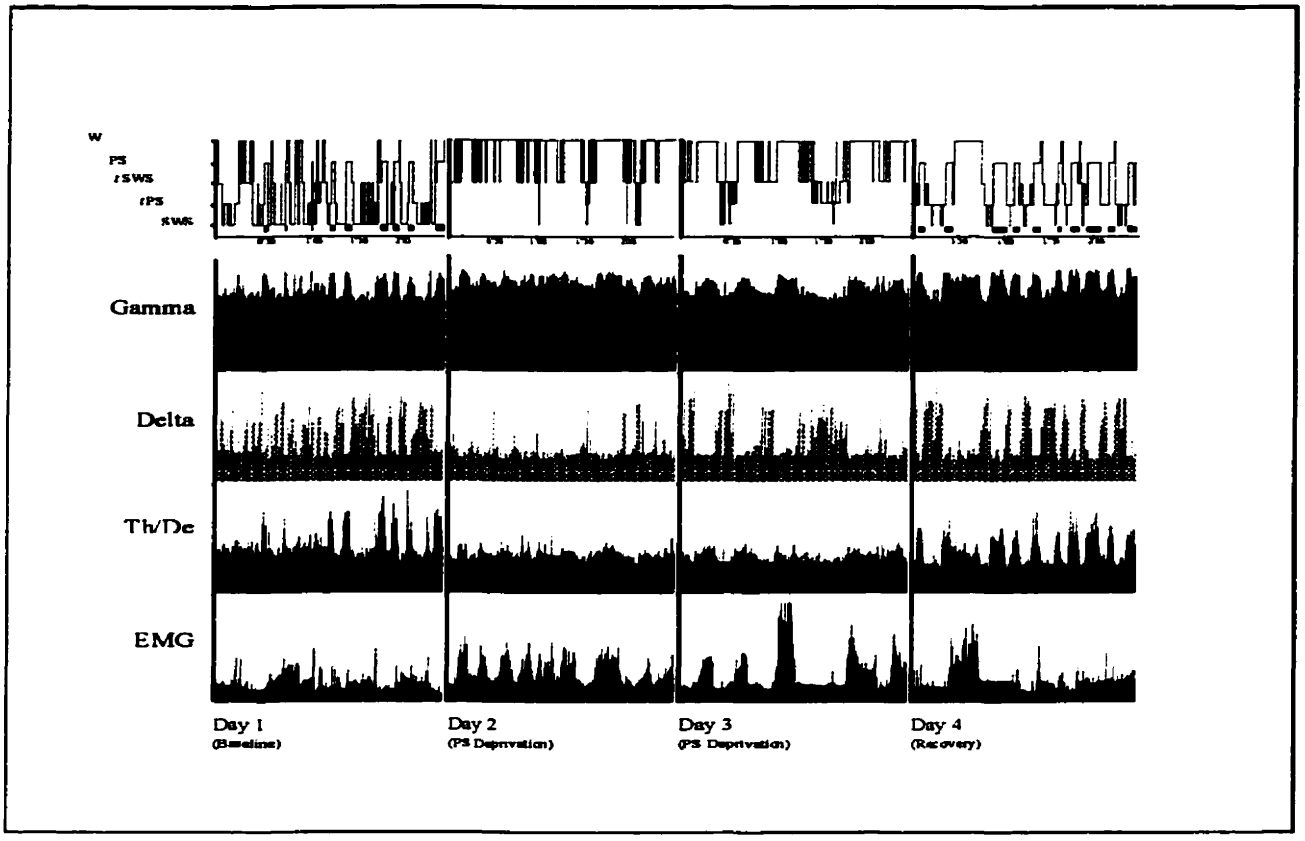
Th/De
EMG
Day 1
(Baseline)
Day 2
(PS Deprivation)
Day 3
(PS Deprivation)
Day 4
(PS Deprivation)

Figure 4. Hypnogram showing sleep-wake states (top row, with PS also indicated by black bars underneath; scored per 20 sec epoch during the 3 hr recording period) over the four experimental days in one representative animal of the PSR condition. Activity in the gamma (30.5-58.0 Hz) and delta (1.5-4.0 Hz) frequency bands and the ratio of theta (4.5-8.5 Hz) over delta (Th/De, indicative of theta activity) are shown for EEG. Total activity (1.5-58 Hz) is shown for EMG. Parallel increases in gamma and theta reflect cortical activation during wake and PS, which is also accompanied by low EMG activity, whereas high delta activity reflects SWS. Note on days 2 and 3, the persistence of SWS marked by high-amplitude delta, despite the deprivation of PS. The recovery and rebound of PS is evident on day 4 by the presence of high-amplitude gamma and theta activity with diminished EMG. Activity displayed as amplitude units scaled to maximum.


W
PS
tSWS
tPS
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Gamma
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Th/De
EMG
Day 1
(Baseline)
Day 2
(PS Deprivation)
Day 3
(PS Deprivation)
Day 4
(Recovery)

Figure 5. Photomicrographs of sections dual-immunostained for c-Fos (blue granular chromogen, BDHC) and ChAT (A), GAD (B), serotonin (C), or TH (D) (brown chromogen, DAB). Black arrowheads indicate double-labeled cells, and white arrowheads indicate examples of adjacent single-labeled c-Fos+ cells. Scale, 25 μm.

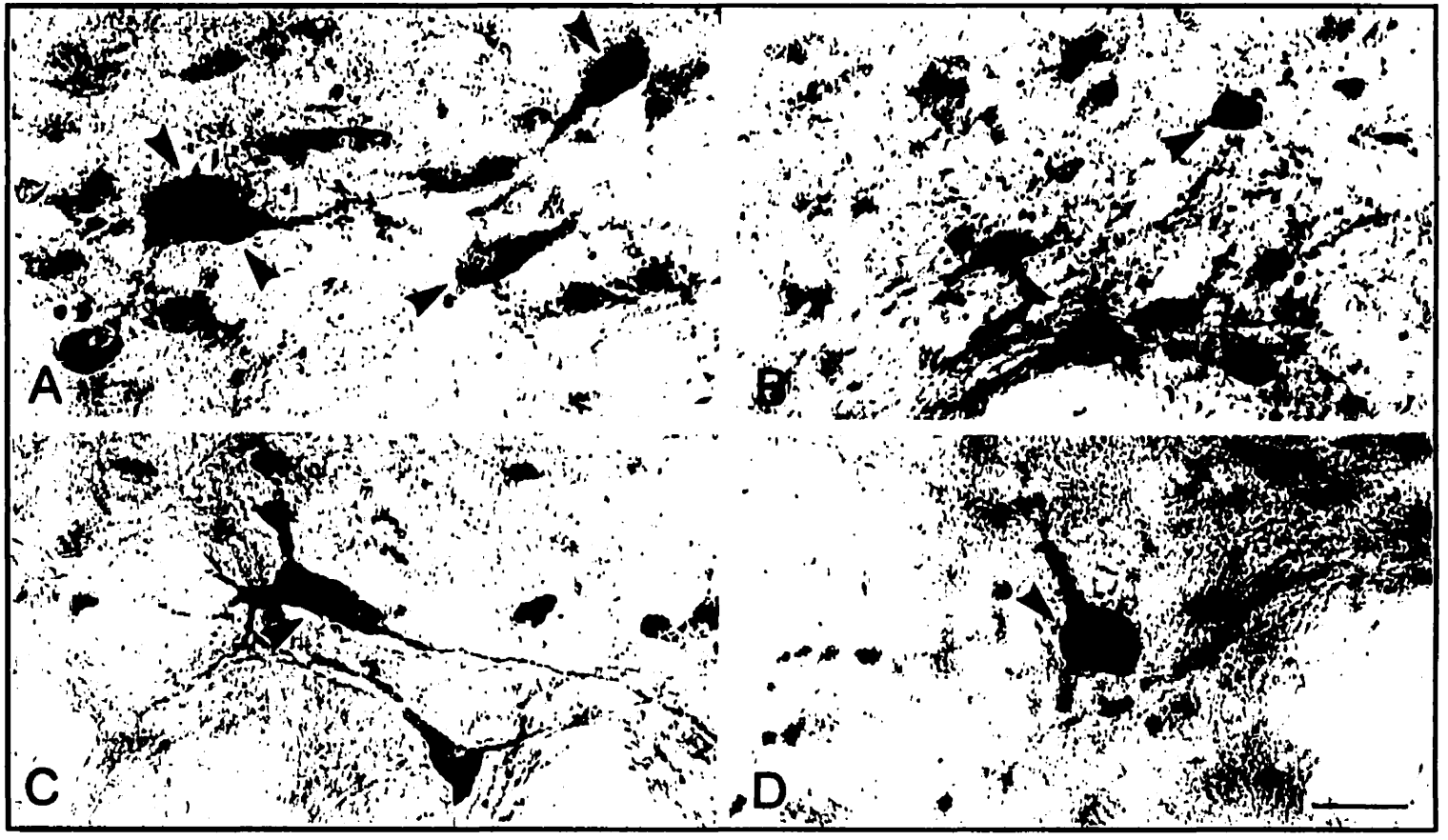
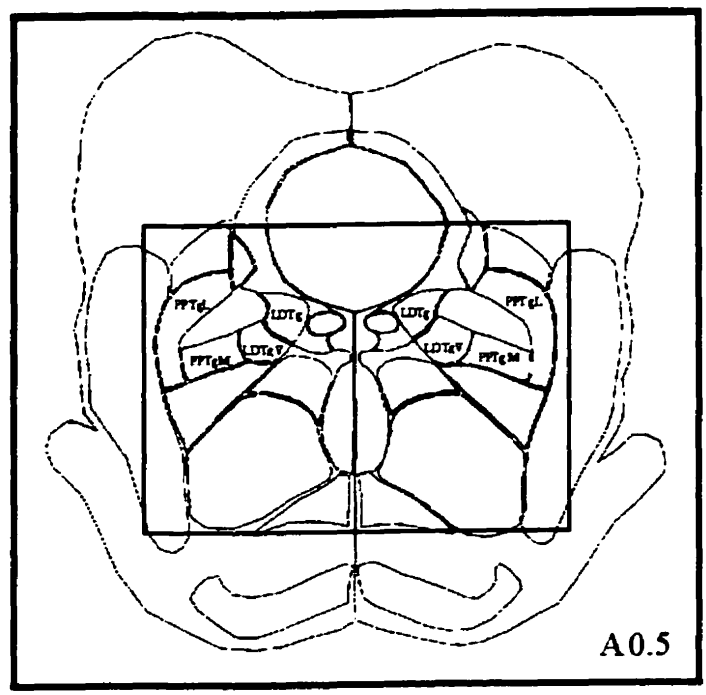

A
B
C
D

Figure 6. Computerized atlas figure through the pontomesencephalic tegmentum (~A 0.5) showing the cholinergic cell group (top) where ChAT+/c-Fos+ cells (circles) and GAD+/c-Fos+ cells (triangles) were mapped (bottom) in representative animals from PSD (left) and PSR (right) groups. Note apparent increase in ChAT+/c-Fos+ cells and GAD+/c-Fos+ cells in the PSR condition compared with the PSD condition. LDTg, Laterodorsal tegmental nucleus; LDTgV, laterodorsal tegmental nucleus, ventral part; PPTgM, pedunculopontine tegmental nucleus, medial part; PPTgL, pedunculopontine tegmental nucleus, lateral part.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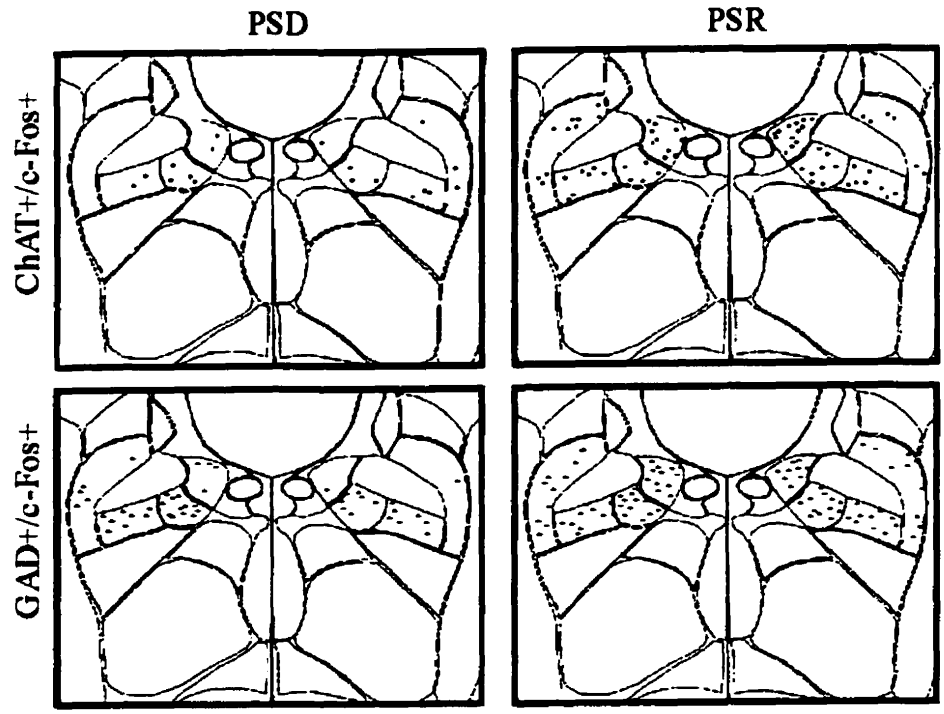
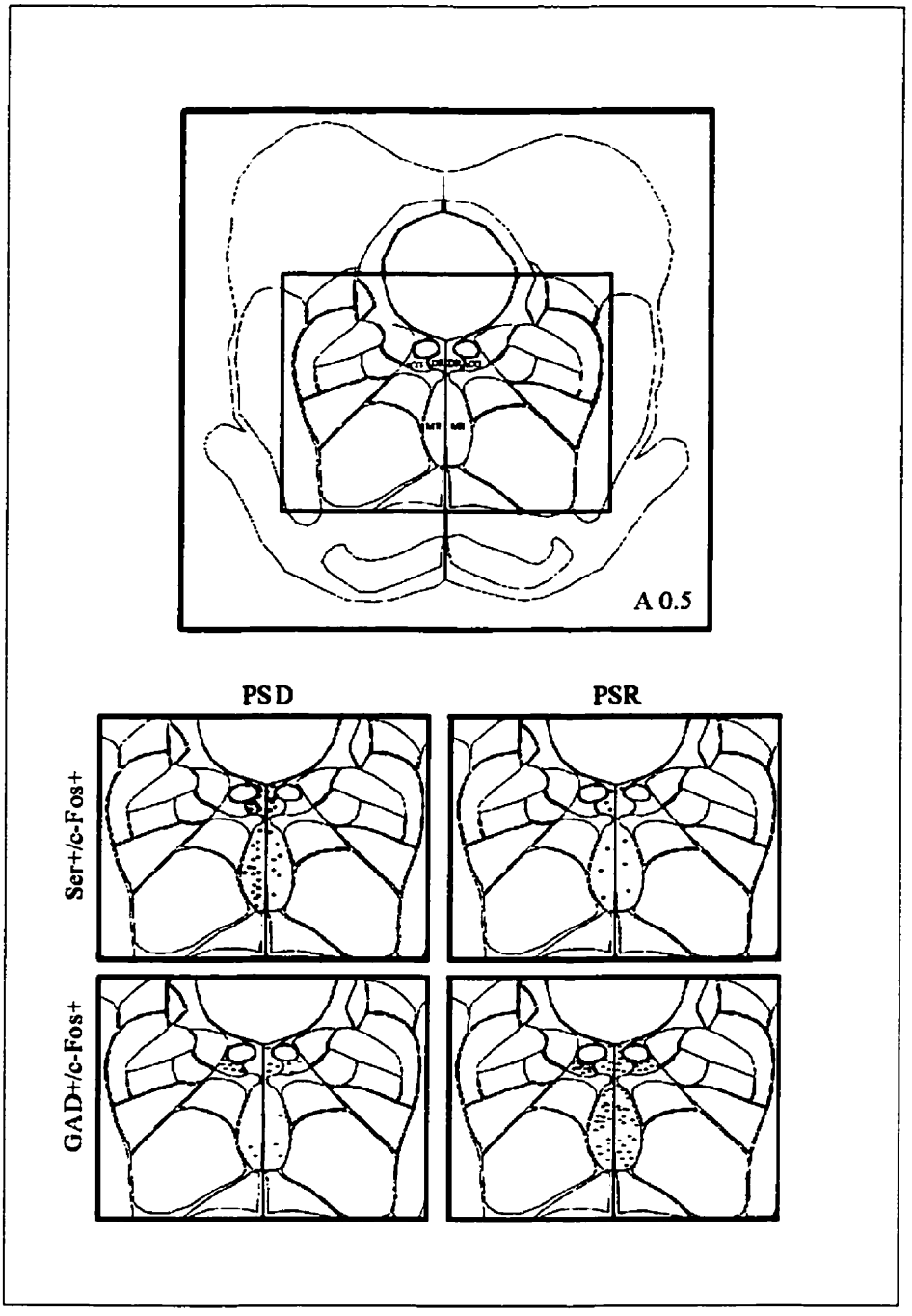

PSD
PSR
ChAT+/c-Fos+
GAD+/c-Fos+

Figure 7. Computerized atlas figure through the pontomesencephalic tegmentum (~A 0.5) showing serotonergic cell group and surrounding rostral central gray area (top) where Ser+/c-Fos+ cells (asterisks) and GAD+/c-Fos+ cells (triangles) were mapped (bottom) in representative animals from the PSD (left) and PSR (right) groups. Note the apparent decrease in Ser+/c-Fos+ cells and increase in GAD+/c-Fos+ cells in the PSR condition compared with the PSD condition. rCG, Rostral central gray; DR, dorsal raphe nucleus; MR, median raphe nucleus.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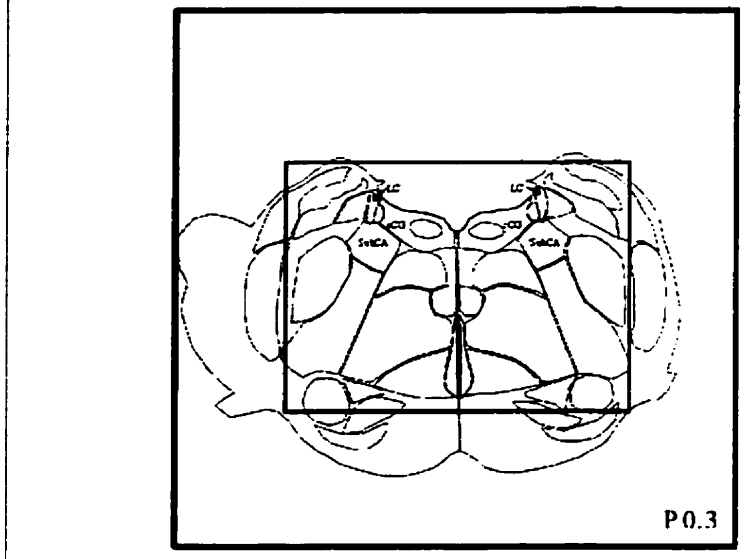
PSD
PSR
Ser+/c-Fos+
GAD+/c-Fos+

Figure 8. Computerized atlas figure through the pontomesencephalic tegmentum (~P 0.3) showing noradrenergic cell group and surrounding caudal central gray (top) where TH+/c-Fos+ cells (squares) and GAD+/c-Fos+ cells (triangles) were mapped (bottom) in representative animals from PSD (left) and PSR (right) groups. Note the apparent decrease in TH+/c-Fos+ cells and increase in GAD+/c-Fos+ cells in the PSR condition compared with the PSD condition. cCG, Caudal central gray; LC, locus coeruleus; SubCA, subcoeruleus nucleus, α part.


LC
LC
SubCA
SubCA
P 0.3

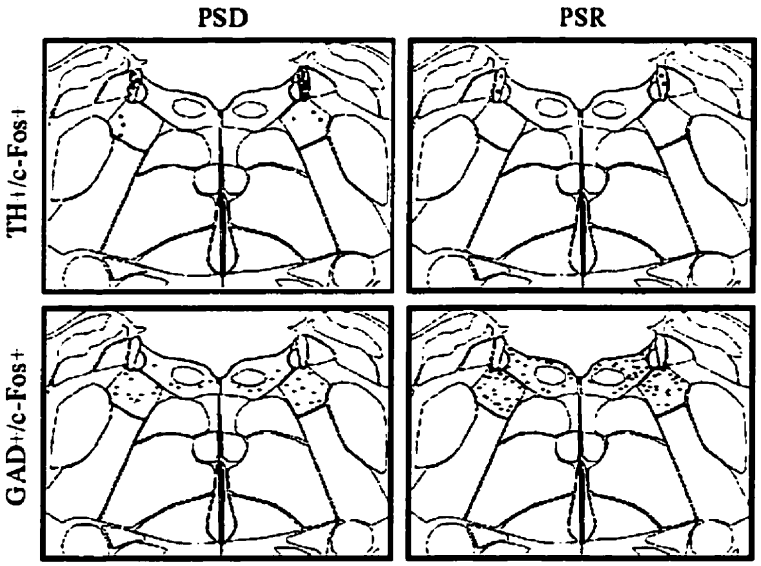
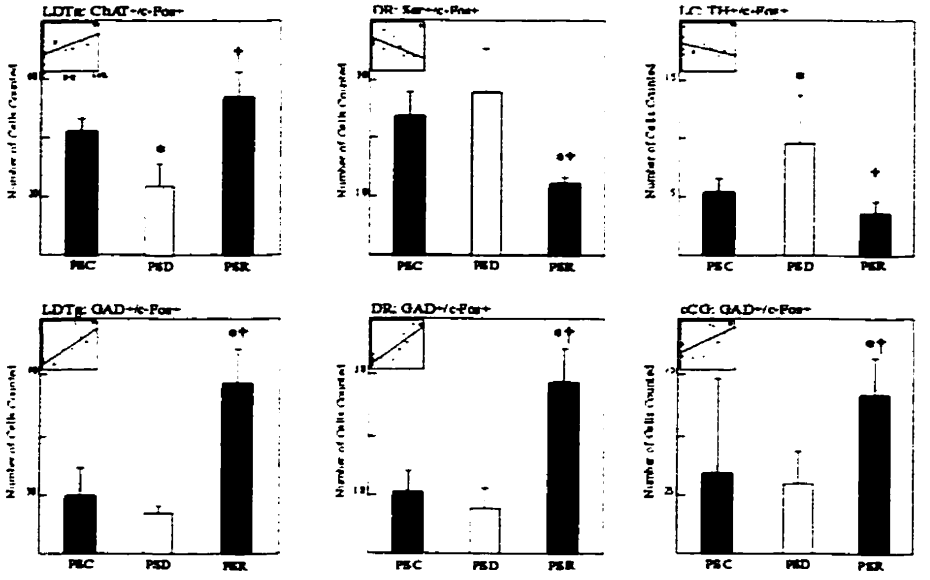

PSD
PSR
TH+/c-Fos+
GAD+/c-Fos+

Figure 9. Numbers of ChAT+/c-Fos+ and GAD+/c-Fos+ neurons in the LDTg, Ser+/c-Fos+, and GAD+/c-Fos+ neurons in the DR, and TH+/c-Fos+ neurons and GAD+/c-Fos+ neurons in the LC and neighboring cCG, respectively, for the three different groups, PSC, PSD, and PSR. As according to the statistics detailed in Table 2, there was a significant main effect of condition in every case shown. According to post hoc tests, there were significant differences between individual conditions as indicated: PSR or PSD versus PSC, *p 0.05; PSR versus PSD, p 0.05. The inserts in the top left corners show linear regression plots and coefficients of cell numbers with the percent of PS across conditions. The scale of the x-axis is consistent for all boxes and represents percent PS (0-40%), as indicated. The scales of the y-axes correspond to the number of cells, as indicated in the respective full scale y-axis for each cell type and nucleus. Correlations between percent time spent in PS and the number of double-labeled cells in each nucleus were examined by multiple linear regression with animal as a covariate (*p 0.05, df = 11). See Table 2 and Materials and Methods for other details.


LDTg: ChAT+/c-Fos+
DR: Sal+/c-Fos+
LC: TH+/c-Fos+
LDTg: GAD+/c-Fos+
DR: GAD+/c-Fos+
cCG: GAD+/c-Fos+
PSC
PSD
PSR

Chapter Three

Pontomedullary Reticular Formation

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3. Pontomedullary Reticular formation

3.1 Introduction

Having confirmed the activation of cholinergic neurons of the pontomesencephalic tegmentum during PS, the next issue approached in this series of studies was whether activity in the pontomedullary brainstem, a projection site of the cholinergic neurons, was altered in PS. There is evidence of a cholinergic induction of the PS characteristic of muscle atonia (Morales et al., 1987), and muscle atonia is believed to be relayed through the medulla since stimulation of this area results in muscle atonia (Magoun and Rhines, 1946) (Chase et al., 1986). Immunohistochemical staining for c-Fos together with the neurotransmitter, serotonin, or the synthetic enzyme for GABA, was used for identification of activation in serotonergic and GABAergic neurons of the reticular and raphe nuclei during PS rebound. The study supports the hypothesis that activation of the pontine reticular nucleus, par oralis (PnO), by possible disinhibition, may activate other systems including GABAergic neurons in caudally located medullary reticular and raphe nuclei which may in turn cause inhibition of motor neurons and/or local inhibition of serotonergic neurons.

3.2 *c-Fos expression in GABAergic, serotonergic, and other neurons of the pontomedullary reticular formation and raphe after paradoxical sleep deprivation and recovery*

3.2.1 Abstract

The brainstem contains the neural systems that are necessary for the generation of the state of paradoxical sleep (PS) and accompanying muscle atonia. Important for its initiation are the pontomesencephalic cholinergic neurons that project into the pontomedullary reticular formation and that we have recently shown increase c-Fos expression as a reflection of neural activity in association with PS rebound after deprivation in rats (Maloney et al., 1999). As a continuation, we examined in the present study c-Fos expression in the pontomedullary reticular and raphe neurons, including importantly GABAergic neurons [immunostained for glutamic acid decarboxylase (GAD)] and serotonergic neurons [immunostained for serotonin (Ser)].

Numbers of single-labeled c-Fos+ neurons were significantly increased with PS rebound only in the pars oralis of the pontine reticular nuclei (PnO), where numbers of GAD+/c-Fos+ neurons were conversely significantly decreased. c-Fos+ neurons were positively correlated with PS, whereas GAD+/c-Fos+ neurons were negatively correlated with PS, suggesting that disinhibition of reticular neurons in the PnO from locally projecting GABAergic neurons may be important in the generation of PS. In contrast, through the caudal pons and medulla, GAD+/c-Fos+ cells were increased with PS rebound, covaried positively with PS and negatively with the electromyogram (EMG). In the raphe pallidus-obscurus, Ser+/c-Fos+ neurons were positively correlated, in a reciprocal manner to GAD+/c-Fos+ cells, with EMG, suggesting that disfacilitation by removal of a serotonergic influence and inhibition by imposition of a GABAergic influence within the lower brainstem and spinal cord may be important in the development of muscle atonia accompanying PS.

3.2.2 Introduction

In early studies, it was demonstrated by Jouvet (1962) that the integrity of the pontomedullary reticular formation was critical for the occurrence of the state of paradoxical [or rapid eye movement (REM)] sleep (PS) and muscle atonia. Subsequent lesion studies indicated that the reticularis pontis caudalis (Jouvet, 1965) was the most important nucleus, yet others indicated that the reticularis pontis oralis was the critical nucleus for the generation of PS (Carli and Zanchetti, 1965). Transections through the upper pons (and not the caudal pons) produced a generalized and enduring muscular atonia, indicating that the oral pontine tegmentum also had the capacity to tonically inhibit muscle tonus (Keller, 1945). This influence was thought to be relayed through the medullary reticular formation, because stimulation therein was shown to produce generalized inhibition of postural tone (Magoun and Rhines, 1946). Indeed, multiple studies have since shown that stimulation in the medullary or pontine reticular formation produces motor inhibition (Chase et al., 1986; Lai and Siegel, 1988; Lai and Siegel, 1991; Kohyama et al., 1998) and that lesions in these areas diminish or eliminate muscle atonia (Jouvet and Delorme, 1965; Henley and Morrison, 1974; Sakai et al., 1979; Hendricks et al., 1982; Friedman and Jones, 1984; Schenkel and Siegel, 1989; Holmes and Jones, 1994). Acetylcholine (ACh) was revealed to be importantly involved (Domino et al., 1968; Karczmar et al., 1970; Jouvet, 1972), and when injected into the pontine reticular formation, its agonist carbachol was shown to elicit PS and/or muscle atonia (George et al., 1964; Mitler and Dement, 1974; Baghdoyan et al., 1984; Morales et al., 1987). Destruction by neurotoxic lesions of the pontomesencephalic cholinergic neurons, which innervate the pontomedullary reticular formation (Jones, 1990, 1991), resulted in a loss of PS and muscle atonia (Jones and Webster, 1988; Webster and Jones, 1988). Substantiating previous claims that presumed cholinergic neurons are active during and thus capable of stimulating PS (ElMansari et al., 1989; Kayama et al., 1992), we recently reported an increase in c-Fos expression, as a reflection of neural activity (Dragunow and Faull, 1989), in choline acetyltransferase (ChAT)-labeled pontomesencephalic neurons in association with PS rebound after deprivation in rats (Maloney et al., 1999).

As a continuation of this research, we sought in the present study to examine c-Fos expression in neurons of the pontomedullary reticular formation and raphe including, importantly, GABAergic and serotonergic neurons in association with PS. GABAergic neurons are codistributed with glutamatergic or serotonergic neurons and include both small locally projecting and larger distantly projecting neurons in some nuclei (Jones et al., 1991; Holmes et al., 1994; Ford et al., 1995; Jones, 1995). In addition to playing a role in the inhibition of pontomesencephalic monoaminergic neurons, as our previous c-Fos study suggested (Maloney et al., 1999), GABAergic neurons within the pontomedullary reticular formation and raphe could play important roles in mediating the central and peripheral changes associated with PS, including muscle atonia (Holmes and Jones, 1994). To assess potentially differential changes in c-Fos expression as a reflection of neural activity according to region and neurotransmitter, we examined c-Fos in glutamic acid decarboxylase (GAD)-immunostained neurons and serotonin (Ser)-immunostained neurons as well as other chemically unidentified neurons in the pontine and medullary reticular formation and raphe after PS deprivation and rebound as executed previously (Maloney and Jones, 1999; Maloney et al., 1999).

3.2.3 Methods and Materials

Animals and surgery. Twelve male Wistar rats (Charles River, Montreal, Quebec, Canada), weighing ~225 gm, were operated under barbiturate anesthesia (Somnotol; 67 mg/kg, i.p.) for the implantation of chronically in-dwelling electrodes for recording the electroencephalogram (EEG) and electromyogram (EMG), as described previously (Maloney et al., 1997, 1999). Animals were allowed 2 or 3 d recovery from surgery in the animal room before being placed in recording chambers for the duration of the experiment.

Recording and experimental procedures. For recording and experimentation, each rat was placed in a Plexiglas box that was contained within a larger electrically shielded recording chamber and maintained on a 12 hr light/dark cycle (with lights on from 7:00 A.M. to 7:00 P.M.). Animals had free access to food and water throughout the experiment.

The EEG and EMG signals were amplified using a Grass model 78D polygraph and subsequently sent to a computer (ALR 386SX) for analog-to-digital conversion, filtering, and storage on hard disk with the aid of Stellate Systems (Montreal, Quebec, Canada) computer software, as described previously (Maloney et al., 1997).

PS deprivation was performed using the flower pot technique that was previously shown to cause a fairly selective deprivation of PS with minimal stress in rats (Mendelson, 1974), as confirmed in its application according to the previously published procedure (Maloney et al., 1999). This deprivation procedure was reported to not be associated with changes in the weight of adrenal glands and thus to not be associated with severe levels of stress (Mendelson, 1974). Each rat was placed on an inverted flower pot that was just large enough (~6.5 cm in diameter) to hold the animal and was surrounded by water. As previously described in detail (Maloney et al., 1999), the experimental protocol was performed over a 4 d period. Recordings were performed in the afternoon (~12:00-3:00 P.M.) during the natural sleep period of the rat, for the 4 consecutive days. On day 1 (the first day before experimental manipulation), a baseline recording was performed on all animals. On the remaining three days of the experiment, the "condition"

was varied for three different groups (with four animals per group): PS control (PSC), PS deprivation (PSD), and PS recovery (PSR). (1) For the control condition, the PSC animals remained on a bed of wood chips in their recording boxes for the 4 d. At the termination of the experiment on day 4, the PSC animals were anesthetized for perfusion (at ~3:00 or 3:30 P.M.) after the afternoon recording period. (2) For the deprivation condition, the PSD animals were placed on flower pots for the second, third, and fourth days of the experiment. On day 4 after the recording period, the PSD animals were anesthetized for perfusion (at ~3:30 P.M.), having been in the deprivation condition for ~53 hr. (3) For the recovery condition, the PSR animals were also placed on flower pots for the second, third, and fourth days like the PSD animals. However, on day 4 after ~50 hr of PS deprivation, the animals were returned to a dry bed of wood chips in their recording boxes to allow for recovery of PS. After the recording period, the animals were anesthetized for perfusion (at ~3:00 P.M.), having been in the PS recovery condition for ~3 hr after PS deprivation of ~50 hr. The experiments were conducted using two recording chambers and thus on two animals at one time, running pairs of PSD and PSR or PSC and PSC animals.

Perfusion and fixation. The animals were killed under barbiturate anesthesia (Somnotol, ~100 mg/kg) by intra-aortic perfusion of a fixative solution (containing 3% paraformaldehyde and 0.2% picric acid). The time between the barbiturate injection and initiation of the perfusion was ~10 min.

Immunohistochemistry. Coronal sections were cut at 25 μm thickness on a freezing microtome. Up to six series of adjacent sections were collected every 200 μm for immunohistochemical processing using the peroxidase-antiperoxidase technique, as previously described (Maloney et al., 1999). For the immunostaining of c-Fos protein, an anti-c-Fos antiserum from sheep (Cambridge Research Biochemicals, Cheshire, UK) was used at a dilution of 1:3000. For neurotransmitter or enzyme immunostaining, rabbit anti-Ser antiserum (1:30,000; Incstar, Stillwater, MN) and rabbit anti-GAD antiserum (1:3000; Chemicon, Temecula, CA) were used. In all brains, one series of sections was immunostained for c-Fos alone using the brown, floccular reaction product 3,3' diaminobenzidine (DAB) as chromogen. In other adjacent sections for the main

experimental series, c-Fos was immunostained in combination with the neurotransmitter or enzyme (revealed with DAB) using a sequential procedure with c-Fos in the second position revealed with the blue granular reaction product benzidine dihydrochloride (BDHC). Controls in the absence of primary antibodies and in the presence of normal sera were routinely run with every single and dual immunostaining procedure to ensure the absence of nonspecific single or dual immunostaining. Brains from sets of PSD-PSR, which were run together experimentally, were processed in the same manner for immunohistochemistry together with an accompanying PSC brain.

Analysis of sleep-wake state data. The EEG was examined by off-line analysis on a computer screen and scored for sleep-wake state by visual assessment of EEG and EMG activity in 20 sec epochs using Eclipse software (Stellate Systems) for each 3 hr recording session, as described previously (Maloney et al., 1999). EMG amplitude was computed (for the total spectrum up to 58.0 Hz). As evaluated by EEG and EMG amplitudes, epochs were scored as one of the three major states wake, slow wave sleep (SWS), or PS or transition (t) stages, tSWS or tPS, between states (Maloney et al., 1999).

The number of epochs scored in each state was calculated as a percentage of total epochs in the 3 hr recording session for each day. An overall statistic was performed using a repeated-measures ANOVA with two trial factors ("state" and "day") and one grouping factor (condition). Data were further analyzed per state by repeated-measures ANOVA tests with one trial factor (day) and one grouping factor (condition). When a main effect of condition was significant, *post hoc* tests were performed per day across groups (PSR or PSD vs PSC; PSR vs PSD) using Fisher's pairwise comparisons. In the case in which there was a significant difference between groups (condition), a repeated-measures ANOVA and a *post hoc* test were performed to examine whether there was a significant difference in state within groups by comparing the final recording day (day 4) and baseline day (day 1). Changes in average EMG activity (for the 3 hr recording period) were also assessed. For this purpose, EMG amplitude values were normalized by using the ratio of day 4 values to those of the baseline, day 1 values for the individual animals. Statistical tests of variance in EMG activity across conditions were performed for day

4 using one-way ANOVA with condition as a grouping factor and *post hoc* tests across conditions performed by Fisher's pairwise comparisons.

Analysis of immunohistochemical data. Cells in which the nucleus was immunostained for c-Fos were counted as c-Fos+, and their numbers were tabulated for each nucleus on each section with the aid of an image analysis system. The sections were viewed with a Leitz Orthoplan microscope equipped with an *x/y* movement-sensitive stage and CCD camera attached to a computer. Single- and dual-immunostained cells were mapped using a computer-based image analysis system (Biocom, Paris, France) with a resident atlas of sections through the pontomedullary reticular formation (Jones, 1995). Single c-Fos-immunostained cells were mapped and counted unilaterally every 400 μm in the pons at representative stereotaxic levels corresponding approximately to anterior (A) 0.9 through to posterior (P) 0.3, and every 800 μm in the medulla at representative levels corresponding approximately to P 1.1 through to P 4.3 depending on the specific nucleus (Paxinos and Watson, 1986; Jones, 1995). Dual-immunostained GAD+/c-Fos+ cells were mapped and counted bilaterally every 400 μm at representative stereotaxic levels corresponding approximately to A 0.9 through to P 0.3, and every 800 μm in the medulla at representative levels corresponding approximately to P 1.1 through to P 4.3, depending on the specific pontine or medullary nuclei. Dual-immunostained Ser+/c-Fos+ cells were mapped and counted bilaterally every 400 μm through the pons and medulla at representative stereotaxic levels corresponding approximately to P 0.7 through to P 4.3 depending on the specific raphe or reticular nucleus. The experimenter (K.J.M.) mapping the cells did not have knowledge of the experimental group (PSD-PSR-PSC) to which the individual brains belonged. She was only given this information after all the data were tabulated on computer spreadsheets, when the condition group was inserted for the statistical analysis of the completed data set. Cell counts were tabulated automatically for each reticular and raphe nucleus and averaged for the pontine and medullary regions.

Whether the number of cells counted over multiple levels (sections) per nucleus per animal varied as a function of condition was examined for the regions of the pons and medulla. A one-way ANOVA was used, with condition and "nucleus" as grouping factors and "section" and "animal" as covariates. In the case of a significant main effect in a

region, statistical differences in the number of cells in individual nuclei within the pontine or medullary regions were subsequently examined using a one-way ANOVA with condition as grouping factor and section and animal as covariates. When there was a significant main effect of condition, differences in cell counts between individual conditions were analyzed by *post hoc* tests using Fisher's pairwise comparisons. When differences in cell counts were significant across conditions in a region or nucleus, a general linear model was used to determine whether cell counts varied as a function of PS or other states. Using an interactive stepwise function with multiple linear regression, PS was entered as the independent variable, testing the hypothesis that it accounted significantly for a proportion of the variance in cell counts across conditions. In the absence of a significant relationship of cell counts with PS, SWS was stepped into the equation, and PS and SWS were evaluated in the model. In the absence of a significant relationship with PS and SWS, PS was stepped out and SWS was examined as a single independent variable. Finally, if these models including PS, PS, and SWS or SWS were not significant, wake was evaluated as the independent variable in the model. In addition, EMG was examined separately as the independent variable. For nuclei in which a significant relationship was found between the number of cells and state or EMG, simple correlations were examined. For these simple correlations, the total number of labeled cells counted was calculated for each reticular and raphe nucleus per animal by adding averaged bilateral counts across sections. To assess the correlation between total cell number and sleep states in the case in which a significant relationship was found with PS and SWS in the model, the standard partial regression coefficient for PS and SWS was calculated, and the residuals [PS(sws)] were plotted in the regression with cell number. All statistics were performed using Systat (version 9) for Windows (Evanston, IL). Figures were prepared for publication using CorelDraw (Ottawa, Ontario, Canada).

3.2.4 Results

Sleep-wake state changes

In comparing the sleep-wake states across the two experimental and control conditions (PSR vs PSD; PSR and PSD vs PSC) or between day 4 (the final experimental day, representing the 3 hr period before anesthesia and perfusion) and day 1 (the first day before experimental manipulation, representing the baseline day or condition), it was apparent that there were marked and significant changes in the amount of time spent in PS (Table 1), as reported previously (Maloney et al., 1999). After ~53 and 50 hr of deprivation, respectively, PS represented 0% in the PSD condition and ~28% in the PSR condition, as compared with ~15% in the PSC condition. There were also changes in the percentage of time spent in SWS and tPS in the PSR condition as compared with the PSD and PSC conditions, but they were not significant when compared with the respective baseline condition within each group (day 1). In the PSD condition, there was an increase in the percentage of time spent in wake as compared with the PSC and PSR conditions, which did represent a significant increase when compared with the respective baseline condition (day 1). However, PS was the one state that was significantly decreased in the deprivation and significantly increased in the recovery condition, as compared with both control and baseline conditions. The parallel changes in SWS and reciprocal changes in wake, nonetheless led us to consider the potential contribution of SWS or wake in addition to PS toward the changes in cell counts across conditions and thus to allow their inclusion in a general linear model used to examine whether changes in c-Fos-expressing cells across conditions were caused by changes in PS or other states.

Average EMG activity was also found to vary significantly across conditions ($F = 4.271$, df = 2, 9, $p \leq 0.05$), being higher in PSD (3.33 ± 0.96) than in PSC (1.18 ± 0.15, $p \leq 0.030$) and PSR (1.26 ± 0.32, $p \leq 0.035$), and not differing between the latter two groups. EMG was also examined independently as potentially contributing to variations in c-Fos-expressing cells across conditions.

c-Fos expression in the pontomedullary reticular formation and raphe

c-Fos expression was evident in single-immunostained cells (c-Fos+) in reticular and raphe nuclei through the pontine and medullary regions of all animals (Fig. 1*A*). Dual-immunostained GAD+/c-Fos+ cells were evident in the oral and caudal pontine reticular nuclei (PnO and PnC), where they were codistributed with single-immunostained c-Fos+ cells (Fig. 1*A*). These GAD+/c-Fos+ cells were relatively small cells. Similar GAD+/c-Fos+ cells were seen among single-immunostained c-Fos+ cells in rostral and caudal medullary gigantocellular reticular nuclei (GiR and GiC). GAD+/c-Fos+ cells were also apparent in the α and ventral parts of the gigantocellular field (GiA and GiV) and in the medially adjacent magnus and pallidus-obscurus raphe nuclei (RM and RPO). In these regions, they included relatively large cells (Fig. 1*B*) and were codistributed with large Ser+/c-Fos+ cells seen in adjacent sections (from RPO) (Fig. 1*C*).

c-Fos expression in the nuclei of the pontine reticular formation

The numbers of single-immunostained c-Fos+ cells in the pontine tegmentum varied significantly as a function of experimental condition and were significantly greater in the PSR condition than in the PSD and PSC conditions (Table 2, c-Fos+, Pons, Average). In the individual nuclei, the number varied significantly across conditions in the rostral, PnO, but not in the caudal, PnC, nucleus. Within the PnO, numbers of c-Fos+ cells were greater in the PSR condition in comparison to the PSD and PSC conditions (Table 2, Fig. 2) (c-Fos+, Pons, PnO). Across the pontine tegmentum (Average) and in the PnO, but not in the PnC, PS was found to account significantly for a portion of the variance in c-Fos+ cell counts and to covary positively with the counts in a linear regression model (Table 3). The number of dual-immunostained GAD+/c-Fos+ cells in the pontine tegmentum also varied significantly as a function of experimental condition yet differentially according to nucleus, as revealed by a significant interaction of condition with nucleus (Table 2, GAD+/c-Fos+, Pons, Average). In the PnO, the number of GAD+/c-Fos+ cells was lower in the PSR condition in comparison to the PSD and PSC

conditions (Table 2, Fig. 2) (GAD+/c-Fos+, PnO). In the PnC, the number of GAD+/c-Fos+ cells was higher in the PSR condition in comparison to the PSD condition (Table 2, Fig. 3). In the PnO, PS alone did not account significantly for variance in GAD+/c-Fos+ cell counts but together with SWS did so in a linear regression model in which %PS covaried negatively and %SWS covaried positively with the cell counts (Table 3). In the PnC, the number of GAD+/c-Fos+ cells covaried positively and significantly with PS alone (Table 3).

The variation of cell counts as a function of PS in the PnO was further substantiated by simple correlations using total cells counted per nucleus per animal. The total number of c-Fos+ cells was positively correlated with %PS, whereas the total number of GAD+/c-Fos+ cells was reciprocally negatively correlated with %PS after partialing out the correlation with %SWS (PS[sws]) (Fig. 4).

c-Fos expression in the nuclei of the medullary reticular formation and raphe

The numbers of single-immunostained c-Fos+ cells in the medullary reticular formation (GiR, GiA_1 GiC, and GiV) and raphe (RM and RPO) did not vary significantly as a function of experimental condition (Table 2, c-Fos+, Medulla).
The numbers of dual-immunostained Ser+/c-Fos+ cells in the medullary reticular and raphe nuclei did vary significantly as a function of experimental condition (Table 2, Ser+/c-Fos+, Medulla, Average). There was a significant increase in the PSD condition in comparison to the PSC condition for the region and in the GiA nucleus as revealed by *post hoc* tests for the individual nuclei. The numbers of Ser+/c-Fos+ cells were also higher in the PSR condition in comparison to the PSC condition for the region and in the GiA nucleus and did not differ significantly from the PSD condition for the region or in any nucleus (presented in Table 2; not shown in schematic figures). In an examination of the relationship between the cell counts and sleep state, it was found that neither for the medulla (Average) nor any of the individual nuclei did sleep state (PS, PS and SWS, or SWS) significantly account for any proportion of the variance in Ser+/c-Fos+ cell counts.

However, wake was found to account for a significant although small proportion of the variance for the medulla (Average) and the GiA nucleus and to covary positively therein with cell counts in a linear regression model. EMG was found to covary positively with the number of Ser+/c-Fos+ cells in the RPO.

The numbers of dual-immunostained GAD+/c-Fos+ cells in the medulla varied significantly as a function of condition. The cell counts for the PSR condition were higher compared with those of the PSD and PSC conditions (Table 2, GAD+/c-Fos+, Medulla, Average). There was also a significant interaction of condition with nucleus. Across the individual nuclei, the numbers varied significantly as a function of condition in the reticular and raphe nuclei of the GiR, GiA, GiC, and RM. The variation was evident as higher numbers of cells in the PSR condition as compared with the PSD and PSC conditions within these nuclei (Table 2, Fig. 3). In a linear regression model, PS significantly accounted for a proportion of the variance in GAD+/c-Fos+ cell counts for the entire medulla (Medulla, Average) and covaried positively with cell counts in the model (Table 3). This relationship was also significant in nuclei within the GiR, GiA, GiC, and RM. In the caudal medulla, cell counts did not vary significantly as a function of PS, PS and SWS or SWS, but did so with wake as the independent variable in the RPO. Across the medulla (Average), in the GiR and GiC reticular nuclei and in the RM and RPO raphe nuclei, EMG amplitude also significantly accounted for a proportion of the variance and covaried negatively with the GAD+/c-Fos+ cell counts in the linear regression model (Table 3).

In the RPO, where EMG covaried significantly with Ser+/c-Fos+ and GAD+/c-Fos+ cell counts, simple correlations involving the total number of cells counted per animal showed a positive correlation of EMG with Ser+/c-Fos+ cell numbers and a reciprocal negative correlation with GAD+/c-Fos+ cell numbers, as illustrated in Figure 5.

3.2.5 Discussion

The present results showed that through the pontomedullary reticular formation and raphe, the one region where c-Fos+ cells were significantly increased in number in association with an increase in PS was the PnO. In this same region, GAD+/c-Fos+ cells were significantly decreased, whereas in other regions including the PnC and medulla, they were significantly increased in association with PS. Through the medullary reticular and raphe nuclei, Ser+/c-Fos+ cells varied in number, but not as a function of PS, instead as a function of wake or EMG, significantly increasing in the RPO with increasing muscle tonus. Reciprocally, GAD+/c-Fos+ cells in the same areas significantly decreased in number with increasing muscle tonus. These results suggest a differential involvement of reticular and raphe nuclei according to both region and neurotransmitter in the generation of sleep-wake states and associated changes in muscle tonus.

To be noted, the present results are interpreted according to the assumption that c-Fos expression reflects increased neuronal discharge, although it can also reflect increases in other calcium-mediated cellular processes (Morgan and Curran, 1986; Dragunow and Faull, 1989). Differences in numbers of c-fos-expressing cells, moreover, were examined and interpreted as a function of the modified sleep-wake states, although they could also be attributable in part to different levels of stress (Cullinan et al., 1995), undoubtedly present, although reportedly attenuated in such chronic experiments (Mendelson, 1974; Stamp and Herbert, 1999).

The oral pontine reticular formation and the state of PS

Since early studies involving lesions of the brainstem in the cat, the pontine reticular formation has been known to be critical for the generation of PS, although whether the reticularis pontis caudalis or oralis is most important was not resolved (Carli and Zanchetti, 1965; Jouvet, 1965). In the present study in rats, the number of c-Fos+ cells was significantly increased and most particularly in the PnO in association with PS

rebound. The PnO is densely innervated by cholinergic fibers originating in the pontomesencephalic cholinergic neurons (Jones, 1990) that play a critical role in initiating PS (Webster and Jones, 1988) and express c-Fos in association with PS rebound (Maloney et al., 1999). It is within the PnO where it was first discovered that the cholinergic agonist carbachol could elicit a PS-like state in the cat (George et al., 1964), and where, albeit in the dorsal, ventral, medial, or lateral part, it has since been confirmed many times to have the capacity to elicit all components of PS in cats and rats, including cortical activation, hippocampal theta, muscle atonia, and phasic activity (Mitler and Dement, 1974; Katayama et al., 1984; Gnadt and Pegram, 1986; Morales et al., 1987; Vanni-Mercier et al., 1989; Elazar and Paz, 1990; Yamamoto et al., 1990; Takakusaki et al., 1993; Vertes et al., 1993; Bourgin et al., 1995; Garzon et al., 1998; Horner and Kubin, 1999). This region, originally called reticularis pontis oralis in rabbit and man (Meesen and Olszewski, 1949; Olszewski and Baxter, 1954) and more recently paralemniscal tegmental field in cat (Berman, 1968), would appear to partially overlap with the region called peri-LC by Sakai (1988) in the cat, where he found carbachol injections to be most effective in eliciting PS (Vanni-Mercier et al., 1989) and where he also recorded a large number of "specific PS-on" neurons. Within the PnO, neurons give rise to projections ascending into the forebrain and others descending into the lower brainstem and/or spinal cord (Jones and Yang, 1985; Jones, 1995), thus being in a position to modulate both forebrain and bulbospinal activities in response to carbachol injections and in the natural generation of PS. In single-unit recording studies, it has been found that a major proportion of neurons in the pontine reticular formation are excited by carbachol and ACh (Greene and Carpenter, 1985), including those that are normally active during PS (Shiromani and McGinty, 1986). Moreover, in studies with carbachol-induced PS, increases in c-Fos-expressing cells have also been reported in the rostral pontine tegmentum of cats (Shiromani et al., 1992; Yamuy et al., 1993).

In the PnO and not in the PnC, the numbers of c-Fos-expressing GABAergic neurons were actually decreased in association with PS rebound. The GABAergic neurons in the PnO could correspond to the minor proportion of pontine neurons that have been shown to be directly inhibited by carbachol and ACh (Greene and Carpenter, 1985; Shiromani and McGinty, 1986; Gerber et al., 1991; Nunez et al., 1997) and become

inactive during carbachol-induced PS-like phenomena (Nunez et al., 1991). Because in the PnO the numbers of c-Fos+ cells were reciprocally increased with PS rebound, our results suggest that reticular neurons may be released from inhibition by local GABAergic neurons during naturally occurring PS. However, although there was a decrease in GABAergic c-Fos-expressing neurons in animals of the PSR group, there was not a significant simple correlation between GAD+/c-Fos+ cells and the %PS. When the variation attributable to SWS was taken into account, however, the variation particular to PS became evident, and the results indicated that GABAergic c-fos-expressing neurons were negatively correlated with PS. One possible explanation for this finding is that in the PnO GABAergic neurons may be active during SWS, as "SWS-on," and become inactive during PS, as "PS-off" cells. Accordingly, local GABAergic neurons could be responsible for inhibiting PnO "PS-on" neurons during SWS and wake and disinhibiting them with PS. Indeed, some PS-on neurons in the pontine tegmentum have been shown to be excited by iontophoretic application of the $GABA_A$ antagonist bicuculline, when applied during SWS (Sakai and Koyama, 1996). Moreover, it has recently been reported by Chase and his colleagues (Xi et al., 1999) that injections of bicuculline into the nucleus reticularis pontis oralis elicit the state of PS, lending credence to the present results and also indicating that GABAergic PnO neurons may play a determining role in PS generation.

The pontomedullary reticular formation - raphe and muscle atonia

Within the medullary reticular and raphe nuclei, as in the PnC, there was no significant variation in numbers of single-immunostained c-Fos+ neurons across conditions. On the other hand, there was a significant increase in dual-immunostained GAD+/c-Fos+ neurons in association with PS rebound. These results would suggest that the small, GABAergic neurons within the caudal pontine and medullary reticular and raphe nuclei may be active during PS and responsible for inhibiting other neurons the activity of which is not compatible with PS. In many nuclei, GABAergic c-Fos-expressing neurons also covaried negatively with EMG. It is thus likely that small GABAergic cells with local projections (Jones et al., 1991; Holmes et al., 1994) serve to inhibit nearby excitatory reticulo-spinal or raphe-spinal projection neurons and thus to

effect a disfacilitation of motor neurons during PS. In the ventral reticular and midline raphe nuclei, there were also relatively large GABAergic c-Fos-expressing neurons. Similarly large GABAergic neurons have been shown to project to the spinal cord (Reichling and Basbaum, 1990; Holstege, 1991; Jones et al., 1991) and postulated to provide a direct inhibitory influence in the dorsal and ventral horns, respectively, coming from the more rostral (RM and GiA) and caudal (RPO and GiV) nuclei and thus, respectively, influencing sensory and motor activity (Basbaum et al., 1978; Skagerberg and Bjorklund, 1985). Identified medullary reticulo-spinal neurons have been found to increase their discharge rate in association with hyperpolarizing potentials recorded from spinal motor neurons during carbachol or PS atonia (Kanamori et al., 1980; Takakusaki et al., 1994). Such reticulo-spinal inhibitory neurons could be GABAergic but could also well be glycinergic (Holstege and Bongers, 1991). There is direct evidence for an important role of glycine in the inhibition of spinal motor neurons during PS atonia (Chase et al., 1989). Yet there is also evidence to suggest that GABA release may be increased in the region of motor neurons during motor suppression and accordingly that GABA in addition to glycine may be involved (Kodama et al., 2000). It is also possible as shown in the spinal cord that neurons may contain and corelease GABA and glycine (Todd and Sullivan, 1990; Jonas et al., 1998).

Although numbers of Ser+/c-Fos+ neurons in the medullary reticular and raphe nuclei were altered by PS deprivation, they were not directly correlated with %PS, for similar reasons perhaps to the reported lack of significant variation in c-Fos-expressing serotonergic neurons reported in association with carbachol-induced PS (Yamuy et al., 1995). Across the medulla, serotonergic c-Fos-expressing neurons covaried positively with wake and moreover in the RPO correlated positively with EMG, where the GABAergic c-Fos-expressing neurons were reciprocally negatively correlated with EMG. These results parallel those reported after neurotoxic lesions of the medullary reticular formation and raphe, after which muscle atonia was disrupted and the degree of muscle tonus during PS was positively correlated with the number of surviving serotonergic neurons and negatively correlated with that of surviving GABAergic neurons (Holmes and Jones, 1994; Holmes et al., 1994). That these relationships emerge here with c-Fos expression in the raphe pallidus-obscurus nuclei may reflect the fact that the major

projections from the serotonergic and nonserotonergic neurons of these caudal nuclei are to the ventral horn (Basbaum et al., 1978; Skagerberg and Bjorklund, 1985) and that the serotonergic neurons therein discharge most particularly in association with motor activities (Jacobs and Fornal, 1991; Veasey et al., 1995). It is also known that serotonin has an excitatory action on spinal and bulbar motor neurons (Fung and Barnes, 1989; White and Fung, 1989). As supported by recent evidence (Kubin et al., 1993; Yamuy et al., 1999; Kodama et al., 2000), our results would suggest that during PS muscle atonia, motor neurons may undergo disfacilitation by removal of a serotonergic influence and inhibition by imposition of a GABAergic, in addition to a glycinergic, influence.

In summary, the present results provide suggestive evidence for differential and important roles of pontomedullary GABAergic cell populations in relation to reticular and serotonergic neurons in the determination of the state of PS and accompanying muscle atonia.

3.3 Conclusions

The pontine reticular nucleus, pars oralis (PnO), demonstrates increased activity during PS, and this increase is hypothesized to be due, in part, to disinhibition by local GABAergic neurons that exhibit a decrease in activity during PS. Previously established as increasing in activation during PS, and known to provide a cholinergic innervation to the PnO, the cholinergic neurons of the pontomesencephalic tegmentum may be responsible for this disinhibition during PS. Activation of the cholinoceptive PnO may initiate the generation of the PS characteristic of muscle atonia by activation of medullary mechanisms hypothesized to be responsible for its generation. Indeed, injections of the cholinergic agonist, carbachol, into the PnO produces muscle atonia (George, 1964), while lesions of the descending cholinergic fibers result in a decrease in muscle atonia (Webster and Jones, 1988). This study further confirms that GABAergic activity is increased in the medullary reticular and raphe nuclei during PS, and in the medial reticular nuclei the number of active GABAergic numbers covaries negatively with EMG. In the raphe pallidus obscurus, where the number of active serotonergic neurons correlates positively with EMG, the number of active GABAergic neurons correlates negatively with EMG. These results support the notion of a medullary generation of the PS characteristic of muscle atonia, which may be initiated by influences from the cholinoceptive PnO region.

3.4 References

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3.5 Tables and Figures

Table 1.

Percentage of time spent in wake, tSWS (transition from wake into SWS), SWS, tPS (transition from SWS to PS), and PS states during the ~3 hr baseline recording period (day 1) and during the final ~3 hr recording period before they were killed (day 4) for animals in each condition (mean ± SEM, n = 4). Significant differences per state between conditions were tested over the 4 d by repeated-measures ANOVA with one trial factor (day) and one grouping factor (condition). When there was a significant main effect for condition for one of the states, post hoc tests were performed per day across conditions using Fisher's pairwise comparisons. In the cases in which a significant difference between conditions was found, another post hoc test was performed by repeated measures to determine whether there was a significant difference between day 4 and day 1 within that group. Significant differences with respect to PSC is indicated by * $p \leq 0.05$ and with respect to PSD by $p \leq 0.05$. Symbols in parentheses indicate a lack of significant difference relative to baseline, and those not in parentheses indicate a significant difference relative to baseline in the repeated-measures tests.

Table 1. The percent of sleep-wake states on day 1 and day 4 for each condition (PSC, PSD and PSR).

CONDITION	STATE	DAY 1	DAY 4
PSC	%Wake	21.82 ± 5.10	21.00 ± 2.00
	% *t*SWS	27.34 ± 1.43	30.00 ± 5.00
	%SWS	11.50 ± 3.14	9.00 ± 3.00
	% *t*PS	25.75 ± 4.21	25.00 ± 4.00
	%PS	11.61 ± 2.31	14.50 ± 6.50
PSD	%Wake	21.65 ± 3.59	56.32 ± 3.87 *
	% *t*SWS	36.92 ± 7.02	34.00 ± 4.74
	%SWS	8.64 ± 2.46	3.04 ± 1.53
	% *t*PS	23.54 ± 5.01	6.25 ± 1.03 (*)
	%PS	12.43 ± 4.57	0.0 ± 0.0 *
PSR	%Wake	20.89 ± 2.88	16.36 ± 1.28 (†)
	% *t*SWS	21.88 ± 3.83	10.75 ± 2.60
	%SWS	25.64 ± 1.67	25.51 ± 1.06 (*,†)
	% *t*PS	18.25 ± 4.42	19.25 ± 1.32 (*,†)
	%PS	12.83 ± 1.23	28.37 ± 1.86 *,†

Table 2.

Cell counts were taken from adjacent series of sections cut at 25 µm and collected at 200 µm intervals. A significant main effect for condition is indicated by •,••,••• or ($p \leq 0.05$, 0.01, or 0.001) in the column under F, and placed in parentheses when there was a significant interaction with nucleus for the Average across nuclei. Individual ANOVAs were also performed for each nucleus with condition as grouping factor and section and animal as covariates (•,••,or •••, $p \leq 0.05$, 0.01, or 0.001). With significant main effects of condition, post hoc tests between individual conditions were performed using Fisher's pairwise comparisons. A significant difference with respect to PSC is indicated by *, **, or *** ($p \leq 0.05$, 0.01, or 0.001) and with respect to PSD by †,††or †††; ($p \leq 0.05$, 0.01, or 0.001).

[a] c-Fos+ cell numbers correspond to the mean ± SEM of the average number of single-immunostained c-Fos+ cells counted unilaterally per section per nucleus in four animals per condition. c-Fos+ cells were counted as follows: in two sections at 400 µm intervals for the PnO (at ~A0.9 and ~A0.5) and PnC (at ~A0.1 and ~P0.3), in three sections at 800 µm intervals for the GiR, GiA, and RM (at ~P1.1, ~P1.9, and ~P2.7), and in two sections at 800 µm intervals for the GiC, GiV, and RPO (at ~P3.5 and ~P4.3).

[b] Ser+/c-Fos+ and GAD+/c-Fos+ cell numbers correspond to the mean ± SEM of the average number of dual-immunostained cells counted bilaterally per side per section per nucleus in three animals per condition. Ser+/c-Fos+ cells were counted as follows: in six sections at 400 µm intervals for the GiA and RM (between ~P0.7 and ~P2.7) and in four sections for the RPO (between ~P3.1 and P4.3). GAD+/c-Fos+ cells were counted as follows: in two sections at 400 µm intervals for the PnO (at ~A0.9 and ~A0.5) and PnC (at ~A0.1 and ~P0.3), in three sections at 800 µm intervals for the GiR, GiA, and RM (at ~P1.1, ~P1.9, and ~P2.7), and in two sections at 800 µm intervals for the GiC and GiV (at ~P3.5 and ~P4.3).

[c] Statistical tests of the variation in the average cell numbers across condition were performed using a one-way ANOVA with condition and nucleus as grouping factors, and section and animal as covariates.

Table 2. Number of c-Fos+, GAD+/c-Fos+ or Ser+/c-Fos+ cells counted per section in nuclei of the pontomedullary reticular formation or raphe across PSC, PSD and PSR groups.

	c-Fos+[a]				Ser+/c-Fos+[b]				GAD+/c-Fos+[b]			
	PSC	PSD	PSR	F	PSC	PSD	PSR	F	PSC	PSD	PSR	F
PONS												
PnO	305.63 +44.71	382.18 +30.12	482.88+17.63 ●●	**,†					26.00+ 2.00	19.08 + 1.40 *	15.42 + 2.01 ●●●	***,†
PnC	226.00 +21.84	222.13 +21.31	269.63+ 30.91						16.50+ 2.46	11.33 + 2.09	20.83+ 3.65 ●	††
Average[c]	*265.81 +26.14*	*302.13+ 27.28*	*376.25 +32.46* ●●●	***,†					*21.25+ 1.84*	*15.21+ 1.37* *	*18.13+ 2.12* (●)	
MEDULLA												
GiR	153.25+ 16.31	147.83 +11.97	178.67 +14.32						11.33+ 2.09	5.50 + 1.06	40.67 +10.40 ●●●	***,††
GiA	66.07 + 8.73	64.25+ 9.61	85.17+12.28		2.90 + 0.29	5.43+ 0.55 ***	5.30 + 0.80 ●●●	***	8.75+ 1.38	7.50+ 2.31	25 42 + 7.05 ●●	**,††
RM	27.83 + 2.66	30.00+ 3.10	26.67+ 3.25		2.31+ 0.23	2.67 + 0.31	2.46+ 0.38		3.22 + 0.48	3.44+ 0.83	6.61 + 0.73 ●●●	***,††
GiC	130.57+16.50	133.50 +11.06	124.86+ 9.89						10.20+ 1.81	7.58 + 1.25	19.00+ 4.11 ●	*,††
GiV	46.14 +10.32	50.86 + 5.07	55.13 + 6.65						4.30 + 1.01	5.17+1.52	4.00 + 0.82	
RPO	14.29+ 2.85	18.25+ 2.73	18.50+ 2.75		3.57+ 0.42	4.21+ 0.51	2.88+ 0.43		3.30 + 0.68	1.83 + 0.39	3.25 + 0.80	
Average[c]	*75.61 + 8.24*	*75.43+ 7.29*	*84.57+ 8.54*		*2.83+ 0.18*	*4.00+ 0.29* ***	*3.52+ 0.36* ●●●	*	*6.63+ 0.65*	*5.04+ 0.57*	*15.73+ 2.49* (●●●)	***,†††

Table 3.

A general linear model was used to test whether cell counts varied significantly as a function of state and/or EMG. Using multiple linear regression with an interactive stepwise procedure for adding or removing variables, a model was examined in all regions in which a significant main effect of condition was found (see Table 2). To test the principal hypothesis that cell counts varied as a function of PS, %PS alone was evaluated first. If not significant, %SWS was stepped in, and the regression for %PS and %SWS was evaluated in the model. If not significant, %SWS alone was evaluated. When none of the sleep states (PS, PS and SWS or SWS alone) were significant, %wake alone was tested in the model. %EMG was also evaluated independently. Significant models are indicated with the state(s) showing the sign (+ or) of the covariation, the squared regression coefficient (variance), and the F value of the model. Dashes indicate a lack of significance for any of the states and EMG tested. Probabilities are indicated as *, **, or ***, ($p \leq 0.05, 0.01$, or 0.001).

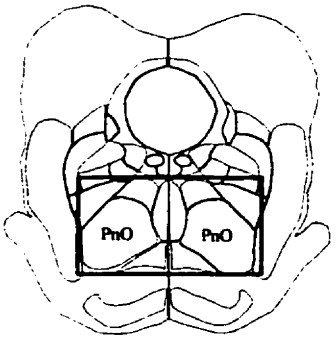
Table 3. Relationship between the number of c-Fos+, GAD+/c-Fos+ or Ser+/c-Fos+ cell counts and states (or EMG) as assessed by a linear regression model (testing for %PS, %PS and %SWS, %SWS or %Wake, or for EMG).

	c-Fos+			Ser+/c-Fos+			GAD+/c-Fos+		
	State	r^2	F	State	r^2	F	State	r^2	F
PONS									
PnO	+PS	0.25	7.03 *				-PS + SWS	0.41	11.64 ***
PnC							+PS	0.13	5.28 *
Average	*+PS*	*0.11*	*5.69* *						
MEDULLA									
GiR							+PS	0.25	11.14 **
							(-EMG	0.25	11.14 **)
GiA				+Wake	0.04	4.05 *	+PS	0.17	6.81 *
RM							+PS	0.13	7.91 **
							(-EMG	0.10	5.70 **)
GiC							+PS	0.16	6.24 *
							(-EMG	0.11	4.09 *)
GiV									
RPO							-Wake	0.12	4.43 *
				(+EMG	0.07	4.86 *)	(-EMG	0.13	4.57 *)
Average				*+Wake*	*0.02*	*5.43* *	*+PS*	*0.08*	*18.39* ***
							(-EMG	*0.22*	*11.59* ***)

Figure 1. Photomicrographs of sections through the pontomedullary reticular formation, dual-immunostained for c-Fos (blue granular chromogen, BDHC) and either GAD (A, B) or serotonin (C) (brown chromogen, DAB). In the PnO (A), single c-Fos+ cells are distributed among dual-immunostained GAD+/c-Fos+ cells, as well as single GAD+ cells. In the RPO (B, C), single c-Fos+ cells are distributed among dual-immunostained GAD+/c-Fos+ (B) and Ser+/c-Fos+ (C) cells. White arrowheads indicate examples of single-immunostained c-Fos+ cells, and black arrowheads indicate dual-immunostained cells. Scale bar, 25 μm.


B

Figure 2. Computerized atlas figure through the oral pons (~A 0.5) showing the PnO, where c-Fos+ cells (indicated by x) and GAD+/c-Fos+ cells (triangles) were mapped in representative animals from PSD (left) and PSR (right) groups. Note apparent greater number of c-Fos+ cells and lesser number of GAD+/c-Fos+ cells in the PSR condition compared with the PSD condition. PnO, Pontine reticular nucleus, pars oralis.



A 0.5

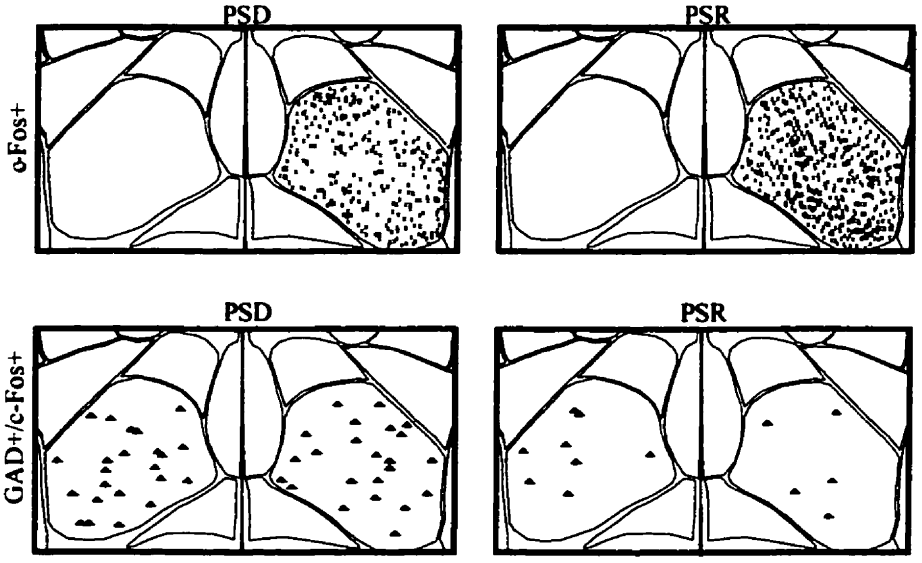
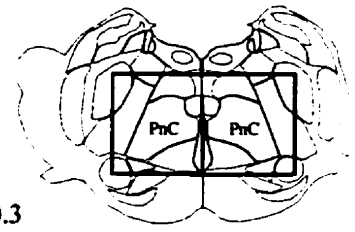
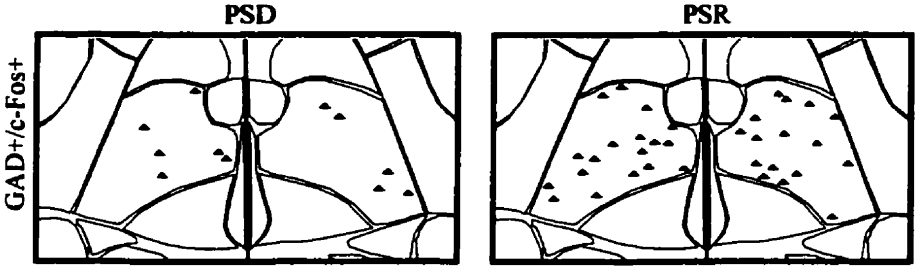
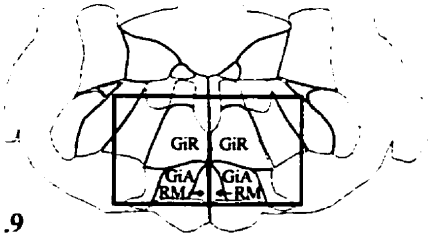


Figure 3. Computerized atlas figure through the caudal pons and medulla at ~P 0.3 (top) showing the PnC nucleus, at ~P 1.9 (middle) showing the GiR, GiA, and RM nuclei, and at ~P 3.5 (bottom) showing the GiC, GiV, and RPO nuclei. GAD+/c-Fos+ cells (triangles) were mapped in a representative animal from PSD (left) and PSR (right) groups. Note the apparent greater number of GAD+/c-Fos+ cells in the PSR condition compared with the PSD condition in most nuclei. PnC, Pontine reticular nucleus, pars caudalis; GiR, gigantocellular reticular nucleus, pars rostralis; GiA, gigantocellular reticular nucleus, pars ; RM, raphe magnus nucleus; GiC, gigantocellular reticular nucleus, pars caudalis; GiV, gigantocellular reticular nucleus, pars ventralis; RPO, raphe pallidus-obscurus nucleus.

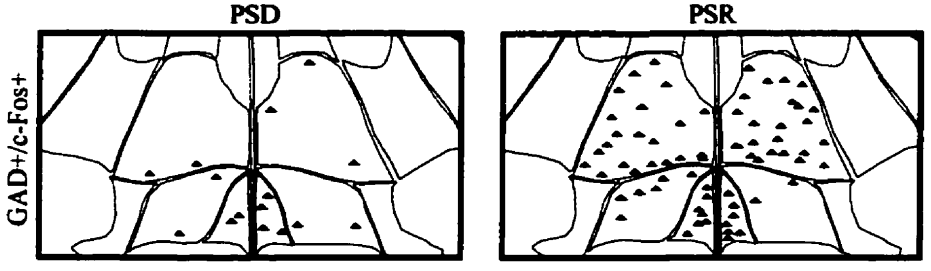

PnC
PnC

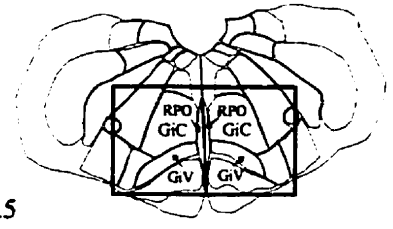
P 0.3


PSD
PSR
GAD+/c-Fos+


GiR
GiR
GiA
RM
GiA
RM

P 1.9


PSD
PSR
GAD+/c-Fos+


RPO
GiC
RPO
GiC
GiV
GiV

P 3.5

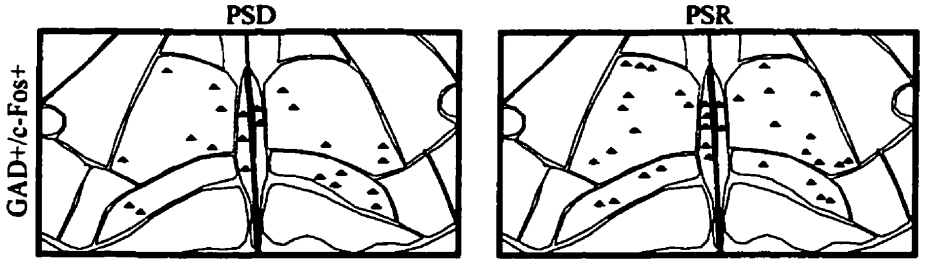

PSD
PSR
GAD+/c-Fos+

Figure 4. Linear regression plots and coefficients (with significance indicated by *p 0.05) for the PnO of the total number of single-immunostained c-Fos+ cells (left) as a function of the percentage of time spent in PS (df = 11) and the total number of dual-immunostained GAD+/c-Fos+ cells (right) as a function of the standardized residuals of PS after partialing out the regression with SWS [PS(sws), df = 9]. The bar charts in the middle show the average total number of c-Fos+ cells (top left) and GAD+/c-Fos+ cells (top right) and the percentage of time spent in PS (bottom) for the three different conditions PSC, PSD, and PSR. According to the statistics detailed in Table 2, there was a significant main effect of condition for the number of c-Fos+ cells and GAD+/c-Fos+ cells, and as detailed in Table 1, for the %PS.

PnO

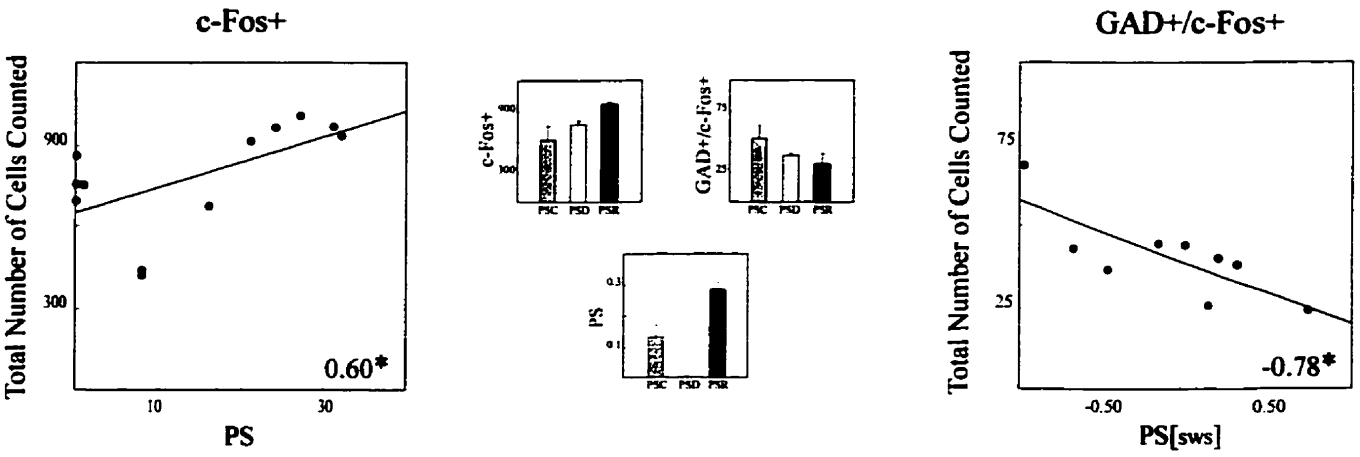
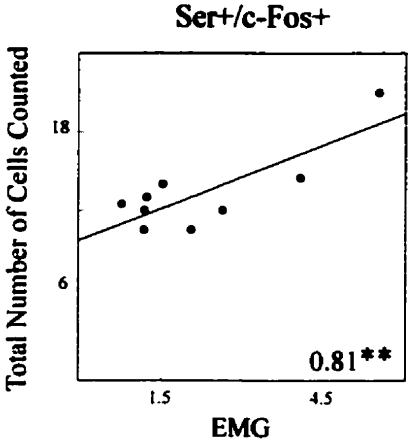
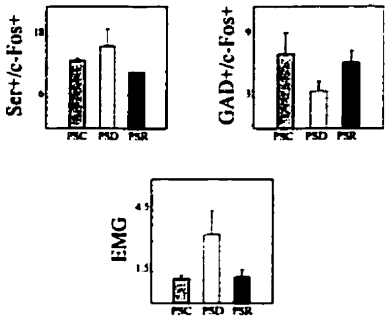
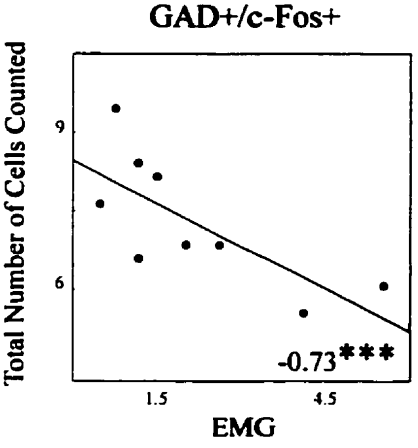

c-Fos+
Total Number of Cells Counted
900
300
10
30
PS
0.60*
c-Fos+
GAD+/c-Fos+
PS
PSC
PSD
PSR
GAD+/c-Fos+
75
25
-0.50
0.50
PS[sws]
-0.78*

Figure 5. Linear regression plots and coefficients (with significance indicated by *, **, *** ($p \leq 0.05$, 0.01, or 0.001) for the RPO of the total number of dual-immunostained Ser+/c-Fos+ cells (left) and GAD+/c-Fos+ cells (right) with EMG activity (df = 9). The bar charts in the middle show the number of Ser+/c-Fos+ cells (top left) and GAD+/c-Fos+ cells (top right) and EMG activity (bottom) for the three different conditionsPSC, PSD, and PSR. As detailed in Table 2, neither the Ser+/c-Fos+ cell counts nor GAD+/c-Fos+ counts differed significantly across conditions, yet they varied significantly as a function of EMG. As presented in the text, EMG values (normalized as a ratio of baseline) differed significantly across conditions and between groups.

RPO


Ser+/c-Fos+
Total Number of Cells Counted
18
6
0.81**
1.5
4.5
EMG


Ser+/c-Fos+
GAD+/c-Fos+
EMG


GAD+/c-Fos+
Total Number of Cells Counted
9
6
-0.73***
1.5
4.5
EMG

Chapter Four

Ventral Mesencephalic Tegmentum

A portion of this chapter was submitted to European Journal of Neuroscience

4. Ventral Mesencephalic Tegmentum

4.1 Introduction

Of the many physiological manifestations of the PS state, such as rapid eye movement and muscle atonia, its cognitive correlate, dreaming, is probably the most widely known. The cholinergic neurons of the pontomesencephalic, known to be important in PS generation and shown earlier to be active during PS, may initiate the PS characteristic of muscle atonia through descending influences, but may also through rostral connections initiate other PS characteristics, such as dreaming. One terminal location of these important cholinergic PS-on neurons is the ventral mesencephalic tegmentum (VMT), where dopaminergic neurons of the ventral tegmental area (VTA) and substantia nigra (SN) reside. Previous research had not fully elucidated the role that dopaminergic neurons play in the sleep-wake states, and in the tradition of utilizing c-Fos expression as a means of immunostaining chemically identified neurons for activation, the following study evaluates dopaminergic and GABAergic neurons located in the VMT for activation during PS. The study supports the notion that activity during PS is different in the two functionally distinct nuclei of the VMT; in the SN there is an increase in GABAergic activity, while in the VTA both dopaminergic and GABAergic neurons increase activity in association with PS. The neurons within these dopaminergic regions, in turn, project to striatal structures, in the case of the SN, and to limbic and cortical structures, in the case of the VTA. Hence, the SN would be in a position to modulate higher order motor occurrences during PS, while the VTA would be in a position to modulate the affective and cognitive aspects associated with the PS characteristic of dreaming.

4.2 c-Fos expression in the substantia nigra and ventral tegmental area after paradoxical sleep deprivation and recovery

4.2.1 Abstract

Lesion and pharmacological studies have indicated that dopaminergic neurons may regulate behavioral arousal and sleep-wake states, yet recording studies have not reported changes in average discharge rate for presumed dopaminergic neurons, although they have for non-dopaminergic neurons of the ventral mesencephalic tegmentum (VMT) across waking, slow wave sleep (SWS) and paradoxical sleep (PS). In the present study in rats, we examined by using c-Fos expression, as a reflection of neural activity, combined with dual-immunostaining for tyrosine-hydroxylase (TH) or glutamic acid decarboxylase (GAD), whether dopamine (DA) and adjacent GABA -synthesizing neurons in the substantia nigra (SN) and ventral tegmental area (VTA) are differentially active in association with PS deprivation and recovery. The number of GAD+/c-Fos+ neurons was higher with PS recovery than PS deprivation and control conditions. In contrast, the number of TH+/c-Fos+ neurons did not change in the SN, however in the VTA, their number was higher with PS recovery than PS deprivation and control. The results suggest that the DA VTA neurons were more active during waking and PS than SWS and most active during PS. Together with evidence from recording studies, it would appear that neither SN nor VTA DA neurons are tonically inhibited by local GABAergic neurons during PS, but they may be submitted to differential inhibitory and excitatory influences such that VTA mesolimbocortical, and not SN nigrostriatal, DA neurons may change their pattern of discharge to be most active in association with PS and therein contribute to the unique physiological and cognitive aspects of that state.

4.2.2 Introduction

Early lesion and pharmacological studies indicated that dopaminergic neurons of the VMT play a role in the regulation of sleep-wake states. Electrolytic and 6-hydroxydopamine (6-OHDA) lesions of the VMT decreased arousal (Ungerstedt, 1971; Jones et al., 1973), and inhibition of catecholamine (CA) synthesis increased SWS and PS (or rapid eye movement sleep, REM) (King and Jewett, 1971). Pharmacological enhancement of extracellular DA conversely increased waking while decreasing sleep (Jones et al., 1977) and proved to be instrumental in maintaining waking for treating narcolepsy (Nishino and Mignot, 1997). Yet, a decrease in PS was recently found following neurotoxic lesions of the VMT (Lai et al., 1999). Such different effects could be due in part to different roles played by SN versus VTA DA neurons. Indeed, in contrast to the hypoactivity produced by 6-OHDA lesions of the SN, hyperactivity was reported to follow lesions of the VTA and was characterized by a loss of attentive and motivational arousal (Galey et al., 1977; Montaron et al., 1982). Accordingly, the SN DA neurons, which give rise to the nigrostriatal projection, are considered to be important in the initiation of motor activity that is impaired in Parkinson's disease, and the VTA DA neurons, which give rise to the mesolimbocortical projection, to be important in the promotion of selective and rewarding arousal that is impaired in attention deficit disorders and involved in schizophrenia (Bunney et al., 1991). It would also appear likely that these DA cell groups would be differentially involved in different sleep-wake states and PS, when muscle atonia occurs together with dreaming, often likened to hallucinations (Yeomans, 1995).

In electrophysiological studies, it was surprisingly found that in contrast to noradrenergic neurons (Hobson et al., 1975), presumed dopaminergic neurons did not change their average discharge rate across sleep-waking states (Miller et al., 1983; Steinfels et al., 1983; Trulson and Preussler, 1984). However, presumed non-dopaminergic neurons discharged at higher rates during active waking and PS than during SWS. Since many of these neurons would be GABAergic and assumed to exert an

inhibitory influence on adjacent dopaminergic neurons (Tepper et al., 1995), the lack of variation in the DA unit discharge was even more surprising.

The VMT DA neurons receive an excitatory input from the cholinergic neurons of the pontomesencephalic tegmentum (PMT) that is thought to be integral to reward circuits and processes (Clarke and Pert, 1985; Beninato and Spencer, 1987; Lacey et al., 1990; Bolam et al., 1991; Corrigall et al., 1994; Yeomans, 1995). The cholinergic neurons are also critical for generating PS (Webster and Jones, 1988; Jones, 1991). In recent studies employing c-Fos immunostaining as a reflection of neural activity combined with choline-acetyltransferase (ChAT)-immunostaining (Maloney et al., 1999), we found that cholinergic PMT neurons were most active in association with PS. Accordingly, they would be expected to activate dopaminergic neurons in turn during this state. In an attempt to resolve whether identified DA and GABAergic SN and/or VTA neurons are active in PS, we thus employed the same PS deprivation and recovery paradigm as for the cholinergic PMT neurons in the VMT and applied dual immunostaining for c-Fos and TH or c-Fos and GAD.

4.2.3 Methods and Materials

Animals and surgery. Twelve male Wistar rats (Charles River Canada, St. Constant, Quebec), weighing approximately 225 grams were employed. All procedures were approved by the McGill University Animal Care Committee and the Canadian Council on Animal Care. Rats were operated under barbiturate anesthesia (Somnotol, 67 mg/kg, i.p.) for the implantation of chronically indwelling electrodes. For the electroencephalogram (EEG), stainless steel screws were threaded into holes drilled in the skull so that the screws were in contact with the dura. They were placed over the left and right retrosplenial, anterior frontal, parietal and occipital cortices, as described previously (Maloney et al., 1997; Maloney et al., 1999). One electrode was placed in the frontal bone rostral to the frontal lobes to be used as a reference. For the electromyogram (EMG), two stainless steel loops were inserted into the muscles of the neck. All leads were connected to a miniature (12 lead) plug, which was cemented to the skull. Animals were allowed two or three days recovery from surgery in the animal room before being placed in recording chambers for the duration of the experiment.

Recording and experimental procedures. For recording and experimentation, each rat was placed in a Plexiglas box that was contained within a larger electrically shielded recording chamber. The rat was connected to a cable that was attached to a commutator and suspended with a balanced boom to allow free movement of the animal within the box. During the baseline day and in the control condition, the floor of the box was covered with woodchips. The animal had free access to food and water in containers, which hung within easy reach on the sides of the box. As was the case in the animal room, a 12-h light/dark cycle was maintained in the recording room (with lights on from 7:00 a.m. to 7:00 p.m.). The rat was placed in the recording box and connected to the cable three days prior to baseline recording to allow for habituation to the recording environment.

The EEG and EMG signals were amplified using a Grass Model 78D Polygraph and subsequently sent to a computer (ALR 386SX) for analog-to-digital conversion, filtering and storage on hard disk with the aid of Stellate Systems (Montreal, Quebec) computer software, as described previously (Maloney et al., 1997).

PS deprivation was performed using the flowerpot technique that has previously been shown in rats to cause a fairly selective deprivation of PS without increases in adrenal weights and thus signs of stress (Mendelson et al., 1974). Each rat was placed on an inverted flowerpot that was just large enough (~6.5 cm in diameter) to hold the animal. The flowerpot was surrounded by water that filled the Plexiglas box to within 1 cm of the inverted pot's surface. In this situation, the animal could engage in SWS but not PS, since the loss of muscle tonus that occurs with PS onset causes the animal to fall into the water and awaken. Food and water containers were positioned to be easily accessible to the animal on the flowerpot.

As previously described in greater detail (Maloney et al., 1999), the experimental protocol was carried out over a four-day period. Recordings were performed in the afternoon (~12:00 - 3:00 p.m.) during the natural sleep period of the rat, for the four consecutive days. On day 1 (the first day prior to experimental manipulation), a baseline recording was performed on all animals. On the remaining three days of the experiment, the 'condition' was varied for three different groups (with 4 animals per group): PS Control (PSC), PS Deprivation (PSD), and PS Recovery (PSR). 1) For the control condition, the PSC animals remained on a bed of woodchips in their recording boxes for the four days. At the termination of the experiment on day 4, the PSC animals were anesthetized for perfusion (at ~3:00 or 3:30 p.m.) following the afternoon recording period. 2) For the deprivation condition, the PSD animals were placed on flowerpots for the second, third and fourth days of the experiment. On day 4 following the recording period, the PSD animals were anesthetized for perfusion (at ~3:30 p.m.), having been in the deprivation condition for ~53 hours. 3) For the recovery condition, the PSR animals were also placed on flowerpots for the second, third and fourth days like the PSD animals. However, on day 4 following ~50 hours of PS deprivation, the animals were

returned to a dry bed of woodchips in their recording boxes to allow for recovery of PS. Following the recording period, the animals were anesthetized for perfusion (at ~3:00 p.m.), having been in the PS recovery condition for ~3 hours after PS deprivation of ~50 hours. The experiments were conducted using two recording chambers and thus on two animals at one time, running pairs of PSD and PSR or PSC and PSC animals.

Perfusion and fixation. The animals were killed under barbiturate anesthesia (Somnotol, ~100 mg/kg) by intra-aortic perfusion of a fixative solution. The time between the barbiturate injection and initiation of the perfusion was ~10 minutes. One liter of 3% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer was perfused for fixation and followed by 250 ml of 10% sucrose in buffer. The brains were immersed in 30% sucrose overnight to complete cryoprotection. The brains were frozen at -50°C and stored at -80°C.

Immunohistochemistry. Coronal sections were cut at 25 μm thickness on a freezing microtome. Up to six series of adjacent sections were collected every 200 μm for immunohistochemical processing. All immunohistochemistry was performed using the peroxidase-antiperoxidase (PAP) technique, according to previously published procedures (Maloney et al., 1999). For the immunostaining of c-Fos protein, an anti-c-Fos antiserum from sheep (Cambridge Research Biochemicals, Cheshire, U.K.) was employed at a dilution of 1:3000. For enzyme immunostaining, the following antibodies were used: rabbit anti-tyrosine hydroxylase (TH) antiserum (1:15,000, Eugene Tech International, Allendale, NJ) and rabbit anti-glutamic acid decarboxylase (GAD) antiserum (1:3000, Chemicon International). Incubations with primary antibodies were carried out at room temperature overnight using a Tris saline solution (0.1M) containing 1% normal donkey serum (NDS) and following incubation with Tris saline containing 6% NDS for blocking. Appropriate secondary antisera and PAP antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were employed after the respective primary antisera. In all brains, one series of sections was immunostained for c-Fos alone using the brown, floccular reaction product, 3,3' diaminobenzidine (DAB) as chromogen. In other adjacent series, c-Fos was immunostained in combination with the

neurotransmitter or enzyme using a sequential procedure staining c-Fos in either the first (for 4 animals) or second (for 8 animals) position. When in the first position, c-Fos was revealed with DAB (or DAB-Ni^{++} in one set of 3 brains), and the neurotransmitter or enzyme was revealed with the blue granular reaction product, benzidine dihydrochloride (BDHC, or DAB in the DAB-Ni^{++}stained brains respectively). However, as revealed by these and other pilot studies (see (Maloney et al., 1999), for greater details) GAD immunostaining in the second position was difficult to clearly discern and for the major experimental series, the enzyme was immunostained and revealed in the first position with DAB, and c-Fos was immunostained and revealed in the second position with BDHC. Controls in the absence of primary antibodies and in the presence of normal sera were routinely run with every single and dual immunostaining procedure to ensure the absence of non-specific single or dual immunostaining. Brains from sets of PSD-PSR, which were run together experimentally, were processed in the same manner for immunohistochemistry together with an accompanying PSC brain.

Analysis of sleep-wake state data. The EEG was examined by off-line analysis on computer screen and scored for sleep-wake state by visual assessment of EEG and EMG activity in 20-second epochs using Eclipse software (Stellate Systems) for each 3-hour recording session (~540 epochs). Fast Fourier Transform (FFT) was performed using Rhythm software (Stellate Systems) to determine amplitude in EEG frequency bands for the 20 sec state-scored epoch data, as described previously (Maloney et al., 1997). EMG amplitude was computed for the total spectrum up to 58.0 Hz. As evaluated by EEG and EMG amplitudes, epochs were scored as one of the three major states (Wake, SWS or PS) or transitional (t) stages between states: 1) wake marked by the presence of low voltage fast activity associated with EMG tonus, 2) transition from wake into slow wave sleep (tSWS) characterized by moderate amplitude slow or mixed slow, spindle and fast activity, 3) slow wave sleep (SWS) marked by continuous high amplitude slow activity, 4) transition from slow wave sleep into PS (tPS) marked by a decrease in high amplitude slow activity and the appearance of spindles and theta waves or 5) PS characterized by a prominence of theta waves, in addition to low voltage fast activity, with low EMG activity.

Analysis of immunohistochemical data. Sections were viewed with a Leitz Orthoplan microscope equipped with an x/y movement-sensitive stage and CCD camera attached to a computer. Single- and dual-immunostained cells were mapped using a computer-based image analysis system (Biocom, Paris). Dual-immunostained TH+/c-Fos+ cells and GAD+/c-Fos+ cells were counted and mapped bilaterally every 400 μm at representative stereotaxic levels corresponding approximately to A 2.9, A 2.5 and A 2.1 (Paxinos and Watson, 1986; Jones, 1995). Single c-Fos immunostained cells were subsequently counted on adjacent sections and mapped unilaterally in regions delineated by the presence of previously counted TH+/c-Fos+ cells as the VTA and the SN on the stereotaxic level corresponding to A2.9. Cell counts were tabulated at each level and for each area, and then counts across levels were added to provide total cells counts per area.

Whether the number of cells counted over multiple levels (sections) per animal varied as a function of 'condition' was examined for the entire VMT and then separately for the SN and the VTA. Analysis of variance tests (ANOVA's) were used with 'condition' as the grouping factor (and in the case of the VMT, with 'area' as a second grouping factor), and 'level' and 'animal' as covariates. In the case of a significant main effect, differences in cell counts between individual conditions were analyzed by post hoc tests using Fisher's pair wise comparisons. When differences in cell counts were significant across conditions in the SN or the VTA, a general linear model was employed to determine if the cell counts varied as a function of (%) PS or other sleep-wake states. A stepwise interactive method was applied examining first whether PS significantly accounted for a proportion of the variance in cell counts across conditions. In the absence of a significant relationship of cell counts with PS, Wake was stepped into the model, and PS together with Wake was evaluated. When the model was significant, simple correlations were performed between total cell counts per area and PS or PS after partialing out Wake (PS[WAKE]). For the correlation of cell counts with PS[WAKE], the standard partial regression coefficient for PS and Wake was calculated and the residuals plotted in the linear regression. General linear models were also employed to test whether EMG covaried with cell counts. All statistics were performed using Systat

(version 9) for Windows (Evanston, Illinois). Figures were prepared for publication using CorelDraw (Ottawa, Ontario).

4.2.4 Results

Sleep-wake state changes

In comparing sleep-wake states between groups submitted to PS deprivation (PSD), recovery (PSR) or control (PSC) conditions or within groups on the last, experimental day and the first, baseline day of recording, it was apparent that there were marked and significant changes in the amount of time spent in PS as a function of the experimental condition (Table 1, as previously reported (Maloney et al., 1999)). After ~53 and ~50 hr of deprivation, respectively, PS represented (of the 3 hour recording period prior to perfusion) 0% in the deprivation condition and ~28% in the recovery condition, as compared to ~15% in the control condition. PS was the one state that was significantly decreased in the deprived (PSD) and significantly increased in the recovery (PSR) groups, as compared between groups to control (PSC) and within groups to baseline conditions. Wake was significantly increased in the deprived (PSD) group, but did not change significantly in the recovery (PSR) group, as compared to control and baseline. Average EMG amplitude also varied significantly across groups being significantly higher in PSD than in PSR and PSC and not differing between PSR and PSC (as previously reported, (Maloney et al., 2000)).

In the assessment of the variation in c-Fos expressing neurons as a function of experimental condition, their numbers were examined between the three experimental groups (below). In addition, given the variation in PS, and also in Wake and EMG amplitude, the cell numbers were examined as a function of these variables across individual animals in a linear regression model and if significant, by simple correlations.

c-Fos expression in the ventral mesencephalic tegmentum

Through the VMT, in both the SN and VTA, c-Fos expression was evident within neurons in sections single-immunostained for c-Fos and in adjacent sections dual-immunostained for c-Fos and TH or for c-Fos and GAD (Fig. 1). In the SN, TH+/c-Fos+ cells were apparent particularly within the pars compacta (SNC), where TH+ cells are most densely distributed. GAD+/c-Fos+ cells were apparent particularly within the pars reticulata (SNR), where GAD+ cells are most numerous. Because the two cell groups do nonetheless overlap and extend across the SNC, SNR and SNL, they were plotted and tabulated across the entire SN considered as one area (Fig. 2). TH+/c-Fos+ and GAD+/c-Fos+ cells were present and distributed relatively evenly across the VTA (Fig. 2). Across the VMT, it was not possible to ascertain by visual examination in single immunostained sections whether the number of c-Fos+ cells was different in the PS recovery (PSR) as compared to the deprived (PSD) and control (PSC) groups. In dual-immunostained sections, it did appear upon visual examination that TH+/c-Fos+ cells and GAD+/c-Fos+ cells were more numerous in brains from the PS recovery (PSR) group than in those from the deprived (PSD) group (Figure 2) and also the control (PSC) group (not shown). According to cell counts in the SN and VTA, the numbers of single immunostained c-Fos+ cells did not differ significantly across groups ($F=3.11$; $df=2$, $df_{error}=17$; $p=0.07$). However, numbers of TH+/c-Fos+ cells ($F=4.24$; $df=2$, $df_{error}=124$; $p=0.016$) and GAD+/c-Fos+ ($F=6.81$; $df=2$, $df_{error}=88$; $p=0.002$) did differ significantly according to ANOVA with group and area as dependent variables (and rat and level as covariates). For both cells types, the largest numbers of c-Fos expressing cells occurred in the PS recovery group. There also appeared to be an interaction of group and area and an indication that the changes in c-Fos expressing cells were not the same in the SN and VTA. Further analysis was performed separately for each area.

c-Fos expression in the substantia nigra

Counted in single-immunostained sections, c-Fos+ cells varied significantly in the SN across groups, being more numerous in the PSR than in the PSD group (Table 2). On the other hand, as counted in the dual-immunostained series, TH+/c-Fos+ cells did not vary significantly across groups and thus conditions. GAD+/c-Fos+ cells did vary significantly, being more numerous in the PSR than in the PSD group (Table 2). In a linear regression model, the numbers of GAD+/c-Fos+ cells covaried positively with the % PS across animals of the different groups (Table 3). They also covaried negatively with EMG amplitude, however not significantly so ($r^2 = 0.065$; F=3.17; df=1,df_{error}=46; p=0.082).

The variation of GAD+/c-Fos+ cell counts as a function of PS was further examined by simple correlations using total cell counts in the SN per animal. In this case, the number of GAD+/c-Fos+ cells was not significantly correlated with %PS (r=0.46, n=8; p=0.251; or EMG: r=-.41; n=8; p=0.31).

c-Fos expression in the ventral tegmental area

Although, the number of single-immunostained c-Fos+ cells in the VTA did not vary significantly as a function of experimental condition (Table 2), the number of dual-immunostained TH+/c-Fos+ cells and GAD+/c-Fos+ cells did (Table 2). TH+/c-Fos+ cells were more numerous in the PSR group than in both the PSD and PSC groups. They were also more numerous in the PSD than in the PSC group, though not significantly so (p=0.12). In a linear regression model, the numbers of TH+/c-Fos+ cells did not covary significantly with %PS alone. However, when %Wake was included in the model, the numbers of TH+/c-Fos+ cells covaried positively with %PS together with %Wake (Table 3). GAD+/c-Fos+ cells were also more numerous in the PSR group than in the PSD and PSC groups and also more numerous in the PSD group than in the PSC group (Table 2). As with the TH+/c-Fos+ cells, the numbers of GAD+/c-Fos+ cells did not covary

significantly with %PS alone. However, when the %Wake in addition to %PS was considered in the linear regression model, the numbers of GAD+/c-Fos+ cells were found to covary positively with %PS and %Wake (Table 3). Neither the TH+/c-Fos+ nor GAD+/c-Fos+ cells covaried with EMG amplitude.

The variation of cell counts as a function of PS was further substantiated by simple correlations using the total number of cells counted in the VTA per animal. The total number of TH+/c-Fos+ cells and the total number of GAD+/c-Fos+ cells were significantly positively correlated with %PS after the variation due to %Wake was partialed out of the regression function (Fig. 3).

4.2.5 Discussion

The present results show significant variations in c-Fos expression in dopaminergic and GABAergic neurons of the VMT in association with PS deprivation and recovery. Across the VMT, the number of c-Fos expressing dopaminergic and GABAergic neurons was greatest in the PS recovery group, suggesting increased activity of these cell groups in PS. Yet, the changes were not parallel in the SN and VTA cell groups, suggesting their differential involvement in sleep-wake states and PS.
As noted in our previous studies, we have employed c-Fos expression as a reflection of increased neural activity, although it can also reflect increases in other cellular processes that commonly involve calcium influx into neurons (Morgan and Curran, 1986; Dragunow and Faull, 1989). It should also be mentioned that although c-Fos expression has been found to be increased in locus coeruleus neurons with chronic stress, which might be evoked in our deprivation paradigm, it has not been reported to be increased in VMT neurons under such conditions (Cullinan et al., 1995).

Substantia Nigra

As similarly reported in a recent pharmacological study (Sastre et al., 2000), enhanced PS, here with PS recovery, was found to be associated with an increase in the number of c-Fos expressing cells in the SN. However, here, the increase was found to be within GABAergic neurons and not dopaminergic neurons. These results corroborate electrophysiological data showing an increase in average discharge rate by non-dopaminergic neurons, and not by dopaminergic neurons, during PS as compared to SWS and waking (Miller et al., 1983; Steinfels et al., 1983). Being concentrated in the SNR and projecting to the thalamus or tegmentum, GABAergic SN neurons could contribute to the phasic activity of PS through phasic discharge (Datta et al., 1991).

c-Fos expressing GABAergic neurons have been shown to increase in other brainstem areas with PS and hypothesized to play a role in inhibiting local monoaminergic neurons during PS (Maloney et al., 1999). Since in the present analysis, there was no decrease in c-Fos expressing DA neurons with PS rebound, it would not appear here, or in single unit recording studies (Miller et al., 1983), that GABAergic SN neurons provide a tonic inhibitory influence to DA SN neurons during PS. However, the inhibition could be reflected in the lack of increase in c-Fos expressing DA neurons, or unit discharge, despite potential excitatory input to them during PS. It is also known that DA SN neurons are under negative feedback control by D_2 autoreceptors, which could be a factor in PS and narcolepsy (Nishino et al., 1991; Honda et al., 1999). These receptors could be activated such as to maintain a relatively low constant discharge rate by these neurons during sleep. How such a mechanism could be selectively active in the SN could be explained by findings that D_2 receptors are most concentrated (Hurd et al., 1994) and exert greatest control on DA release from the SN DA neurons (Cragg and Greenfield, 1997).

Ventral tegmental area

In the VTA, PS recovery resulted in an increase in the number of dopaminergic and GABAergic cells expressing c-Fos. Their numbers were higher in the recovery than in the deprivation condition and also somewhat higher in the deprivation than in the control condition. Since the recovery was associated with an increase in PS, whereas the deprivation was associated with an increase in waking, these results suggest their increased activity during each of these states. Indeed, using linear regression models, the activity of dopaminergic and GABAergic neurons was found to covary with the amount of time spent in PS and Waking and to correlate with PS once the correlation with Wake had been partialed out. The present results thus suggest that both the dopaminergic and GABAergic neurons in the VTA are more active during PS and Wake than during SWS and most active in association with PS. They appear to be different from electrophysiological data for presumed dopaminergic neurons in VTA that showed no

significant change in average discharge rate during PS relative to SWS and quiet or active waking, whereas they are similar to those for presumed non-dopaminergic neurons that showed a significant increase in rate during PS relative to quiet waking or SWS (Miller et al., 1983; Trulson and Preussler, 1984).

These results evoke the question of how c-Fos expression could indicate increased activity in the DA VTA cell group during PS and waking, whereas recording studies have not revealed significant changes in average discharge rate across the sleep-wake states. There are several possible explanations. First, most recording studies have analyzed the activity of SN neurons (Steinfels et al., 1983; Strecker and Jacobs, 1985) or VTA neurons grouped together with SN neurons (Miller et al., 1983). Second, recording studies in freely moving animals have not identified the recorded neurons as dopaminergic by immunostaining, which particularly in the VTA, is important since dopaminergic neurons are intermingled there with numerous non-dopaminergic neurons (Swanson, 1982; Ford et al., 1995). Third, single unit recording studies can only sample individual units and thus cannot reliably assess whether neuronal populations are active in association with a behavioral state. Fourth, and perhaps most pertinent, analysis of the average rate of discharge in these recording studies does not reveal changes in the pattern of discharge that may occur during a behavioral state. Indeed, it is known that dopaminergic neurons tend to discharge in bursts of spikes and do so during active or stimulated waking conditions (Freeman et al., 1985; Horvitz et al., 1997) and during behaviorally significant and rewarding situations (Schultz, 1986; Mirenowicz and Schultz, 1996; Overton and Clark, 1997). In one recording study, it was reported that the variance of the interspike interval was significantly different across sleep-wake states for dopaminergic VMT neurons, such as to indicate that increased bursting occurred in these neurons during PS as compared to SWS (Miller et al., 1983). Here, the increased c-Fos expression in the DA VTA neurons in association with PS could be due to increased burst discharge, which is associated with increased calcium influx (Overton and Clark, 1997). Interestingly, there is evidence that in anesthetized rats a much larger percentage of presumed DA VTA neurons (73%) than DA SN neurons (18%) discharge in bursts (Freeman and Bunney, 1987; Grenhoff et al., 1988). Since dopaminergic VTA neurons do not exhibit burst firing in vitro, it is likely not generated by intrinsic properties but instead evoked by

afferent input to the neurons (Grace, 1988). Accordingly, either because of differential excitatory input or differential inhibitory control (above), the DA VTA neurons may be more active or discharge more in bursts than the DA SN neurons during PS.

The increased number of GABAergic neurons expressing c-Fos in the VTA following deprivation and recovery suggests that these neurons are more active during waking and PS than during SWS and most active during PS. This finding is in accordance with recording studies of presumed non-dopaminergic (Miller et al., 1983) and more recent studies of immunohistochemically identified GABAergic VTA neurons that were found to discharge at their highest rates during PS (Lee et al., 1997; Steffensen et al., 1998). GABAergic VTA neurons give rise to long ascending projections into the posterior hypothalamus (Ford et al., 1995) and forward to limbic and cortical targets (Steffensen et al., 1998) where they may influence activity in parallel with the dopaminergic neurons by acting upon different target neurons or by potentially phasically modulating the same target neurons. It would appear that they do not cause a tonic inhibition of the neighboring dopaminergic VTA neurons during PS, but could exert a phasic influence on them also, which could promote their discharge in bursts (Overton and Clark, 1997).

The burst discharge of VTA neurons has been shown to depend upon excitatory input, which comes from glutamatergic (Overton and Clark, 1997) and also cholinergic input, which originates in the PMT (Grenhoff et al., 1986; Gronier and Rasmussen, 1998). Acetylcholine (ACh) induces bursting through nicotinic receptors on both VTA and SN DA neurons, however, notably, also induces bursting through muscarinic receptors on VTA but not SN DA neurons. Hence, cholinergic PMT neurons could stimulate burst firing, particularly in VTA DA neurons during specific waking conditions and during PS when the cholinergic neurons are maximally active (ElMansari et al., 1989; Maloney et al., 1999). Enhancement of extracellular ACh in the VTA by local inhibition of cholinesterase increases DA release in the limbic forebrain, an effect that depends upon input from the laterodorsal tegmental (LDTg) but not from the pedunculopontine tegmental (PPTg) nucleus (Blaha et al., 1996). In our previous study

(Maloney et al., 1999), cholinergic c-Fos expressing neurons were significantly increased during PS recovery and significantly correlated with PS within the LDTg nucleus. Accordingly, the cholinergic LDTg neurons could stimulate burst discharge in the dopaminergic VTA neurons during PS.

Increases in DA VTA neuronal activity could be responsible for the activation of limbic forebrain areas during PS (Maquet et al., 1996; Sastre et al., 2000), as during rewarding brain stimulation (Flores et al., 1997; Arvanitogiannis et al., 2000). It could also subserve a role in learning and memory during PS (Winson, 1993; Karni et al., 1994), as during waking (Ljungberg et al., 1992; Schultz, 1998). And, it could play a role in the human cognitive correlate of PS, dreaming.

4.3 Conclusions

As inferred here by c-Fos expression, activity in GABAergic neurons is increased during PS in the VMT. In the SN, increased GABAergic activity may play a role in the inhibition of motor movements and a permissive role in induction of muscle atonia. In the VTA, both GABAergic and dopaminergic activity were increased in association with PS. The VTA projects to limbic and cortical structures and hence, by its connections, may contribute to the affective and cognitive aspects associated with the PS characteristic of dreaming. Increased activity in the dopaminergic VTA system has been associated with the hallucinogenic aspects associated with psychosis [Davis, 1974 #1953], which in turn have been likened to dreaming. The notion that the differences between these areas are a result of differential activation by cholinergic input from the pontomesencephalic tegmentum has been discussed. The cholinergic pontomesencephalic tegmentum has been shown to be active during PS. Through efferent projections, these neurons can influence descending systems from the PnO, which may in turn modulate muscle atonia, while through ascending projections, they may modulate cholinoceptive neurons in VMT differentially. In the SN, this cholinergic effect may provide via activation of GABAergic neurons a parallel inhibition of motor activity in the striatal structure. In the VTA, this cholinergic effect may provide via activation of both dopaminergic and GABAergic neurons modulation in limbic and cortical structures considered to be important in reward mechanisms, learning, and the induction of dreaming.

4.5 References

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4.2.7 Tables and Figures

Table 1.

Percentage of time spent in Wake, tSWS (transition from Wake into SWS), SWS, tPS (transition from SWS to PS) and PS states during the final ~3 hour recording period prior to sacrifice (day 4 of the experimental paradigm) for animals in each condition (mean ± s.e.m., n=4). Significant differences per state between conditions were tested by repeated measures ANOVA with one trial factor ('day') and one grouping factor ('condition'). When there was a significant main effect for condition for one of the states, post hoc tests were performed per day across conditions using Fisher's pair wise comparisons. In the cases where a significant difference between conditions was found, another post hoc test was performed by repeated measures to determine whether there was a significant difference between day 4 and day 1 within that group. Only differences that were significant both with respect to the control group (PSC) and relative to the baseline condition (day 1) are indicated. Differences with respect to PSC are indicated by * p□0.05. Differences with respect to PSD are indicated by † p□0.05. (After (Maloney et al., 1999)).

Table 1. Sleep-wake states on the last experimental day (day 4) for PSC, PSD, and PSR.

Condition	State (%)	
PSC	Wake	21.00±2.00
	tSWS	30.00±5.00
	SWS	9.00±3.00
	tPS	25.00±4.00
	PS	14.50±6.50
PSD	Wake	56.32±3.87*
	tSWS	34.00±4.74
	SWS	3.04±1.53
	tPS	6.25±1.03
	PS	0.0±0.0 *
PSR	Wake	16.36±1.28
	tSWS	10.75±2.60
	SWS	25.51±1.06
	tPS	19.25±1.32
	PS	28.37±1.86*†

Table 2.

Cell counts were taken from adjacent series of sections cut at 25 μm and collected at 200 μm intervals.

[a] c-Fos+ cell numbers correspond to the mean ± s.e.m. of the total number of cells counted unilaterally in single-immunostained sections at one level (~A2.9) in 4 animals per condition.

[b] TH+/c-Fos+ and GAD+/c-Fos+ cell numbers correspond to the mean ± s.e.m. of the total number of cells counted bilaterally in dual-immunostained sections at 3 levels (~A2.9, ~A2.5 and ~A2.1) in 4 animals per condition.

An ANOVA was performed with cell numbers as dependent variable, condition as grouping variable and rat and level as covariates. A significant main effect for condition is indicated by •,•• or ••• ($p \leq 0.05$, 0.01 or 0.001) in the column under F. With significant main effects of condition, post hoc tests between individual conditions were performed using Fisher's pair wise comparisons. A significant difference with respect to PSC is indicated by *, ** or *** ($p \leq 0.05$, 0.01 or 0.001) and with respect to PSD by †, †† or ††† ($p \leq 0.05$, 0.01 or 0.001).

Table 2. Number of c-Fos+, TH+/c-Fos+, or GAD+/c-Fos+ cells counted in the ventral tegmental area and the substantia nigra in PS control (PSC), deprived (PSD), or recovery (PSR) groups.

	c-Fos+[a]				TH+/c-Fos+[b]				GAD+/c-Fos+[b]			
	PSC	PSD	PSR	F	PSC	PSD	PSR	F	PSC	PSD	PSR	F
SN	616.75 ± 83.90	540.00 ± 68.80	706.50 ± 58.42†	●	70.50 ± 13.36	86.50 ± 29.72	88.33 ± 12.32		249.33 ± 43.18	190.33 ± 18.66	295.00 ± 87.00†	●
VTA	314.00 ± 102.46	343.75 ± 60.60	431.75 ± 75.22		38.25 ± 3.82	62.00 ± 20.49	96.00 ± 24.01**†	●●	14.33 ± 4.98	44.33 ± 7.51**	77.50 ± 18.50**††	●●●

Table 3.

A general linear model was employed to test whether cell counts varied significantly as a function of state. Using multiple linear regression with an interactive stepwise procedure for adding or removing variables, a model was examined in all regions where a significant main effect of condition was found (see Table 2). To test the principal hypothesis that cell counts varied as a function of PS, %PS alone was evaluated first. If not significant, %Wake was stepped in and the regression for %PS and %Wake was evaluated in the model. Significant models are indicated with the state(s) showing the signs (+ or -) of the individual coefficients, the squared regression coefficient (variance) and the F value of the model. Dashes indicate a lack of significance. Probabilities are indicated as *, ** or *** ($p \leq 0.05$, 0.01 or 0.001).

Table 3. Relationship between the number of c-Fos+, TH+/c-Fos+, or GAD+/c-Fos+ cell counts and %PS or %PS with % Wake as assessed by linear regression models

	c-Fos+			TH+/c-Fos+				GAD+/c-Fos+			
	State	r^2	F	State	r^2	F		State	r^2	F	
SN								+ PS	0.08	4.03	*
VTA				+ PS + WAKE	0.20	7.71	***	+ PS + WAKE	0.33	11.29	***

Figure 1. Photomicrographs of sections through the substantia nigra (SN) and the ventral tegmental area (VTA) with dual-immunostained c-Fos (blue granular chromogen, BDHC) and either TH (upper) or GAD (lower) (brown chromogen, DAB). Black arrowheads indicate single-immunostained c-Fos+ cells; white arrowheads indicate examples of single-immunostained TH+ or GAD+ cells; and paired black & white arrowheads indicate dual-immunostained cells. Scale bar, 25 μm.

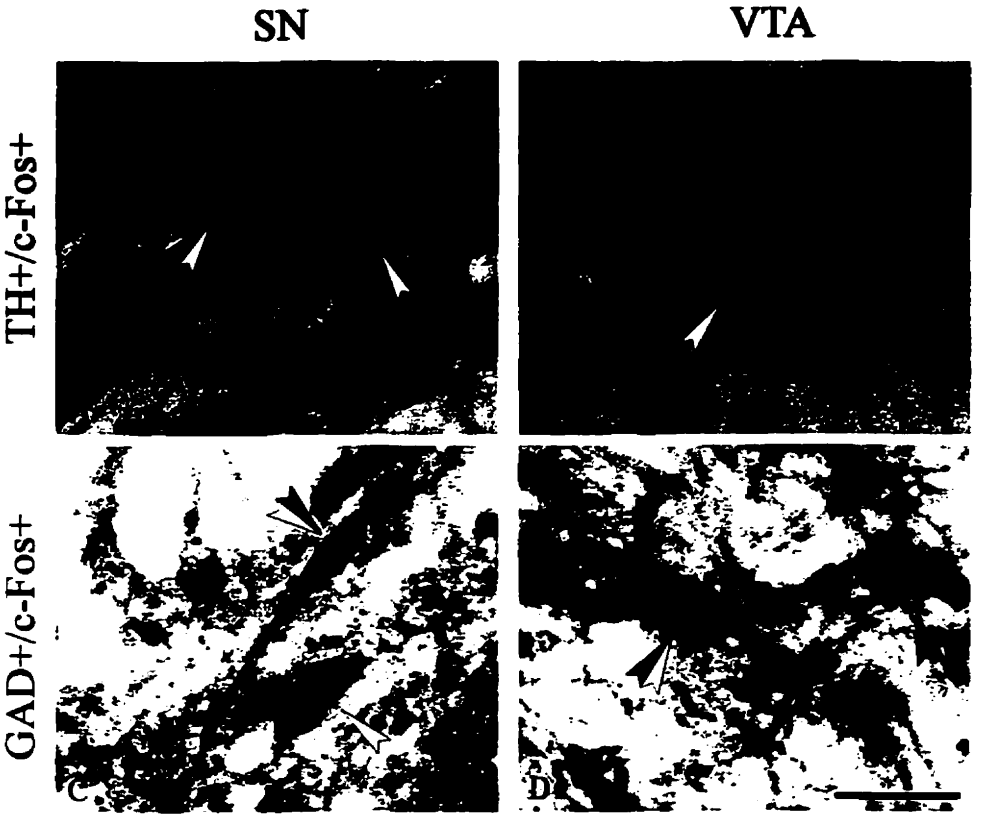
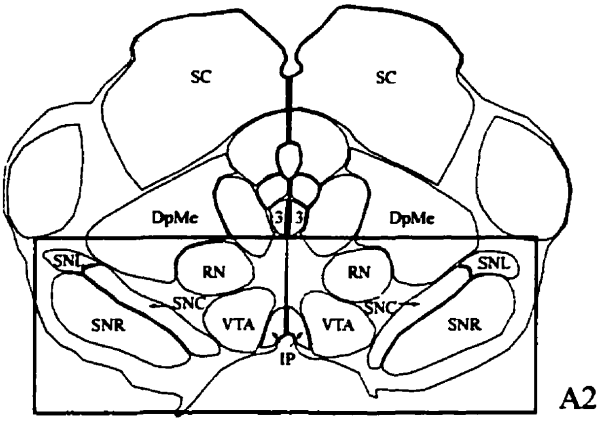

SN
VTA
TH+/c-Fos+
GAD+/c-Fos+
C
D

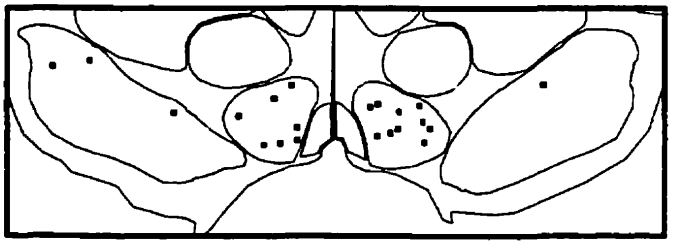
Figure 2. Computerized atlas figure through the mesencephalon (at ~A2.9, (Paxinos and Watson, 1986)). TH+/c-Fos+ cells (squares) and GAD+/c-Fos+ cells (triangles) were mapped in the SN and VTA in representative animals from PSD (left) and PSR (right) groups. Note the apparent greater number of GAD+/c-Fos+ cells and TH+/c-Fos+ cells in the PSR animal as compared to the PSD animal. DpMe, deep mesencephalic nucleus; IP, interpeduncular nucleus; RN, red nucleus; SC, superior colliculus; SNC, substantia nigra, pars compacta; SNR, substantia nigra, pars reticulata; SNL, substantia nigra, pars lateralis; VTA, ventral tegmental area; 3, oculomotor nucleus.



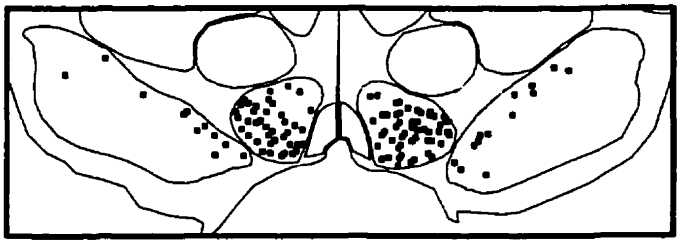
A2.9

PSD **PSR**

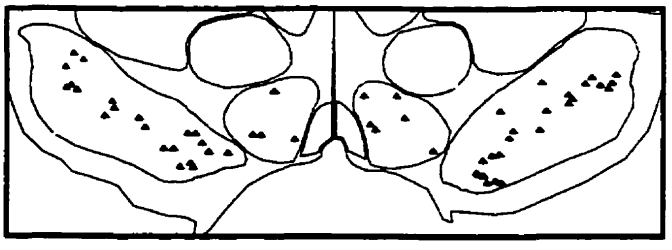
TH+/c-Fos+



TH+/c-Fos+



GAD+/c-Fos+



GAD+/c-Fos+

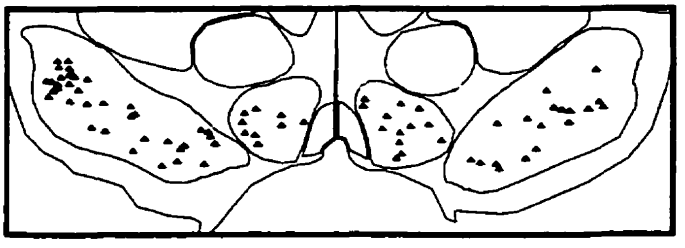
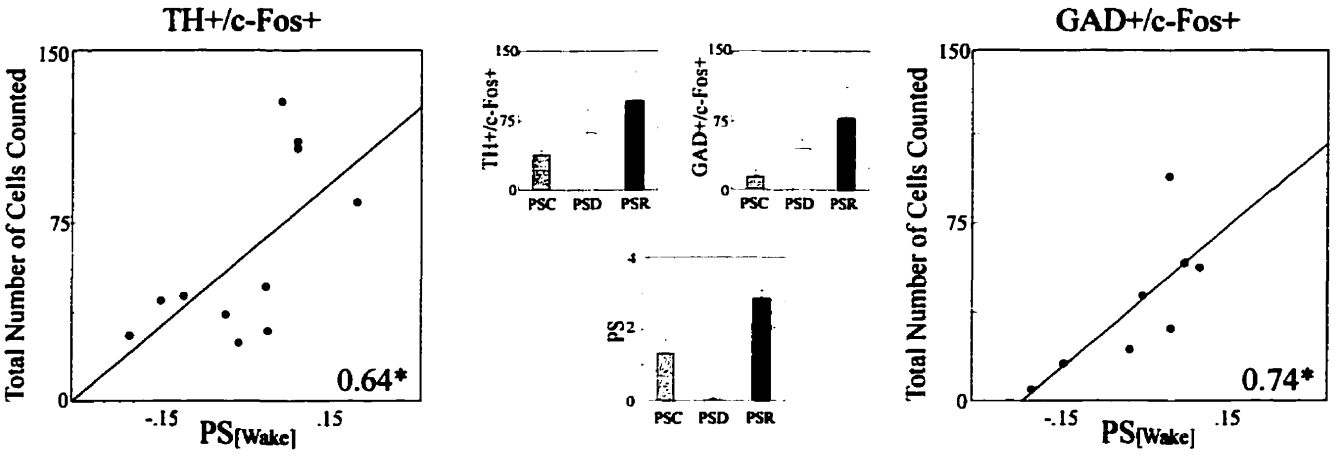


Figure 3. Linear regression plots and coefficients (with significance indicated by * p□ 0.05) for the VTA of the total number of dual-immunostained TH+/c-Fos+ cells (left) (df=11) and GAD+/c-Fos+ cells (right) (df=8) as a function of the standardized residuals of %PS after partialing out the regression with %Wake (PS[Wake]). The bar charts in the middle show the average total number of TH+/c-Fos+ cells (top left) and GAD+/c-Fos+ cells (top right) and the percentage of time spent in PS (bottom) for the three different conditions, PSC, PSD, and PSR. According to the statistics detailed in Table 2, there was a significant main effect of condition for the number of TH+/c-Fos+ and GAD+/c-Fos+ cells.

VTA


TH+/c-Fos+
Total Number of Cells Counted
150
75
0
-.15
.15
PS[Wake]
0.64*
TH+/c-Fos+
GAD+/c-Fos+
PSC
PSD
PSR
PS
GAD+/c-Fos+
0.74*

Chapter Five
Discussion

5. Discussion

5.1 Discussion

Although the range of physiological events that underlie the unique state of paradoxical sleep (PS) would undoubtedly involve complex mechanisms and dynamic processes throughout the central nervous system, the pontomesencephalic brainstem, and in particular the cholinergic neurons within the tegmentum, appear to play a particularly important role in the initiation of PS. Through modulation of descending and ascending systems, the cholinergic pontomesencephalic tegmental (PMT) neurons, have been hypothesized to elicit many PS characteristics, including muscle atonia and even perhaps the cognitive correlate of PS, dreaming (Hobson, 1992). Pharmacological, lesion and single unit recording studies of "presumed" cholinergic neurons have provided evidence for a cholinergic involvement in PS, however, confirmation of increased activity in chemically defined cholinergic neuronal populations within the PMT during PS was lacking. Similarly, pharmacological, lesion and single unit recording studies of "presumed" monoaminergic neurons have provided evidence that monoaminergic neurons located within the pontomesencephalic brainstem may antagonize the occurrence of PS, and their hypothetical cessation of activity was considered to be critical in the induction of PS. However, again, without verification of the chemical identity of single unit recording studies the inactivity of these monoaminergic neuronal populations remained hypothetical, until now.

The three studies presented in this thesis confront these previous limitations by utilizing a dual immunohistochemical procedure, for c-Fos expression and neurotransmitters or their enzymes, in order to immunochemically visualize activity in a population of chemically defined neurons during PS rebound. c-Fos expression may be induced to high levels by physiological stimuli and has been reported to be transiently expressed in neurons after synaptic stimulation (Hunt et al., 1987; Robertson et al., 1991). To induce an enhancement in the duration of PS, and therefore an enhancement in the physiological stimuli underlining that state, the present studies also utilized, successfully, the established phenomena of PS rebound after PS deprivation (Morden et al., 1967). By implementing these immunohistochemical and behavioural procedures, the results

presented in this series of studies not only offer authentication of suggestions inferred by lesion and single-unit recording studies, but provide potential insights into the possible mechanisms involved in invoking such fluctuations in activity within and between neuronal populations during PS.

Within the laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) nuclei of the PMT, cholinergic neurons are intermingled with GABAergic neurons (Ford et al., 1995). The hypothetical increase in activation of cholinergic neurons populating this region during PS was confirmed here by an increase in c-Fos expression in cholinergically identified neurons during PS rebound. Interestingly, it was further found that GABAergic neurons co-distributed with the cholinergic neurons in the LDT/PPT nuclei also exhibit an increase in c-Fos expression during PS rebound. It has been shown that a significant proportion of non-cholinergic LDT/PPT neurons are active during PS (Merchant-Nancy et al., 1992; Shiromani et al., 1992), and it may now be suggested that this population of neurons is GABAergic. Injection of the muscarinic receptor agonist, carbachol, into the central pontomesencephalic region, the pontine reticular nucleus, pars oralis (PnO), induces a state similar to PS and also is associated with an increase in c-Fos expression in GABAergic neurons in the LDT/PPT nuclei in the side ipsilateral to the injection (Torterolo et al., 2001). The GABAergic cells presumed active during PS and carbachol-induced PS may also correspond to non-cholinergic neurons excited by carbachol microinjections *in vivo* (Sakai et al., 1986). Furthermore, microinjections of GABA agonists into the cholinergic LDT/PPT nuclei dose-dependently reduce behavioral arousal (Miller et al., 1991), while injections of GABA antagonist increase arousal (Sanford et al., 1998). The functional significance of this increase in GABAergic activity in the LDT/PPT cholinergic area during PS may include an inhibition of a subset of cholinergic neurons that are selectively active during wake (Thakkar et al., 1998). Other hypothetical functional significance of this GABAergic activation may involve the projection of inhibition to monoaminergic neurons in close proximity, to other neurons in the pontomesencephalic tegmentum, or even involve the pacing cholinergic neurons as is suggested in other systems (Steriade and Deschenes, 1984). In sum, the once hypothetical increased activity of cholinergic neurons populating the LDT/PPT nuclei during PS is supported here by the demonstration that cholinergic neurons exhibit an

increase in c-Fos expression during PS. The findings presented here also demonstrate an increase in GABAergic activity within the same regions that may provide a potential mechanism by which certain cholinergic neurons are selectively activated, or how cholinergic neurons may induce indirect inhibition of local monoaminergic neurons during PS.

Receiving projections from the cholinergic LDT/PPT region are the dorsal and median raphe nuclei. The raphe nuclei contain both serotonergic and GABAergic neurons. Providing support that serotonergic neurons are inactive during PS, it was demonstrated here that there was a decrease in c-Fos expression in serotonergic neurons during PS. Furthermore, it was found that GABAergic neurons of the raphe nuclei expressed higher levels of c-Fos in association with PS rebound suggesting that they are active during PS. Cholinergic LDT/PPT stimulation can cause an inhibition of serotonergic neurons that is likely indirect (Sakai, 1988; Li et al., 1998), and hence potentially mediated through local PMT and/or raphe GABAergic neurons. Studies using carbachol to induce a state similar to PS have shown that the state is associated with an increase in c-Fos expression in non-serotonergic DR neurons (Yamuy et al., 1995), which we may now suggest are GABAergic. In this way, naturally occurring PS or carbachol-induced PS, may activate GABAergic neurons which in turn suppress serotonergic neurons of the raphe nuclei. Within the dorsal raphe, GABA immunoreactive terminals contact serotonergically identified neurons (Wang et al., 1997), that have also been shown to express $GABA_A$ receptors (Gao et al., 1993). Microdialysis studies show that GABA release in the raphe nuclei is maximal during PS in comparison to SWS and Wake (Nitz and Siegel, 1997b). Iontophoretic application of GABA agonists cause a reduction in dorsal raphe serotonergic activity (Gallager and Aghajanian, 1976) and an increase in PS (Lancel et al., 1996) (Nitz and Siegel, 1997a), while iontophoretic application of GABA antagonist, bicuculline, reverses the suppression of serotonergic neurons observed in SWS (Levine and Jacobs, 1992). Together, these results suggest that GABAergic neurons projecting to and/or in the dorsal or median raphe nuclei, potentially excited by cholinergic activity, inhibit surrounding serotonergic neurons during PS.

The locus coeruleus, populated by noradrenergic neurons, and surrounded by GABAergic neurons of the central gray, also receive projections from the LDT/PPT

cholinergic nuclei. Noradrenergic neuronal firing has been hypothesized to virtually cease during PS, and this assumption is supported here by the observation of a decrease in c-Fos expression in noradrenergic neurons during PS. Furthermore, it was found that GABAergic neurons surrounding the locus coeruleus expressed higher levels of c-Fos in association with PS rebound, suggesting an increase in GABAergic activity during PS. In support of increased activity in GABAergic neurons innervating the locus coeruleus during PS, biochemical studies have found greater release of GABA in the locus coeruleus during PS than during SWS or waking (Nitz and Siegel, 1997b). Moreover, the cessation of discharge by locus coeruleus neurons during this state can be reversed by microinjection of $GABA_A$ antagonist bicuculline (Gervasoni et al., 1998), which also results in a decrease in PS (Kaur et al., 1997). These results indicate that neighboring GABAergic neurons could inhibit noradrenergic locus coeruleus neurons during PS.

In the initiation and generation of PS, the cholinergic projections from the PMT to the central pontomesencephalic tegmentum, the pontine reticular nucleus, pars oralis (PnO), is considered to be of particular importance. Microdialysis studies have shown that there is an increase acetylcholine (ACh) release in the PnO during PS (Kodama et al., 1990) and, as previously mentioned, injections of the cholinergic muscarinic receptor agonist, carbachol, into the PnO induces a state very similar to PS with muscle atonia (George et al., 1964; Baghdoyan et al., 1984; Morales et al., 1987; Vanni-Mercier et al., 1991). Studies using carbachol injections into the PnO as a means of inducing a state similar to PS have shown a small increase or no change in the number of cholinergically-identified neurons exhibiting c-Fos in the LDT/PPT nuclei (Shiromani et al., 1996; Yamuy et al., 1998). However, because carbachol acts directly on cholinoceptive target neurons (Vanni-Mercier et al., 1989; Jones, 1990), its pharmacological effect would not depend on increased activity in cholinergic cells, i.e. cholinergic neurons would be 'downstream' to the cascading events of its effect. In fact, carbachol exerts an inhibitory effect on LDT/PPT cholinergic neurons (Sakai and Koyama, 1996), and hence cholinergic activity cannot be reliably evaluated under such a paradigm. Hence, carbachol-induced PS demonstrates the importance of the cholinoceptive PnO neurons in the generation of PS but cannot be used to evaluate all neuronal events underlying the natural state of PS.

In the PnO, our results demonstrated an increase in c-Fos expressing neurons populating that region during PS, and a decrease in c-Fos expression in the local GABAergic neurons located there within. This type of configuration in neuronal activity would support a model of disinhibition by local GABAergic neurons during PS, and it should be mentioned that of all the regions examined in these studies only this region, the PnO, exhibited a decrease in GABAergic activity. In support of a decrease in GABAergic activity in the PnO during PS, injections of GABA or GABA agonists in this region reduces PS, while injections of GABA antagonist excite PS-on neurons (Sakai and Koyama, 1996), and increases PS (Xi et al., 1999). Activation of this cholinoceptive region by carbachol injections has been shown to induce a state very similar to PS with muscle atonia (George et al., 1964; Torterolo et al., 2001), which we now suggest is due in part to a disinhibition by GABAergic neurons.

Through activation of descending pathways, the PnO is in a position to induce the PS characteristic, muscle atonia. Transections through the upper pons produce a generalized and enduring muscle atonia, indicating that the oral pons has the capacity to tonically inhibit muscle tonus (Keller, 1945). The influence of the PnO in the generation of muscle atonia is thought to be relayed through the medullary reticular formation since stimulation of this region results in muscle atonia (Magoun and Rhines, 1946). GABAergic neurons of the medullary reticular formation have been shown to project to the spinal cord (Jones, 1991), where it is suggested that they provide, either directly or indirectly, muscular inhibition during PS. Supporting this notion, our results found an increase in c-Fos expression in GABAergic neurons in the reticular nucleus, pars caudalis (PnC), and in most gigantocellular reticular nuclei in the medullary reticular formation. Hence, it can be presented that the cholinoceptive PnO region released from disinhibition, during PS, may provide excitation to local and projecting GABAergic neurons in the medullary reticular formation, which in turn may initiate the PS characteristic of muscle atonia.

Early neurochemical studies assessing the presumed 'cholinergic' content of the reticular activating system revealed two ascending routes, a dorsal relay through the thalamus that was later confirmed to be cholinergic, and a ventral relay through the basal forebrain that was later found not to be primarily cholinergic but dopaminergic (Jones

and Cuello, 1989). This dopaminergic system arises from the dopamine neurons located in the ventral mesencephalic tegmentum (VMT); substantia nigra and the ventral tegmental area. The results of our study show that activity may be different in these two nuclei during PS. In the substantia nigra, GABAergic neurons are shown to increase in c-Fos expression as would be expected in a system involved in initiation of motor movement, while in the ventral tegmental area, both dopaminergic and GABAergic neurons increase in c-Fos expression during PS. The role that GABAergic neurons play during sleep in the VMT has not been made clear by previous research. Non-dopaminergic neurons, and possibly GABAergic neurons, have been shown to increase their mean average discharge rate during PS (Miller et al., 1983; Steinfels et al., 1983). In the substantia nigra, it can be hypothesized that these GABAergic neurons may exert an inhibitory influence on adjacent dopaminergic neurons (Tepper et al., 1995), or inhibitory influence on distal striatal structures to which they project. Together, the dopaminergic and GABAergic neurons, both local and distally projecting, may mediate a role in permitting the muscle atonia that accompanies PS to occur. GABAergic influence over muscle activity in the substantia nigra may be appreciated by studies showing that injections of muscimol (GABA agonist) abolish bethanechol-induced muscle rigidity (Schulz and Macdonald, 1981), while injections of bicuculline (GABA antagonist) produce tonic activity in the EMG (Havemann et al., 1983). Increased activity in GABAergic neurons may inhibit or attenuate activity in the striatal motor system which if disturbed may antagonize PS. The substantia nigra neurons also send non-dopaminergic projections to the pontomesencephalic tegmentum cholinergic cell area, which have been defined by intracellular recording studies as being inhibitory (Noda and Oka, 1986). Hence, the GABAergic neurons of the substantia nigra may potentially modulate incoming signals from the LDT/PPT cholinergic nuclei or communicate motor information to lower brainstem structures via the LDT/PPT cholinergic cell area.

In this study, an increase in c-Fos expression was observed in both GABAergic and dopaminergic neurons of the ventral tegmental area during PS. Electrophysiological studies of ventral tegmental area dopaminergic neurons over the sleep-waking cycle have demonstrated variance in interspike interval suggestive of bursting activity during PS (Miller et al., 1983), and an increase in c-Fos expression may indicate such a change in

firing pattern (Overton and Clark, 1997). Cholinergic influence on ventral tegmental area GABAergic mechanisms and/or direct excitatory influences on dopaminergic neurons may be responsible for this hypothetical change in activity during PS. However, since bursting in dopaminergic neurons is associated with an increase of dopamine release in its terminal locations (Gonon, 1988), an increase in c-Fos expression if reflecting a change in firing pattern may also reflect an increase in dopamine release in limbic and cortical structures to which this system projects. Given the role these terminal structures play in PS, and the role the ventral tegmental area plays in learning, reward, and even hallucinations or psychotic thought, all of which have been linked to PS and dreaming, the final findings in this series of studies are provocative. It is presented here, that the increase or change in dopaminergic activity in the ventral tegmental area observed during PS, may induce via limbic and cortical structures the cognitive correlate of PS, dreaming, and provide insight into its functional significance.

In the pontomesencephalic tegmentum the cholinergic neurons, and GABAergic neurons co-distributed in this regions are shown to increase activity in association with PS, while monoamiengic neurons are shown to decrease activity in association with PS and this decrease may be elicited by cholinergic mechanisms. If the cholinergic neurons of the LDT/PPT are the initiators of PS, then the cholinoceptive PnO region appears to be one of the generators of PS and PS characteristics. Activation in the PnO, by a possible disinhibition through local GABAergic neurons, is increased during PS, and may contribute to the medullary mediation of muscle atonia. The LDT/PPT projection to the VMT, may act differentially on the neurons of the substantia nigra and ventral tegmental area, which may result in an increase in GABAergic activity in the both the SN and VTA, but only an increase (or change of firing pattern) in the VTA dopaminergic neurons. This dopaminergic activation may be associated with inducing via limbic and cortical structure, to which it projects, the cognitive correlate of PS, dreaming.

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Appendix A

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