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Development of REM sleep drive and clinical implications

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Abstract

REM sleep in the human declines from about 50% of total sleep time (~8 hours) in the newborn to about 15% of total sleep time (~1 hour) in the adult, and this decrease takes place mainly between birth and the end of puberty. We hypothesize that, *if this developmental decrease in REM drive does not occur, lifelong increases in REM sleep drive may ensue*. In the rat, the developmental decrease in REM sleep occurs between 10 and 30 days after birth, declining from over 70% of total sleep time in the newborn to the adult level of about 15% of sleep time during this period. Rats aged 12–21 days were anaesthetized with Ketamine, decapitated and brainstem slices cut for intracellular recordings. We found that excitatory responses of pedunculopontine nucleus (PPN) neurons to NMDA decrease, while responses to kainic acid increase, over this critical period. Serotonergic type 1 agonists have increasing inhibitory responses, while serotonergic type 2 agonists do not change, during this developmental period. The results suggest that, as PPN neurons develop, they are increasingly activated by kainic acid and increasingly inhibited by serotonergic type 1 receptors. These processes may be related to the developmental decrease in REM sleep. Developmental disturbances in each of these systems could induce differential increases in REM sleep drive, accounting for the post-pubertal onset of a number of different disorders manifesting increases in REM sleep drive. Examination of modulation by PPN projections to ascending and descending targets revealed the presence of common signals modulating both ascending arousal-related functions and descending postural/locomotor-related functions.

Keywords

Arousal; kainic acid; NMDA; pedunculopontine nucleus; serotonin

Introduction

Fifty years ago, Aserinsky and Kleitman (Aserinsky and Kleitman 1953) provided the first comprehensive description of a distinct phase of sleep characterized by rapid eye movements (REM). Soon thereafter, the nomenclature of “paradoxical sleep” for this state was proposed (Jouvet 1962), and the idea was advanced that cholinergic mechanisms in the midbrain and pons generated REM sleep (Hernandez-Peon et al. 1963; George et al 1964).

A systematic study of the development of REM sleep in man soon followed (Roffwarg et al. 1966). Basically, REM sleep in the human declines from about 50% of total sleep time (~8 hours) in the newborn to about 15% of total sleep time (~1 hour) in the adult, and this decrease takes place mainly between birth and the end of puberty. Comparative investigations in the rat, cat and guinea pig were then described (Jouvet-Mounier et al. 1970). In the rat, the developmental decrease in REM sleep occurs between 10 and 30 days after birth, declining from over 70% of total sleep time in the newborn to the adult level of about 15% of sleep time during this period.

A number of excellent, recent books (Steriade and MacCarley 1990; Lydic and Baghdoyan 1999) and reviews (Scarnati and Florio 1997; Sakai et al. 2001; Hobson and Pace-Schott 2002) describe the considerable progress in the field of sleep-wake mechanisms. We are particularly interested in the organization of the development of REM sleep and have recently reviewed this area of research (Garcia-Rill et al. 2003). It was previously suggested that the developmental decrease in REM sleep indicates that there is a REM sleep inhibitory process (RIP) that arises during the first two weeks of life in the rat (Vogel et al. 2000). We have been investigating factors that may lead to the proposed RIP because of the potential clinical implications. We hypothesize that, *if this developmental decrease in REM drive does not occur post-pubertally, lifelong increases in REM sleep drive may ensue* (Garcia-Rill et al. 2003). We believe that, if there is a developmental dysregulation that prevents the RIP from occurring, the resulting condition will be marked by increased REM sleep drive. We assume that the greater the degree of excessive REM sleep drive, the more pronounced the severity of subsequent REM sleep drive symptomatology, which may include hallucinations [which have been proposed to represent REM sleep intrusion into waking (Dement 1967)], frequent nocturnal arousals, exaggerated reflexes and hypervigilance. A number of disorders exhibit increases in REM sleep drive, including such post-pubertal onset diseases as schizophrenia, panic attacks, bipolar disorder and obsessive-compulsive disorder. Moreover, changes in REM sleep regulation later in life are evident in depression, insomnia, and such degenerative conditions as Alzheimer's, Huntington's and Parkinson's Diseases (Garcia-Rill, 1997).

The pedunculopontine nucleus (PPN), as the cholinergic arm of the Reticular Activating System (RAS), is known to modulate waking and REM sleep. PPN neurons increase their firing rates during synchronization of fast rhythms in waking and REM sleep (i.e. show tonic activity in waking, bursting activity during REM sleep and reduced activity during slow wave sleep) (Sakai et al. 1990; Steriade et al. 1990a, b). We have been studying the changes in PPN neuronal properties, synaptic inputs and neurochemical control during the most rapid decrease in REM sleep in the rat, 12–21 days post-natally. This work in brainstem slices has revealed important principles of developmental regulation of arousal and sleep-wake cycles at the cellular level. PPN neurons are known to have excitatory glutamate receptors, and inhibitory serotonergic, cholinergic, noradrenergic and gabaergic inputs (see Garcia-Rill et al. 2003). As stated above, we hypothesize that developmental dysregulation of glutamatergic and serotonergic inputs to PPN may lead to increases in REM sleep drive. The studies described below document the normal changes occurring in glutamatergic and serotonergic modulation of PPN neurons during this critical period in

development. In addition, we have been studying the main ascending target of the PPN, the intralaminar thalamus (ILT) (involved in thalamocortical activation), and the main descending targets in the pontomedullary reticular formation (involved in reflex regulation, resetting postural tone and locomotion), especially the medioventral medulla (MED). We used electrical stimulation of the PPN in these slices in order to study the effects of PPN efferents on ascending (ILT) and descending (MED) synaptic targets. This approach has allowed us to discover certain potential common mechanisms by which this system may exercise its modulation. Disturbances in these mechanisms also may underlie the manifestations observed in sleep-wake dysregulation in the disorders mentioned.

Methods

Subjects

Timed-pregnant Sprague-Dawley rats (280–350 g) were used and the litters culled to 10. At 12–21 days of age, pups were anaesthetized using Ketamine (70 mg/Kg, i.m.) until tail pinch and corneal reflexes were absent, then were rapidly decapitated. The brains were dissected free under cooled (4°C), oxygenated (95% O₂, 5% CO₂) artificial cerebrospinal fluid (aCSF) and three different types of slices cut: 1) semi-horizontal brainstem slices along the long axis of the PPN for recording of developmental changes in local properties, 2) semi-sagittal slices containing the PPN and an ascending target, the ILT, for recordings in thalamus following PPN stimulation, and 3) semi-horizontal slices containing the PPN and a descending target, the MED, for recordings in medulla following PPN stimulation. The block of tissue was glued onto a stage and 400 µm slices cut with a Vibroslicer (Campden Inst., U.K.) under cooled, oxygenated aCSF, and then allowed to equilibrate for 1 hr in oxygenated aCSF at room temperature before recording. The composition of the aCSF was (in mM): NaCl 122.8; KCl 5; MgSO₄ 1.2; CaCl₂ 2.5; NaH₂PO₄ 1.2; NaHCO₃ 25; and dextrose 10. Only 1–2 of the 400 µm slices from each brain contained the PPN.

We used 86 pups to generate the data on glutamatergic agonists, 40 pups for the serotonergic agonist data, 45 for the ILT and 46 for the MED data. All animal use procedures were approved by the Institutional Animal Care and Use Committee.

Recording Procedures

The recording chamber allowed the slice to be suspended on a nylon mesh so that oxygenated aCSF could flow all around the slice. The gravity-fed aCSF flowed through a sleeve of circulating warmed water so that the temperature of the aCSF in the chamber was 30±1°C. The outflow was removed by suction and the flow adjusted to 2–3 ml/min. Microelectrodes were pulled in a Sutter Instruments puller using Omega-Dot™, thin-wall borosilicate glass and filled with 3 M K⁺ acetate and 1% biocytin and had a resistance of 70–90 MΩ. Signals were amplified with an Axoclamp 2B amplifier (Axon Inst., CA) in the current clamp mode. Neurons were impaled and allowed to stabilize for about 5 min before testing. Neurons which showed a stable resting membrane potential (RMP) –50 mV and action potentials 50 mV and which had stable, long-term recordings were accepted for data analysis. The RMPs were verified and adjusted when the electrode was withdrawn at the end of recordings (usually only 1–2 mV difference, sometimes >5 mV especially after

biocytin injection). In bridge mode, a series of hyperpolarizing and depolarizing current steps of 0.1–1.0 nA at RMP were applied to determine membrane properties. These current steps also allowed the computation of a preliminary I–V curve during the linear range of voltage deflections using SuperScope software (GW Inst., MA).

Stimulation Procedures

Electrical stimulation was carried out using bipolar electrodes and the parameters described below. Neuroactive agents were applied via a manifold with 6 perfusion ports, hence multiple gravity-fed solutions could be applied for pharmacological characterization of neuronal properties. The concentrations of the superfused neuroactive agents in aCSF were as follows: 2-amino-5-phosphonopentanoic acid (AP5) (50 μ M), a NMDA antagonist; 5-carboxyamido-tryptamine (5-CT) (5 μ M), a serotonin (5-HT₁) agonist; $\pm$ 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) (20 μ M), a 5-HT₂ agonist; gamma-d-glutamylaminomethyl sulfonic acid (GAMS) (50 μ M), a KA antagonist; scopolamine (SCOP) (30 μ M), a cholinergic antagonist; and tetrodotoxin (TTX) (0.3 μ M), a sodium channel blocker. The concentrations of these agents were adjusted so that effects were evident using superfusion times of 1 min. The concentrations of micropressure-applied agents in aCSF were as follows: carbachol (CAR) (50 μ M), a cholinergic agonist; kainic acid (KA) (100 μ M); and n-methyl-d-aspartic acid (NMDA) (300 μ M). The same number of puffs or superfusion time was used to test cells of different ages. Direct effects of these agents on recorded PPN neurons were confirmed before, during and after washout/recovery from TTX superfusion.

The presence of three types of PPN neurons was reported in the guinea pig, namely neurons with a low threshold spike (“LTS”) (type I), an “A” current (type II) and both “A+LTS” (type III) (Leonard and Llinas 1990). Most type II and III neurons were identified as cholinergic. It should be noted that even newborn guinea-pigs show adult-like REM sleep percent, so that their REM sleep drive is more like that of the adult rat, and they undergo no major changes in sleep/wake control across postnatal development (Jouvet-Mounier et al. 1970). In general, there appears to be agreement across laboratories that, in the rat, there are three types of PPN neurons, type I (LTS, non-cholinergic), type II (A, 2/3 cholinergic) and type III (A+LTS, 1/3 cholinergic) (Kang and Kitai 1990; Kamondi et al. 1992; Leonard and Llinas 1990; Takakusaki and Kitai 1997; Takakusaki et al. 1997). Therefore, we first determined the type of PPN neuron being studied, injected it intracellularly, and later confirmed if it was cholinergic or not.

Histological Procedures

At the end of the recording period, each neuron was injected with biocytin using intracellular depolarizing pulses adjusted to elicit a train of action potentials (about 0.5–1.0 nA) of 500 msec duration at 1 Hz for 10–15 min. Such injections yielded well-filled neurons. All of the slices were processed for NADPH diaphorase histochemistry for selective labeling of cholinergic mesopontine (PPN) neurons (Vincent et al. 1983). Briefly, slices were fixed in 4% buffered paraformaldehyde for 1–2 hr, cryoprotected in 20 % sucrose and cut in a cryostat at 50 μ m. Sections were incubated in 1 mg/ml NADPH and 0.1 mg/ml nitroblue tetrazolium in PBS at 37°C for 30–60 min. For intracellularly labeled PPN

neurons, Texas Red-avidin immunocytochemistry (fluorescence microscopy) was carried out preceding NADPH diaphorase histochemistry. Double-labeled neurons were assumed to be cholinergic PPN cells. Texas Red-labeled NADPH diaphorase-negative cells were assumed to be non-cholinergic PPN cells as long as they were in the vicinity of NADPH diaphorase-positive (cholinergic PPN) cells. For intracellularly labeled ILT and MED neurons, NADPH diaphorase histochemistry was carried out to verify the stimulation sites in the PPN, and intracellular labeling was verified using avidin-biotin immunocytochemistry (diaminobenzidine, DAB, chromogen, light microscopy).

Statistical Procedures

For comparison of data between the different age groups and cell types in each experiment, measures were tested using one factor, two factor or multifactor analysis of variance (ANOVA) to conclude whether any of the factors (neuroactive agent) had a significant effect on the magnitude of the measure (depolarization or hyperpolarization) and also whether the interaction of the factors significantly affected the measure (age vs agent). Differences were considered significant at values of $p \leq 0.05$. If a statistical significance was present, a post hoc test (Newman-Keuls) was used to compare between groups.

Results

Developmental shifts in transmitter control of PPN neurons

NMDA and KA receptors—The following results are from a population of 51 intracellularly recorded neurons, all of which were identified as type II (presence of an “A” current) and as cholinergic (Texas Red and NADPH diaphorase-positive). Figure 1 is an example of a type II PPN neuron injected intracellularly. On the left is a fluorescence photomicrograph of the cell injected with biocytin and processed for Texas Red-avidin immunocytochemistry. On the right is the same view under light microscopy showing that the same cell, as well as others in the vicinity, were histochemically labeled by NADPH diaphorase, indicative of their cholinergic nature (Vincent et al. 1983).

In order to avoid the magnesium (Mg) block on the NMDA receptor, these recordings were carried out in Mg-free aCSF. Type II PPN cholinergic neurons were exposed to micropressure-applied NMDA (300 μ M, 3 puffs) and KA (100 μ M, 3 puffs). Previous surveys had established that these concentrations and amounts were adequate for obtaining reproducible responses (Garcia-Rill et al. 2003). Of the 51 type II cholinergic PPN cells tested, 51 responded to NMDA and 46 responded to KA, all by depolarization.

Figure 2 shows the responses of two type II cholinergic PPN neurons, one 12 days of age and the other 21 days of age. The day 12 cell showed an “A” type current (delayed return to RMP after release from hyperpolarization followed by a single action potential), an “ I_h ” current (slow depolarization during hyperpolarizing steps), and did not accommodate during depolarizing pulses. TTX was then applied in order to block sodium channels and action potential generation. The day 12 cell’s responses following application of NMDA showed a high amplitude prolonged depolarization, while application of KA elicited a low amplitude prolonged depolarization. These responses were blocked by the antagonists AP5 and

GAMS, respectively, in the cells tested (n=4, not shown). Conversely, the day 21 cell, which showed an “A” current, no “I_h” current and accommodated, was depolarized modestly by NMDA while KA induced a high amplitude depolarization. Again, such responses were blocked by the NMDA and KA antagonists AP5 and GAMS, respectively, in the cells tested (n=4, not shown).

The amplitude of the peak of the depolarization was measured for every cell of each age between 12 and 21 days for each agent. Figure 3A shows the average peak depolarization and standard error (for clarity in the figure) of the depolarization for cells of each age. Briefly, the mean ($\pm$ standard deviation) depolarization induced by NMDA in 12-day cells was 12.0 ± 6.2 mV, while KA induced a much lower amplitude depolarization of 4.0 ± 1.0 mV. The mean amplitude of the depolarization induced by the same concentration and amount of NMDA gradually decreased with age (13-day 9.8 ± 3.3 mV; 14-day 8.4 ± 3.4 mV; 15-day 7.6 ± 3.4 mV; 16-day 7.6 ± 2.5 mV; 17-day 7.2 ± 2.4 mV; 18-day 6.0 ± 3.0 mV; 19-day 5.3 ± 3.7 mV; 20-day 3.3 ± 3.5 mV; 21-day 3.2 ± 3.0 mV). Conversely, the depolarization induced by the same concentration and amount of KA gradually increased with age (13-day 3.6 ± 1.1 mV; 14-day 3.9 ± 3.2 mV; 15-day 4.0 ± 3.2 mV; 16-day 7.0 ± 2.7 mV; 17-day 6.5 ± 2.1 mV; 18-day 8.4 ± 4.6 mV; 19-day 8.7 ± 4.9 mV; 20-day 11.2 ± 3.1 mV; 21-day 13.1 ± 2.0 mV).

Statistical analysis showed that type II cholinergic PPN neurons had a significant decrease in response to NMDA over age (ANOVA $F=2.87$, $p=0.01$). Post hoc testing revealed that the response to NMDA of 12-day cells was significantly higher than responses to NMDA of 20-day ($p<0.001$) and 21-day ($p<0.001$) cells. Similarly, type II cholinergic PPN neurons had a significant increase in response to KA over age (ANOVA $F=5.18$, $p=0.0001$). Post hoc testing revealed that the response to KA of 12-day and 13-day cells was significantly lower than responses to KA of 21-day cells ($p<0.001$). Responses to KA of 14-day cells were lower than those of 20-day and 21-day cells ($p<0.001$), as were responses of 15-day cells compared to 21-day cells ($p<0.001$). The distributions of mean peak amplitudes across age were significantly different for responses to NMDA compared to responses to KA using a Chi-square test ($df=9$, $F=21.63$, $p<0.01$).

Serotonergic type 1 and type 2 receptors—The following results are from a population of 91 intracellularly recorded neurons, all of which were identified as type II (presence of an “A” current). However, in cells tested for responses to the 5-HT₁ agonist 5-CT, we did not identify the cells as cholinergic or not, we merely injected them intracellularly and processed the tissue for DAB-biotin label (and ensured their location in the vicinity of NADPH diaphorase-positive neurons). Briefly, the amplitude of the peak of the hyperpolarization was measured for every cell of each age between 12 and 21 days. Figure 3B shows the average peak hyperpolarization and standard error (for clarity in the figure) of the hyperpolarization for cells of each age. 5-CT induced a mean ($\pm$ standard deviation) hyperpolarization in 12-day cells of -0.6 ± 3.0 mV, 13-day -2.9 ± 2.2 mV, 14-day -3.6 ± 2.6 mV, 15-day -4.0 ± 1.7 mV, 16-day -2.0 ± 1.7 mV, 17-day -5.5 ± 3.3 mV, 18-day -5.2 ± 3.9 mV; 19-day -6.7 ± 2.9 mV, 20-day -7.2 ± 3.3 mV and 21-day -7.0 ± 1.4 mV.

Statistical analysis of response of type II PPN neurons across age showed that there was a significant increase in the hyperpolarization induced by the 5-HT₁ agonist 5-CT over the ages tested (ANOVA $F=3.55$, $p<0.003$). Post hoc analysis showed that the 5-CT-induced hyperpolarization was significantly greater in 19- and 20-day cells than in 12-day cells (post hoc $p<0.001$).

A total of 42 type II PPN neurons tested for responses to the 5-HT₂ agonist DOI were identified as type II (presence of an “A” current), and were also classified as cholinergic ($n=30$) or non-cholinergic ($n=12$). All of these cells were tested for direct effects using TTX. Of the 30 cholinergic neurons, 21 did not respond following DOI exposure, while 9 did respond. However, the hyperpolarization induced in these 9 cells was only -2.0 ± 0.6 mV, indicating minimal hyperpolarization. The amplitude of this hyperpolarization did not appear to change with age (see Figure 3B), although this represents a small sample. Of the 12 non-cholinergic type II PPN neurons studied, all 12 were hyperpolarized by DOI, but to a greater extent (-8.3 ± 1.8 mV) than cholinergic neurons. The amplitude of the hyperpolarization did not appear to change with age (see Figure 3B), although, again, the sample to date is quite small.

In general, our findings suggest that a) serotonergic 5-HT₁ inputs to the PPN induce increasing hyperpolarization with age; and b) serotonergic 5-HT₂ inputs are preferentially aimed at non-cholinergic type II PPN neurons and exercise a similar inhibitory effect over age, while the small number of cholinergic type II PPN neurons that are affected are only minimally inhibited.

Common signals modulating arousal and movement

Descending projections to MED—Stimulation of the PPN is known to induce changes in arousal and postural/locomotor states (Reese et al. 1995). The distribution and type of various descending cholinergic projection targets have been functionally described (Lydic and Baghdoyan 1993). Previously, PPN stimulation was reported to induce prolonged responses (PRs) in extracellularly-recorded caudal pontine (PnC) neurons in the decerebrate cat (Garcia-Rill et al. 2001). Intracellular recordings from PnC cells in semi-horizontal slices revealed that the longest mean duration PRs were induced by stimulation at 60 Hz compared to 10, 30 or 90 Hz (Homma et al. 2002). Maximal firing rates in PnC cells during PRs were induced by PPN stimulation at 60 Hz compared to 10, 30 or 90 Hz. The muscarinic cholinergic agonist carbachol (CAR) induced depolarization in most PR neurons tested, and the muscarinic cholinergic antagonist scopolamine reduced or blocked PPN stimulation-induced PRs in some PnC neurons, suggesting that some PRs may be due to muscarinic receptor activation. Interestingly, PRs were induced in small, apparently interneurons, and not on giant, presumably reticulospinal neurons involved in the startle response (Homma et al. 2002). A hypothesis was proposed suggesting that descending PPN projections to PnC “push” towards induction of locomotion (prolonged excitation of interneurons) and simultaneously “pull” away from decreased muscle tone (inhibition of giant neurons involved in the SR). Questions remain regarding the pathway via which locomotor driving may occur, since stimulation of the PnC does not lead to locomotion. However, stimulation

of the more caudally located MED is known to induce controlled locomotion on a treadmill in the rat and cat (Kinjo et al. 1990; Skinner et al. 1990).

Preliminary studies were carried out using the same stimulation protocol as in PnC, except that recordings were made in the MED, a region known to induce locomotion when stimulated electrically (reviewed in Reese et al. 1995). Intracellular recordings in semi-horizontal slices from neonatal rat brain showed that 40/81 neurons studied (49%) responded by depolarization following PPN stimulation using 1 sec trains of 0.5 msec pulses, such as those used in the preceding study in PnC (Homma et al. 2002). Additional studies are needed to increase the sample described, but some tentative comparisons can be made. The duration of the depolarization induced in 34 of the MED neurons studied was 11 ± 5 sec, which was similar to the duration of PRs in PnC neurons previously studied (14 ± 4 sec, $n=91$). The depolarization observed in MED neurons was slightly lower (3.6 ± 1.1 mV, $n=38$) compared to that in PnC neurons (4.8 ± 1.3 mV, $n=24$). None of the differences between MED and PnC neurons were statistically significant.

Figure 4A is a representative example of a MED neuron recorded intracellularly which showed a prolonged depolarization following PPN stimulation. In this case, the duration of the depolarization was approximately 3 sec, and generated action potentials for 2.5 sec. The same neuron was depolarized by application of the cholinergic agonist CAR (50 μ M), which induced a depolarization lasting over 20 sec, and generated action potentials for 2.5 sec (Fig. 4B). The effect of CAR was blocked by superfusion of SCOP, indicating that the CAR effect was probably mediated by muscarinic cholinergic receptors; but was present after superfusion with TTX, indicating that the CAR effect was directly on the MED neuron recorded (not shown). These effects were replicated in all 18/25 neurons tested with CAR. In the other 7 neurons (mostly recorded in the early stages of the study) showing PPN stimulation-induced depolarization, the effects of CAR could not be reliably established.

Our previous study on responses of PnC neurons following PPN stimulation using trains of various frequencies had established that there was a firing frequency-dependent activation of PnC cells (Homma et al. 2002). Using the same stimulus parameters, the responses of MED cells were compared when using 10, 30, 60 or 90 Hz stimulation of the PPN. Figure 5A is a graph of the mean $\pm$ S.D. of the firing frequency induced in MED neurons (defined as firing frequency during the 2nd second of the induced train of action potentials) following stimulation of the PPN at various frequencies. As in the PnC, MED cells showed the highest firing frequency when the PPN was stimulated at 60 Hz compared to 10, 30 and 90 Hz. However, the maximal frequency induced in MED cells (4 ± 0.5 /sec) studied to date was numerically lower than that induced in PnC cells (10 ± 3 /sec) following stimulation of the PPN using the same parameters. Figure 5B is a graph of the depolarization induced in MED cells following stimulation of the PPN at different frequencies. The greatest depolarization induced was after 60 Hz stimulation compared to 10, 30 or 90 Hz stimulation. All of these findings are in keeping with those observed in the PnC, showing maximal activation of MED cells after PPN stimulation at 60 Hz (see figure legend for statistical results).

We also carried out a preliminary morphometric analysis on the cell body area of recorded MED neurons, and compared 15 cells that showed PRs following PPN stimulation

(Responsive cells) to 15 cells that showed no response following PPN stimulation (Non-responsive cells). Figure 6 shows representative examples of each type of cell, suggesting that Responsive cells (A, left side) had significantly larger cell areas (558 ± 37 sq μm , $n=15$) compared to Non-responsive cells (B, right side) (382 ± 38 sq μm , $n=15$) (ANOVA $df=29$, $F=11.19$, $p<0.002$). These results on MED cells were reversed compared to our previous study on PnC cells which suggested that PPN input was directed mainly to smaller, perhaps interneuronal, elements (Homma et al. 2002).

In summary, our studies of descending PPN projections suggest that a) a lower percentage of neurons were activated by PPN efferents in more caudal brainstem regions (65% in PnC, 49% in MED), in keeping with morphological studies describing gradually decreasing density of caudal PPN projections (Reese et al. 1995); b) the optimal frequency of PPN stimulation for inducing PRs in all descending targets was 60 Hz, which led to peak firing frequencies in these targets of 4–10 Hz; and c) PPN inputs to PnC are aimed at smaller, perhaps interneurons (which may relay their output to neurons in the region or in the MED), but are aimed at larger neurons of the MED, which may represent reticulospinal projection cells since stimulation of the MED does lead to locomotion (Kinjo et al. 1990; Skinner et al. 1990).

Ascending projections to ILT—Stimulation of the PPN potentiates the appearance of fast (20–40 Hz) oscillations in the EEG, outlasting stimulation by 10–20 seconds (Steriade et al. 1991), indicative of the induction of *prolonged responses eliciting changes in state by the cholinergic arm of the RAS*. We investigated the effects of PPN stimulation at various frequencies on the responses of ILT neurons across development. The semi-sagittal slice used allowed recordings in the posterior ILT, immediately caudal to the fasciculus retroflexus, in the region of the Parafascicular nucleus (Pf, $n=20$), and in the anterior ILT, lateral and anterior to the fasciculus retroflexus, in the region of the Centrolateral nucleus (CL, $n=25$).

Using the same stimulus parameters, the responses of CL and Pf cells were compared when using 10, 30, 60 or 90 Hz stimulation of the PPN. Figure 7A is a graph of the mean $\pm$ S.D. of the firing frequency induced in CL neurons (defined as firing frequency during the 2nd second of the induced train of action potentials) following stimulation of the PPN at various frequencies. Briefly, stimulation at 60 Hz induced the highest firing frequency in CL neurons ($6.6 \pm 2.5/\text{sec}$) compared to stimulation at 10 ($0.4 \pm 0.2/\text{sec}$), 30 ($1.0 \pm 0.4/\text{sec}$) or 90 ($4.1 \pm 1.6/\text{sec}$) Hz. This was similar to the effects observed on PnC and MED neurons (see above). However, the effects of PPN stimulation at different frequencies on responses of Pf cells were less clear. Figure 7B is a graph of the firing frequency induced in Pf cells following stimulation of the PPN at various frequencies. Although the highest frequency was induced by 60 Hz stimulation ($3.5 \pm 0.8/\text{sec}$), 10 ($3.0 \pm 1.1/\text{sec}$), 30 ($3.3 \pm 0.8/\text{sec}$) and 90 ($2.0 \pm 0.6/\text{sec}$) Hz stimulation induced similar firing frequencies.

One additional trend we observed was that the threshold for inducing responses in both CL and Pf cells appeared to increase with age. Figures 8A and 8B show that threshold for depolarization in ILT neurons was higher in slices from older brains. The reason for this effect needs to be explored further, and may or may not have a physiological basis. Because

the slices used were semi-sagittal, the three-dimensional relationship between PPN and the ILT may have changed with age. Studies using different types of slices could help resolve this issue. Moreover, the variability across preparations of the locations of stimulating and recording sites may have contributed to these differences. For the present, this remains an interesting observation that may or may not be confirmed with further experimentation.

In general, our preliminary results in ILT neurons show that, just as with descending PPN projections, stimulation at 60 Hz induced the most pronounced responses in CL neurons. However, Pf neurons did not show such firing frequency-dependent effects, suggesting a different organization for this ascending target of the PPN may be involved. The Pf is more caudal than CL, so that this difference may not be ascribed to a decrease in projection density with greater distance away from the PPN. Rather, the two ILT nuclei may differ in the manner in which they relay PPN information to the cortex.

Discussion

Basic Findings

Developmental decrease in REM sleep—It has been suggested that REM sleep has the biological function of serving to direct the course of brain maturation (Marks et al. 1995). This is in keeping with evidence suggesting that activity-dependent development may be a widespread mechanism directing neural connectivity throughout the brain (Llinas, 1984; Marks et al. 1995). Under this hypothesis, REM sleep could provide endogenous stimulation at a time when the brain has little or no exogenous input. High frequency brainstem activation, especially in the form of PGO waves, could contribute to the maturation of ascending RAS-induced activation of thalamocortical pathways (Marks et al. 1995). A similar effect could be in place in terms of descending RAS projections, given recent results suggesting that spontaneous muscle twitches during sleep help guide spinal self-organization (Petersson et al. 2003).

It has been suggested that the direction of the developmental decrease in REM sleep indicates that there is a REM sleep inhibitory process (RIP) that develops during the first two weeks of life in the rat (Vogel et al. 2000), which may or may not be equivalent to the decrease seen in the human across puberty. This hypothesis predicts that 1) one or more inhibitory process becomes progressively stronger during this period (as outlined above, the PPN receives excitatory glutamatergic, and inhibitory serotonergic, noradrenergic, cholinergic and gabaergic inputs, all likely candidates), and 2) stimulation or blockade of this process will decrease or increase, respectively, the manifestations of REM sleep (Vogel et al. 2000). The same workers found that REM rebound after REM sleep deprivation in the rat was absent at two weeks of age, small at three weeks and larger at four weeks (Feng et al. 2001). That is, the ontogeny of REM rebound was related to the ontogeny of baseline REM sleep. If the RIP were blocked, the result could be a condition characterized by increased REM sleep drive. We hypothesize that, if this developmental decrease in REM drive does not occur post-pubertally, lifelong increases in REM sleep drive may ensue. Our initial studies have addressed glutamatergic and serotonergic inputs to PPN neurons.

NMDA and KA responses—PPN neurons may receive glutamatergic inputs from the reticular formation (Steininger et al. 1992; Stevens et al. 1992), while some cholinergic cells also release glutamate (Clements and Grant 1990). Responses in guinea-pig LDT neurons appear to be mediated by both NMDA and non-NMDA receptors (Sanchez and Leonard 1994, 1996). Injections of glutamate (which activates both NMDA and KA receptors) into the PPN are known to induce increased duration of REM sleep and wakefulness in the rat (Datta and Siwek 1997; Datta et al. 2001). However, NMDA receptors appear to be involved in the induction of increased duration of wakefulness (Datta and Siwek 2001), while KA receptors may be involved in the induction of increased duration of REM sleep (Datta 2002).

Our initial results are in agreement with these findings in the behaving rat and suggest that there is a developmental shift from high amplitude to low amplitude NMDA-induced depolarization, and from low amplitude to high amplitude KA-induced depolarization (Figure 3A). This modulation of depolarization appears to shift around the 15–16 day period, suggesting that KA becomes increasingly important in modulating type II cholinergic PPN neurons from that time on. It should be noted that these are suggestive findings pending recordings from additional numbers of identified neurons. The present results were obtained on 51 neurons across a 10-day period, with some individual days having as few as four neurons. While this allowed statistical comparisons, further sampling is needed to fortify confidence in the results.

It is not clear if the more pronounced KA modulation persists, making sampling at pre- and post-pubertal time points necessary as well. If this shift does persist into adulthood, it suggests that when glutamatergic inputs to the PPN activate KA receptors, there is marked excitation, whereas activation of NMDA receptors does not lead to as great an excitatory effect. This interesting suggestion needs to be tested in the behaving preparation to attempt to dissect the dynamic consequences in the whole animal of this observation in the *in vitro* preparation.

Should these effects be observed in the whole animal, what can this shift tell us about normal and abnormal development? The developmental decrease in REM sleep appears to parallel the decrease in NMDA responsiveness, suggesting that REM sleep becomes the purview of KA modulation. It is not clear at present if the changes in these responses represent changes in receptor number and/or affinity. Future receptor expression and binding studies may reveal if either or both mechanisms are involved in this process. Exploration of the relationship between these two receptor types (as well as the potential role of AMPA receptors) and the control of wakefulness and REM sleep promises to provide intriguing and surprising concepts. For example, these results are in keeping with the work of Datta on intact animals showing that NMDA appears to promote waking, while KA promotes REM sleep, and glutamate promotes both (Datta 2002). However, it is not clear how these results are related to findings showing that there are populations of “REM on”, “Wake” and “Wake/REM on” neurons in the region of the PPN (without being identified as cholinergic or non-cholinergic) (see e.g. Thakkar et al. 1998). Are “REM on” neurons the ones excited by KA receptor activation while “Wake” neurons are the ones excited by NMDA receptor activation and “Wake/REM on” cells by activation of both receptor sub-types? It does not seem far-fetched that the differential activation of these populations of PPN neurons may be

related to the receptor sub-types present on their membranes. Unfortunately, functional and morphological identification of recorded neurons in vivo will be required to answer this question satisfactorily.

From a clinical point of view, despite the partial investigation of the developmental reorganization reported herein, we can make some suggestions regarding the control of REM sleep. For example, the simplest conclusion we can reach at the moment is that REM sleep ultimately comes under the, perhaps exclusive, control of KA receptor activation. Therefore, clinical conditions in which there is an increase in REM sleep drive could be treated with agents that block or reduce KA receptor activation. While this might be undesired during waking hours given the widespread distribution and functions of these receptors in the brain, a KA blocker could be administered before bedtime to reduce REM sleep drive during sleeping hours. This may have the desired effect of reducing the frequent nighttime awakenings that the increased REM sleep drive brings. Such an effect might lead to increased slow wave sleep, hopefully waning by morning. Future studies may identify specific KA receptor sub-types involved in the control of REM sleep, allowing treatment with more specific, targeted blockade of PPN KA receptors. Such a “magic bullet” may some day be used to treat increased REM sleep drive during waking hours, i.e. hallucinations. However, much additional research is essential to determine the details of the different receptors affecting activity in PPN neurons, and their afferents.

Serotonergic responses—In over half of PPN cholinergic neurons, 5-HT induced a TTX-resistant hyperpolarization (Leonard and Llinas 1990; Luebke et al. 1992), but it affected only 25% of non-cholinergic neurons in this way (Greene and Rainnie, 1999). Injections of 5-HT into the mesopontine region suppressed REM sleep (Horner et al. 1997). On the other hand, injection of a 5-HT_{1A} agonist into the PPN did not affect PGO wave induction, and the PPN was found to have relatively few 5-HT_{1A} binding sites compared to LDT (Sanford et al., 1996). Earlier studies concluded that only about 12% of the 5-HT terminals in PPN synapsed on cholinergic cells (Steininger et al. 1992). Others reported that 5-HT₂ receptors were found on cholinergic neurons (Morilak and Ciaranello, 1993), whereas more recent studies suggested that 5-HT₂ receptors were found instead on non-cholinergic neurons (Fay and Kubin, 2000). The preliminary results described here suggest that, 1) 5-HT₁ receptors on PPN neurons induce increasingly greater hyperpolarization during the critical period in development we studied, suggesting that these receptors play a role in the developmental decrease in REM sleep; and 2) type II PPN neurons were hyperpolarized by the 5-HT₂ agonist DOI, and that effect did not change during the period in development studied. Although additional cells are needed, recordings suggest that 5-HT₂ receptors were present on virtually all non-cholinergic type II PPN neurons, but only in a minority of cholinergic type II neurons, and then only induced minimal hyperpolarization in these cells.

A recent study described the inhibition of extracellularly-recorded “REM-on” neurons in the mesopontine region by a 5-HT_{1A} agonist, which had minimal effect on “Wake/REM-on” neurons (Thakkar et al. 1998). The authors hypothesized that, during waking, “REM-on” neurons are inhibited by 5-HT but “Wake/REM-on” neurons are not affected and desynchronization is maintained. They suggested that 5-HT raphe neurons slow their firing in drowsiness and SWS, and less inhibition via 5HT_{1A} receptors on cholinergic PPN “REM-

on” cells leads to increased firing to promote REM sleep (see also Strecker et al. 1999). This suggests that some cholinergic PPN neurons will be inhibited while others will not be affected by 5-HT. Our results do show that some cholinergic PPN neurons were hyperpolarized by a 5-HT₁ agonist, only a minority by a 5-HT₂ agonist, and then only minimally, and some cholinergic neurons were not affected. A greater proportion of non-cholinergic neurons were hyperpolarized by a 5-HT₂ agonist. Overall, these results lend support to the hypothesis advanced, although further studies will be needed to confirm the proposed hypothesis. Moreover, in the future, we may be able to determine if the PPN neurons affected by 5-HT₁ agonists are differentially activated by NMDA and/or KA, and if these are cholinergic or not. This would go a long way towards determining the intricacies of glutamatergic and serotonergic interactions at the level of the PPN. Studies in vitro are ideally suited to investigate such multiple transmitter interactions.

Common signals modulating arousal and movement

During the largest decrease in REM sleep in the rat, 12–21 days (Jouvet-Mounier et al. 1970), there is a significant hypertrophy in PPN neurons (Skinner et al. 1989). It was postulated that the transient increase in cell size was related to increased metabolic needs related to growth of axonal projections. A recent report described an increase in choline acetyltransferase activity during the developmental decrease in REM sleep percent in the rat (Ninomiya et al. 2001), in keeping with the hypertrophy described earlier (Skinner et al. 1989). Muscle twitching during REM sleep is more intense in neonates (in both human and rodent), suggesting that motor inhibition may be less effective early on (Siegel 1999). As mentioned above, recent results suggesting that spontaneous muscle twitches during sleep help guide spinal self-organization (Petersson et al. 2003). In addition, the auditory startle response (SR) in the rat becomes functional after ear opening also around 15 days of age (Sheets et al. 1988; Kungel et al. 1996). These findings suggest that this is a critical stage in the development of ascending and descending components of arousal.

The activation of ascending systems to alert the cortex appears to be coupled with a simultaneous activation of descending systems, resetting motor programs, especially if a SR is elicited by a sensory event (Garcia-Rill et al. 2003). This coherent mechanism can be expected to promote survival, allowing more concerted formulation of fight-or-flight responses. Our results suggest that there may be a common signal elicited by ascending and descending projections of the PPN, and that these are elicited by a frequency-dependent activation of the PPN. Stimulation at ~60 Hz was the optimal stimulation frequency for inducing maximal responses in both ascending (CL and to a lesser extent Pf) and descending (PnC and MED) targets. It is not clear how PPN neurons could be activated physiologically in a manner equivalent to ~60 Hz stimulation (but not at higher or lower frequencies). Regardless of the origin, such activation of the PPN induced a similar peak firing frequency in its targets, suggesting that activation of the PPN at its “preferred” frequency (~60 Hz) induces in its targets a similar, optimal, level of firing (4–10 Hz).

What would be the role of such a common signal? Although movements seem smooth and continuous to us, they are not. They are generated and controlled discontinuously through time, in a pulsatile fashion (Llinas 2001). This periodic control signal is reflected in our

muscles as the 8–12 Hz physiological tremor, which occurs both during movement and rest. This pulsatile control signal is thought to save time and computational overhead, and serves to synchronize all elements of the motor apparatus so that all elements hear the command signal and operate as a single construct (Bernstein 1967). Briefly, 10 Hz is the mean frequency of physiological tremor, the upper limit of individual movements, and is thought to originate in the pontomedullary region as a descending command (Goodman and Kelso 1983; Llinas 2001; Vallbo and Wessberg 1993). This command is thought to act as a cueing function for synchronizing motoneurons, to provide inertia for overcoming friction and viscosity in muscles, and as a control system for binding inputs and outputs in time.

Future studies will explore the possibility that the PPN, as a crucial nexus in the control of both ascending arousal and descending postural and movement regulation, generates the necessary background of activity, a PR, in some or all of its other targets. The advantage behind PRs is that a long-lasting change in state can be induced by a brief input without the need for continuous synaptic drive, and that they can then be reset to return to the previous state, i.e. they have bistable properties. Such a background of activity could subserve an ascending pre-attentional process, modulating orientation and selective attention, as well as a descending pre-motor process, modulating sympathetic discharges, postural adjustments and voluntary movements. *Such integration would help synchronize the disparate systems activated, for example, in fight-or-flight responses induced by an arousing stimulus.*

Clinical implications

Whether or not some or all of these potential functions of REM sleep turn out to be correct, the fact remains that certain sleep pathologies have a developmental etiology, and a number of devastating disorders are marked by a virtually permanent developmental increase in REM sleep drive. In schizophrenia, anxiety disorders and bipolar and unipolar depression, increased REM sleep drive (increased REM duration, decreased REM latency, hypervigilance, etc., usually coupled with decreases in slow wave sleep) is a major, incapacitating symptom (Garcia-Rill 1997; Garcia-Rill et al. 1995). One plausible idea is that this effect is a regression to a previous developmental state. Interestingly, most patients with schizophrenia, bipolar depression, male obsessive-compulsive disorder (OCD), and panic attacks develop the disorder during puberty [~80% between the ages of 15 and 25, *during the normal decrease in REM sleep* shown in humans (Roffwarg et al. 1966)], while unipolar depression in adolescents is very high (Garcia-Rill 1997). One study reported that neonates and endogenous depressives have the same distinctive features of baseline REM sleep (Feng et al. 2001), adding to the suggestion that the REM sleep abnormalities of endogenous depression represent an immature, underdeveloped REM sleep system (Vogel et al. 1997). In general, then, *the increased REM sleep drive in the disorders mentioned may represent a developmental disturbance which normally tends to reduce REM sleep drive.*

In conclusion, our studies revealed that PPN neurons showed increased responsiveness to KA, and decreasing responsiveness to NMDA, during the critical period in development in which REM sleep duration decreases. In addition, responsiveness to a serotonergic type 1 receptor agonist induced increased inhibition over the same period, while a serotonergic type 2 agonist had little effect on cholinergic PPN neurons and did not change during this time

frame. These changes in the regulation of PPN activity may be related to the developmental decrease in REM sleep that occurs during this time.

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Figure 1.

Left side. Photomicrograph of a fluorescent neuron intracellularly injected with biocytin and processed for Texas Red-avidin immunocytochemistry. Right side. Light microscopy of the same cell showing NADPH diaphorase histochemical labeling, suggesting that the recorded PPN neuron was cholinergic. Calibration bar 50 μ m.

Figure 2.

Left side. Responses of intracellularly-recorded day 12 and day 21 type II PPN cholinergic cells to intracellular current pulses. Both cells showed an “A current” (delay in return to baseline) following release from hyperpolarization, making these type II PPN cells. Subsequent Texas Red-avidin and NADPH diaphorase histochemistry revealed that both cells were cholinergic. The resting membrane potential (RMP) of both cells was -61 mV. Calibration bars vertical 20 mV, horizontal 50 msec. Right side, top. Responses of the day 12 type II cholinergic PPN cell showing depolarization from RMP after application of NMDA (300 μ M) or KA (100 μ M). Note the higher amplitude response following NMDA compared to KA. These responses were observed in Mg-free aCSF and under the influence of TTX (0.3 μ M). Right side, bottom. Responses of the day 21 type II cholinergic PPN cell showing lower amplitude depolarization from RMP after application of NMDA compared to KA. Calibration bars vertical 5 mV, horizontal 1 sec.

Figure 3.

A. Responses of type II cholinergic PPN cells to NMDA and KA over age. The changes in membrane potential (mean $\pm$ S.E.) after NMDA (filled triangle) or KA (filled square) are plotted for cells aged 12–21 days. These responses were observed in Mg-free aCSF and under the influence of TTX. Note the gradual increase in depolarization observed for KA over age, with a gradual decrease in depolarization over age for NMDA. B. Responses of type II PPN cells to serotonergic agents over age. The 5-HT₁ receptor agonist, 5-CT (5 μ M), was found to hyperpolarize type II PPN neurons increasingly with age (Xs), although these cells were not identified as cholinergic or not. The 5-HT₂ receptor agonist, DOI (20 μ M), was found to hyperpolarize all type II non-cholinergic PPN cells recorded, but the effect did not change with age (open squares). DOI did not affect the majority of type II cholinergic cells, but in the minority that responded, the hyperpolarization induced was minimal (filled circles), and the effect did not change with age.

Figure 4.

A. Response of a representative MED cell following PPN stimulation (60 Hz train of 0.5 msec pulses for 1 sec). Note the 3 sec duration depolarization induced following the end of stimulation. B. Response of the same MED neuron after application of the cholinergic agonist CAR (50 μ M) showing depolarization and action potential train. The RMP of this neuron was exceptionally high at -42 mV, which was observed in some young (day 12–14) MED neurons. Prolonged stability and consistency of responses, including IV relationship, suggested such cells were healthy. Calibration bars vertical 10 mV, horizontal 2 sec.

Figure 5.

A. Maximal firing frequency (firing rate during the 2nd second of the evoked action potential train) of MED cells induced by PPN stimulation at various frequencies. Note that the highest firing frequency was induced by stimulation at 60 Hz (ANOVA $df=71$, $F=16.81$, $p<0.0001$) compared to 10 Hz ($p<0.01$), 30 Hz ($p<0.01$) or 90 Hz ($p<0.05$). B. Maximal depolarization of MED cells induced by PPN stimulation at various frequencies. Note that the highest amplitude depolarization was induced by stimulation at 60 Hz (ANOVA $df=65$, $F=15.26$, $p<0.0001$) compared to 10 Hz ($p<0.01$), 30 Hz ($p<0.01$) or 90 Hz ($p<0.01$).

Figure 6.

A. Photomicrograph of an intracellularly injected MED neuron processed for biocytin-avidin immunocytochemistry which responded following PPN stimulation. Responsive cells were significantly larger than Non-responsive MED cells. B. Photomicrograph of an intracellularly injected MED neuron which did not respond following PPN stimulation. Calibration bars 50 μm .

Figure 7.

A. Maximal firing frequency (firing rate during the 2nd second of the evoked action potential train) of CL cells induced by PPN stimulation at various frequencies. Note that the highest firing frequency was induced by stimulation at 60 Hz (ANOVA $df=27$, $F=3.20$, $p<0.04$) compared to 10 Hz ($p<0.05$), 30 Hz ($p<0.05$) or 90 Hz ($p<0.05$). B. Maximal firing frequency of Pf cells induced by PPN stimulation at various frequencies. There were no statistically significant differences in the firing frequency induced by different frequencies of stimulation.

Figure 8.

A. Threshold for inducing depolarization in CL cells following PPN stimulation across age. When the threshold for eliciting depolarization in 12–16 day cells was compared to that for inducing a response in 16–21 day cells, there was a statistically significant difference (ANOVA $df=24$, $F=7.84$, $p<0.01^{**}$). B. Threshold for inducing depolarization in Pf cells following PPN stimulation across age. When the threshold for eliciting depolarization in 12–16 day cells was compared to that for inducing a response in 16–21 day cells, there was a statistically significant difference (ANOVA $df=19$, $F=21.73$, $p<0.001^{**}$).