Development of REM sleep drive and clinical implications

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Kobayashi, T., C. Good, K. Mamiya, R. D. Skinner, and E. Garcia-Rill. Development of REM sleep drive and clinical implications. J Appl Physiol 96: 735–746, 2004. First published October 3, 2003; 10.1152/japplphysiol.00908.2003.—Rapid eye movement (REM) sleep in the human declines from ∼50% of total sleep time (∼8 h) in the newborn to ∼15% of total sleep time (∼1 h) in the adult, and this decrease takes place mainly between birth and the end of puberty. We hypothesize that without this developmental decrease in REM sleep drive, lifelong increases in REM sleep drive may ensue. In the rat, the developmental decrease in REM sleep occurs 10–30 days after birth, declining from >70% of total sleep time in the newborn to the adult level of ∼15% of sleep time during this period. Rats at 12–21 days of age were anesthetized with ketamine and decapitated, and brain stem slices were cut for intracellular recordings. We found that excitatory responses of pedunculopontine nucleus (PPN) neurons to N-methyl-d-aspartic acid decrease, while responses to kainic acid increase, over this critical period. During this developmental period, inhibitory responses to serotonergic type 1 agonists increase but responses to serotonergic type 2 agonists do not change. 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 postpubertal 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 ascending arousal-related functions and descending postural/locomotor-related functions. A number of excellent, recent books (32, 52) and reviews (18, 39, 44) describe the considerable progress in the field of sleep-wakefulness mechanisms. We are particularly interested in the organization of the development of REM sleep and have recently reviewed this area of research (12). It was previously suggested that the developmental decrease in REM sleep indicates that there is an REM sleep inhibitory process (RIP) that arises during the first 2 wk of life in the rat (62). 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 sleep drive does not occur postpubertally, lifelong increases in REM sleep drive may ensue (12). We believe that if there is a developmental dysregulation that prevents the RIP, 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 will be, which may include hallucinations [which have been proposed to represent REM sleep intrusion into wakefulness (7)], frequent nocturnal arousals, exaggerated reflexes, and hypervigilance. A number of disorders exhibit increases in REM sleep drive, including such postpubertal-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 (10).

The pedunculopontine nucleus (PPN), as the cholinergic arm of the reticular-activating system (RAS), is known to modulate wakefulness and REM sleep. PPN neurons increase their firing rates during synchronization of fast rhythms in wakefulness and REM sleep (i.e., show tonic activity in wakefulness, tonic and bursting activity during REM sleep, and reduced activity during slow-wave sleep) (40, 51, 53). 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, i.e., postnatal days 12–21. This work in brain stem slices has revealed important principles of developmental regulation of arousal and sleep-wakefulness cycles at the cellular level. PPN neurons are known to have excitatory glutamate receptors and inhibitory serotonergic, cholinergic, noradrenergic, and GABAergic inputs (12). 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 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, kainic acid; N-methyl-d-aspartic acid; pedunculopontine nucleus; serotonin

Fifty years ago, Aserinsky and Kleitman (1) 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 (21), and the idea was advanced that cholinergic mechanisms in the midbrain and pons generated REM sleep (14, 17). A systematic study of the development of REM sleep in humans soon followed (38). Basically, REM sleep in the human declines from ∼50% of total sleep time (∼8 h) in the newborn to ∼15% of total sleep time (∼1 h) 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 (22). In the rat, the developmental decrease in REM sleep occurs 10–30 days after birth, declining from >70% of total sleep time in the newborn to the adult level of ∼15% of sleep time during this period.

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the intralaminar thalami (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 mediusentral medulla (MED). We used electrical stimulation of the PPN in these slices 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-wakefulness dysregulation in the disorders mentioned above.

METHODS

Animals

Timed-pregnant Sprague-Dawley rats (280–350 g) were used, and the litters were culled to 10. At 12–21 days of age, pups were anesthetized with ketamine (70 mg/kg im) until tail pinch and corneal reflexes were absent; then they 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 were cut: 1) semihorizontal brain stem slices along the long axis of the PPN for recording of developmental changes in local properties, 2) semisagittal slices containing the PPN and an ascending target, the ILT, for recordings in the thalamus after PPN stimulation, and 3) semihorizontal slices containing the PPN and a descending target, the MED, for recordings in the medulla after PPN stimulation. The block of tissue was glued onto a stage, and 400-µm slices were cut with a Vibroslicer (Campden Instruments) under cooled, oxygenated aCSF and then allowed to equilibrate for 1 h in oxygenated aCSF at room temperature before recording. The composition of the aCSF was as follows (in mM): 122.8 NaCl, 5 KCl, 1.2 MgSO₄, 2.5 CaCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, and 10 dextrose. Only one or two of the 400-µm slices from each brain contained the PPN.

We used 86 pups to generate the data on glutamatergic agonists, 40 for the serotonergic agonist data, 45 for the ILT data, 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 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 was adjusted to 2–3 ml/min. Microelectrodes were pulled in a Sutter Instruments puller using Omega-Dot thin-walled borosilicate glass and filled with 3 M potassium acetate and 1% biocytin; resistance was 70–90 MΩ. Signals were amplified with an Axoclamp 2B amplifier (Axon Instruments) in the current-clamp mode. Neurons were impaled and allowed to stabilize for ~5 min before testing. Neurons that showed a stable resting membrane potential (RMP) of no more than ~50 mV and action potentials ±50 mV and 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- to 2-mV difference, sometimes >5-mV difference, 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 computation of a preliminary current-voltage curve during the linear range of voltage deflections using SuperScope software (GW Instruments).

Stimulation Procedures

Electrical stimulation was carried out using bipolar electrodes, and the parameters are described below. Neuroactive agents were applied via a manifold with six 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: 50 µM 2-amino-5-phosphono nopenic acid (AP5), an N-methyl-D-aspartic acid (NMDA) antagonist; 5 µM 5-carboxamidotryptamine (5-CT), a serotonin (5-HT₁) agonist; 20 µM (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino propionic hydrochloride (DOI), a 5-HT₂ agonist; 50 µM y-glutamylaminomethyl sulfonic acid (GAMS), a kainic acid (KA) antagonist; 30 µM scopolamine (SCOP), a cholinergic antagonist; and 0.3 µM tetrodotoxin (TTX), an Na⁺ channel blocker. The concentrations of these agents were adjusted so that their effects were evident when superfusion times of 1 min were used. The concentrations of micropressure-applied agents in aCSF were as follows: 50 µM carbachol (CAR), a cholinergic agonist; 100 µM KA; and 300 µM NMDA. The same number of puffs or the same 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 “A + LTS” (type III) (27). Most type II and III neurons were identified as cholinergic. Even newborn guinea pigs show an adult-like percentage of REM sleep, so their REM sleep drive is more like that of the adult rat, and they undergo no major changes in sleep-wakefulness control across postnatal development (22). In general, there appears to be agreement across laboratories that, in the rat, there are three types of PPN neurons, type I (LTS, noncholinergic), type II (A, two-thirds of them cholinergic), and type III (A + LTS, one-third of them cholinergic) (23, 24, 27, 56, 57). Therefore, we first determined the type of PPN neuron, injected it intracelullarly, and later confirmed whether it was cholinergic.

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 (~0.5–1.0 nA) of 500-ms duration at 1 Hz for 10–15 min. Such injections yielded well-filled neurons. All the slices were processed for NADPH diaphorase histochemistry for selective labeling of cholinergic mesopontine (PPN) neurons (60). Briefly, slices were fixed in 4% buffered paraformaldehyde for 1–2 h, cryoprotected in 20% sucrose, and cut in a cryostat at 50 µm. Sections were incubated in 1 mg/ml NADPH and 0.1 mg/ml nitro blue tetrazolium in PBS at 37°C for 30–60 min. For intracellularly labeled PPN neurons, Texas red-avidin immunocytochemistry (fluorescence microscopy) was carried out before NADPH diaphorase histochemistry. Double-labeled neurons were assumed to be cholinergic PPN cells. Texas red-labeled NADPH diaphorase-negative cells were assumed to be noncholinergic 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-biocytin immunocytochemistry (diaminobenzidine, 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
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 shows a type II PPN neuron injected intracellularly. The fluorescence photomicrograph shows a cell injected with biocytin and processed for Texas red-avidin immunocytochemistry. The same view under light microscopy shows the same cell, as well as others in the vicinity, histochemically labeled by NADPH diaphorase, indicative of the cholinergic nature of the cell(s) (60).

To avoid the Mg\(^{2+}\) block on the NMDA receptor, the recordings were carried out in Mg\(^{2+}\)-free aCSF. Type II PPN cholinergic neurons were exposed to micropressure-applied NMDA (300 \(\mu\)M, 3 puffs) and KA (100 \(\mu\)M, 3 puffs). Previous surveys established that these concentrations and amounts were adequate for obtaining reproducible responses (12). 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 on day 12 and the other on day 21. On day 12, the cell showed an A-type current (delayed return to RMP after release from hyperpolarization followed by a single action potential) and an “Ih” current (slow depolarization during hyperpolarizing steps) and did not accommodate during depolarizing pulses. TTX was then applied to block Na\(^+\) channels and action potential generation. After application of NMDA, the cell on day 12 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 (\(n = 4\), not shown). Conversely, on day 21, the cell, which showed an A current, no Ih 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 (\(n = 4\), not shown).

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

Statistical analysis showed a significant decrease in response to NMDA in type II cholinergic PPN neurons with age (\(F = 2.87, P = 0.01\) by ANOVA). Post hoc testing revealed that the response to NMDA was significantly higher in cells on day 12 than on days 20 (\(P < 0.001\)) and 21 (\(P < 0.001\)). Similarly, the response to KA in type II cholinergic PPN neurons significantly increased with age (\(F = 5.18, P = 0.0001\) by ANOVA). Post hoc testing revealed that the response to KA was signif-

Texas Red Fluorescence

NADPH Diaphorase

Fig. 1. Left: photomicrograph of a fluorescent neuron intracellularly injected with biocytin and processed for Texas red-avidin immunocytochemistry. Right: light-microscopic image of cell at left showing NADPH diaphorase histochemical labeling. Image at left suggests that the recorded pediculopontine nucleus (PPN) neuron was cholinergic. Calibration bar, 50 \(\mu\)m.
Fig. 2. Left: responses of intracellularly recorded type II PPN cholinergic cells to intracellular current pulses on days 12 and 21. Both cells showed an “A current” (delay in return to baseline) after release from hyperpolarization, indicating that these are type II PPN cells. Subsequent Texas red-avidin and NADPH diaphorase histochemistry revealed that both cells were cholinergic. Resting membrane potential (RMP) of both cells was −61 mV. Right, top: depolarization from RMP after application of 300 μM N-methyl-D-aspartic acid (NMDA) or 100 μM kainic acid (KA) in type II cholinergic PPN cell on day 12. Note higher amplitude response after NMDA than after KA. Responses were observed in Mg2+-free artificial cerebrospinal fluid (aCSF) and under the influence of 0.3 μM TTX. Right, bottom: lower amplitude depolarization from RMP after application of NMDA than after KA in type II cholinergic PPN cell on day 21.

Significantly lower in cells on days 12 and 13 than on day 21 (P < 0.001). Responses to KA were lower in cells on day 14 than on days 20 and 21 (P < 0.001), as were responses of cells on day 15 compared with day 21 (P < 0.001). The distributions of mean peak amplitudes across age for responses to NMDA were significantly different from those for responses to KA (df = 9, F = 21.63, P < 0.01 by χ2 test).

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-type current). However, in cells tested for responses to the 5-HT1 agonist 5-CT, we did not identify the cells as cholinergic or noncholinergic; we merely injected them intracellularly and processed the tissue for diaminobenzidine-biotinyl 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 days 12 and 21. Figure 3B shows the average peak hyperpolarization and standard error (for clarity) for cells of each age. 5-CT induced a hyperpolarization (mean ± SD) as follows: −0.6 ± 3.0, −2.9 ± 2.2, −3.6 ± 2.6, −4.0 ± 1.7, −2.0 ± 1.7, −5.5 ± 3.3, −5.2 ± 3.9, −6.7 ± 2.9, −7.2 ± 3.3, and −7.0 ± 1.4 mV on days 12, 13, 14, 15, 16, 17, 18, 19, 20, and 21, respectively.

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

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 noncholinergic (n = 12). All these cells were tested for direct effects using TTX. Of the 30 cholinergic neurons, 21 did not respond after DOI exposure and 9 did respond. However, the hyperpolarization induced in these nine cells was only −2.0 ± 0.6 mV, indicating minimal hyperpolarization. The amplitude of this hyperpolarization did not appear to change with age (Fig. 3B), although the sample is small. All 12 noncholinergic type II PPN neurons 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 (Fig. 3B), although, again, the sample was quite small.

In general, our findings suggest that 1) serotonergic 5-HT1 inputs to the PPN induce increasing hyperpolarization with age and 2) serotonergic 5-HT2 inputs are preferentially aimed at noncholinergic 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 (37). The distribution and type of various descending cholinergic projection targets have been functionally described (31). Previously, PPN stimulation was reported to induce prolonged responses (PRs) in extracellularly recorded caudal pontine (PnC) neurons in the decerebrate cat (13). Intracellular recordings from PnC cells in semihorizontal slices revealed that the longest mean duration PRs were induced by stimulation at 60 Hz compared with 10, 30, or 90 Hz (19). Maximal firing rates in PnC cells during PRs were induced by PPN stimulation at 60 Hz compared with 10, 30, or 90 Hz. The muscarinic cholinergic agonist CAR induced depolarization in most PR neurons tested, and the muscarinic cholinergic antagonist SCOP reduced or blocked PPN stimulation-induced PRs in some PnC neurons, suggesting that some PRs may be due to muscarinic receptor activation. Interestingly, some PRs were induced in small, apparently interneurons and not on giant, presumably reticulospinal neurons involved in the startle response (19). A hypothesis was proposed suggesting that descending PPN projections to PnC “push” toward induction of locomotion (prolonged excitation of interneurons) and simultaneously “pull” away from decreased muscle tone (inhibition of giant neurons involved in the startle response). Questions remain regarding the pathway by which locomotor driving may occur, because 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 (25, 48).

Preliminary studies were carried out using the stimulation protocol used for PnC, except recordings were made in the MED, a region known to induce locomotion when stimulated electrically (for review see Ref. 37). Intracellular recordings in semihorizontal slices from neonatal rat brain showed that 40 of 81 neurons (49%) responded by depolarization after PPN stimulation using 1-s trains of 0.5-ms pulses, such as those used in an earlier study in PnC (19). 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 was 11 ± 5 s, which was similar to the duration of PRs in PnC neurons previously studied (14 ± 4 s, n = 91). The depolarization was slightly lower in MED neurons (3.6 ± 1.1 mV, n = 38) than in PnC neurons (4.8 ± 1.3 mV, n = 24). None of the differences between MED and PnC neurons were statistically significant.

In Fig. 4A, a MED neuron recorded intracellularly shows a prolonged depolarization following PPN stimulation. The duration of the depolarization was ~3 s, and action potentials
were generated for 2.5 s. The same neuron was depolarized by application of the cholinergic agonist CAR (50 μM), which induced a depolarization lasting 20 s and generated action potentials for 2.5 s (Fig. 4B). Because the effect of CAR was blocked by superfusion of SCOP, it was probably mediated by muscarinic cholinergic receptors but was present after superfusion with TTX, indicating that CAR had a direct effect on the MED neuron (not shown). These effects were replicated in all 18 of 25 neurons tested with CAR. In the other seven 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 laboratory’s previous study on responses of PnC neurons after PPN stimulation using trains of various frequencies established that there was a firing frequency-dependent activation of PnC cells (19). The same stimulus parameters were used to compare the responses of MED cells with 10-, 30-, 60-, or 90-Hz stimulation of the PPN. Figure 5A shows the firing frequency (mean ± SD) induced in MED neurons (defined as firing frequency during the second of the induced train of action potentials) after 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 with 10, 30, and 90 Hz. However, the maximal frequency induced in MED cells (4 ± 0.5/s) studied to date was numerically lower than that induced in PnC cells (10 ± 3/s) after stimulation of the PPN using the same parameters. Figure 5B shows the depolarization induced in MED cells after stimulation of the PPN at different frequencies. The greatest depolarization was induced after 60-Hz stimulation compared with 10, 30, or 90 Hz. 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 after PPN stimulation (responsive cells) with 15 cells that showed no response after PPN stimulation (nonresponsive cells). Figure 6, which shows representative examples of each type of cell, suggests that responsive cells (A) had significantly larger cell areas (558 ± 37 μm², n = 15) than nonresponsive cells (B: 382 ± 38 μm², n = 15, df = 29, F = 11.19, P < 0.002 by ANOVA). These results on MED cells were reversed compared with results of our previous study on PnC cells, which suggested that PPN input was directed mainly to smaller, perhaps interneuronal, elements (19).

In summary, our studies of descending PPN projections suggest that 1) a lower percentage of neurons are activated by PPN efferents in more caudal brain stem regions (65% in PnC, 49% in MED), in keeping with morphological studies describing gradually decreasing density of caudal PPN projections (37); 2) the optimal frequency of PPN stimulation for inducing PRs in all descending targets is 60 Hz, which leads to peak firing frequencies in these targets of 4–10 Hz; and 3) 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, because stimulation of the MED does lead to locomotion (25, 48).

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 s (50), indicative of the induction of PRs 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 semisagittal slice allowed recordings in the posterior ILT, immediately caudal to the fasciculus retroflexus, in the region of the parafascicular (Pf) nucleus (n = 20), and in the anterior ILT, lateral and anterior to the fasciculus retroflexus, in the region of the centrolateral (CL) nucleus (n = 25).

The same stimulus parameters were used to compare the responses of CL and Pf cells with 10-, 30-, 60-, or 90-Hz stimulation of the PPN. Figure 7A shows the firing frequency (mean ± SD) induced in CL neurons (defined as firing frequency during the second of the induced train of action potentials) after stimulation of the PPN at various frequencies. Briefly, stimulation at 60 Hz induced the highest firing frequency in CL neurons (6.6 ± 2.5/s) compared with stimulation at 10, 30, and 90 Hz (0.4 ± 0.2, 1.0 ± 0.4, and 4.1 ± 1.6/s, respectively). This was similar to the effects observed on PnC and MED neurons (see Descending projections to MED). However, the effects of PPN stimulation at different frequencies on responses of Pf cells were less clear. Figure 7B shows the firing frequency induced in Pf cells after stimulation of the PPN at various frequencies. Although the highest frequency was induced by 60-Hz stimulation (3.5 ± 0.8/s), stimulation at 10, 30, and 90 Hz (3.0 ± 1.1, 3.3 ± 0.8, and 2.0 ± 0.6/s, respectively) induced similar firing frequencies.

One additional trend we observed was that the threshold for inducing responses in CL and Pf cells appeared to increase with age. Figure 8 shows that the 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 were semisagittal, the three-dimensional relation 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. 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 that a different organization for this ascending target of the PPN may be involved. The Pf neuron is more caudal than the CL neuron, so 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

Fig. 6. A: photomicrograph of an intracellularly injected MED neuron processed for biocytin-avidin immunocytochemistry. Neuron responded after PPN stimulation. Responsive cells were significantly larger than nonresponsive cells. B: photomicrograph of an intracellularly injected MED neuron that did not respond after PPN stimulation. Calibration bars, 50 μm.
direct the course of brain maturation (33). This is in keeping with evidence suggesting that activity-dependent development may be a widespread mechanism directing neural connectivity throughout the brain (28, 33). Under this hypothesis, REM sleep could provide endogenous stimulation at a time when the brain has little or no exogenous input. High-frequency brain stem activation, especially in the form of pontogeniculooccipital (PGO) waves, could contribute to the maturation of ascending RAS-induced activation of thalamocortical pathways (33). 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 (36).

It has been suggested that the direction of the developmental decrease in REM sleep indicates the existence of an RIP that develops during the first 2 wk of life in the rat (62), which may or may not be equivalent to the decrease 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 (62). The same workers found that REM rebound after REM sleep deprivation in the rat was absent at 2 wk of age, small at 3 wk, and larger at 4 wk (9). 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 postpubertally, 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 (49, 54), while some cholinergic cells also release glutamate (3). Responses in guinea pig laterodorsal tegmental (LDT) neurons appear to be mediated by NMDA and non-NMDA receptors (41, 42). Injections of glutamate (which activates NMDA and KA receptors) into the PPN are known to induce increased duration of REM sleep and wakefulness in the rat (5, 6). However, NMDA receptors appear to be involved in the induction of increased duration of wakefulness (5), whereas KA receptors may be involved in the induction of increased duration of REM sleep (4).

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**Fig. 7.** A: maximal firing frequency (firing rate during the second of evoked action potential train) of centrolateral nucleus (CL) cells induced by PPN stimulation at various frequencies. Highest firing frequency was induced by stimulation at 60 Hz (df = 27, F = 3.20, P < 0.04 by ANOVA) compared with 10 Hz (P < 0.05), 30 Hz (P < 0.05), or 90 Hz (P < 0.05). B: maximal firing frequency of parafascicular nucleus (PF) cells induced by PPN stimulation at various frequencies. There were no statistically significant differences in firing frequency induced by different frequencies of stimulation.
Our initial results are in agreement with these findings in the behaving rat and suggest a developmental shift from high- to low-amplitude NMDA-induced depolarization and from low- to high-amplitude KA-induced depolarization (Fig. 3A). This modulation of depolarization appears to shift at around days 15–16, suggesting that KA becomes increasingly important in modulating type II cholinergic PPN neurons from that time on. 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 as few as 4 neurons on some individual days. Although this allowed statistical comparisons, further sampling is needed to support the results.

It is not clear whether the more pronounced KA modulation persists, making sampling at pre- and postpubertal time points necessary as well. Persistence of this shift into adulthood would suggest 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 whether the changes in these responses represent changes in receptor number and/or affinity. Future receptor expression and binding studies may reveal whether either mechanism or both mechanisms are involved in this process. Exploration of the relation between these two receptor types (as well as the potential role of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors) and the control of wakefulness and REM sleep promises to provide intriguing and surprising concepts. For example, these results are consistent with the work of Datta (4) on intact animals showing that NMDA appears to promote wakefulness, while KA promotes REM sleep, and glutamate promotes both. However, it is not clear how these results are related to findings of populations of
“REM-on,” “wake,” and “wake/REM-on” neurons in the region of the PPN (without being identified as cholinergic or noncholinergic) (58). Are REM-on neurons excited by KA receptor activation and wake neurons excited by NMDA receptor activation and wake/REM-on cells excited by activation of both receptor subtypes? It does not seem far-fetched that the differential activation of these populations of PPN neurons may be related to the receptor subtypes on their membranes. Unfortunately, functional and morphological identification of recorded neurons in vivo is required to answer this question satisfactorily.

From a clinical point of view, despite the partial investigation of the developmental reorganization reported here, we can make some suggestions regarding the control of REM sleep. For example, the simplest conclusion we can reach 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. Although this might be undesirable during wakefulness, 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 sleep. 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 subtypes 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 wakefulness, 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. 5-HT induced a TTX-resistant hyperpolarization in over half of PPN cholinergic neurons (27, 30), but it had this effect in only 25% of noncholinergic neurons (16). Injections of 5-HT into the mesopontine region suppressed REM sleep (20). On the other hand, injection of a 5-HT1A agonist into the PPN did not affect pontogeniculocippital wave induction, and the PPN was found to have relatively few 5-HT1A binding sites compared with the laterodorsal tegmental nucleus (43). Earlier studies concluded that only about 12% of the 5-HT terminals in PPN synapsed on cholinergic cells (49). Others reported that 5-HT2 receptors were found on cholinergic neurons (34), whereas more recent studies suggested that 5-HT2 receptors were found instead on noncholinergic neurons (8). 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 of development studied. Although additional cells are needed, recordings suggest that 5-HT2 receptors were present on virtually all noncholinergic 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-HT1A agonist, which had a minimal effect on wake/REM-on neurons (58). The authors hypothesized that, during wakefulness, 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 slow-wave sleep, and less inhibition via 5-HT1A receptors on cholinergic PPN REM-on cells leads to increased firing to promote REM sleep (55). This suggests that some cholinergic PPN neurons will be inhibited while others will not be affected by 5-HT. Our results show that some cholinergic PPN neurons were hyperpolarized by a 5-HT1 agonist and only a minority by a 5-HT2 agonist, and then only minimally, and some cholinergic neurons were not affected. A greater proportion of noncholinergic neurons were hyperpolarized by a 5-HT2 agonist. Overall, these results lend support to the hypothesis advanced, although further studies are needed to confirm the proposed hypothesis. Moreover, in the future, we may be able to determine whether the PPN neurons affected by 5-HT1 agonists are differentially activated by NMDA and/or KA and whether these are cholinergic or noncholinergic. This would go a long way toward 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, days 12–21 (22), there is a significant hypertrophy in PPN neurons (47). 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 percentage of REM sleep in the rat (35), in keeping with the hypertrophy described earlier (47). Muscle twitching during REM sleep is more intense in neonates (in humans and rodents), suggesting that motor inhibition may be less effective early in development (46). As mentioned above, recent results suggest that spontaneous muscle twitches during sleep help guide spinal self-organization (36). In addition, the auditory startle response in the rat becomes functional after ear opening, also at around day 15 (26, 45). 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 startle response is elicited by a sensory event (12). This coherent mechanism can be expected to promote survival, allowing more concerted formulation of flight-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 optimal for inducing maximal responses in ascending (CL and, to a lesser extent, PF neurons) 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 (29). This periodic control signal is reflected in our muscles as the 8- to 12-Hz physiological tremor that occurs 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 (2). 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 (15, 29, 59). 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 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 of 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 preattentional process, modulating orientation and selective attention, as well as a descending premotor process, modulating sympathetic discharges, postural adjustments, and voluntary movements. Such integration would help synchronize the disparate systems activated, e.g., 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 (e.g., increased REM duration, decreased REM latency, and hypervigilance, usually coupled with decreases in slow-wave sleep) is a major, incapacitating symptom (10, 11). 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, and panic attacks develop the disorder during puberty (~80% between 15 and 25 yr of age, during the normal decrease in REM sleep in humans (38)), while unipolar depression in adolescents is very high (10). One study reported that neonates and endogenous depressives have the same distinctive features of baseline REM sleep (9), adding to the suggestion that the REM sleep abnormalities of endogenous depression represent an immature, underdeveloped REM sleep system (61). In general, then, the increased REM sleep drive in the disorders mentioned above may represent a developmental disturbance that 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 serotoninergic 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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