Behavioral State-Related Changes of Extracellular Serotonin Concentration in the Pedunculopontine Tegmental Nucleus: A Microdialysis Study in Freely Moving Animals

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Neurons of the cholinergic mesopontine tegmentum preferentially discharge during REM sleep and are thought to promote this state. It has been hypothesized they are inhibited during wakefulness by serotonergic input. The present study used the microdialysis sampling procedure coupled to microbore HPLC to measure extracellular serotonin levels in the pedunculopontine tegmental nucleus (PPT) in naturally sleeping cats. Extracellular serotonin levels were found to be highest during periods of wakefulness, lower during slow wave sleep, and lowest during periods of REM sleep. During wakefulness serotonin levels measured in 10 µl samples were 1.14 ± 0.13 fmol/sample, whereas during slow wave sleep serotonin levels declined significantly to 72% of the wakefulness baseline (0.85 ± 0.11 fmol/sample), and dropped further to 45% of the wakefulness baseline in REM samples (0.52 ± 0.10 fmol/sample; all p’s<0.003). The decrease in PPT serotonin levels during REM sleep may be an important determinant in the timing of REM sleep cyclicity. The data support the hypothesis that, during slow wave sleep and REM sleep, the declining levels of serotonin release the PPT REM-promoting neurons from serotonergic inhibition, which, in turn, leads to increases in acetylcholine release in terminal areas, facilitating the emergence of REM sleep.

CURRENT CLAIM: Extracellular serotonin levels in the pedunculopontine tegmental nucleus were found to be highest during wakefulness, to decline during slow wave sleep, and to be lowest during periods of REM sleep.

Serotonergic (5-HT) neurons of the dorsal raphe nucleus (DRN) and cholinergic neurons in the adjacent laterodorsal (LDT) and pedunculopontine tegmental nuclei (PPT) have together been implicated in the regulation and production of REM sleep (reviewed in McCarley et al., 1995; Jones, 1993). Several lines of evidence indicate that the activity of cholinergic neurons in the PPT is involved in the generation of REM sleep via projections to the pontine reticular formation and thalamus. For example, electrophysiological studies of PPT neurons reveal sub-populations that discharge preferentially just before and during REM sleep (termed REM-on neurons), or discharge during both wakefulness (W) and REM sleep (referred to as Wake/REM-on neurons) (El Mansari et al., 1989; Steriade et al., 1990; Kayama et al., 1992; Thakkar et al., 1998).

In contrast to mesopontine neurons that preferentially discharge in REM sleep, monoaminergic neurons in the noradrenergic locus coeruleus and serotonergic DRN exhibit a pattern of discharge activity that is nearly opposite to that of the cholinergic PPT neurons: discharge is greatest during waking, declines during slow wave sleep (SWS) and virtually ceases prior to and during REM sleep for both DRN (McCinty and Harper, 1976; Lydic et al., 1987; Jacobs and Fornal, 1991) and locus coeruleus (Hobson et al., 1975; Foote et al., 1983). This inverse correlation with REM sleep led to suggestions that norepinephrine (McCarley and Hobson, 1975) and 5-HT activity (McCinty and Harper, 1976) might suppress REM sleep, and formed the basis of a structural and mathematical model of REM sleep control, the reciprocal interaction model (McCarley and Hobson, 1975), that had as one of its postulates that the REM-off neurons inhibited the REM-promoting, REM-on neurons (McCarley and Massaquoi, 1992; McCarley et al., 1995). For many years this was regarded as a highly controversial postulate. However, in recent years, in vitro work has supported this hypothesis, revealing that 5-HT directly inhibits identified cholinergic neurons of the LDT and PPT (Mühlethaler et al., 1990; Luebke et al., 1992; Leonard and Llinás, 1994). Furthermore, there is anatomical evidence indicating that the DRN sends 5-HT projections to both LDT and PPT (Semb and Fibiger, 1992; Honda and Semb, 1994; Steininger et al., 1997). Other in vivo work has demonstrated that the 5-HT inhibitory control of the LDT/PPT REM-generating region is sufficiently strong to influence the behavioral expression of REM sleep (Cespuglio et al., 1979; Portas et al., 1996; Sanford et al., 1994; Horner et al., 1997).

In summary, previous work supports the following model of 5-HT involvement in REM sleep generation: when the discharge activity of 5-HT DRN neurons slows in drowsiness and SWS, less 5-HT is released from the 5-HT terminals onto inhibitory 5HT1a receptors on cholinergic neurons of the PPT, leading to decreased output from LDT/PPT to the thalamus and cortex, facilitating the appearance of REM sleep.

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causing the PPT neurons to be disinhibited. This, in turn, leads to increased discharge of the PPT neurons, which themselves promote REM sleep via their projections to REM sleep effector neurons in the pontine reticular formation and thalamus. The present study was designed to test an important part of this model: the prediction that spontaneous extracellular 5-HT levels in the PPT are highest in W, lower in SWS, and lowest during REM sleep. A preliminary account of this work has been reported in abstract form (Strecker et al., 1998).

METHODS

Experimental animals and surgery

Adult male cats were housed under constant temperature, with ad libitum access to food and water. Under pentobarbital anesthesia, animals were implanted with electrodes for recording electroencephalogram (EEG), electrooculogram (EOG), electromyogram (EMG) and ponto-geniculocortical (PGO) waves for determination of behavioral state. Intracerebral guide cannulas, for later insertion of the microdialysis probe, were implanted at a 45 degree angle above and posterior to the PPT target area. This angled approach avoided the bony tentorium and allowed the microdialysis probe tract to parallel the cholinergic cell region of the PPT (in and around the brachium conjunctivum). According to the atlas of Berman (1968), the target coordinates of the rostro-ventral tip of the dialyzing region were: AP +1.0, ML 3.0, DV -2.5. Histological verification of the probe site was performed using previously published procedures and included stains specific for mesopontine cholinergic neurons (ChAT & NADPH diaphorase), as well as nuclear stains (neutral red & cresyl violet) (Luebke et al., 1992; Porkka-Heiskanen et al., 1997; Thakkar et al., 1998). All animals were treated in accordance with the American Association for Accreditation of Laboratory Animal Care's policy on care and use of laboratory animals.

Microdialysis sampling procedures

Microdialysis perfusion was used in the PPT to collect samples for measurement of extracellular 5-HT levels during different behavioral states. Microdialysis experiments were conducted in a sound-attenuated chamber that had the same temperature and light conditions as the animals’ home cages, with food and water available ad libitum. CMA 10 probes (CMA/ Microdialysis; Acton, MA) with a 2 mm length of polycarbonate dialyzing membrane (20,000 Dalton cutoff and a 500 μm outer diameter) were used. In vitro recovery data indicate that 5-HT recoveries for 2 mm CMA 10 probes are 18-24%. At least 16 hours prior to the beginning of the experiment, the microdialysis probe was inserted through the guide cannula into the PPT, and the probe secured to the guide cannula. Subjects were connected to an electrical polygraph cable for recording of behavioral state and then to the probe inlet and outlet tubing (1m pieces of low dead volume FEP tubing; CMA/microdialysis). Artificial cerebrospinal fluid (ACSF = NaCl 147 mM, KCl 3 mM, CaCl2 1.2 mM, MgCl2 1.0 mM, pH 7.2) was perfused at a flow rate of 1.5 μl/min. Samples were collected from the outlet tubing after exiting the cage. Timing marks were put on the EEG paper record at the time of collection of each sample. Sample volumes were 10 to 15 μl/sample allowing a desirably short sampling interval of 7.5 to 10 minutes (10 min x 1.5 μl/min flow rate = 15 μl). For all experiments the time delay due to the dead volume of the system (fluid contained in the output perfusate tubing and the probe) was taken into account in correlating neurochemical readings with the EEG recording of behavioral state.

Behavioral state was divided into 3 categories (for details, see Thakkar et al., 1998): (1) Wake, which included both active and quiet waking; (2) SWS and (3) REM sleep. For measuring state-specific correlations with the biochemical measurements, we primarily used those behavioral epochs which consisted of a single behavioral state. Samples were labeled as single state if the behavioral state that occurred concurrently was >90% of that state. Samples collected during a mixture of W, SWS, & REM states were not included in the final analysis; hence, it could take 4 h to obtain 2 h of consecutive single state samples (see Figure 3). Several samples were collected from each probe for each behavioral state; hence, mean extracellular 5-HT levels across state were calculated for each probe and these means were used in subsequent analysis of the group data. For the analysis of group data, a sleep cycle was defined as a continuous period that contained consecutive samples collected during all of three behavioral states (W, SWS, REM) and began and ended with waking periods. All parts of the microdialysis sampling system were washed regularly with 70% ethanol.

Neurochemical analysis of serotonin

The samples were analyzed with a microbore high performance liquid chromatography (HPLC) system (Bioanalytical Systems, W. Lafayette, IN), using dual electrochemical detectors (BAS, model LC-4C). The glassy carbon working electrodes were set at applied potentials of 550 mV and 475 mV relative to a Ag/AgCl reference electrode. Observed peak heights for 5-HT were maximal at the high potential and half maximal at the lower potential; thus, the two detectors provide a peak height ratio that is unique for 5-HT, adding to the other criteria used for the identification of the 5-HT peak (i.e., retention time & "sample spiking," the addition of a known amount of 5-HT to a sample). Mobile phase, consisting of ethylenediaminetetraacetic acid 0.5mM (EDTA), sodium octyl sulfate 0.15 g/l, sodium phosphate 0.1 M, acetonitrile 8%, pH ~5, was delivered through the microbore column (BAS MF-8949, 1 x 100 mm, with ODS C18 packing of 3 μm particle size) at 0.1 ml/min. Ten μl of each sample was injected through a Rheodyne model 9125. Identification and quantification of 5-HT in samples was achieved by comparison to the retention time and height of a 10 fmol 5-HT standard. Assay retention times for 5-HT peaks were stable during each day, but ranged from 5 to 6 min for the different experiments. The lower detection limit of the assay was consistently <0.25 fmol (45 fg) per injection, based on a signal to noise ratio of 2:1. Nonetheless, in some cases, especially REM samples, 5-HT peaks were near the limit of assay detection. For those few REM samples where the 5-HT signal was very low, a peak height of 1 mm was assigned, as this height typically

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represented a 2:1 signal-to-noise ratio and thus provided a conservative estimate of the decline in extracellular 5-HT observed during REM sleep.

**RESULTS**

PPT microdialysis samples were collected for 5-HT analysis across the sleep-wakefulness cycle from 9 probes in 9 subjects. Reliable estimates of 5-HT levels during wakefulness were obtained from 8 probes, and data across all 3 behavioral states were obtained from 7 probes. Serotonin levels (mean ± SEM) in samples collected during W were 1.14 ± 0.13 fmol/sample (0.20 ± 0.023 pg/sample, N=8), and in SWS were 0.85 ± 0.11 fmol/sample (N=7), and in REM were 0.52 ± 0.10 fmol/sample (N=7). Figure 1 shows raw chromatographic data from a standard injection (left) and from samples collected from an individual probe during the three behavioral state conditions. Note that the 5-HT peaks produced by 10 µl sample injections were small, despite the use of microbore HPLC. Multiple state-specific samples were assayed for each probe; for W, a mean ± SEM of 7.1 ± 1.35 samples/probe were analyzed, for SWS, 3.9 ± 0.51 samples/probe were analyzed, and for REM, 2.0 ± 0.38 samples/probe. Subsequent data analysis for each probe used the mean of 5-HT measurements from the several samples collected during each of the three behavioral states.

Histological analysis confirmed that probes were located in the targeted area of the mesopontine tegmentum. The dotted line in Figure 2A illustrates the approximate range of cholinergic cells (NADPH diaphorase-positive) observed in a single subject. The 5-HT data from the 2 probe sites located at the anterior edge of this zone did not differ from the data of the other more centrally located probes.

Figure 3 illustrates 5-HT concentrations in consecutive samples from a representative probe during W, SWS, and REM sleep episodes (these episodes were relatively pure in that each 10 min sample had at least 90% of a single state). Note that levels are highest during wakefulness, and lower during sleep, with REM lower than SWS.

**Figure 1.** Chromatograms of a 5-HT standard (10 fmol; left panel) and PPT microdialysis samples collected from an individual microdialysis probe during periods of wakefulness, SWS, and REM sleep. The standard serves to identify the 5-HT peak with a retention time of 5 min; the other arrows indicate sample peaks identified as 5-HT by the same retention time. The vertical lines to the left of each 5-HT peak are caused by the solvents in the sample passing through the electrode shortly after the sample injection. Applied electrode potential = 475 mV; see text for other chromatographic details.
The mean 5-HT level for each of the 7 probes across the three behavioral states is shown in Figure 4A. In each of the 7 cases 5-HT levels declined from W basal levels to SWS and REM sleep, a group effect that was highly significant with repeated measures ANOVA (F[2,6] = 33.0, \(p < 0.0001\)). The histogram in Figure 4B shows the mean 5-HT levels in each state for the whole group (N=7); all comparisons were significant \((p's < 0.003)\) using paired \(t\)-tests (with a Bonferroni correction the probability value required for significance was \(p < 0.017\)), including a comparison of the 5-HT levels measured during SWS versus REM \((t[6] = 4.96, p < 0.003)\).

DISCUSSION

Spontaneous levels of extracellular 5-HT in the PPT were found to be highest during periods of wakefulness, lower in slow wave sleep, and lowest during REM sleep, a state in which levels declined to only 45% of W baseline. Previous microdialysis studies have found that extracellular 5-HT levels are lower during sleep relative to wakefulness in both the DRN itself (Portas and McCarley, 1994; Portas et al., 1998), and in distant projection sites of the DRN/5-HT neurons (Kalén et al., 1989; Wilkinson et al., 1991; Iwakiri et al., 1993; Portas et al., 1998). It should be noted, however, that PPT 5-HT levels do not necessarily track the discharge rate of DRN 5-HT neurons, nor levels in other DRN projection zones, as presynaptic inhibition/facilitation might influence the 5-HT output per discharge differently in PPT than elsewhere. Thus, the present study data are essential in determining whether 5-HT levels follow the same pattern of W > SWS > REM in the cholinergic zone of the PPT as observed elsewhere in the brain. The present findings empirically demonstrate what has previously been an important assumption in the existing model of the brainstem regulation of the REM sleep cycle (McCarley et al., 1995), namely that 5-HT levels declined in the mesopontine cholinergic zone as the REM phase is approached and entered. The model further postulates that decreases in 5-HT levels in the cholinergic zone of the mesopontine tegmentum during the approach to a REM sleep episode releases the REM-promoting neurons in this area from 5-HT inhibition, thereby allowing the emergence of REM sleep.

With respect to the intrinsic neurons of the PPT and adjacent LDT, there is now considerable evidence in support of a REM-promoting role for a cholinergic sub-population of these neurons. These cholinergic neurons have projections to areas in the pontine reticular formation (Mitani et al., 1988) in which local injections of cholinergic agonists produce a REM-like state (George et al., 1964; Amatruda et al., 1975). In addition, \textit{in vitro} application of cholinergic agonists to pontine reticular
formation neurons produces the same enhanced excitability and depolarization (Greene et al., 1989) seen in intracellular in vivo studies during REM sleep (Ito and McCarley, 1984). Lesions (Webster and Jones, 1988) and electrical stimulation (Thakkar et al., 1996) of the PPT/LDT produce, respectively, a decrease and an increase in REM sleep. As predicted, microdialysis measurements of pontine acetylcholine reveal higher levels during REM sleep (Kodama et al., 1992; Leonard and Lydic, 1997).

Direct support for 5-HT inhibition of PPT/LDT neurons came first from in vitro studies that found that 5-HT directly inhibits identified cholinergic neurons of the LDT and PPT (Mühlethaler et al., 1990; Luebke et al., 1992; Leonard and Llinas, 1994). More recently we reported that the discharge activity of PPT/LDT neurons identified in vivo as discharging preferentially in REM sleep was almost completely suppressed by local microdialysis perfusion of the selective 5HT1a receptor agonist 8-OHDPAT, whereas this agonist had almost no effect on the Wake/REM-on neurons in this region (Thakkar et al., 1998). Combined with the present data, this finding indicates that the decreases in PPT levels of 5-HT observed during sleep would disinhibit the REM-on subpopulation of neurons allowing their spontaneous discharge rate to increase, but would not alter the discharge of the Wake/REM-on population of PPT neurons (see Thakkar et al., 1998 for further discussion). Thus, the data support the conclusions that the PPT cholinergic REM-on cells do not fire in waking because they are inhibited by 5-HT, and the decrease in PPT 5-HT levels in SWS releases these cells from 5-HT inhibition and permits the emergence of REM sleep. In contrast, the higher PPT levels of 5-HT observed during wakefulness would act to suppress REM sleep. This last point is supported by studies finding that local microinjections, or microdialysis perfusion, of serotonin agonists in the PPT/LDT region produces a decrease in REM sleep and an increase in wakefulness (Horner et al., 1997; Sanford et al., 1994; Strecker et al., 1998).

The preceding discussion describes a local circuit mechanism wherein 5-HT inhibits REM sleep via 5-HT projections to the REM-promoting PPT/LDT region. 5-HT has been implicated in a wide variety of behavioral and physiological processes, presumably mediated by 5-HT action at other brain sites. For example, micro injections of 5-HT in hypothalamic sites or the basal forebrain facilitates SWS and reduces gamma wave activity associated with wakefulness (Denoyer et al., 1989; Cape and Jones, 1998). Further, the present data are, in general, compatible with the proposal of Jacobs and Fornal (1993) that 5-HT has a global functional role of facilitating motor output while concurrently inhibiting sensory information processing. Thus, activity of some 5-HT neurons is highest during those periods of wakefulness that are accompanied by motor activity, particularly repetitive types of motor behavior, such as grooming. Our circuit model predicts that under these conditions 5-HT release in the PPT/LDT would also be higher and this would inhibit REM sleep. Future work can address this issue by examining extracellular 5-HT levels in PPT/LDT during a variety of waking states including quiet and active wakefulness, and grooming behavior. Our findings (Thakkar et al., 1998) of two populations of cholinergic neurons, REM-on (inhibited by 5-HT) and Wake/REM-on (not inhibited by 5-HT) would predict different roles for each of the two populations in motor and sensory control according to the Jacobs and Fornal functional model of 5-HT influences, a prediction that is experimentally testable. Interestingly, 5-HT unit activity does not decline during REM sleep in animals that experience REM sleep without atonia (due to a lesion in the dorsomedial pons) (Trulson et al., 1981). These data suggest that 5-HT is not the only neurotransmitter that regulates REM sleep via an action in the PPT/LDT; for example, we have also postulated that norepinephrine acting through alpha-2 receptors would have 5-HT-like inhibitory effects in the PPT/LDT region (Thakkar et al., 1998).

It has long been known that 5-HT neurons of the DRN are maximally active in W, diminish their activity in drowsiness and SWS, and virtually cease discharge in REM sleep (McGinty and Harper, 1976; Lydic et al., 1987; Jacobs and Fornal, 1991). Interestingly, the magnitude of the decline in extracellular 5-HT levels during REM sleep compared with W is substantially less than the percentage decrease in discharge rate of presumptively serotonergic DRN neurons. This may occur as a result of several non-exclusive mechanisms. Microdialysis probes sample the 5-HT level in the extracellular space, rather than in the synaptic cleft. The 5-HT measured with this method is thought to derive from "overflow" from synaptic transmission; a hypothesis that assumes a relatively inefficient and slow clearance of extracellular 5-HT by the mechanisms of metabolism and re-uptake. Thus, compared to unit recording of 5-HT neurons, microdialysis provides a poorer time resolution. REM episodes in the cat are relatively brief and microdialysis samples are from a relatively large extracellular space; this likely leads to a residual presence of serotonin levels from previous states in the microdialysis samples, lessening the contrast between REM and other states. Another factor might be that 5-HT continues to be released from terminals despite the absence of axonal discharge. Local perfusion with specific Na+ channel blockers that prevent axonal discharge generally do not reduce 5-HT levels below 40% of baseline (Westerink et al., 1987; Kalén et al., 1988; Portas et al., 1996; Auerbach et al., 1989). Finally, although evidence indicates that 5-HT measured in microdialysis studies is predominantly derived from neuronal sources (Kalén et al., 1988; Auerbach et al., 1989), it remains possible that minimum basal levels of 5-HT are elevated as an artifact of the microdialysis procedure itself; the presence of the probe itself, or injury produced by the probe could elevate the minimal basal levels observed. Thus, the high levels of 5-HT observed immediately after probe insertion have been attributed to mechanical tissue stimulation and 5-HT derived from the blood (Kalén et al., 1988). The possibility that these potential sources of 5-HT contribute to elevated basal levels cannot be completely ruled out, even though we waited a minimum of 12 hours post insertion for data collection, and many collections were performed 2-3 days after probe insertion.

In conclusion, the present finding that PPT 5-HT levels are markedly reduced in SWS and REM sleep provides further
support for the following hypotheses: PPT cholinergic REM-promoting neurons do not discharge during wakefulness because they are inhibited by 5-HT. Furthermore, the decrease in PPT 5-HT levels during sleep releases the REM-promoting neurons from 5-HT inhibition, which, in turn, leads to an increase in cholinergic neuronal activity, and an increased acetylcholine release from the cholinergic projections to the pontine reticular formation (PRF). These increases in acetylcholine levels depolarize the PRF neurons (Ito and McCarley, 1984; Greene et al., 1989; Imon et al., 1996), thereby activating the efferent pathways involved in the phasic (rapid eye movements, muscle twitches and PGO waves) and tonic (muscle atonia, EEG activation, respiratory depression) events of REM sleep.

ACKNOWLEDGMENTS

This work was supported by the Department of Veterans Affairs (RES and RWM) and by the National Institutes of Health Grant R37MH39683 (RWM). We thank Michael Gray and John Franco for providing care for the animals and Melissa Mudrick and Russell Delgiacco for art work, and Sid Auerbach for HPLC consultation.

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