Spontaneous REM Sleep Is Modulated By the Activation of the Pedunculopontine Tegmental GABA_B Receptors in the Freely Moving Rat

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INTRODUCTION

The pedunculopontine tegmentum (PPT), situated in the dorsolateral mesopontine tegmentum, contains a prominent group of cholinergic neurons, which project widely throughout the brain stem and forebrain (for review, see Datta 1995). Single-cell recordings from the PPT of behaving cats and rats have identified several different classes of cells the firing rates of which correlate with both wakefulness and rapid-eye-movement (REM) sleep (Datta 1995; Datta and Siwek 2002; El-Mansari et al. 1989; Saito et al. 1977; Steriade et al. 1990). Some of these PPT neurons, called REM-ON cells, progressively increase their firing rates as the animal moves from wakefulness (W) to slow-wave sleep (SWS) and then to REM sleep (Datta 1995; Datta and Siwek 2002; El-Mansari et al. 1989; Saito et al. 1977; Steriade et al. 1990; Thakkar et al. 1998). Others, constituting the majority of these cells in the PPT, called wake-REM-ON cells, are tonically active during both W and REM sleep (Datta 1995; Datta and Siwek 2002). Microinjection studies in cats and rats have demonstrated that chemical excitation of PPT cells increased both wakefulness and REM sleep by eliminating SWS (Datta and Siwek 1997; Datta et al. 2001a,b). More recent studies have shown that the activation of PPT kainate receptors induces REM sleep (Datta 2002; Datta et al. 2002), whereas activation of PPT N-methyl-D-aspartate (NMDA) receptors is involved in awake-locomotor activity behavior (Datta et al. 2001a; Garcia-Rill 1991; Garcia-Rill et al. 1990, 2001).

Neurotransmitter-mediated excitation and inhibition of brain stem cells are important processes for the regulation of wakefulness and REM sleep (Datta 1995; Thakkar et al. 2002). Recently, considerable progress has been made in identifying the neurotransmitters and receptors involved in the excitation of PPT cells that induce REM sleep (Datta 2002; Datta et al. 2002). However, the identification of neurotransmitters involved in the inhibition of PPT cells and suppression of REM sleep in freely moving rats and cats remains to be discovered. Based on some indirect studies and on theoretical prediction, it has long been suggested that neurotransmitters like serotonin, norepinephrine, and adenosine are involved in the regulation of REM sleep by inhibiting PPT cell activity (Steriade and McCarley 1990). On the contrary, one of our recent microinjection studies has demonstrated that the serotonin, norepinephrine, and adenosine in the PPT are not involved in the regulation of REM sleep in the rat (Datta et al. 2003).

Recently, pharmacological studies have demonstrated that the neurotransmitter GABA is involved in different parts of the brain in the regulation of sleep-wake cycle (Arnaud et al. 2001; Manfridi et al. 2001; Sanford et al. 2003; Torterolo et al. 2001; Xi et al. 1999a,b). Because the neurotransmitter GABA plays a role in the regulation of sleep-wake cycle and we have shown that the serotonin, norepinephrine, and adenosine may not be involved in the PPT for the regulation of REM sleep (Datta et al. 2003), it is likely that GABA is involved in the PPT for the regulation of REM sleep. This proposed role for GABA in the PPT is supported by the fact that the PPT receives GABAergic inputs from the substantia nigra, local neurons, and many other parts of the brain (Beckstead et al. 1979; Carpenter et al. 1981; Jackson and Crossman 1981; Kosaka et al. 1987; Moon-Edley and Graybiel 1983; Mugnaini and Oertel 1985; Scarnati et al. 1988). In addition to these GABAergic projections, different types of GABA receptors are also present in the PPT (Bowery et al. 1987; Chu et al. 1990). The general hypothesis of this
study is that the activation of selective GABA receptors in the PPT cholinergic cell compartment inhibits spontaneous REM sleep in freely moving rats.

To identify the specific GABA receptor that has a physiological REM sleep suppressing effect in freely moving rats, we examined polygraphic wake-sleep signs after microinjections of control vehicle, GABA_A receptor-specific agonist, GABA_B receptor-specific agonist, and GABA_A receptor-specific agonist into the PPT. By comparing the alterations in patterns of REM sleep after injections of control vehicle and selective GABA receptor-specific agonists, contributions made by each receptor subtype in REM sleep suppression were evaluated. In this study, in a limited number of cells, we also examined whether REM sleep suppressing GABA receptor specific agonist suppresses single cell activity of PPT REM-on cell in the freely moving rat.

METHODS

Subjects and housing

Experiments were performed on 29 male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing between 250 and 350 g each. The rats were housed individually at 24°C with food and water provided ad libitum with lights on from 07:00 to 19:00 h (light cycle) and off from 19:00 to 07:00 h (dark cycle). Principles for the care and the use of laboratory animals in research, as outlined by the National Institute of Health publication 85-23 (1985), were strictly followed.

Drugs and vehicle for microinjection

The drugs used included GABA_A receptor agonist, isoguvacine hydrochloride (ISGV; molecular weight: 163.6), GABA_B receptor agonist, baclofen hydrochloride (baclofen; molecular weight: 250.13), and GABA_A receptor agonist, cis-4-aminomorotic acid (CACA; molecular weight: 101.11). All of these drugs were Sigma-RBI brand saline. This 0.9% saline was also used for the control vehicle microinjections. Control saline and drug solutions were freshly prepared under sterile conditions before each use. The selection of agonists was based on the selective agonistic effects on specific types of GABA receptors (Bowery 1993; Bowery et al. 1978; Enna and Maggi 1979; Falch et al. 1986; Feigenspan et al. 1993; Fromm et al. 1990; Hill and Bowery 1981; Hong and Henry 1991; Johnston et al. 1975; Kerr and Ong 1995; Krosgaard-Larsen and Johnston 1978; Lukasiewicz and Werblin 1994; Matthews et al. 1994; Misgeld et al. 1995; Paredes and Agmo 1989; Qian and Dowling 1993). In addition to their selectivity, these drugs are also water soluble and accessible to extracellular receptors.

Surgical procedures and implantation of electrodes

Treatment of the animals and surgical procedures were in accordance with an approved institutional animal welfare protocol (No. 00-196). Rats were anesthetized with pentobarbital (40 mg/kg ip), placed in the stereotax apparatus, and secured using blunt rodent ear bars. With the use of sterile procedures, cortical electroencephalogram (EEG), dorsal neck muscle electromyogram (EMG), electrooculogram (EOG), and hippocampal EEG (to record theta wave) recording electrodes were chronically implanted in all 29 rats as described elsewhere (Datta et al. 2001a,b; Datta et al. 2002). In 21 of these 29 rats, in addition to EEG, EMG, and EOG electrodes, bilateral stainless steel guide tubes (26 gauge) with a fitted stylette of equal length inside were stereotaxically implanted 2 mm above the PPT (A:1.0; L:1.8; H:3.0) (Paxinos and Watson 1997) as described previously (Datta 2002; Datta et al. 2002). In the remaining eight rats, in addition to EEG, EMG, and EOG electrodes, one chemitrode (assembly of 12 microwires for single-cell recording and a guide tube for microinjection) was stereotaxically implanted to microinject saline and baclofen into the PPT while recording extracellular single-cell activities from microinjection site as detailed in our earlier publications (Datta 2002; Datta and Siwek 2002). Rats were postsurgically treated with butyrophene (0.2 mg/kg im) to control any possible pain on recovery from anesthesia.

Habituation and polygraphic recordings

During recovery, habituation, and free-moving recording periods, all rats were housed under a 12/12-h light/dark cycle with free access to food and water. After a postsurgical recovery period of 3–7 days, rats were habituated to a sound attenuated recording cage (size: 2.5 × 1.5 × 1.5 ft) and free-moving polygraphic (Grass Polygraph, model #79, Grass Instrument, Quincy, MA) and single-cell recording conditions for 10 days. All adaptation-recording sessions were performed between 10:00 and 16:00 h, when rats are normally sleeping.

Intracerebral microinjections and experimental design for the identification of the GABA receptor type or types in the PPT that are involved in the regulation of sleep

After the adaptation recording sessions were completed, experimental recording sessions were started. During experimental sessions, animals were connected to the polygraphic recording system 15 min before a microinjection into the PPT. The microinjection system and procedures for microinjection in freely moving rats were same as described elsewhere (Datta 2002; Datta et al. 2002; Mavanji and Datta 2003). Six-hour (between 10:00 and 16:00 h) microinjection recording sessions were begun after a single, unilateral microinjection of 100 nl control saline or one of the four different doses (0.5, 1.0, 1.5, or 3.0 nmol in 100 nl saline) of three different GABA receptor-selective agonists (ISGV, baclofen, and CACA) into the PPT. During microinjection, animals were free to move around the cage with the cannula in place. Because of the extended PE tubing, the injections were made while the animals were moving around. In individual rats, each microinjection was separated by ≥2 days. Each PPT site received no more than three microinjections in three different recording sessions ≥96 h apart. In these three recording sessions, microinjections of control saline or any one of the GABA agonists were never repeated in a single site. The sequence of these microinjections was random. At the end of all experimental sessions and 30 min before perfusion, with the use of the same injector used for GABA agonists and saline, 100 nl black ink was microinjected to each injection site for histological identification of microinjection sites.

Experimental design to study the effects of baclofen application on the single cell activity patterns of REM-on cells

After the adaptation-recording sessions, experimental recording sessions began. During a recording session, each animal was connected to the recording and fluid delivery systems. In the next room, polygraphic signs of the sleep-wake cycle and spontaneous PPT neuronal spikes were continuously monitored on a computer monitor and an oscilloscope and digitized with a computer (between 10:00 and 16:00 h) as described in our earlier publication (Datta and Siwek 2002).

The fluid delivery system consisted of a 32-gauge stainless steel injector cannula with a 26-gauge collar that extended 2.0 mm beyond the implanted guide. The tip of the injector cannula delivered baclofen or saline within 50-µm distances of the chemitrode (12 microwires, each 20 µm in diameter) tips, already implanted into the PPT. The collar of the injector cannula was connected to one end of the fluid swivel with PE 20 tubing. The other end of the fluid swivel was
connected to a long PE tubing attached to a 1.0-µl motor-driven microsyringe located outside of the recording chamber. At the beginning of recording sessions, the injector cannula and 20 mm of the connecting tube contained baclofen solution (concentration: 0.5 nmol/50 nl). The rest of the fluid-delivery system was filled with saline. A 2-mm distance filled with mineral oil separated the saline and baclofen solutions within the fluid delivery system from each other.

To identify good single-cell recording electrode, signals (unitary spike-to-noise >2:1) from each of the micro-wires were recorded for at least two complete sleep-wake cycles as described in our earlier publication (Datta and Siwek 2002). Once good quality, presumed somatic unitary spike potentials (biphasic or negative >1 ms) were identified, signals from those individual micro-wires were recorded continuously for at least two sleep-waking cycles to positively identify REM-ON cell. REM-ON cells exhibited increased (>50%) firing discharge in REM sleep compared with W and SWS (Datta 1995; Datta and Siwek 2002). Normally, the PPT REM-ON cells in the rat are almost silent during wakefulness and SWS. The average firing rates are in a range between 0.1 and 0.3 Hz (Datta 1995; Datta and Siwek 2002). Five to 10 s before the onset of REM sleep, the firing rate of REM-ON cells began to increase and reached their maximal rate at the beginning of REM sleep. This firing rate remains high (between 5 and 15 Hz) throughout the entire REM sleep episode. Five to 8 s prior to the REM sleep, their firing rate drops dramatically and the neurons cease firing as soon as the rat enters into wakefulness or SWS. Once a REM-ON cell was identified, during SWS, 50-nl volume of baclofen was slowly (over a 60-s period) microinjected into the recording site. After microinjection of baclofen, polygraphic signs and single-cell activity were recorded until those cells began to fire in a normal state-dependent manner (like before microinjection of baclofen). After this recovery, the injector cannula was replaced with a same size injector cannula filled with saline. Again, when the animal entered into the SWS state, 50-nl volume of saline was microinjected into the same site where baclofen was microinjected. After microinjection of saline, single-cell activity and polygraphic recordings were continued for at least one complete sleep-wake cycle. At the end of all recording sessions and 30 min before perfusion, 100 nl black ink was microinjected into each injection site for localizing microinjection sites.

Unitary spikes and square-wave pulses triggered by well-discriminated unit action potentials (via window discriminator) as well as polygraphic signals of polygraphic sleep-wake signs were stored in a computer for subsequent off-line analyses. Amplified original single cell recording signals were also recorded on a digital tape recorder (DTR) for off-line computerized analysis using “Experimenters Work Bench” software (DataWave Technologies, Longmont, CO).

Histological localization of single-cell recording and microinjection site

At the conclusion of all recording sessions, rats were killed with pentobarbital (60 mg/kg ip) and perfused transcardially with heparinized cold phosphate buffer (0.1 M, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and processed for NADPH-diaphorase staining and histological localization of single-cell recording and microinjection sites as described earlier (Datta and Hobson 1994; Datta and Siwek 2002; Datta et al. 2001a,b).

Scoring of behavioral states and analysis of neuronal activity

Polygraphic data were scored visually for W, SWS, and REM sleep as described in our earlier publications (Datta 2000, 2002; Datta and Siwek 2002). Action potentials were collected from the DTR via a DataWave acquisition system and stored in the computer with proper labeling of cell number and behavioral state. These stored action potentials were first discriminated on the basis of their height, duration, and shape to make sure that they were from the same cell body before they were subjected to further analysis. The discriminated unitary action potentials were analyzed to determine firing rate with the use of the Experimenters Work Bench software of the Data Wave System as described earlier (Datta and Hobson 1994; Datta and Siwek 2002). The mean firing rate (in Hz) for each cell was computed separately during the epochs of W, SWS, and REM sleep. Paired t-test was used to calculate statistical differences in firing rate between before microinjection of baclofen and after microinjection of saline during W, SWS, and REM sleep.

Statistical analyses

For the purposes of determining possible effects on sleep and wakefulness, three behavioral states were distinguished based on the visual scoring of polygraphic records as described earlier (Datta 2000, 2002; Datta and Siwek 2002). The behavioral states of W, SWS, and REM sleep were scored in successive 10-s epochs. The polygraphic measures provided the following dependent variables that are quantified for each trial: percentage of recording time spent in W, SWS, and REM sleep; latencies to the onset of the first episode of REM sleep after the onset of injection; total number of REM sleep episodes in 6-h recording session; and mean duration of REM sleep episodes in 6-h recording session. To identify the type of GABA receptor that could be involved in the regulation of sleep, the effects of the 13 different treatments (saline; 3 agonist × 4 doses = 12 drug treatment groups) on the total percentages of W, SWS, and REM sleep were statistically analyzed using one-factor ANOVA. For detailed analysis of baclofen effects, the effects of the five different treatments (saline and 0.5, 1.0, 1.5, and 3.0 nmol baclofen) on the percentages of W, SWS, and REM sleep were statistically analyzed using a two-way ANOVA with time as a repeated measure within subject variable (6 levels corresponding to 6 1-h epochs after injections) and treatment as a between-subject variable (5 levels corresponding to the 5 different treatments). After a two-way ANOVA, post hoc Scheffe F tests were performed to determine the individual levels of significant difference between control (saline) and the four different baclofen doses at six individual data points. Statistical analyses (1-factor ANOVA, 2-way ANOVA, Scheffe F test) were performed with the use of StatView statistical software (Abacus Concepts, Berkeley, CA).

RESULTS

Effects of three different GABA receptor specific agonists microinjection into the PPT on wakefulness and sleep

Immediately after microinjection of all four different doses of baclofen, animals assumed a lying down position in one corner of the recording chamber. Although, we did not quantify any individual waking behavior (like, exploratory, grooming, feeding, and drinking behaviors), based on our qualitative observation, after baclofen microinjection, the animals’ normal active behaviors were minimal compared with those after microinjections of saline control, ISGV, or CACA. These behavioral observations indicated that the baclofen microinjections into the PPT induce presomnic behavior in the rat.

To identify the GABA receptor type or types in the PPT that are involved in the regulation of sleep, analyses were conducted to in the following text quantify the different microinjection effects on the total percentages of time spent in W, SWS, and REM sleep in a 6-h postinjection recording sessions. The results of that analyses are summarized in Fig. 1. One-factor ANOVAs indicated a significant effect of treatment on total percentage of time spent in W [F(12,92) = 4.55, P <
effects of baclofen microinjection into the PPT in the six different time points of each 6-h recording session.

Effects of baclofen microinjection into the PPT on W

The changes in the percentage of time spent in W after microinjection of saline control and the different baclofen doses are summarized in Fig. 2. Two-way ANOVA indicated a significant main effect of treatment \((F(4,44) = 14.09, P < 0.0001)\), time \((F(5,44) = 30.60, P < 0.0001)\), and a significant treatment × time interaction \((F(20,44) = 10.84, P < 0.0001)\) on total percentage of time spent in W. The results of post hoc analysis (Scheffe F test) on total percentage of time spent in W are presented in Fig. 2. Compared with saline control microinjections, all four doses of baclofen (0.5, 1.0, 1.5, and 3.0 nmol) microinjection into the PPT caused a significant reduction of the total percentage of W. This baclofen-induced reduction in the total percentage of W was dose dependent. The reduction in W after 0.5 and 1.0 nmol doses lasted for the first 2 h of recordings. The total percentage of W from the third hour to the end of 6-h recordings after lower doses of baclofen (0.5 and 1.0 nmol) remained comparable to the after saline microinjection. The W reducing effect of baclofen microinjection at higher doses (1.5 and 3.0 nmol) lasted for the 3 h of recordings. Postinjection values for the percentage of W during the fourth, fifth, and sixth hour after application of these
higher doses of baclofen were not significantly different when compared with the saline control values.

**Effects of baclofen microinjection into the PPT on SWS**

The changes in the percentage of time spent in SWS after microinjection of control saline and different baclofen doses are summarized in Fig. 3. Two-way ANOVA indicated a significant main effect of treatment \( F(4,44) = 36.56, P < 0.0001 \), time \( F(5,44) = 21.04, P < 0.0001 \), and a significant treatment \( \times \) time interaction \( F(20,44) = 12.66, P < 0.0001 \) on total percentage of time spent in SWS. The results of post hoc analysis (Scheffe \( F \) test) on total percentage of time spent in SWS are presented in Fig. 3. After microinjection of baclofen, there was a dose-dependent increase in the total percentage of time spent in SWS. Compared with the saline, after lower doses (0.5 and 1.0 nmol) of baclofen, animals spent more time in the SWS for the first 2 h of recordings. As the dose of baclofen increased to 1.5 nmol, SWS remained significantly higher for the first 3 h of recordings. When the baclofen dose was further increased to 3.0 nmol from 1.5, increased total percentage of SWS after 3.0 nmol dose remained comparable to that after 1.5 nmol dose. Comparison between the 1.5 and 3.0 nmol doses of baclofen suggests that the 1.5 nmol dose of baclofen is optimum for the maximum change of SWS.

**Effects of baclofen microinjection into the PPT on REM sleep**

The changes in the percentage of time spent in REM sleep after microinjection of saline and different dosages of baclofen are summarized in Fig. 4. Two-way ANOVA indicated a significant main effect of treatment \( F(4,44) = 80.52, P < 0.0001 \), time \( F(5,44) = 215.96, P < 0.0001 \), and a significant treatment \( \times \) time interaction \( F(20,44) = 21.15, P < 0.0001 \) on total percentage of time spent in REM sleep. The results of post hoc analysis (Scheffe \( F \) test) on total percentage of time spent in REM are presented in Fig. 4. Compared with post-control-injection recordings, after microinjection of baclofen, there was a dose-dependent decrease in the total percentage of time spent in REM sleep. Post hoc analysis indicated that the total percentages of REM sleep after microinjection of 4 different doses of baclofen and control saline into the PPT. Bars represent percentages (means \( \pm \) SE) of wakefulness during each of the 6-h periods after injection of control saline and 0.5, 1.0, 1.5, and 3.0 nmol baclofen. Note dose-dependent decrease of wakefulness after baclofen microinjection. \( * \), the levels of statistical significance (Scheffe \( F \) test) of the differences relative to control saline: \( * P < 0.05; ** P < 0.01; *** P < 0.001 \).
Figure 4. REM sleep after microinjections of 4 different doses of baclofen and control saline into the PPT. Bars represent percentages (means ± SE) of REM sleep during each of the 6-h periods after injection of control saline and 0.5, 1.0, 1.5, and 3.0 nmol baclofen. Note dose-dependent decrease of REM sleep after baclofen microinjection. *p, the levels of statistical significance (Scheffe F test) of the differences relative to control saline: *p < 0.05; **p < 0.01; ***p < 0.001.

Effects of baclofen application on the single-cell activity patterns of REM-ON cells

Having documented that the baclofen microinjection into the PPT suppress REM sleep, we next tested the effect of baclofen on spontaneous unitary activity in 12 REM-ON cells in the PPT. Figure 5 illustrates single cell activity patterns of a typical PPT REM-ON cell before and after diffusion of baclofen. Before diffusion of saline or baclofen to the REM-ON cells, these cells fired slowly during W (0.17 ± 0.12 Hz) and SWS (0.29 ± 0.23 Hz). Five to 10 s before the onset of REM sleep, the firing rate of these cells began to increase and reached their maximum rate at the beginning of REM sleep. This firing rate remains high throughout the entire REM sleep. The mean firing rate during REM sleep was 6.70 ± 2.50 Hz. While recording single-cell activity from the REM-ON cell, 50 nl volume of 0.5 nmol baclofen was injected close to the recording electrode. Immediately after microinjection (50 ± 14 s) of baclofen solution, 11 of those 12 REM-ON cells stopped firing. This low dose of baclofen solution did not significantly delay the next episode of REM sleep; however, these REM-ON cells remained relatively silent for a long period of time (130 ± 12 min, after the end of baclofen diffusion). After that time, cells began to fire in a state-dependent manner. To rule out the possibility that these REM-ON cells silencing effect is simply due to a mechanical disturbance, caused by the diffusion of 50 nl volume of fluid, rather than the pharmacological effect of baclofen, we microinjected 50 nl volume of saline into the same site where baclofen was microinjected. Microinjection of saline control, after recovery from the baclofen microinjection, did not stop or reduce firing rates of those REM-ON cells. Paired t-test revealed no significant differences in the firing rate during W (0.17 ± 0.12 vs. 0.30 ± 0.20 Hz), SWS (0.29 ± 0.23 vs. 0.36 ± 0.34 Hz), and REM sleep (6.70 ± 2.50 vs. 7.15 ± 1.9 Hz) between before and after microinjection of saline in the recording site. These results indicate that the application of baclofen caused PPT REM-ON cells to reduce firing.

DISCUSSION

The principal findings of this study are that 1) microinjections of GABA$_B$ receptor-selective agonist, baclofen, into the PPT decreases REM sleep in a dose-dependent manner, 2) baclofen suppresses extracellular unitary activity of PPT REM-ON cells, 3) baclofen microinjection into the PPT also reduces the total amount of wakefulness in a dose-dependent manner, 4) and microinjections of GABA$_A$ and GABA$_C$ receptors selective agonists, ISGV and CACA, into the PPT do not change wakefulness or sleep parameters. As a consequence of the baclofen-induced decrease in wakefulness and REM sleep, slow-wave sleep is increased. The results presented here strengthen and extend hypothesis that neurotransmitter-medi-
ated activation and inhibition of the cholinergic cell compartment of the PPT are critical processes for regulating wakefulness and REM sleep (Datta 1995). The findings are discussed in relation to ongoing efforts to understand the mechanisms by which activation and inhibition of the PPT contributes to the regulation of REM sleep.

A major limitation of the microinjection method relates to the diversity in the neurochemical nature of the neuronal population affected by the drug application. In the present study, all of our microinjection as well as single-cell recording sites were in the middle of the NADPH-diaphorase-positive cell compartment of the PPT. In the rat, this part of the PPT is called pars compacta, where most cells are known to be cholinergic (Mesulam et al. 1983; Datta 1995). These cholinergic cells are also capable of synthesizing nitric oxide (Datta 1995). Because the spike durations and firing rates of these REM-on cells were similar to those of identified cholinergic cells in the PPT and LDT (Datta and Siwek 2002; Kayama et al. 1992); it is likely that the phenotype of those REM-on cells recorded in this study were also cholinergic. In the cat, some PPT cells, especially those in the caudal part of the PPT, were also shown to be aminergic (Datta 1995). A recent immunohistochemical study in the cat showed that 50% of the PPT cholinergic cells also contain GABA (Jia et al. 2003). Thus it is clear that each of these PPT cells is capable of synthesizing more than one neurotransmitter. We acknowledge that if there are noncholinergic cells located within these cholinergic cell groups, they will be also affected by the application of GABAergic drugs. Another limitation, which can be confirmed and extended by future studies, is to identify the role of endogenous GABA and its specific receptors in the PPT in the regulation of spontaneous REM sleep. In this study, we chose specific agonists to activate different GABA receptors to identify the involvement of each GABA receptor in the suppression of REM sleep. The use of agonists permitted identification of the consequences of activation of a particular receptor type in a restricted brain region in the modulation of REM sleep. The present results point to the need for future studies using GABA receptor specific antagonists to confirm the receptor specificity of the present study and to identify the role of endogenous GABA and its specific receptors in the PPT in the regulation of physiological REM sleep.

In the last 5 yr, evidence has accumulated indicating that the neurotransmitter glutamate is the excitatory input for the activation of PPT cells and induction of both wakefulness and REM sleep (Datta and Siwek 1997; Datta et al. 2001a,b). Most importantly, those studies have demonstrated that the tonic activation of PPT kainate receptors induces REM sleep and activation of PPT NMDA receptors induces wakefulness (Datta 2002; Datta et al. 2001b, 2002). Because neurotransmitter-mediated excitation and inhibition of PPT cells have been suggested to be important processes for the regulation of wakefulness and REM sleep, we began our examination to identify neurotransmitters and their receptors that may be involved in the inhibition of PPT cells and REM sleep. On that quest, we have shown in our earlier study that serotonin, norepinephrine, and adenosine may not be involved in the PPT for the regulation of REM sleep (Datta et al. 2003). Recent pharmacological
and immuno-histochemical studies have shown that the GABAergic system in the brain stem (caudal and rostral parts of the nucleus pontis reticularis) is involved in the modulation of wakefulness and REM sleep (Sanford et al. 2003; Torterolo et al. 2001; Xi et al. 1999a,b, 2001). Because the PPT receives inhibitory GABAergic inputs from the substantia nigra, local neurons, and many other parts of the brain (Beckstead et al. 1982; Carpenter et al. 1981; Jackson and Crossman 1981; Jia et al. 2003; Moon-Edley and Graybiel 1983; Reese et al. 1995; Scarnati and Florio 1997; Scarnati et al. 1988; Steininger et al. 1992), PPT cell activity may also be modulated by GABAergic neurotransmission. The possibility that GABAergic neurotransmission in the PPT may modulate PPT cell activity is also supported by the fact that different types of GABA receptors and GABAergic fibers are present in the PPT (Bowery et al. 1987; Chu et al. 1990; Kosaka et al. 1987; Mugnaini and Oertel 1985). Consistent with the suggestion that the GABAergic system could modulate the activity of PPT cells, the results of the present study provide the first direct evidence that the activation of GABA B receptors within the PPT causes suppression of REM sleep partly by inhibiting the extracellular activity of the REM-ON cell. In addition to the suppression of REM sleep, our results demonstrated that the activation of GABA B receptors in the PPT reduces the total percentages of wakefulness. This interpretation is also supported by another intra-cerebro-ventricular injection study that has shown that the application of GABA A receptor antagonist increases wakefulness and REM sleep in the rat (Gauthier et al. 1997).

Because we are mainly focusing on the regulation of REM sleep, in this study we did not study the effects of baclofen microinjection on the extracellular activities of wake-REM-ON or wake-ON cells. Based on the behavioral effects of baclofen microinjection, it is expected that the application of baclofen would also inhibit wake-REM-ON and wake-ON cells. This anticipated result would suggest that the GABA B receptors in the PPT are also involved in the modulation/regularation of wakefulness. In this respect, the major function of the GABA B receptors in the PPT cholinergic cell compartment of the PPT is to suppress wakefulness. Besides the results of this study, there are two other lines of evidence implicating involvement of the PPT cholinergic cells in wakefulness. The first comes from extracellular single-cell recording studies. One group of PPT cholinergic cells is active both during wakefulness and REM sleep (Datta 1995; Datta and Siwek 2002; El-Mansari et al. 1989). These cells have a much higher firing rate during W than during REM sleep (Datta and Siwek 2002). The second line of evidence comes from the receptor activation studies. Activation of postsynaptic NMDA receptors in the cholinergic cell compartment of the PPT induces locomotor activity, wakefulness, and cortical activation (Datta and Siwek 1997; Datta et al. 2001b; Garcia-Rill et al. 1990). To better understand the mechanisms of baclofen-induced and/or GABA B receptor-induced reduction of wakefulness, future studies may require to examine the effects of baclofen on the extracellular activities of wake-REM-ON and wake-ON cells of the PPT.

In the present study, microinjections of GABA A or GABA C receptor agonist into the PPT did not produce any significant changes in the wakefulness and/or sleep. These results indicate that the GABA A and GABA C receptors in the PPT may not be involved in the modulation of wakefulness and sleep. Indeed, another study in the freely moving cat has shown that the GABA A receptor antagonist, bicuculline, microinjection into the PPT does not have any significant effect on wakefulness and sleep (Sanford et al. 1998). It is interesting to note that application of GABA A receptor agonist and antagonists were effective in changing REM sleep in the both cats and rodents when they were applied into the nucleus pontis reticularis (Pollack and Mislberger 2003; Sanford et al. 2003; Torterolo et al. 2001; Xi et al. 1999a,b, 2001), where carbachol microinjections induce REM sleep (Bourgin et al. 1995; Datta 1995; Marks and Birabil 1998, 2001; Shiromani and McGinty 1986). All of these GABAergic receptor manipulation studies have shown that activation of the GABA A receptor in the nucleus pontis reticularis, by application of GABA A receptor agonist, suppresses REM sleep. Conversely, the application of GABA A receptor-selective antagonist increases REM sleep. The lack of effect of GABA A receptor agonist on REM sleep, when microinjected into the PPT, compared with the REM sleep suppressing effect of GABA A receptor agonist, when microinjected into the nucleus pontis reticularis, indicates that there may be a considerable regional specificity for different types of GABA receptors involved in the regulation of REM sleep. In summary, activation of both GABA A receptors in the nucleus pontis reticularis and GABA B receptors in the PPT suppresses REM sleep. There is no comparable study using GABA C receptor manipulation in the PPT and/or nucleus pontis reticularis; nonetheless, one study in the rat has shown that the intra-cerebro-ventricular injection of GABA C receptor antagonist increases wakefulness and decrease both SWS and REM sleep (Arnaud et al. 2001). Because we have demonstrated that activation of GABA C receptors in the PPT does not cause any changes in the wakefulness and/or sleep, it is likely that the intra-cerebro-ventricular injection of GABA C antagonist might have acted yet in another site to induce its effects on the wakefulness and sleep.

In conclusion, the present study shows for the first time that activation of GABA B receptors in the PPT is a critical step for the regulation of REM sleep. The data provide a novel perspective on the regulatory aspect of PPT cells’ activity in the regulation of REM sleep. The results also suggest that the different REM sleep regulatory sites in the brain may use different types of GABA receptors for the regulation of REM sleep.

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