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Increased Brain Serotonin Metabolism During Rebound Sleep in Sleep-Deprived Rats

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TORU, M., H. MITSUSHIO, N. MATAGA, M. TAKASHIMA AND H. ARITO. *Increased brain serotonin metabolism during rebound sleep in sleep-deprived rats*. PHARMACOL BIOCHEM BEHAV **20**(5) 757–761, 1984.—Adult male Wistar rats were almost totally deprived of sleep by handling for 24 hr. 5-Hydroxyindolacetic acid concentrations in the dorsal raphe nucleus area and thalamus increased by 140–180%, immediately after sleep deprivation and when the rats had a 3- or 30-min rebound sleep. The higher levels of 5-hydroxyindolacetic acid were still observed after the rats were awakening from a 4-hr sleep. The concentrations of 5-hydroxytryptamine (serotonin) decreased after sleep deprivation and increased during and after sleep, but the differences were not significant. Tryptophan accumulated in the dorsal raphe area and thalamus after sleep deprivation, and an elevated level did not return to baseline concentrations until the rats were awakening. Tryptophan hydroxylase activity did not change in the dorsal raphe area during and after sleep deprivation. These results suggest that the release and synthesis of 5-hydroxytryptamine in the dorsal raphe area and thalamus increased when the rats had a sleep pressure or a rebound sleep after total sleep deprivation. An increased transport of tryptophan into the brain may be closely involved in sleep-inducing mechanisms.

Total sleep deprivation 5-Hydroxytryptamine (Serotonin) Tryptophan 5-Hydroxyindolacetic acid Dorsal raphe Thalamus Tryptophan hydroxylase

WHILE it has been suggested that brain monoamines play a role in the regulation of the sleep-waking cycle [8], few biochemical studies have been made to clarify the relationship between sleep states and amines in the discrete brain areas [14]. The biogenic amine, 5-hydroxytryptamine has been thought to be most closely related to sleep mechanisms [8].

In our previous studies [16,19] an increased metabolism of 5-hydroxytryptamine in the thalamus of rats was correlated with a short period of sleep following 24-hr total sleep deprivation. The present investigation extends these findings by studying the effects of total sleep deprivation and subsequent rebound sleep on 5-hydroxytryptamine metabolism in the dorsal raphe nucleus area, thalamus and hypothalamus of rats.

METHOD

Sleep Deprivation Procedures

Male Wistar rats (230–300 g) were deprived almost completely of sleep for 24 hr starting at 9:30 a.m. The animals were kept in the usual grid cage and stimulated by handling or shifting their position to keep them awake. During the dark period from 7:00 p.m. to 7:00 a.m., a dim red light was used. After the 24-hr sleep deprivation, one group of rats was killed by decapitation (SD group). Following the 24-hr sleep deprivation, three groups of rats were left in the cage to sleep. They started to sleep within 6 min on the average. One group of rats was killed 3 min after the onset of sleep (SDS-3 group), one group was killed 30 min after sleep onset (SDS-30 group), during which period the characteristic phenomena of REM sleep, i.e., twitching movements of muscles were usually observed on the face and paws of the animals. Another group was killed 10 min after awakening from a continuous sleep (SDSA group) which lasted approximately 4 hr. Other rats which were kept in a room at 24°C with the light on from 7:00 a.m. to 7:00 p.m. served as controls (C group) and were killed at the same time as the SD group.

Electrophysiological Studies

Five rats were implanted under Equithesin (100 ml contains 750 mg pentobarbital sodium, 4.25 g of chloral hydrate, 2.125 g of $MgSO_4$, 11.5 ml of alcohol and 44.34 ml of propylene glycol, 2 mg/kg, IP) anesthesia with a screw electrode into the skull above the parieto-occipital cortex (1 mm lateral and 3.5 mm posterior to bregma) and a reference electrode above the cerebellar cortex for electroencephalographic (EEG) recording from the hippocampal area. Stainless steel insulated wire electrodes with uninsulated tips were inserted bilaterally in the dorsal neck muscles to record

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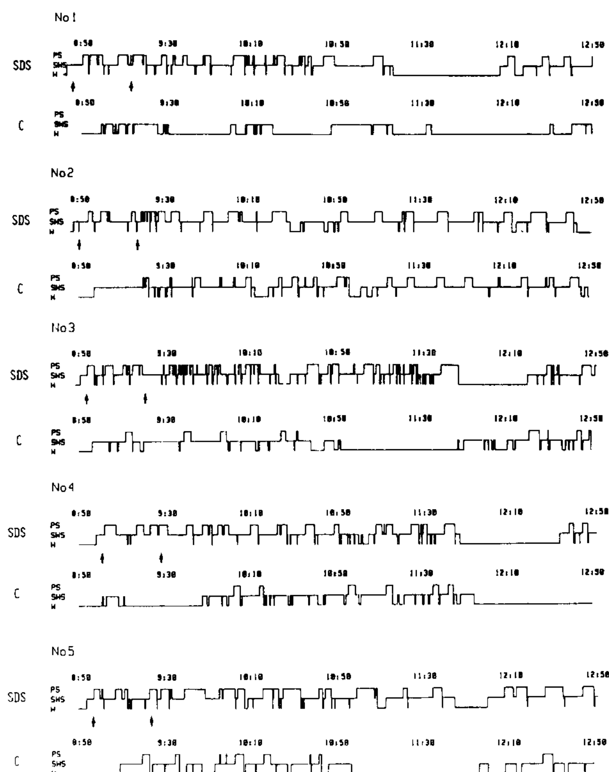

FIG. 1. Sleep stage diagrams of 5 rats following total sleep deprivation (SDS) compared with baseline records (C). Wakefulness (W) is classified according to the criteria of low amplitude fast waves of EEG and sustained tonic activity of EMG. Non-REM sleep (SWS) is characterized by irregular slow waves of large amplitude and progressive reduction of EMG tone. REM sleep (PS) is characterized by regular theta-like waves in the parieto-occipital cortex and lack of EMG tone. Arrows indicate at 3 and 30 min after the onset of sleep.

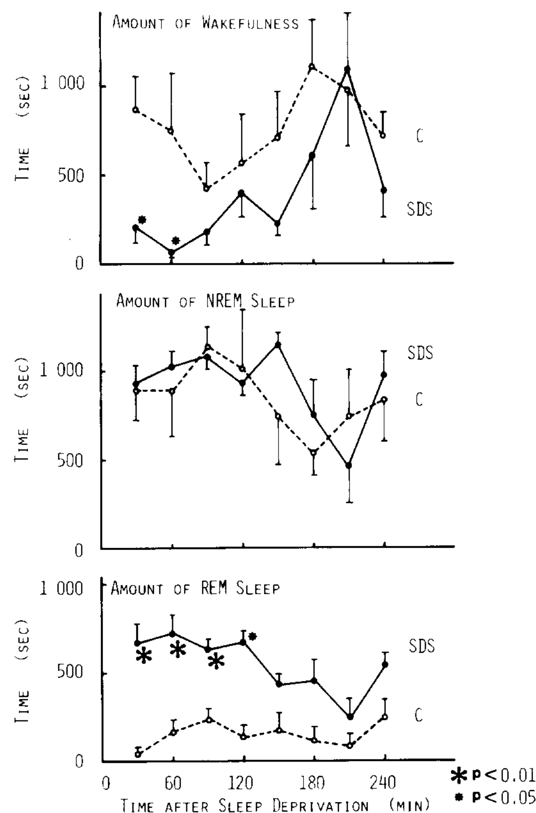

FIG. 2. Half-hourly distribution of the amounts of wakefulness, NREM and REM sleep of 5 rats following total sleep deprivation (SDS) compared with baseline distribution (C). Vertical bars indicate SEM. Statistical comparisons were made between values of SDS and C at corresponding time.

electromyogram (EMG). The electrodes were fixed to the skull with dental acrylic cement.

EEG and EMG activity of the rats was recorded for 4 hr from around 8:45 with 13 channel electroencephalograph, immediately after sleep deprivation and compared with the baseline records which were made 11 days after the experiment.

Sample Preparations

The decapitated heads were chilled in crushed ice, the brains removed in a cold box at 0°C, and immediately frozen at −80°C. Unless otherwise stated, brains were sectioned in the frontal plane at 600 μm on a freezing microtome (−10°C). Dissection of brain structures was carried out in a cold box maintained at −15°C. The thalamus was obtained from two slices caudal to A 5340 μm according to the atlas of König and Klippel [10]. The hypothalamus was dissected from three slices caudal to A 6060 μm. The dorsal raphe area was punched out with a 1.5 mm punch from three slices of 450 μm thickness caudal to P 0.1 mm according to the atlas of Palkovitz and Jacobowitz [13].

Biochemical Measurements

Each tissue was sonicated in solution containing 25 μl of 0.4 N perchloric acid, 40 μl of 0.2 N Tris-HCl (pH 8.5), and 10 μl of 0.1 M EDTA·2Na after the addition of 10 ng p-hydroxyphenylacetic acid (PHPA) dissolved in 10 μl 0.1 N perchloric acid as an internal standard. After 10 μl was taken for protein assay, 50 μl chloroform was added to the homogenates, the mixture was whirled for 15 sec and centrifuged at 8,800 × g for 15 min at 4°C. Fifty μl chloroform was added again to a 70 μl portion of the supernatant, and the mixture was centrifuged at 8,800 × g for 15 min at 4°C. The supernatant was applied to a high-performance liquid chromatograph (Yanaco L 2000) with electrochemical detection (Yanaco VMD 101). A stainless-steel reversed phase column (25 cm × 4.6 mm ID) Nucleosil 7C 18 was used. The potential of the electrode was maintained at +0.8 V versus an Ag/AgCl reference electrode. Phosphate buffer (0.05 M) containing 20% methanol and 0.3 mM hexanesulfonic acid, pH 5.1, was used as the mobile phase. The flow-rate was 0.8 ml/min.

Tryptophan hydroxylase was measured by a $^{14}CO_2$ trapping method as previously described [7,15].

Protein was determined by the method of Lowry *et al.* [11].

Statistical Analysis

Results were analysed statistically using Ryan's multiple comparison test following one-way analysis of variance. For

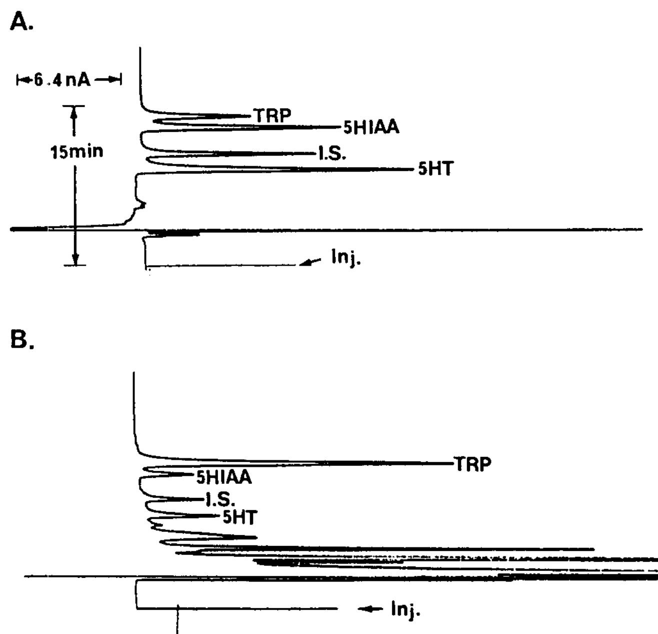

FIG. 3. Chromatograms of tryptophan (TRP), 5-hydroxytryptamine (5HT) and 5-hydroxyindolacetic acid (5HIAA) with internal standard (I.S.=p-hydroxyphenylacetic acid). A: Authentic standards without brain tissue, 10 ng of each standard was injected. B: Extracts from the rat thalamus. Chromatographic condition: Column; Nucleosil 7C18. Mobile phase; 0.05 M phosphate buffer containing 15% methanol, pH 5.10. Flow rate; 0.8 ml/min. Electrode potential; 0.8 V vs. Ag/AgCl.

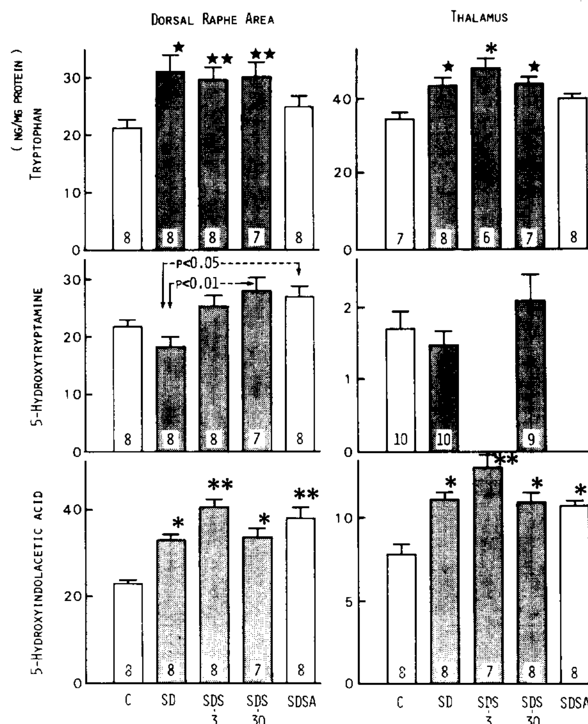

FIG. 4. Tryptophan, 5-hydroxytryptamine and 5-hydroxyindolacetic acid concentrations in the dorsal raphe area and thalamus of rats before, during and after 24-hr total sleep deprivation. Each column indicates the mean±SEM of the number of animals shown in the columns. C: control, SD: immediately after 24-hr sleep deprivation, SDS-3: after a 3-min sleep following sleep deprivation, SDS-30: after a 30 min sleep following sleep deprivation, SDSA: 10 min after awakening from sleep. ★$p<0.05$, ★★$p<0.02$, *$p<0.01$, **$p<0.001$ vs. C, when compared using actual values of concentration of the substances.

comparisons of electrophysiological parameters, Student's t-test was used.

RESULTS

Rebound Sleep

Electrophysiological studies confirmed that no REM sleep was observed in the sleep-deprived rats within 3 min after the onset of sleep (Fig. 1). All the sleep-deprived rats had REM sleep during 30 min after the onset of sleep (Fig. 1). The amount of wakefulness of sleep-deprived rats after the cessation of handling were smaller than that of baseline (Fig. 2). The decrease was significant during the first 60 min. Sleep-deprived rats had a markedly increased amount of REM sleep while their NREM sleep amount did not change from the baseline (Fig. 2).

Chromatography

A chromatogram showing 5-hydroxytryptamine, 5-hydroxyindolacetic acid and tryptophan with p-hydroxyphenylacetic acid as an internal standard is compared to one of an extract of thalamus in Fig. 3. The three substances were present in the extract.

Tryptophan (Trp) in the Brain

Trp accumulated in the dorsal raphe area immediately after total sleep deprivation, and an elevated level (29.7–31.0 ng/mg protein or 140–146% of controls) did not return to baseline levels until the rats were awakening (Fig. 4). Trp was still elevated in the SDSA group (118%) but the level did not differ significantly from the C group. The same changes were obtained in the thalamus (Fig. 4).

5-Hydroxytryptamine (5HT)

The level of 5HT in the dorsal raphe area (Fig. 4) decreased to 18.4 ng/mg protein or 84% after sleep deprivation (SD group), but the change was not significant. A 30-min period of sleep following sleep deprivation (SDS-30) raised the 5HT level to 128% of control, and the level was the same after awakening (SDSA). These changes were significant when compared with the SD group but not significant compared with the controls. Similar changes were seen in thalamic 5HT levels (Fig. 4) or in the hypothalamus (Table 1).

5-Hydroxyindolacetic Acid (5HIAA)

Concentrations of 5HIAA increased significantly in the dorsal raphe area in the SD (33.1 ng/mg protein or 144%), SDS-3 (178%) and SDS-30 (146%) groups, and the high levels were still present in rats awakening from a 4-hr sleep (166%) (Fig. 4). Significantly higher 5HIAA levels also were found in the thalamus (Fig. 4) in all experimental groups. The highest 5HIAA values were found in the SDS-3 group in these two brain areas (Fig. 4). In the hypothalamus the 5HIAA content increased only in the SD group (Table 1).

TABLE 1

EFFECTS OF SLEEP DEPRIVATION (SD) AND 30-MIN SLEEP FOLLOWING SLEEP DEPRIVATION (SDS-30) ON 5-HYDROXYTRYPTAMINE METABOLISM IN THE HYPOTHALAMUS OF RATS

	C	SD	SDS-30
5-Hydroxytryptamine	2.64 ± 0.35 (10)	2.20 ± 0.31 (10)	2.99 ± 0.47 (9)
5-Hydroxyindolacetic acid	6.29 ± 0.52 (10)	8.12 ± 0.46* (10)	7.03 ± 0.24 (9)

Values are mean ± S.E.M. ng/mg protein with numbers of animals in parentheses.
*$p<0.05$ Compared with controls (C) by one-way ANOVA following by multiple comparison.

TABLE 2

EFFECTS OF SLEEP DEPRIVATION (SD) AND 30-MIN SLEEP FOLLOWING SLEEP DEPRIVATIONS (SDS-30) ON TRYPTOPHAN HYDROXYLASE ACTIVITY IN THE DORSAL RAPHE AREA OF RATS

	C	SD	SDS-30
Tryptophan hydroxylase	318.8 ± 23.3 (8)	320.3 ± 49.3 (9)	325.0 ± 17.1 (7)

Values are mean ± S.E.M. $^{14}CO_2$ evolved pmoles/mg protein/min with numbers of animals in parentheses.
All values are N.S.

Tryptophan Hydroxylase Activity

The levels of this enzyme in the SD and SDS-30 groups were not altered (Table 2).

DISCUSSION

Three brain structures have been postulated to be involved in the NREM sleep mechanisms [8]; the lower brain stem, thalamus and anterior hypothalamus. The finding that insomnia was experimentally induced by p-chlorophenylalanine [3, 9, 20] or destruction of the raphe nuclei [8], suggests that cerebral 5-hydroxytryptamine (serotonin, 5HT) may either initiate or sustain NREM (slow wave) sleep.

We have reported [16, 17, 19] that 5HT metabolism increases in the thalamus of rats after a short period of sleep following 24-hr total sleep deprivation.

In the present study, sleep deprivation was employed to elucidate further the relationship between 5HT metabolism and sleep mechanisms. Sleep deprivation was started at 9:30 a.m. because the influence of the changing point of darkness to light (7:00 a.m.) on the restorative sleep should be avoided. To minimize the effect of stress, which usually accompanies sleep deprivation, this study focused on the effects of the rebound sleep which closely followed the 24-hr total sleep deprivation. Four points were studied before and during the sleep: *immediately after sleep deprivation* (SD) when the animals had an intensive pressure to sleep; *3 min after the onset of sleep* (SDS-3) when the rats were asleep but the REM sleep had not yet appeared; *30 min after the onset of sleep* (SDS-30) when the REM phenomena had appeared; and *10 min after the rats were awakening* (SDSA).

There were only slight changes in 5HT concentration in three brain areas before, during and after sleep. A tendency of thalamic 5HT to decrease in the SD group and increase in the SDS-30 group confirmed previous findings [16,19]. The decreased levels of 5HT found in the dorsal raphe and thalamus in the SD group, accompanied with elevated levels of 5HIAA, may indicate that 5HT turnover began to increase during or immediately after total sleep deprivation. The high ratios of 5HIAA to 5HT in the SD group suggest that the release of 5HT in these brain areas may play a role when sleep initiates. The elevated levels of 5HIAA observed in the two brain areas in the SDS-3 group may support this inference. The finding that the level remained high in the SDSA group is, however, difficult to explain. It is possible that the metabolite remained in the CNS for a time after the increased release of 5HT was terminated, or, that the rats had not completely recovered vigilance at this time.

As to circadian changes of the substances, we confirmed that the values of Trp, 5HT and 5HIAA in the dorsal raphe area or thalamus in another control rats sacrificed at the same time of SDSA group did not significantly differ from those of the C group (Data not shown).

The experiments in which p-chlorophenylalanine-induced insomnia was restored by 5-hydroxytryptophan [9,12], show that the sleep mechanism is dependent upon the synthesis of 5HT. Héry *et al.* [6] showed REM sleep deprivation increases ^{3}H-5HT synthesis in the brain stem-mesencephalon of the rat after intracisternal injection of ^{3}H-Trp. We also found formation of ^{3}H-5HT and ^{3}H-5HIAA from intraperitoneally injected ^{3}H-Trp in the thalamus of rats after a short period of sleep following 24-hr total sleep deprivation [16]. Thus, an increase of the synthesis and release of 5HT related to the rebound sleep.

Few biochemical analyses of the dorsal raphe nucleus during sleep, have been published. The unit activity of the dorsal raphe of cats is depressed during NREM and REM sleep [18]. It has been suggested that the dorsal raphe nucleus receives projections from other raphe nuclei [2]. Serotonergic nerve terminals have been found in the area of the raphe system [1]. The increased 5HT metabolism in the dorsal raphe area found in this study may occur in the nerve terminals located in the dorsal raphe nucleus.

Since the thalamus is innervated by the ventral bundle of serotonergic neurons from the dorsal raphe nucleus, the increased 5HT metabolism may indicate increased release of 5HT in the nerve terminals in these areas.

In the hypothalamus, the 5HT metabolism did not increase in the SDS-30 group. The results, which are consistent with our previous finding [16], may suggest that serotonergic neurons in this area are not mainly involved in the mechanisms which induce rebound sleep.

Another interesting finding is the accumulation of Trp in the dorsal raphe area and thalamus. An elevated Trp level was observed immediately after sleep deprivation (SD group), when the 5HIAA levels were elevated. The biosynthesis of 5HT in the brain is partly regulated by the availability of Trp [5]. The absence of changes in Trp hydroxylase activity in the dorsal raphe area of the SD and SDS-30 group support the role of Trp in 5HT synthesis, although monoamine oxidase activity was not measured in the present study. Spearman rank correlation coefficients show that there exists a highly significant positive correlation ($r=0.6060$, $n=39$, $p<0.0005$) between Trp and 5HIAA values in the dorsal raphe area whereas no significant correlation is found between Trp and 5HT or 5HT and 5HIAA values. Since 5HIAA values may partly reflect the biosynthesis rate of 5HT, the increased accumulation of Trp may be related closely to the increased release and synthesis of 5HT.

The present results are consistent with our previous findings [16,17] which showed a markedly increased accumulation of intraperitoneally given ^{3}H-Trp in all brain areas of rats after a short period of sleep following total sleep deprivation. An important finding in the present experiment is that Trp, in both areas of rats awakening from sleep (SDSA group), reached levels not significantly different from the controls. These results indicate that an accumulation of Trp in both areas closely correlates with sleep. However, little is known about the mechanisms which control the active transport of Trp into serotonergic neurons in the brain.

An increased transport of Trp into brain slices of rats deprived of REM sleep has been shown by Héry *et al.* [6]. The concentration of free Trp in plasma is considered to be one of the factors controlling transport of Trp into the brain [5]. As suggested by Fernstrom and Wurtman [4], the changes in concentrations of other neutral amino acids in the plasma may influence the Trp transport mechanisms. Uptake studies, using brain homogenates of sleep-deprived rats, are now in progress in this laboratory.

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