Endogenous neuropeptide S tone influences sleep–wake rhythm in rats
（内因性神経ペプチドSはラットにおける睡眠覚醒リズムに影響を与える）

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（内因性神経ペプチドSがラットにおける睡眠覚醒リズムに影響を与える）

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Abstract

Neuropeptide S (NPS) is an endogenous peptide that exerts wakefulness promoting, analgesic, and anxiolytic effects when administered exogenously. However, it remains to be determined if endogenous NPS tone is involved in the control of the diurnal sleep–wake cycle, or spontanous behavior.

In this study, we examined the effects of the NPS receptor antagonist [D-Cys(tBu)5]NPS (2 and 20 nmol, icv) on physiological sleep and spontaneous locomotor behavior. The higher dose of [D-Cys(tBu)5]NPS decreased the amount of time spent in wakefulness [control 782.5 ± 25.5 min, treatment 751.7 ± 28.1 min; $p < 0.05$] and increased the time spent in NREMS [control 572.6 ± 17.2 min, treatment 600.2 ± 26.1 min; $p < 0.05$]. There was no statistically significant difference in time spent in REMS. There were no behavioral changes including abnormal gross motor behavior in response to [D-Cys(tBu)5]NPS administration. Collectively these data suggest an involvement of the endogenous NPS/NPS receptor system in physiological sleep architecture.

1. Introduction

Neuropeptide S (NPS) and its receptor were first identified by reverse pharmacology in 2004 [29]. Rat NPS precursor mRNA is discretely expressed in a few brain areas with the highest expression in the LC. Moderate expression is also found in the dorsomedial hypothalamic nucleus and the amygdala. On the other hand, the NPS receptor (NPSR) protein is widely distributed in the brain. High

levels of mRNA expression are found in the cortex, olfactory nuclei, thalamus, hypothalamus, amygdala, and subiculum [28] and [29]. Neuropeptide S is involved in the control of various functions. Exogenous central NPS produced anxiolytic-like effects [6], [17], [21] and [29]. Neuropeptide S facilitated memory consolidation with noradrenergic interaction [9] and also improved N-methyl-d-aspartate (NMDA)-induced memory impairment [10]. Alcohol dependent rats increased NPS receptor mRNA expression in different brain areas [22]. Neuropeptide S may have protective effects against the neurotoxic and behavioral changes produced by NMDA receptor antagonists [19]. Recent reports showed an involvement of the NPS system in the regulation of the neuroendocrine stress response in humans [14] and in drug addiction [12].

Among these physiological effects, NPS regulates sleep–wakefulness. For example, exogenous NPS induced arousal and reduced all stages of sleep [29]. Neuropeptide S also decreased diazepam-induced sleep time in mice. The NPSR could be involved in this NPS effect because it is counteracted by the selective NPSR antagonist, [D-Cys(tBu)5]NPS [5]. Moreover, NPSR knockout mice (NPSR(−/−) showed attenuated arousal and increased anxiety-like behaviors [7]. The neuropeptide S receptor antagonist, [D-Cys(tBu)5]NPS selectively antagonized NPS stimulated intracellular calcium mobilization ([Ca++]i) in a concentration-dependent manner.[5] and counteracted various NPS actions. For example, NPS reduced stress-induced defecation that is counteracted by [D-Cys(tBu)5]NPS. Neuropeptide S activated synaptic inhibition of amygdala output to suppress pain behavior; [D-Cys(tBu)5]NPS blocked these pain-induced electrophysiological and behavioral effects of NPS [20].

In sleep, the single nucleotide polymorphism (SNP) rs324981 in NPSR1 had a significant effect on duration of sleep. Subjects with the homozygote T/T genotype had significantly shorter duration of both sleep and rest compared to the A-allele carriers [26]. This study suggests that endogenous NPS could be involved in sleep–wake regulation. The neuropeptide S receptor antagonist, [D-Cys(tBu)5]NPS itself did not change diazepam-induced sleeping time; while it prevented the arousal effect of NPS on sleep time in mice [5]. These results indicated that the NPS/NPSR system may not be involved in the control of pharmacological sleep induced by hypnotics. However, pharmacological sleep is different from physiological sleep [1] and [3]. It remained to be determined if NPS/NPSR system is

involved in physiological sleep architecture. In this study, we therefore tested the effects of [D-Cys(tBu)5]NPS on physiological sleep archtecture and behavior in rats.

2. Materials and methods

With the approval of the institutional committee on animal research of Hirosaki University Graduate School of Medicine, a total 37 male Sprague–Dawley rats weighing 350–430 g were used. All rats were housed for at least 1 week before surgery and were maintained in a 12-h light:12-h dark cycle environment (lights on 8:00–20:00 h) at a temperature of 24 ± 0.5 °C and 40% humidity. The animals were provided free access to food and water. The NPS receptor antagonist, [D-Cys(tBu)5]NPS, was synthesized according to published methods; details of which have been described previously [8].

2.1. Surgery and EEG recording

All surgery was performed under ketamine-xylazine (35 and 5 mg/kg) anesthesia as previously described [15]. In sleep recording, the rats were surgically implanted with a lateral intracerebroventricular (icv) cannula, electroencephalogram (EEG) electrodes, and electromyogram (EMG) electrodes. The animals were placed in sleep-recording chambers (MIR553, Sanyo, Osaka, Japan) immediately after the surgery. Rats were habituated to the recording procedure for 10 days; during this period, they were connected to recording cables. The animals were allowed relatively unrestricted movement inside the recording cages that were kept on a 12-h light:12-h dark cycle (lights on at 08:00 h) at 24 ± 0.5 °C ambient temperature. Water and food were available ad libitum throughout the experiment. A flexible tether connected the electrodes and thermistor leads to an electronic swivel (SL6C; Plastics one Roanoke, VA, USA). The leads from the electronic swivel were routed to LEG1000 polygraphs (Nihon Koden, Tokyo, Japan) to record the EEG and EMG. The EEG was filtered below 0.5 and above 40 Hz. The amplified signals were digitized at a frequency of 128 Hz for the EEG. The data were stored on a computer in 10-s intervals. Online Fourier analysis of EEG data was performed. Vigilance states were determined off-line in 10-s epochs. Vigilance states of wakefulness (W), non-rapid eye movement sleep (NREMS), and REMS were visually identified in 10-s epochs using criteria previously reported [15]. The amount of time spent in each vigilance state was calculated for 1-h intervals and for the entire recording period. The amount of time spent in each vigilance state was used for statistical analyses. In behavioral studies, the animals were implanted with 21G icv cannula and were

kept on a 12-h light:12-h dark cycle (lights on at 17:00 h) under the same conditions as the sleep study. The animals were allowed a 10-day recovery period.

2.2. Experimental protocol

In the sleep study, each animal received an injection of 4 µl pyrogen free sterilized physiologic saline solution (PFS) icv to obtain control values. On a separate day, the same animals were then injected icv with one of three doses of the [D-Cys(tBu)5]NPS: 0 nmol (n = 7); 2.0 nmol (n = 7); 20.0 nmol (n = 7). The injections took place at the onset of darkness (20:00 h).

In the behavioral study rats were moved to the testing room 1 h before the test to acclimate. Animals received 20 nmol of [D-Cys(tBu)5]NPS (n = 8) or the PFS (n = 8). The animals were placed in a square plastic cage (60 cm × 60 cm) 30 min after the icv injection and ambulatory behavior was monitored for 5 min. The central zone of the open field was defined as a central 30 cm × 30 cm square. Horizontal activity was monitored by a video capturing and archiving system (Clever system Inc. Reston, VA, USA). Four rats were monitored in parallel in each experiment. The parameters measured were the total distance moved across the entire open field and the time spent in the central zone.

All values were expressed as mean ± SEM. Statistical analyses in the sleep time course were performed with two-way analysis of variance for repeated measures (ANOVA) across the entire recording period followed by Bonferroni's multiple comparisons test. Data in the locomotor activity test were analyzed with Student's t-test. All statistical analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). A significance level of $p < 0.05$ was accepted.

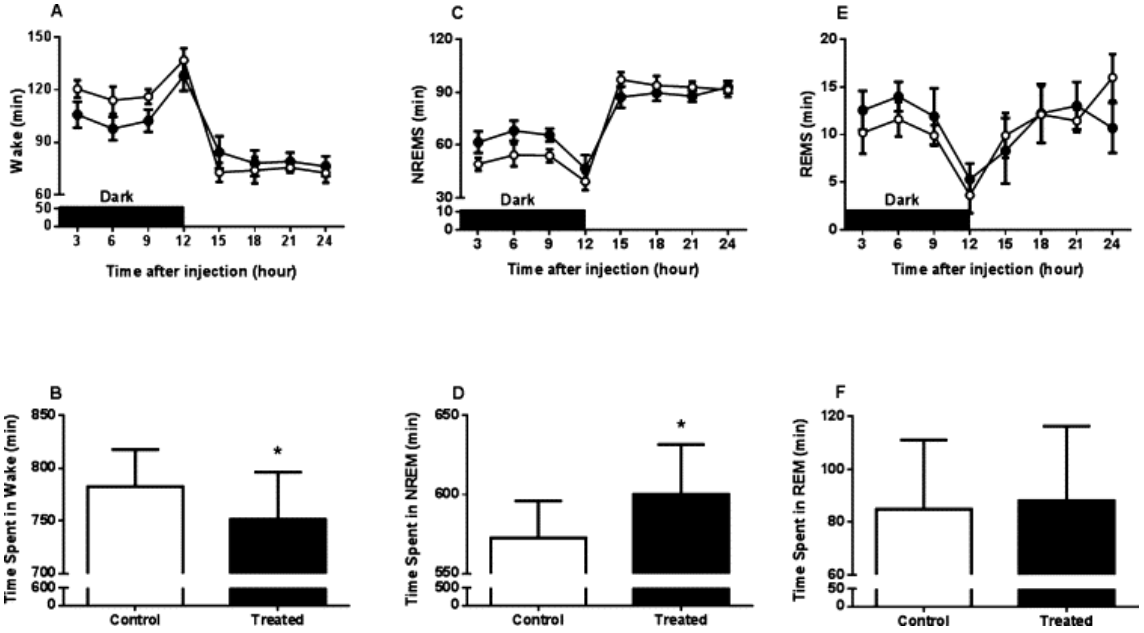
3. Results

Rats given physiological saline continued to exhibit the normal diurnal variations in sleep characteristics of this species. Zero or 2 nmol of [D-Cys(tBu)5]NPS failed to affect the parameters measured in this study. Twenty nmol of [D-Cys(tBu)5]NPS decreased the amount of time spent in wakefulness [782.5 ± 25.5 min in control vs 751.7 ± 28.2 min in treatment group; ANOVA treatment effect; $F(1, 6) = 12.89$, $p = 0.012$]. This dose also increased time spent in NREMS [572.6 ± 17.2 min in control vs 600.2 ± 26.1 min in treatment group; ANOVA treatment effect; $F(1, 6) = 8.40$, p

= 0.028]. There was no statistically significant difference in time spent in REMS [84.9 ± 26.2 min in control vs 88.1 ± 28.3 min in treatment group; ANOVA treatment effect; $F(1, 6) = 0.27$, $p = 0.628$] (Fig. 1).

Total distance moved was 14.8 ± 1.0 m (control) and 15.2 ± 0.9 m (treatment). The time spent in the central zone was 26.0 ± 4.4 s (control) and 19.7 ± 5.1 s (treatment). There were no statistically significant differences in these behavioral responses. No grossly abnormal motor behavior was observed in the [D-Cys(tBu)5]NPS group.

Fig. 1.



Twenty-four hour post intracerebroventricular (icv) injection time course of 20 nmol [D-Cys(tBu)5]NPS on wakefulness (panel A), non-rapid eye movement sleep (NREMS; panel C), and rapid eye movement sleep (REMS; panel E) in rats. Total time spent over 24 h following icv injection of 20 nmol of [D-Cys(tBu)5]NPS in wakefulness (panel B), NREMS (panel D) and REMS (panel F). Error bars indicate SEM. Open symbols (o) show results after vehicle treatment (control); closed symbols (•) represent results after [D-Cys(tBu)5]NPS treatment. Horizontal dark bars denote the dark phase of the day. Intracerebroventricular [D-Cys(tBu)5]NPS decreased the amount of time spent awake and increased NREMS. Symbol (*) denotes $p < 0.05$ vs control.

4. Discussion

The major finding of this study is that [D-Cys(tBu)5]NPS decreased wakefulness with an increase in NREMS. Previous studies reported exogenous NPS decreased physiological sleep [29], and pharmacological sleep time included by sedatives [21] or anesthetics [16]. These results indicate that NPS has some property of promoting wakefulness. In this study, inhibition of NPS receptor activation by endogenous NPS with a selective antagonist, [D-Cys(tBu)5]NPS, decreased wakefulness and increased NREMS; therefore, the NPSR antagonist disrupted the normal role for endogenous NPS in physiological sleep–wake regulation. Other studies showed that diazepam-induced sleep time was not affected by an NPSR antagonist [24] and [25] or in NPS knockout mice [23]. In addition, we previously reported that NPS decreased thiopental and ketamine anesthesia time; while, [D-Cys(tBu)5]NPS increased anesthesia time [16]. Based on the above results, it is suggested that the role of NPS and NPS/NPSR in pharmacological sleep may be different depending on receptor-based pharmacological properties of each agent. Diazepam and thiopental acts via the GABAA receptor, and ketamine acts via NMDA receptor. Exact differences in the roles of NPS/NPSR on physiological and pharmacological sleep remain to be determined.

The NPS receptor antagonist, [D-Cys(tBu)5]NPS increased the amount of NREMS but not REMS. This NPS effect on sleep–wake regulation was smaller compared to orexin; a well-established endogenous arousal-promoting peptide. An orexin antagonist promoted both NREMS and REMS in rats, dogs, and humans [2]. Rats administered TCS-OX2-293, a selective orexin-2 receptor antagonist, decreased wakefulness due to a decrease in the average wake episode duration and an increased REMS due to an increase in the number of REMS episodes [13]. One study indicated that NPS enhanced c-Fos expression in histaminergic and orexinergic neurons of the hypothalamus where mRNA of NPSR was densely expressed [30]. Therefore promotion of wakefulness by NPS could be through these neuronal activities. This is a possible reason NPS effects on sleep–wake cycle regulation was smaller compared to orexin. On the other hand, orexin exerts its arousal effect, in part, by directly activating noradrenergic neuron in the locus coeruleus (LC) [27]. The LC noradrenergic neurons are electrically silent during REMS [11]. Neuropeptide S is co-expressed in glutamate-producing neurons located rostral to the LC (pre-coeruleus region) [28]. This anatomical property suggests interaction of NPS with brain noradrenergic neurons. Indeed, blockade of adrenergic signaling by propranolol attenuated NPS-induced memory enhancement

[18]. Therefore, NPS could exert its physiological effects via brain adrenergic receptors.

The effect of [D-Cys(tBu)5]NPS on NREMS was dominant to REMS. In humans, physiological sleep develops by progression from light NREMS to deeper NREMS. The first REMS episode usually occurs 90 min after the first NREMS. This 90 min cycle of NREMS and REMS continues through the night with the proportion of REMS steadily increasing. In lower mammals such as cats, mice and rats, this sleep architecture is similar to humans although the length of each sleep cycle is shorter. Non-rapid eye movement sleep develops after wakefulness and REMS follows a period of NREMS [4]. In other words, NREMS is necessary to generate REMS in physiological conditions. Therefore the dominant effect of [D-Cys(tBu)5]NPS on NREMS indicates that NPS is involved in the mechanisms used to generate natural sleep architecture.

We recorded 5 min of locomotor activity after 30 min [D-Cys(tBu)5]NPS icv to clarify if this antagonist exerted acute behavioural effects. The 30 min time-lag seemed to be sufficient to allow the centrally administrated drug to produce a behavioral effect. We found that [D-Cys(tBu)5]NPS itself did not affect locomotion activity in rats. Another NPS receptor antagonist, [tBu-D-Gly5]NPS, prevented NPS-induced hyper locomotion in mice; however [tBu-D-Gly5]NPS itself did not affect locomotion [24]. Similar findings were obtained with a further NPS receptor antagonist, SHA 68 [25]. There was no difference in stereotypic behavior between NPS receptor knockout mice and wild type mice [7]. These results suggest that endogenous NPS/NPSR pathways do not exert an active control of locomotion in various species. Moreover, there were no differences in locomotor activity and sensitivity to diazepam in mice lacking the NPSR gene [23]. The available evidence [5] suggests that [D-Cys(tBu)5]NPS behaves as a selective NPSR antagonist. This peptide has been used by several research groups for investigating different biological actions of NPS and no evidence for off target effects have been reported. Importantly, [D-Cys(tBu)5]NPS has been obtained by mutating a single residue of a 20 mer peptide sequence (NPS) that is extremely selective for the NPSR receptor. Indeed, NPS has repeatedly been found to be inactive in mice knockout for the NPSR gene. Thus, although we do not have direct evidence demonstrating that the decrease in wakefulness induced by [D-Cys(tBu)5]NPS is due to NPSR blockade, we consider the possibility of off target effects of the antagonist extremely unlikely.

5. Conclusion

In conclusion, NPS/NPSReceptor system is involved in physiological sleep architecture but not tonic locomotor activity. Our data provide evidence in support of the application of NPS/NPSReceptor system in sleep regulation without abnormal behavioral responses.

Conflicts of interest

The authors declare no conflicts of interest.

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