SPS-B, A PHYSIOLOGICAL SLEEP REGULATOR, FROM THE BRAINSTEMS OF SLEEP-DEPRIVED RATS, IDENTIFIED AS OXIDIZED GLUTATHIONE

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We previously reported regarding the "sleep-promoting substance (SPS)," which was isolated from the brainstem extract of sleep-deprived rats, the existence of multiple active components including uridine and SPS-B. Intracerebroventricular infusion of crude SPS-B exhibited significant enhancing effects on both slow wave sleep and paradoxical sleep in unrestrained rats. Further investigation of SPS-B has resulted in its final identification as oxidized glutathione (GSSG, gamma-glutamylcysteinylglycine disulfide). Authentic GSSG similarly administered in rats also significantly enhanced sleep which was indistinguishable from normal physiological sleep. We propose GSSG as a candidate endogenous sleep substance.

KEYWORDS electroencephalogram; oxidized glutathione; paradoxical sleep; physiological sleep; rat brainstem; sleep-promoting substance; sleep substance; slow wave sleep

Many bioactive substances have been proposed during the past decade as a candidate for an endogenous sleep substance.[1-3] However, only three substances, delta-sleep-inducing peptide,[4] a muramyl tetrapeptide[5] and uridine,[6-9] have been isolated and identified directly from the tissues and body fluids of sleeping or sleep-deprived animals and humans. This was based on assays for a somnogenic activity during the course of purification. Sleep-promoting substance (SPS) was originally purified from brainstem extract of 24-h sleep-deprived rats. We previously reported the existence of multiple active components including uridine and SPS-B in SPS.[6-9] Here we describe the final identification of SPS-B as oxidized glutathione (GSSG, gamma-glutamylcysteinylglycine disulfide), based on both chemical evidence and somnogenic effects.[10]

To purify SPS, brainstem extract was first subjected to cation exchange column chromatography on SP Sephadex to give fractions containing SPS. Secondly, the combined SPS was fractionated by gel filtration on Sephadex G-10. Sleep-promoting activities were found in two different parts, designated SPS-A and SPS-B. SPS-A was finally purified to afford uridine as an active component.[6]

As shown in Table I, the combined crude SPS-B significantly increased both slow wave sleep (SWS) (38.6% above the baseline) and paradoxical sleep (PS) (49.9%).[7-9] Since the increments were caused by an elevated occurrence of SWS and PS episodes but not their prolongation, the enhanced sleep appeared to be normal. Therefore, an active component was further purified from the crude SPS-B by reverse-phase preparative HPLC. A sample of the crude SPS-B was separated into 11 fractions as shown in Fig. 1.[11] Only fraction 9, eluted between 34.7 and 40.5 min on the chromatogram, significantly increased both SWS (14.2% above the baseline) and PS (45.9%) as shown in Table I, and it showed a single peak with an elution time of 36.0 min on analytical HPLC under the same conditions as employed in Fig. 1.

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So we considered that this single peak may correspond to the sleep-promoting component of SPS-B. Consequently, to get enough pure SPS-B for structural elucidation, preparative HPLC on a large scale was carried out with the crude SPS-B under the same conditions as above, collecting the eluent corresponded to the single peak of fraction 9 in Fig. 1. The final yield of the pure SPS-B was 0.9 mg from the crude SPS-B of 470 brainstem equivalents.

Table I. Sleep-Promoting Effects of Crude SPS-B, Pure SPS-B and GSSG[10)]

	Baseline		Experiment	
	SWS	PS	SWS	PS
Crude SPS-B at 2 units/200 μl (n=9)				
Total time (min)	246.1±15.4	47.7±8.3	341.0±20.4**	71.5±9.5*
Pure SPS-B at 5 units/100 μl (n=7)				
Total time (min)	213.2±11.6	33.8±2.6	243.4±14.3	49.3±6.6*
GSSG at 25 nmol/100 μl (n=5)				
Total time (min)	202.9± 5.3	25.5±3.4	271.8± 9.4***	44.4±6.1*

Mean ± SEM, * : $P<0.05$, ** : $P<0.01$, *** : $P<0.001$.

Spectral data of the pure SPS-B were obtained as follows.[12)] ^{1}H-NMR (270 MHz in D_2O, 50 °C) δ: 2.18 (2H, double d-like, J=14.0 and 7.3, Glu-$C_\beta H_2$), 2.55 (2H, m, Glu-$C\gamma H_2$), 3.02 (1H, double d, J=14.2 and 8.9, Cys-C_βH), 3.28 (1H, double d, 14.2 and 5.0, Cys-C_βH), 3.85 (1H, t, J=6.4, Glu-CαH), 4.00 (2H, s, Gly-CH_2), 4.75 (1H, double d, J=8.9 and 5.0, Cys-CαH); ^{13}C-NMR (67.8 MHz in D_2O, 30 °C) δ: 28.8 (t, Glu-C_β), 34.1 (t, Glu-Cγ), 41.5 (t, Cys-C_β), 44.3 (t, Gly-C), 55.4 (d, Cys-Cα), 56.4 (d, Glu-Cα), 175.4 (s, Cys-CONH), 176.1 (s, Glu-COOH), 177.6 (s, Glu-CONH, Gly-COOH); FAB-MS, m/z: 613 $(M+H)^+$. These data from the pure SPS-B indicated that this substance should be GSSG, and this was confirmed by direct comparison with an authentic sample. In order to ascertain this in respect to sleep-enhancing property in rats, we intracerebroventricularly infused 25 nmol (42 pmol per min) of authentic GSSG. This caused a significant increase in both SWS (34.0% above the baseline) and PS (74.1%) as shown in Table I. These effects of authentic GSSG were indistinguishable from normal sleep since both SWS and PS increased and the increase was largely due to the increased occurrence of sleep episodes. The weaker potency, especially in SWS, of the pure SPS-B (see above) may be due to a slightly lower dose than the optimum 25 nmol. The content of GSSG in the rat third ventricle is unknown although that in the rat cerebral cortex has been reported to be 14 nmol per gram wet weight.[13)]

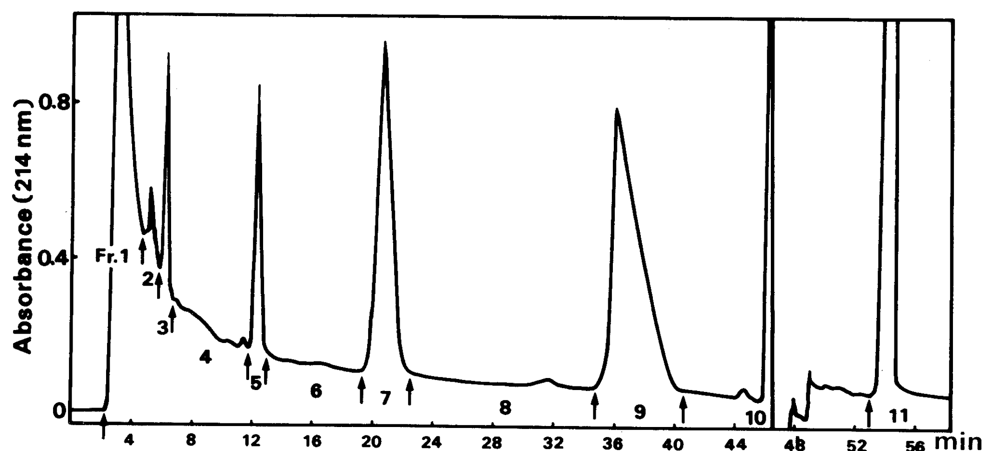

Fig. 1. Chromatogram of Preparative HPLC of the Crude SPS-B[11)]

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Recently, both GSSG and reduced glutathione (GSH) have been reported to significantly inhibit the binding of Glu in synaptic membrane preparations from the rat brain,[14] and to have specific binding sites in synaptic membranes.[15] Glu has been considered to be a potential candidate for an excitatory neurotransmitter in the mammalian central nervous system. It is suggested that GSSG and GSH may in part participate in the putative Glu-ergic synaptic transmission in the central nervous system by interfering with its receptor system and/or its inactivation mechanism.[14] Furthermore, riluzole, an antagonist of Glu-ergic receptor, increased the amount of both SWS and PS in rats.[16] Hence, it appears likely that the sleep-promoting activity of GSSG is related to the inhibitory activity in the Glu-ergic synaptic transmission. Taking all these into consideration, we propose that GSSG is a new candidate endogenous sleep substance. Further investigation is required to understand the sleep-regulatory mechanism resulting from GSSG. Experiments to investigate a somnogenic activity of GSH are now in progress.

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10) Every fraction obtained from each step of the purification of SPS-B was tested for a somnogenic activity in freely behaving rats to select the active fractions.[7-9] The animals had been implanted with cortical electrodes for electroencephalogram (EEG), nuchal electrodes for electromyogram, and a cannula in the third ventricle for continuous infusion. An appropriate amount (1-5 brainstem equivalents units; one brainstem equivalent corresponds to an amount obtained from one brainstem) of each fraction dissolved in 100 or 200 μl of physiological saline was continuously infused during a 10-h period (19:00-5:00). Otherwise the rats were continuously infused with saline at the same rate. Total time of SWS and PS during the 12-h dark period (20:00-8:00) were compared with that of the previous night (the baseline), when saline was continuously infused, and statistically analyzed by Student's t-test.
11) The crude SPS-B of 50 brainstem equivalents was dissolved in 10 μl of 0.1% trifluoroacetic acid (TFA) and injected into a μBondasphere C_{18} column (Waters, 5 μ, 3.9 x 150 mm 30°C) eluted with 0.1% TFA (0-42 min), CH_3CN/0.1% TFA (50:50, 42-49 min) and then (95:5, 49-58 min) at a flow rate of 0.5 ml/min. The eluent was collected and separated into 11 fractions according to the absorbance at 214 nm. An appropriate amount of each fraction was tested to evaluate sleep-promoting activities in rats.[10]
12) NMR spectra were recorded on a JEOL JNM-GX270 NMR spectrometer. Chemical shifts (δ) are reported in ppm downfield from external 3-(trimethylsilyl)propionic acid-d4 sodium salt, and the following abbreviations are used: s=singlet, d=doublet, t=triplet and m= multiplet. The coupling constants (J) are shown in Hz. Assignments of proton signals were based on ^1H-^1H correlation spectroscopy. FAB-MS was performed on a JEOL JMS-HX100 mass spectrometer. Glycerol was used as the matrix.
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