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Structure-Activity Relationship in the Effects of Delta-Sleep-Inducing Peptide (DSIP) on Rat Sleep

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OBAL, F , JR , V M KOVALZON, V N KALIKHEVICH, A TOROK, P ALFOLDI, G SÁRY, M HAJÓS AND B PENKE *Structure-activity relationship in the effects of delta-sleep-inducing peptide (DSIP) on rat sleep* PHARMACOL BIOCHEM BEHAV 24(4) 889–894, 1986 —DSIP and its analogues, [D-Trp¹]-DSIP, [D-Tyr¹]-DSIP, and [D-Trp¹]-DSIP$_{1-6}$, were injected ICV (7 nmol/kg) into rats at dark onset, and the sleep-wake activity was recorded during the 12-hr dark period and the subsequent 12-hr light period The effects were evaluated with respect to baseline records obtained after artificial CSF injections DSIP did not increase sleep, whereas both [D-Trp¹]-DSIP and [D-Tyr¹]-DSIP promoted sleep in the first part of the night. [D-Trp¹]-DSIP$_{1-6}$ had a prompt arousing effect It is suggested that the sleep-promoting analogues act by facilitating slight endogenous sleep tendencies at some time after dark onset, while DSIP is degraded quickly and is therefore not effective The increase of W after [D-Trp¹]-DSIP$_{1-6}$ may indicate that DSIP contains a fragment with an arousing effect The results corroborate the notion that the active DSIP molecule has a pseudo-cyclic structure

DSIP Sleep Sleep factors

THE delta-sleep-inducing peptide (DSIP), a nonapeptide (Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu), was isolated from the cerebral venous blood of rabbits with the aim of finding a physiological sleep-promoting factor [21,27] The possible physiological importance of DSIP was supported by the fact that DSIP-like immunoreactivity has been demonstrated in different tissues and fluids of a number of species (see [6] for a review), including man [13]. The neuronal network containing DSIP-like material has recently been mapped in the rat brain [5]. The successful synthesis of DSIP [20, 27, 28] prompted a great number of experiments to reveal the biological actions of the substance It seems that DSIP may affect various physiological and behavioral variables, but the increase of sleep is still regarded as its main function [6,26]

The promotion of sleep in response to DSIP administration has repeatedly been reported in various animals, nevertheless, the results, and particularly those obtained in rats, are controversial Thus, Kafi *et al* [11] and Ursin and Larsen [30] found an increase of sleep after intravenous and intracerebroventricular (ICV) administration of DSIP, respectively In contrast, Tobler and Borbély [29], Mendelson *et al* [16], and Kovalzon and Tsibulsky [15] failed to observe a sleep-promoting effect of DSIP in rats Inoué *et al.* [8,9] reported that the response to DSIP depended on the diurnal period, i.e., the circadian rhythm of sleep-wake activity

In our earlier experiments [23], sleep did not increase when DSIP and a structural analogue, ω-aminocaprilyl-DSIP, were injected ICV at dark onset into rats Instead, an increase of wakefulness (W) was found 6 to 9 hours after the treatment Since Inoué *et al* [9] demonstrated that sleep increased in response to the continuous infusion of DSIP to rats at night, we suggested that the differences in the effects of a single ICV injection and continuous infusion might be due to a quick degradation and elimination of the peptide after the injection In fact, Kastin *et al* [14] showed that the increased DSIP-like immunoreactivity induced in the rat brain by peripheral injection of the peptide rapidly returned to the preinjection level, suggesting

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a quick breakdown of the substance. Experiments with DSIP incubated in rat brain homogenates provided evidence that degradation of the peptide started with release of the amino terminal Trp in a few minutes [7] Therefore, it seemed worthwhile to study the effects of DSIP analogues which might be more resistant than the original molecule to enzymic splitting Thus, we tested the effects of peptides in which the amino terminal Trp had been replaced by D-Trp or D-Tyr. The rats were injected ICV with DSIP, [D-Trp^1]-DSIP, [D-Tyr^1]-DSIP or [D-Trp^1]-DSIP$_{1-6}$ (a hexapeptide without the carboxy terminal tripeptide of DSIP) at the onset of the dark period, and the sleep-wake activity was followed for 24 hr (12-hr dark and 12-hr light periods) The results were evaluated with respect to the 24-hr records obtained after the ICV injection of artificial cerebrospinal fluid (CSF) the day before treatment with the peptides

METHOD

Male CFY rats weighing between 300 and 350 g were used The numbers of animals evaluated in the groups designed to characterize the effects of the various substances were as follows 11 rats for DSIP, 11 rats for [D-Trp^1]-DSIP, 8 rats for [D-Tyr^1]-DSIP, and 8 rats for [D-Trp^1]-DSIP$_{1-6}$

The methods, the experimental conditions and the schedules were exactly the same as those used in our earlier studies [23] Under pentobarbital anaesthesia (50 mg/kg), golden jewellery screws were cemented over the frontal and parietal cortices and over the cerebellum for EEG recording The implantation of the ICV cannula into the left lateral ventricle was carried out according to the description provided by Tobler and Borbély [29] Stainless steel 33-gauge needles served for the ICV injection

The placement of the cannula and the drainage of the ventricle was tested by means of the drinking response elicited by angiotensin assuming that the CSF circulates normally, then angiotensin (100 ng, 1 μl) injected into the lateral ventricle will reach the third ventricle and elicit drinking in 2 min via the stimulation of preoptic structures [4] Each animal was tested 5 days before, and immediately after the sleep-wake recordings After the termination of the experiments, the placement of the cannula was checked in frozen sections of the brain The present results, and the numbers of animals in the groups as given above, relate only to those rats in which the angiotensin test was positive both before and after the experiments, and in which examination of the brain sections confirmed the proper position of the cannula

The animals were adapted to the experimental conditions for at least 10 days after surgery During this period they lived connected to the flexible recording cable in individual Plexiglas cages in the sound attenuated recording chambers The ambient temperature was regulated at 21°C, and low-level continuous noise was provided by means of loudspeakers Sun spectrum-emitting light-tubes provided light The animals were raised on a light-dark (LD) of 12 hr each, with lights on from 8 30 to 20 30 The same LD was maintained during the experiments with DSIP and [D-Trp^1]-DSIP For the rats injected with [D-Tyr^1]-DSIP and [D-Trp^1]-DSIP$_{1-6}$, the LD was reversed (lights on from 20 30 to 8 30) 21 to 24 days before the experiments.

The animals received ICV artificial CSF for 5 days before the experiments Both during this habituation period and in the experiments, artificial CSF and the peptides were injected 15 min before dark onset The composition of the artificial CSF followed the prescription proposed by Myers

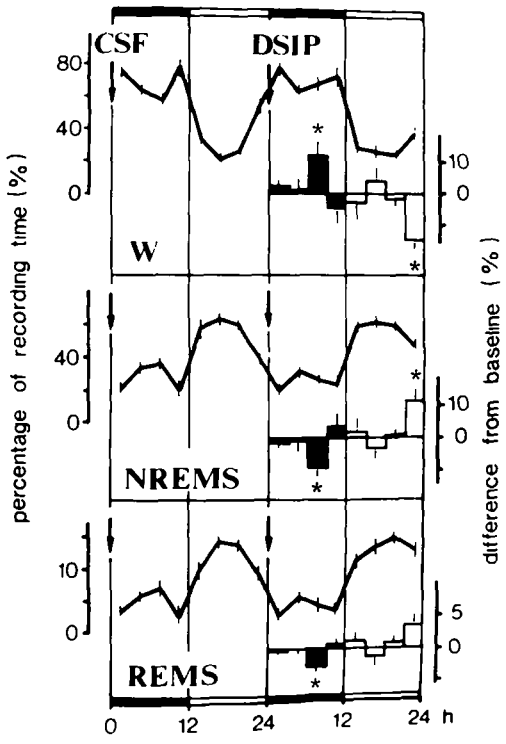

FIG 1 Effects of ICV injection of DSIP (7 nmol/kg) on the sleep-wake activity of rats (n=11), as compared to the sleep-wake activity after the injection of artificial CSF The mean values (±SE) for each vigilance state were computed for consecutive 3-hr periods after the injection of artificial CSF (baseline) and DSIP, and expressed as percentages of the recording time Histograms show mean differences (±SE) from the baseline values (as percentages of recording time) Black and open columns indicate dark and light periods, respectively Asterisks denote significant differences (at least $p<0$ 05, paried t-test, 2-sided) with respect to the baseline values

[22] The artificial CSF and the substances were administered in a volume of 3–3 5 μl in about 1 to 2 min The dry substances, stored at 4 to 5°C, were dissolved in artificial CSF immediately prior to their use The peptide dose was invariably 7 nmol/kg The experimenters used coded substances distributed in test tubes, the structures of the peptides were learned only after results obtained with the substances in the various series of test tubes had been calculated

After the habituation period, the sleep-wake activity was recorded for 24 hr following the ICV injection of artificial CSF on day 6. The next day, DSIP or one of its analogues was injected, and the recording was continued for another 24 hr

The EEG and (by means of monitoring cable movements via piezoelectric force recorders) the motor activity of the rats were recorded on paper charts (5 mm/sec) The vigilance states were scored as W, non-REMS sleep (NREMS) and REM sleep (REMS) according to conventional criteria in 40-sec intervals, and the percentages of each state were calculated for 1-hr, 3-hr and 12-hr periods. The sleep-wake records obtained after the injection of artificial CSF were regarded as baseline Analysis of variance was used to compare the baseline sleep-wake patterns of the various groups The effects of the peptides were evaluated by comparing the

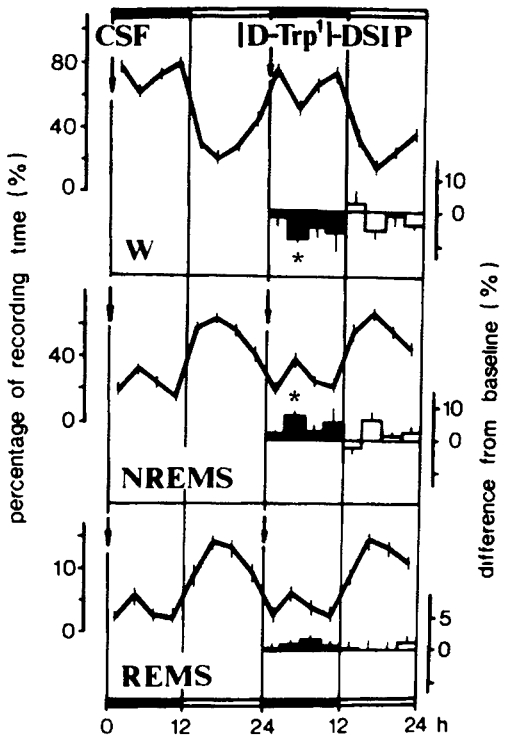

FIG 2 Effects of ICV injection of [D-Trp¹]-DSIP on the sleep-wake activity of rats (n=11), as compared to the sleep-wake activity after the injection of artificial CSF See Fig 1 legend for details

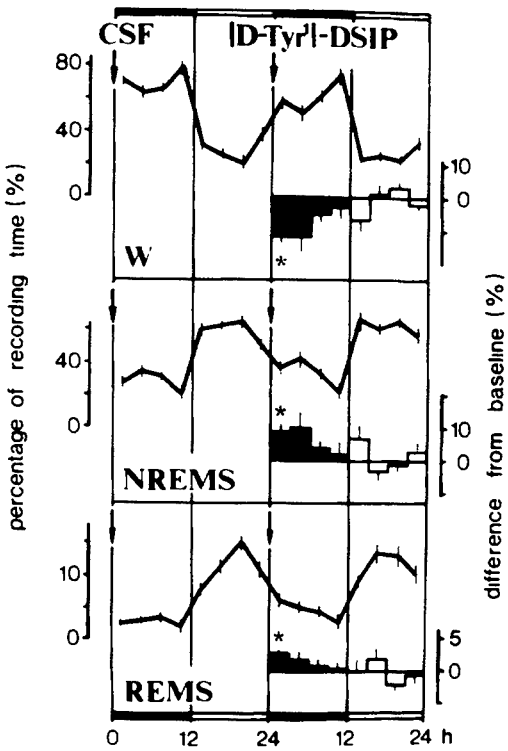

FIG 3 Effects of ICV injection of [D-Tyr¹]-DSIP on the sleep-wake activity of rats (n=8), as compared to the sleep-wake activity after the injection of artificial CSF See Fig 1 legend for details

percentages of the vigilance states with the corresponding baseline values by means of the paired *t*-test (two-sided).

DSIP was synthetized by two of the authors (A.T and B.P) in a standard solid-phase synthesis via DCC coupling of the amino acids [23] Its analogues were synthetized by Kalikhevich *et al.* [12] via classical liquid-phase method from two fragments (1–4 and 5–9) using the benzyloxycarbonyl group for N-protection and p-nitrophenyl esters or dicyclohexylcarbodiimide for coupling After cleavage of the protecting groups by means of catalytic hydrogenolysis, the nonapeptides were purified by ion-exchange chromatography on a DEAE A-25 Sephadex column using ammonium acetate buffer (gradient 0 01 to 0 5 M). Pure fractions were pooled, lyophilized and characterized through amino acid analysis, electrophoretic running properties, optical rotation values and retention factors on Merck Silica TLC plates (System A=n-butanol-pyridine-acetic acid-water 10 5:6 1 7 5, System B=isopropanol-ammonium hydroxie-water 7 1 2, System C=acetone-water 7·3)

RESULTS

The sleep-wake activities during the 24-hr baseline records in the four groups of rats were subjected to analysis of variance by using the percentages of the vigilance states calculated for consecutive 1-hr periods In agreement with the large diurnal variations (Figs. 1–4), significant time effects were obtained for each vigilance state, W $F(23,864)=49.9$, $p<0.05$, NREMS· $F(23,864)=43.4$, $p<0.05$; REMS. $F(23,864)=33\ 8$, $p<0.05$ Although the rats on the reversed LD (Figs 3–4) tended to sleep more in the first few hours after dark onset than the animals on the normal LD (Figs. 1 and 2), the time effects, W: $F(3,864)=2.0$; NREMS: $F(3,864)=2\ 2$; REMS. $F(3,864)=2.5$, and the interactions, W: $F(69,864)=1\ 4$; NREMS $F(69,864)=2.0$; REMS $F(69,864)=1\ 3$, were not significant.

Effects of DSIP

The ICV injection of 7 nmol/kg DSIP did not affect the sleep-wake activity for 6 hr (Fig. 1). A significant increase of W and a decrease of both NREMS and REMS were observed 6 to 9 hr after the treatment. The percentages of the vigilance states then returned to the baseline levels. A significant increase of NREMS at the expense of W was noted only at the end of the light period, i.e., 21 to 24 hr after the injection of DSIP

Effects of [D-Trp¹]-DSIP

[D-Trp¹]-DSIP increased sleep at night (Fig. 2). NREMS was significantly higher and W significantly less than the corresponding baseline value as early as the first 1-hr postinjection period. This reaction, however, was transient, and the percentages of the vigilance states calculated for the first 3-hr period did not reveal significant changes with respect to the baseline values. A significant increase of NREMS and a reduction of W were observed 3 to 6 hr after the injection

W tended to decrease throughout the second half of the night, but the differences calculated for the individual 3-hr periods did not reach the level of significance. Compared to the result of the baseline night, the percentage of W calcu-

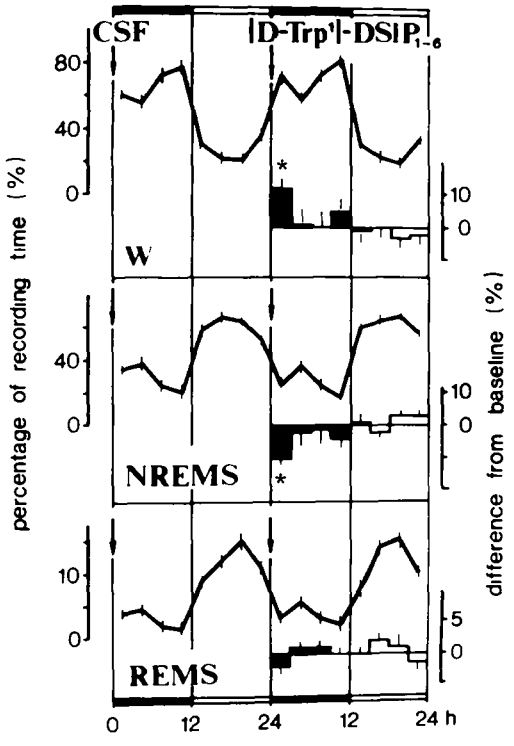

FIG 4 Effects of ICV injection of [D-Trp¹]-DSIP$_{1-6}$ on the sleep-wake activity of rats (n=8), as compared to the sleep-wake activity after the injection of artificial CSF See Fig 1 legend for details

lated for the total 12-hr dark period after [D-Trp¹]-DSIP was significantly decreased (mean±SE, Student t-test 73 3±1 2 vs 68 2±1 5, $p<0$ 05) and NREMS increased (23 3±1 0 vs 27 7±1 5, $p<0$ 05) The sleep-wake activity in the light period did not differ from the baseline values

[D-Tyr¹]-DSIP

Injection of [D-Tyr¹]-DSIP induced a pronounced increase of sleep (Fig 3) Although the percentages of the vigilance states in the first postinjection hour were not yet different from the baseline values, a large reduction of W and increases of both NREMS and REMS were found in the subsequent hours. As a result, the decrease of W, and the increase of NREMS and REMS were significant for the first 3-hr period. The tendency to increased sleep gradually declined towards light onset, the changes in the vigilance states in the individual 3-hr periods during the remainder of the dark period did not reach the level of significance The animals spent much more times both in NREMS (Student's t-test) (28 0±2 1 vs 34 5±2 1, $p<0$ 05) and in REMS (2 8±0 5 vs 4 6±0 5, $p<0.05$), and less in W (69 1±2 0 vs 60 9±3 3, $p<0$ 05) than during the baseline recording when the percentages of the vigilance states calculated for the 12-hr dark period were considered Alterations in the sleep-wake activity were not observed during the subsequent light period

[D-Trp¹]-DSIP$_{1-6}$

[D-Trp¹]-DSIP$_{1-6}$ did not promote sleep. On the contrary, an increase of W at the expense of NREMS was observed (Fig 4) The increase of W and the reduction of NREMS were prompt effects and the alterations in the vigilance states were significant for the 3-hr postinjection period Thereafter, the percentages of the vigilance states returned to the baseline values and remained at these levels throughout the dark period and the subsequent light period The amounts of the vigilance states calculated for 12-hr periods did not reveal significant differences with respect to the corresponding baseline values

DISCUSSION

The effects of the four substances on the sleep-wake activity of rats were different DSIP did not promote sleep, two of the analogues, [D-Trp¹]-DSIP and [D-Tyr¹]-DSIP, increased sleep, and another peptide, [D-Trp¹]-DSIP$_{1-6}$, increased W Besides an evaluation of the structural differences between the peptides, consideration of the experimental conditions, which were essentially the same as in our earlier studies [23], also appears to be of importance for a discussion of the results

(1) The animals were habituated both to the recording conditions and to the treatment, in order to reduce stress reactions which have been reported to influence the effects of DSIP [25] (2) The dry substances were dissolved immediately prior to their use, as suggested by Ursin and Larsen [30] (3) A volume of 3 to 3 5 μl was injected to avoid the non-specific effects of large ICV injections [3] (4) The drinking response to ICV angiotensin before and after the sleep-experiments yielded evidence of the functioning drainage of the ventricle (5) All the substances were administered in the dose recommended for experiments with DSIP [19] This dose, 7 nmol/kg, corresponds to the peak of the dose-response curve reported for the delta-sleep-inducing effect of DSIP on ICV administration [26] It should be noted that doses about three times higher than the one we injected were used by Tobler and Borbély [29] without effects on sleep in rats at night (6) Our results might be complicated by the fact that animals on normal and on reversed LD were used However, significant differences in sleep profile were not found between the rats on the two LD Moreover, of the two substances which increased sleep, one ([D-Trp¹]-DSIP) was injected to rats on normal LD, while the other ([D-Tyr¹]-DSIP) was administered to animals on reversed LD Finally, the present result with DSIP in animals on normal LD, i e , an increase of W 6 to 9 hr after the injection, was exactly the same as the finding obtained with another sample of DSIP (Hoffman-LaRoche, donated by Dr Schoenenberger) in rats on reversed LD in our earlier experiments [23] Therefore, both the failure of DSIP to promote sleep on injection at dark onset, and the sleep-inducing capacity of some analogues are results independent of the LD A tendency to increased sleep was found 21 to 24 hr after the injection of DSIP in the present experiments, which was not observed in our previous studies If this finding has any biological significance, it might be related to the long-term effects of DSIP treatment which were reported earlier [30] (7) The animals received the substances at dark onset, i e , peptide injection was timed for the "Zeitgeber" that normally indicated the onset of the diurnal active period The repetition of the injection procedure over several days before the actual experiment may contribute to the "Zeitgeber" effect of dark onset The fact that the administration of the peptides was timed for the onset of the dark period was, of course, not favourable for sleep induction It was assumed, however, that a trigger

substance of sleep, as DSIP was regarded by Inoué *et al.* [9], may elicit sleep even under these conditions. Nevertheless, in our experiments DSIP was ineffective, while (apart from a slight increase of sleep in the first postinjection hour after [D-Trp^1]-DSIP) both active analogues increased sleep only long after the injection. It is characteristic of rats that feeding, a prevailing activity at the beginning of the night, is followed by short periods of sleep [2]. DSIP analogues relatively resistant to enzymic splitting may facilitate these spontaneous sleep periods at a time when DSIP has been broken down. The assumption that the effect of the peptide requires an already existing sleep pressure, i.e., it acts by facilitating the endogenous sleep process instead of triggering sleep, is as speculative as any other description of the effects of DSIP. It may explain, however, the controversies and inconsistencies in the responses to DSIP administration reported from various laboratories, and also corroborates the notion that the substance exerts an "optimizing" effect on sleep disturbed sleep can be improved by facilitating sleep process but when the sleep requirement is fully satisfied sleep cannot be further increased [6,26].

It was suggested earlier that DSIP may contain a fragment which increases W instead of sleep [23] The finding that [D-Trp^1]-DSIP$_{1-6}$ had an arousing effect seems to support this proposal These results, however, should be interpreted with care, because the substance was tested only in rats on reversed LD with relatively low percentages of W at the beginning of dark onset. Further, peptides with W-increasing character might be shorter or longer than the hexapeptide studied in our experiments The results obtained through the injection of [D-Trp^1]-DSIP$_{1-6}$, however, demonstrated that removal of the carboxy terminal of an active DSIP analogue, [D-Trp^1]-DSIP, evidently abolished the sleep-promoting property

To summarize, the experiments showed that the sleep-increasing activity of DSIP is related to both the amino terminal and the carboxy terminal of the nonapeptide, and thus our findings may provide indirect evidence of the significance of the pseudo-cyclic structure of DSIP, which had been predicted as a result of theoretical considerations [1,24]. Spectral analysis studies supported this idea [17] Finally, DSIP elicited membrane hyperpolarization on a snail neuron, only analogues with a cyclic structure sharing this property [10,18] The proposed pseudo-cyclic structure is assumed to be stabilized by interactions between the side-chains of the amino terminal Trp and the carboxy terminal Glu On the removal of the carboxy terminal tripeptide, this structure is obviously disrupted and this may explain why [D-Trp^1]-DSIP$_{1-6}$ failed to induce sleep. The sleep-promoting activities of [D-Trp^1]-DSIP and [D-Tyr^1]-DSIP might also be attributed to the preservation of the active structure, which is otherwise broken down quickly by the enzymic splitting of Trp from the original molecule. The increase of sleep on [D-Tyr^1]-DSIP implies that the Tyr side-chain possesses the ability to establish an interaction with the amino terminal, by forming an H-bond between the phenolic OH and the gamma-carboxylate anion of Glu.

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