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Chronic, Low-Level Exposure to the Cholinesterase Inhibitor DFP. II. Time Course of Behavioral State Changes in Rats

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DEURVEILHER, S., B. HARS AND E. HENNEVIN. *Chronic, low-level exposure to the cholinesterase inhibitor DFP. II. Time course of behavioral state changes in rats.* PHARMACOL BIOCHEM BEHAV. **64**(1) 105–114, 1999.—Rats were repeatedly administered with low doses of diisopropylfluorophosphate (DFP; 0.2 mg/kg/day, SC), an irreversible cholinesterase (ChE) inhibitor. Control rats received a daily injection of oil vehicle or of saline. Recordings of the sleep–wake states were obtained in the 6 h following 1, 3, 6, 9, 13, 17, and 21 injections, as well as 2, 4, and 19 days after 9-day treatment. DFP administration increased waking at the expense of slow–wave sleep (SWS), but not of paradoxical sleep (PS); as a result, the PS/SWS ratio was strongly enhanced. These changes developed across days, were maximal after six to nine injections, and were then maintained at that level until cessation of treatment. This time course of behavioral state alterations paralleled the time course of ChE inhibition in the mesopontine cholinergic nuclei and the pontine reticular formation described in the companion article. In contrast, after DFP withdrawal, behavioral states returned to control values more rapidly (in 2–4 days) than did ChE activity. These results are discussed regarding the promoting role of cholinergic neurotransmission in brain-activated states. © 1999 Elsevier Science Inc.

DFP Irreversible cholinesterase inhibitor Chronic low-level exposure Rat
Acetylcholine neurotransmission Sleep–waking states Time course of changes during dosing and after withdrawal

BRAIN stem cholinergic mechanisms have long been thought to be involved in the regulation of behavioral states, and more recent studies have highlighted the critical role that mesopontine cholinergic neurons play in the control of the two brain-activated states, i.e., wakefulness and paradoxical sleep [PS; see, for reviews (23,30,42). These neurons are distributed in the pedunculopontine (PPT) and laterodorsal (LDT) tegmental nuclei, and they innervate a number of forebrain structures, as well as the pontine and medullary reticular formation [reviewed in (42)]. There is strong evidence that these nuclei have an important role in cortical arousal. For example, LDT/PPT stimulation produces cortical EEG activation; it potentiates fast oscillations and reduces slow oscillations in thalamocortical systems (46,47). Consistent with this activating role, the majority of LDT/PPT neurons increase their discharges and excitability during waking and PS, compared to slow-wave sleep [SWS; (15,25,48)]. There is also accumulating evidence suggesting that mesopontine cholinergic neurons play a primary role in the generation of PS. According to a widely prevailing model (30), PS would be induced by increased activity of LDT/PPT cholinergic neurons; the resultant increase of acetylcholine (ACh) release in the pontine reticular formation (PRF) depolarizes PRF effector neurons, thereby activating the efferent pathways involved in phasic and tonic events of PS. In support of this view, it has been shown for example that (a) ACh concentration in the PRF is higher during PS than it is during SWS or waking (26,27); (b) low-level stimulation of the LDT/PPT can increase PS time (9,51); and (c) direct administration of cholinomimetics into the PRF produces a state that very closely mimics natural PS [reviewed in (2)].

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Thus, all of the above underscores the importance of cholinergic transmission in the pontomesencephalic tegmentum (PMT) in the control of the sleep–wake states.

As reported in the companion article, we have studied the time course of changes in cholinesterase (ChE) activity occurring in the PMT during and after chronic administration of a low dose of diisopropylfluorophosphate (DFP), an organophosphosphorous (OP) anticholinesterase compound. By inhibiting acetylcholinesterase (AChE), the hydrolytic enzyme of ACh, DFP prolongs the action of ACh and augments ACh levels in the extracellular space, leading to increased cholinergic neurotransmission (39). The advantage of using DFP compared to other OPs is its low profile of toxicity. We have shown that ChE activity, histochemically quantified in discrete PMT areas (including the LDT/PPT and the PRF), was progressively reduced during the first days of DFP exposure; it was maximally inhibited after 6 days, and then maintained at that level until cessation of treatment; it slowly recovered after DFP withdrawal. Because AChE is by far the most predominant class of brain ChEs, all these changes essentially represented changes in AChE activity.

Thus, chronic low-level exposure to DFP allows fine and gradual manipulation of cholinergic function. We took advantage of that in the present study (a) to examine how progressive alteration in ChE activity modified the sleep–wake cycle, and (b) to evaluate whether the time course of behavioral state changes paralleled the time course of neurochemical changes observed in the PMT. For this, rats received the same DFP treatment regimen as rats used in the neurochemical study, i.e., 0.2 mg/kg/day for 9 or 21 consecutive days, and their sleep–wake states were recorded across days, during and after DFP exposure. This study is the first to describe the kinetics of behavioral state alterations as a function of progressively changing cholinergic transmission. Indeed, whereas the effects of acute injection of cholinergic agents on the sleep–wake states are well documented, there are only three reports on the effects of chronic cholinergic treatment (18,43,49), and the only one that used repeated administration of ChE inhibitor (18) provided no information on the time course of the effects, given that ChE activity was maximally inhibited as early as the first injection.

METHOD

The subjects were male Wistar rats (Iffa-Credo, France) weighing 330–380 g at the onset of drug treatment. They were housed in individual Plexiglas cages in a temperature-controlled colony room (23 ± 1°C) under a 12 L:12 D cycle (0700–1900 h light). Food and water were available ad lib. All the experimental procedures used are in compliance with the European legislation (86/609/EEC) on animal experimentation.

Surgical Preparation of Animals

Under pentobarbital anesthesia (60 mg/kg IP), two supradural cortical electrodes, made of small silver spheres, were placed along the interhemispheric suture with a large frontoparietal contralateral derivation for the recording of electrocorticographic (EEG) activity. This derivation was selected to maximize recording of hippocampal theta activity in the surface EEG. Two silver wires were inserted into the dorsal neck muscles for the recording of electromyographic (EMG) activity. A small silver sphere placed between the dura and the parietal bone was used as a ground. All electrodes were connected to a miniature socket, fixed onto the skull with two stainless steel screws and dental acrylic cement.

Recording and Scoring of Behavioral States

Sleep–waking states were recorded for each rat in its individual home cage via a flexible cable relayed at the top of the cage through a multichannel rotating connector. During each recording session, EEG and EMG activity were continuously monitored on a polygraph (Grass, Model 78D; Quincy, MA), with paper running out at 2.5 mm/s. Four to six rats were recorded at the same time. For four of them, behavioral state scoring was performed on line by an automatic state analyzer (ICPI; Lyon, France), as described previously (8). Briefly, the behavioral state was scored in consecutive 10-s epochs with each epoch classified as waking, SWS, or PS, based on four indices: the EEG mean frequency, the EEG amplitude dispersion, the energy in the EEG theta band relative to that in the delta band, and the integrated EMG amplitude. State scoring used animal-specific, user-determined thresholds for each of these indices. Waking was defined by the presence of low-voltage, fast EEG activity, and a moderate to high EMG. SWS was identified when the EEG displayed high-voltage, slow activity while EMG tone was decreased. PS was recognized by the presence of EEG low-voltage activity, high amount of theta activity, and EMG atonia. When automatic scoring did not fit with visual scoring (less than 5% over the totality of the records), scores from visual examination were retained. For the remaining one or two rats, polygraphic recordings were scored manually in 10-s epochs.

Drug Treatment and Experimental Protocol

DFP was purchased from Sigma Chemical Co. (St. Louis, MO). It was first dissolved in peanut oil (Sigma) at a concentration of 2 mg/ml, aliquoted, and kept refrigerated in darkened tubes. It was dissolved in oil at 0.2 mg/ml immediately before injection. DFP-treated rats received a subcutaneous administration of DFP at a dose of 0.2 mg/kg once daily. This treatment regimen was chosen on the basis of previous data showing that it caused substantial ChE inhibition without inducing behavioral symptoms of cholinergic intoxication (6). Control rats received a daily injection of oil vehicle or of saline (NaCl 0.9%). All injections were made at the upper level of the rat's back, and were given in a volume of 1 ml/kg. They were done in a room adjacent to the vivarium.

One week after surgery, animals were familiarized for 4 days with the injection and recording conditions. Each day, the rat was weighed, then hand restrained for a sham injection (no needle insertion). It was immediately returned to its home cage and connected to the recording system for 7 h. On the last 2 days, vigilance state recordings were obtained to set the appropriate thresholds of the automatic state analyzer.

After this adaptation period, a baseline sleep recording (D0) was first performed: each rat was weighed and received an injection of oil; immediately thereafter, it was returned to its home cage and connected to the recording cable; recording of behavioral states started 30–40 min later and continued for 6 h. Rats were continuously observed throughout the recording session. Drug administration regimen began on the following day using the same procedure, except that rats received DFP, oil, or saline injection, according to the treatment groups. Two separate experimental series were conducted.

The first one was conducted in parallel with the first experimental series reported in the companion article. Rats were divided into two matched groups, equalized on the basis of mean total sleep time at baseline recording. Rats treated with DFP and oil controls were given a daily injection for 9 consecutive days (D1 to D9). Vigilance state recordings were obtained at D1, D3, D6, and D9. In addition, to study drug with-

drawal effects, a subset of animals from each of the two groups was recorded 2, 4, and 19 days after completion of the 9-day exposure (D9 + 2 days, D9 + 4 days, and D9+ 19 days, respectively).

The second experimental series was conducted in parallel with the second experimental series reported in the companion article. Rats were divided into three matched groups. Rats treated with DFP and oil controls were daily injected with DFP or with oil for 21 consecutive days (D1 to D21). Vigilance state recordings were obtained at D1, D3, D6, D9, D13, D17, and D21 (because of technical problems, one rat in the oil group was only recorded until D9). Rats in a second control group received a daily injection of saline for 9 days and were recorded at D1, D3, D6, and D9.

In both series, all injections were made between 0950 and 1000 h. All recordings began 30–40 min after injection and were thus conducted between 1030 and 1630 h. Between the recording days, rats continued to be weighed, drug injected (during the treatment period) or sham injected (during the drug withdrawal period), and connected to recording cables for 7 h each day.

Data Analysis

Sleep–waking parameters were quantified for the entire 6-h recording period. Measures included the amount of time spent in waking, SWS and PS; the percentage of time spent in PS relative to time spent in SWS (%PS/SWS); the number of waking, SWS and PS episodes, and the mean duration of these episodes (calculated by dividing the total time spent in a state over 6 h by the total number of episodes numbered for that state). Latency to sleep onset was defined as the interval between the start of recording and the first SWS episode of at least 30-s duration. To determine at which time point the differences between the DFP and oil control groups became maximal, for each vigilance state and each recording day the mean value obtained in the oil group was subtracted from the value obtained in each DFP-treated rat. Contrast analysis of variance, S <G> *T, was used for all statistical comparisons [VAR3 software package; (37)].

RESULTS

The present results were obtained from a total of 48 animals. Twenty-one out of them were daily injected with DFP for 9 or 21 days; the others received control injections of oil vehicle ($n = 21$) or of saline ($n = 6$). Whatever the duration of DFP exposure, no overt signs of toxicity were detected by home-cage observation. The gain in weight above baseline (D0) was comparable in DFP-treated rats and controls, whether after 9 days of treatment [+42 g on average for DFP-treated rats, +47 g for oil controls, and +46 g for saline controls; $F(2, 45) = 1.673$, NS] or after 21 days [+93 g for DFP rats and +102 g for oil controls; $F(1, 13) = 1.178$, NS]. Nonetheless, DFP treatment caused marked alterations in the sleep–wake states.

First Experimental Series: 9-Day DFP Exposure

Sleep–waking states during DFP exposure. Twelve rats were injected with DFP and 14 with oil for 9 days. Vigilance state recordings were obtained after one, three, six, and nine injections. The data are presented in Fig. 1.

Rats injected with oil exhibited changes in waking and SWS amounts across days, $F(4, 52) = 6.004$, and $F(4, 52) = 7.370$, respectively; $p < 0.001$. However, both states significantly differed from baseline only after the ninth injection ($p < 0.025$, in

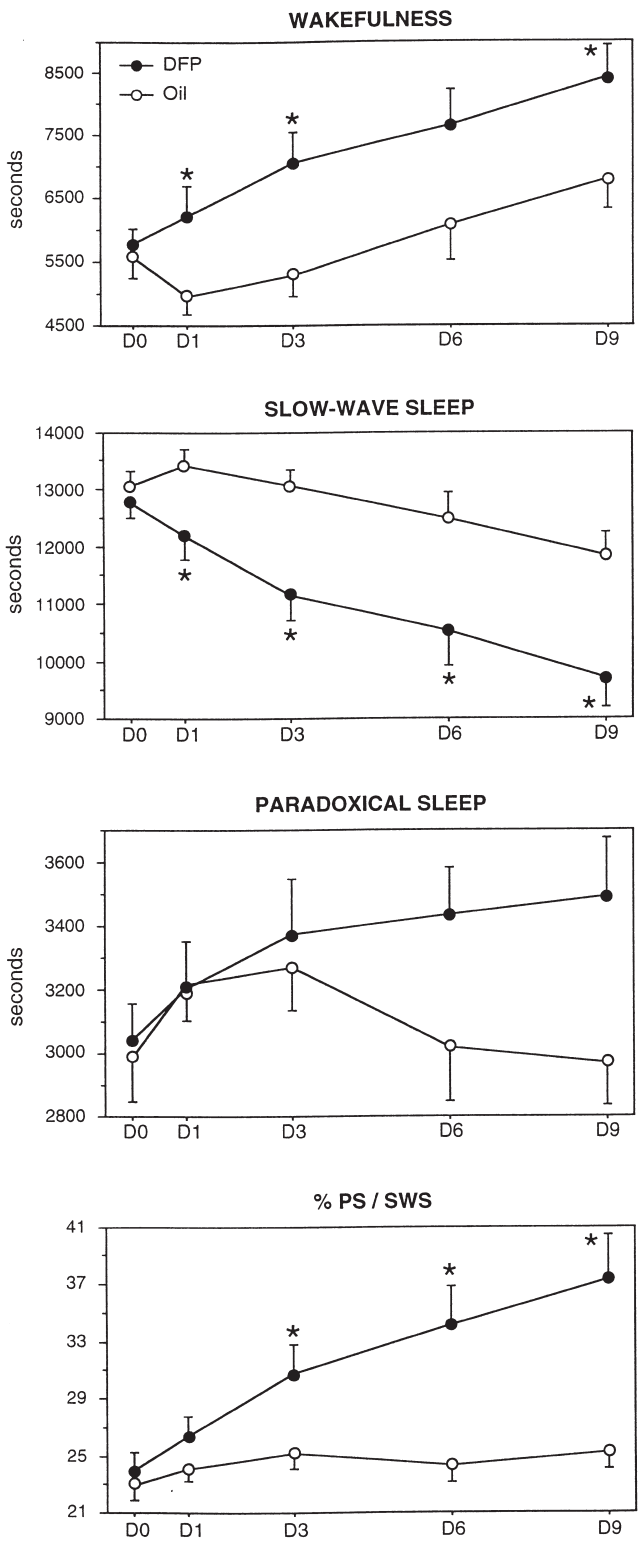


FIG. 1. Time course of the sleep–wake changes during 9-day DFP exposure. Data are from 12 rats that received a daily injection of DFP for 9 consecutive days (D1–D9), and from 14 rats that received a daily injection of oil vehicle. A baseline recording was performed on D0. Mean duration (in seconds) of waking, slow-wave sleep (SWS) and paradoxical sleep (PS), and mean percentage of time spent in PS relative to SWS (%PS/SWS), obtained over the 6-h recording period, are represented ± SEM. *Different from oil controls; all $p < 0.05$ or less.

TABLE 1

NUMBER AND DURATION OF EPISODES OF WAKING, SLOW-WAVE SLEEP (SWS), AND PARADOXICAL SLEEP (PS) DURING A 9-DAY DFP EXPOSURE

			D0	D1	D3	D6	D9
Waking	DFP	*f*	108 (±17)	98 (±17)*	119 (±22)	127 (±25)	141 (±29)
		d	54 (±10)	64 (±17)*	59 (±8)*	60 (±8)	60 (±10)
	Oil	*f*	118 (±19)	115 (±18)	126 (±21)	115 (±28)	125 (±28)
		d	48 (±10)	43 (±5)	42 (±6)	53 (±14)	56 (±14)
SWS	DFP	*f*	107 (±16)	97 (±16)*	116 (±21)	126 (±25)	140 (±27)
		d	121 (±19)	130 (±32)	100 (±32)	89 (±31)*	73 (±27)*
	Oil	*f*	117 (±20)	111 (±18)	124 (±18)	112 (±28)	122 (±29)
		d	116 (±27)	124 (±27)	108 (±23)	120 (±42)	104 (±36)
PS	DFP	*f*	33 (± 4)	34 (±3)	39 (±11)	41 (±11)*	46 (±13)*
		d	92 (±14)	94 (±13)	89 (±15)	86 (±14)	78 (±13)
	Oil	*f*	31 (±5)	36 (±5)	36 (±7)	32 (±8)	31 (±6)
		d	97 (±19)	91 (±13)	93 (±18)	95 (±12)	95 (±13)

Values (mean ± standard deviation) are from 12 rats that received a daily injection of DFP for 9 consecutive days (D1–D9), and from 14 rats that received a daily injection of oil vehicle. A baseline recording was performed on D0. For each vigilance state are indicated the total number of episodes (*f*) obtained over the 6-h recording period and the mean duration (in seconds) of these episodes (*d*).

*Different from oil controls; all $p < 0.05$ or less.

both cases). Neither PS amount nor PS/SWS ratio was significantly altered, $F(4, 52) = 1.368$, and $F(4, 52) = 1.21$, NS.

Rats treated with DFP demonstrated much more pronounced modifications. Compared to controls, they exhibited a larger amount of waking, $F(1, 24) = 7.11$, $p < 0.025$, for the group effect; this difference was already present at D1 ($p < 0.05$). Waking progressively increased across days, becoming significantly different from baseline level from D3 ($p < 0.025$). It no longer differed between D6 and D9 ($p = 0.150$). Waking augmentation was due to an increased number of waking episodes [see Table 1; $F(4, 44) = 16.738$, $p < 0.0001$, for the changes across days in the DFP group; and $F(4, 96) = 6.247$, $p < 0.001$, for the group × day interaction]. Mean duration of waking episodes was not significantly modified across days in DFP rats, $F(4, 44) = 1.684$, NS, although it was longer than that in controls ($p < 0.01$, both for the group effect and the group × day interaction).

This awaking effect of DFP was accompanied by a parallel reduction in SWS. SWS amount was lower in DFP-treated rats than in controls, $F(1, 24) = 10.294$, for the group effect; and $F(4, 96) = 4.592$, for the group × day interaction; $p < 0.005$. The two groups already differed at D1 ($p < 0.025$). SWS progressively decreased across days, becoming significantly different from baseline level from D3 ($p < 0.005$). It no longer significantly differed between D6 and D9 ($p = 0.685$). This SWS decrement was due to a shortening in the mean duration of SWS episodes, $F(4, 44) = 20.413$, $p < 0.0001$, for the changes across days in the DFP group; and $F(4, 96) = 6.508$, $p = 0.0001$, for the group × day interaction. Latency to SWS onset from the start of recording did not change across days ($p = 0.136$) and did not differ from control values ($p = 0.961$).

Despite the decrease and fragmentation of SWS, PS was not reduced in DFP-treated rats. It even showed a moderate elevation across days, $F(4, 44) = 2.296$, $p = 0.074$, and its mean value during the 9 days of exposure was larger than baseline level, $F(1, 11) = 5.166$, $p < 0.05$. PS increase was manifested after the sixth and ninth injections ($p \leqslant 0.05$). Nonetheless, PS did not significantly differ between the DFP and oil groups ($p = 0.115$ for the group × day interaction). In fact, while the number of PS episodes increased across days above control values, $F(4, 96) = 6.528$, $p = 0.0001$, this increase was partly counterbalanced by a shortening in the mean duration of PS episodes, $F(4, 96) = 2.198$, $p = 0.07$.

As a result of both decreased SWS and slightly increased PS, the DFP group exhibited a largely enhanced PS/SWS ratio, as reflected by a highly significant group × day interaction, $F(4, 96) = 7.906$, $p < 0.0001$. This increase was manifested from D3 ($p < 0.005$, compared to baseline), and it continued until D9 ($p = 0.05$, for the comparison between D6 and D9).

Thus, repeated administration of DFP yielded a progressive increase in waking and to a lesser extent in PS, whereas SWS was progressively reduced. As a result, the PS/SWS ratio was strongly enhanced.

Sleep–waking states after DFP withdrawaI. Two subsets of animals from the DFP group ($n = 7$) and the oil group ($n = 8$) were recorded 2, 4, and 19 days after the end of the 9-day exposure. As can be seen in Fig. 2, the changes in vigilance states induced by DFP rapidly lessened when the treatment was stopped. By 2 days after the end of DFP administration (D9 + 2 days), SWS had already reincreased, while waking, PS and %PS/SWS had redecreased: for all parameters, the values obtained at D9 + 2 days differed from those obtained at D9 (all $p < 0.025$), which was not the case in control rats (all $p > 0.05$). As a consequence, at D9 + 2 days, waking and SWS no longer differed between the two groups. On that day, PS amount and PS/SWS ratio were still higher in rats that had been treated with DFP than in controls, $F(1, 13) = 7.054$, and $F(1, 13) = 16.116$, respectively; $p < 0.025$, but they were no longer 2 days later. Thus, the effects of repeated DFP injections on vigilance states persisted no longer than 2–4 days after the end of 9-day exposure.

Second ExperimentaI Series: 21-Day DFP Exposure

Among the 22 rats used here, 9 were exposed to DFP for 21 consecutive days, and 7 received oil injections; 6 additional rats were injected with saline to evaluate whether the changes in waking and SWS observed in oil controls were due to oil administration per se. Recordings were performed after 1, 3, 6, 9, 13,

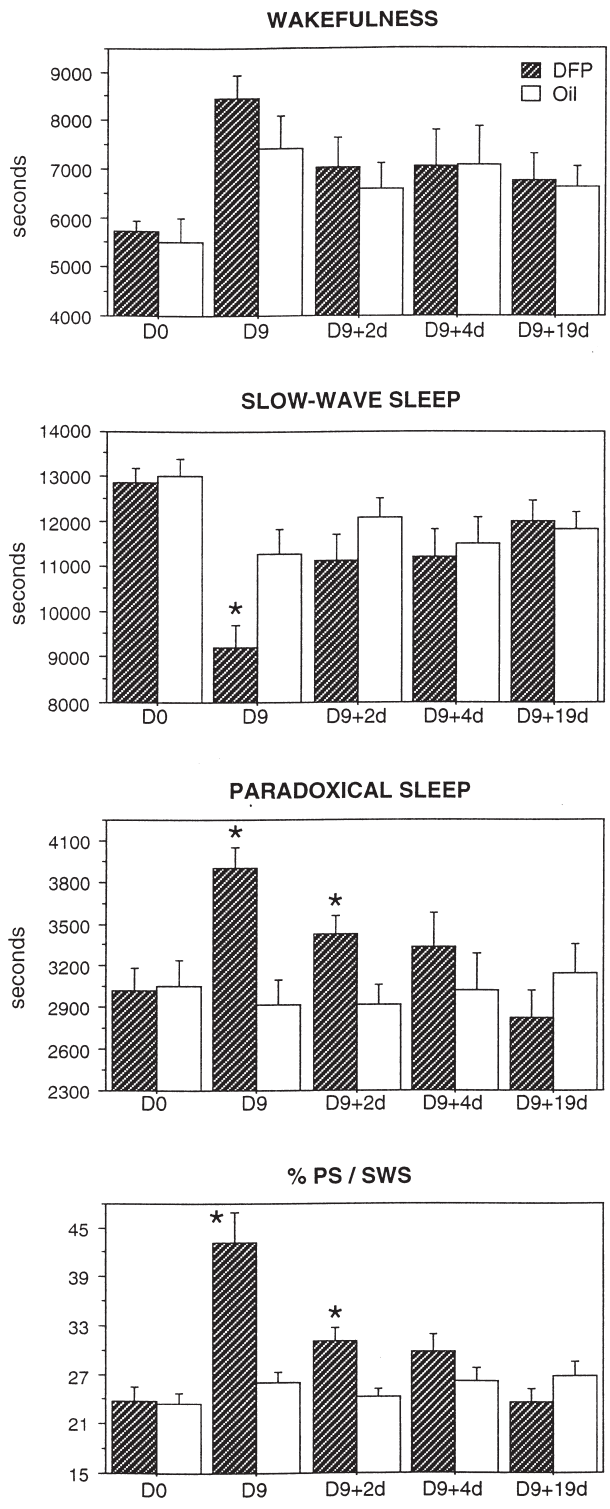


FIG. 2. Rapid lessening of DFP-induced effects on the sleep–wake states after withdrawal from 9-day DFP treatment. All values (mean + SEM) are from animals that received a daily injection of DFP ($n = 7$) or of oil ($n = 8$) for 9 consecutive days, and from which vigilance states recordings were obtained 2, 4, and 19 days after the end of treatment (D9 + 2 days, D9 + 4 days, and D9 + 19 days, respectively). Duration of waking, slow-wave sleep (SWS), and paradoxical sleep (PS), and percentage of time spent in PS relative to SWS (%PS/SWS), obtained over 6 h, are shown at baseline (D0), on the last injection day (D9), and at D9 + 2 days, D9 + 4 days, and D9 + 19 days. *Different from oil controls; all $p < 0.05$ or less.

17, and 21 injections of DFP or of oil, and after one, three, six, and nine injections of saline. The data are presented in Fig. 3.

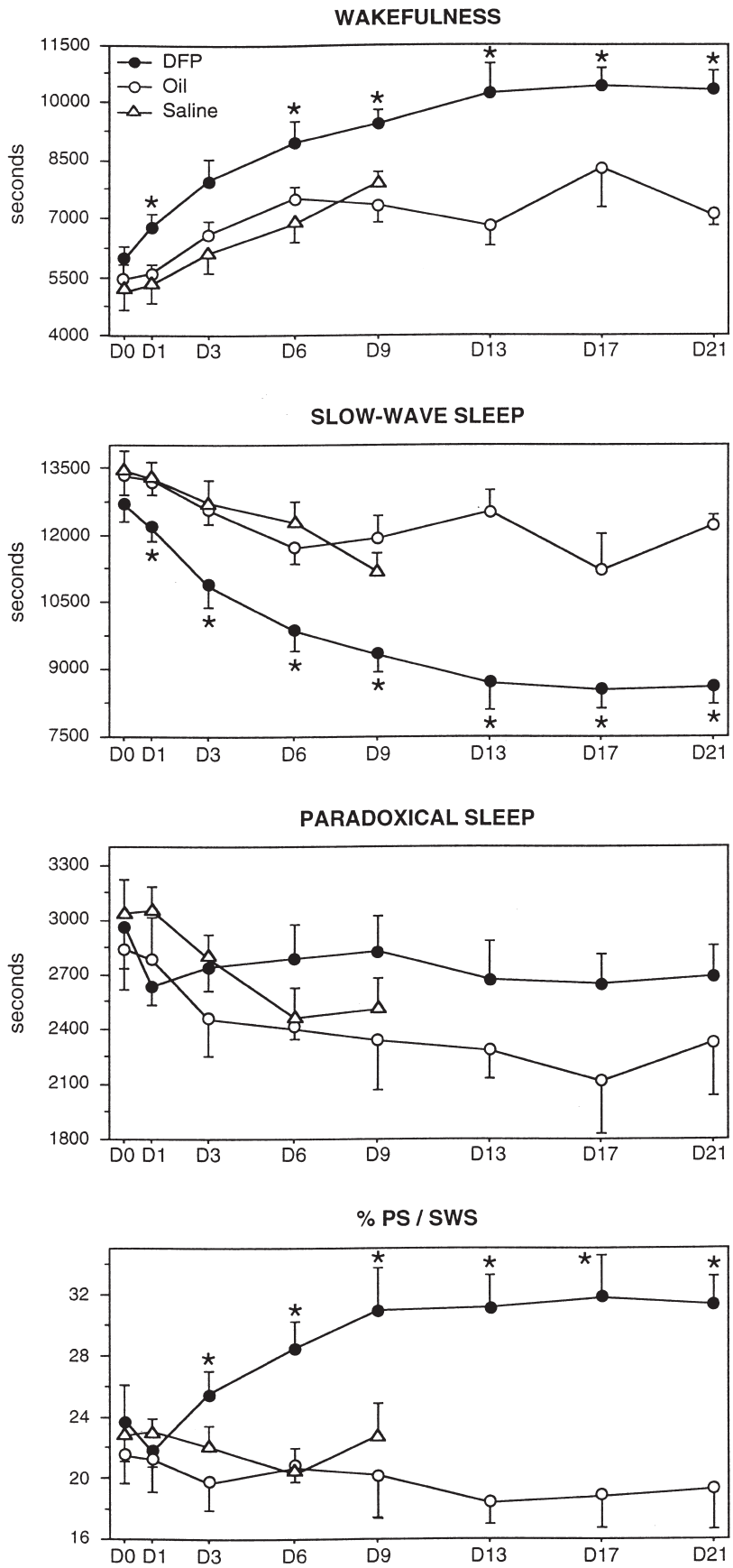
The changes in sleep–waking states exhibited by oil controls were similar to those displayed by rats injected with saline ($p > 0.30$, for all comparisons from D0 to D9). Thus, they did not result from oil administration per se, but they were due to repeated injections and/or to some uncontrolled factors. Over the 21 days of treatment, oil controls showed significant alterations in waking and SWS, $F(7, 35) = 3.934$, $p < 0.005$, and $F(7, 35) = 3.294$, $p < 0.01$, respectively. Their PS amount decreased across days, $F(7, 35) = 2.424$, $p < 0.05$, but their PS/SWS ratio was not significantly affected, $F(7, 35) = 1.118$, NS.

As in the previous experimental series, rats injected with DFP exhibited much more pronounced changes. Their waking amount was higher than that of oil controls, $F(1, 13) = 29.469$, $p < 0.0001$, for the group effect; and $F(7, 91) = 2.08$, $p = 0.0535$, for the group × day interaction. As shown in Table 2, this was due to an increased number of waking episodes in the DFP group, $F(7, 91) = 3{,}122$, $p = 0.005$; mean episode duration was unchanged, $F(7, 91) = 1.025$, NS. Consequently, DFP-treated rats had a lower amount of SWS than oil controls, $F(1, 13) = 29.640$, for the group effect; and $F(7, 91) = 4.361$, for the group × day interaction; $p < 0.001$, which was due to a shortening in the mean duration of SWS episodes, $F(7, 91) = 3.066$, $p < 0.01$ (see Table 2). Latency to SWS onset was not modified across days ($p = 0.408$), and was comparable to that of controls ($p = 0.590$). Concerning PS, its amount did not change across days in DFP-treated rats, $F(7, 56) < 1$. It did not significantly differ from control values, $F(7, 91) = 1.254$, NS, for the group × day interaction; yet, the number of PS episodes was greater in the DFP group than in the control group, $F(7, 91) = 3.134$ $p < 0.01$, for the group × day interaction. Thus, PS time was not increased, but it was not decreased either; consequently, given SWS reduction, PS/SWS ratio was strongly enhanced, $F(7, 91) = 6.401$, $p < 0.0001$, for the group × day interaction.

The changes in waking, SWS, and PS/SWS ratio displayed by DFP-treated rats progressively developed across days (see Fig. 3). To determine at which time point they reached their maximal level and stabilized, two types of comparisons were performed. First, when the raw values obtained in the DFP group were considered, it appeared that waking, SWS and %PS/SWS still changed from D3 to D21 [respectively, $F(5, 40) = 4.711$; $F(5, 40) = 7.212$; $F(5, 40) = 3.520$; all $p < 0.01$], whereas they no longer did significantly from D6 to D21 [respectively, $F(4, 32) = 1.863$; $F(4, 32) = 2.429$; $F(4, 32) = 1.063$; lowest p-value = 0.0680]. Second, when the changes relative to controls were considered (see the Method section for details), it appeared that the differences between the two groups still increased from D6 to D21 [$F(4, 32) = 3.295$; $F(4, 32) = 5.016$; $F(4, 32) = 2.857$, for waking, SWS and %PS/SWS, respectively; all $p < 0.05$], whereas they no longer significantly progressed from D9 to D21 [respectively, $F(3, 24) = 2.190$; $F(3, 24) = 1.758$; $F(3, 24) < 1$; lowest p-value = 0.115]. Thus, the behavioral state alterations caused by DFP became maximal and stabilized after six injections if we only consider the results of the DFP group, or after nine injections if we also take into account the changes occurring in oil controls. In any case, it is clear that when DFP exposure was prolonged for 12 additional days after D9, the sleep–waking alterations persisted and presented the same characteristics as those observed after nine injections.

DISCUSSION

The present study demonstrates that chronic exposure to a subtoxic dose of DFP increased waking at the expense of SWS


WAKEFULNESS
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PARADOXICAL SLEEP
seconds
3300
3000
2700
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2100
1800
D0 D1 D3 D6 D9 D13 D17 D21
% PS / SWS
32
28
24
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16
D0 D1 D3 D6 D9 D13 D17 D21

TABLE 2

NUMBER AND DURATION OF EPISODES OF WAKING, SLOW-WAVE SLEEP (SWS), AND PARADOXICAL SLEEP (PS) DURING A 21-DAY DFP EXPOSURE

			D0	D1	D3	D6	D9	D13	D17	D21
Waking	DFP	*f*	121 (±23)	125 (±22)*	129 (±20)	150 (±30)*	158 (±31)*	160 (±41)*	166 (±28)*	165 (±34)*
		d	50 (±8)	55 (±6)	63 (±17)	63 (±21)	61 (±8)	68 (±27)	64 (±12)	65 (±15)
	Oil	*f*	107 (±17)	100 (±21)	108 (±24)	102 (±19)	116 (±14)	123 (±11)	118 (±27)	114 (±13)
		d	52 (±15)	58 (±11)	64 (±18)	76 (±15)	64 (±12)	56 (±14)	72 (±27)	61 (±7)
SWS	DFP	*f*	116 (±23)	123 (±21)*	124 (±17)	147 (±27)*	155 (±30)*	158 (±40)*	164 (±26)*	164 (±31)*
		d	114 (±29)	102 (±22)*	89 (±17)*	68 (±12)*	63 (±18)*	57 (±15)*	53 (±11)*	54 (±12)*
	Oil	*f*	105 (±17)	97 (±20)	108 (±23)	100 (±17)	114 (±12)	120 (±11)	117 (±27)	113 (±13)
		d	129 (±25)	143 (±42)	121 (±28)	120 (±25)	106 (±20)	105 (±12)	101 (±35)	109 (±11)
PS	DFP	*f*	34 (±9)	28 (±5)	32 (±8)	33 (±10)	34 (±10)	35 (±11)	35 (±5)*	34 (±9)*
		d	87 (±11)	95 (±15)	89 (±15)	86 (±15)	86 (±18)	77 (±7)	76 (±12)	82 (±13)
	Oil	*f*	32 (±5)	30 (±6)	25 (±4)	27 (±4)	25 (±7)	27 (±4)	22 (±8)	25 (±3)
		d	88 (±12)	91 (±7)	98 (±15)	90 (±14)	92 (±15)	86 (±13)	101 (±18)	94 (±22)

Values (mean ± SD) are from nine rats that received a daily injection of DFP for 21 consecutive days (D1–D21), and from seven rats that received a daily injection of oil vehicle. A baseline recording was performed on D0. For each vigilance state are indicated the total number of episodes (*f*) obtained over the 6-h recording period and the mean duration (in seconds) of these episodes (*d*).

*Different from oil controls; all $p < 0.05$ or less.

and enhanced the PS/SWS ratio. These changes progressively developed across days, were maximal after six to nine injections, and then maintained at that level until cessation of DFP administration. They rapidly dissipated when the treatment was stopped, persisting no longer than 2–4 days.

These results are first evaluated with regard to literature data on the effects of cholinomimetics on the sleep–wake states. The time course of the behavioral state changes is then compared with that of concomitant neurochemical changes described in the companion article. Hence, hypotheses about the potential mechanisms by which DFP modified the behavioral states are considered and discussed.

Comparison With Previous Studies

Converging lines of evidence support a role of cholinergic transmission in promoting brain-activated states. For example, cholinergic agonists increase cortical activation [e.g., (34)]; they augment waking and reduce SWS [review in (35)]; they facilitate the induction of PS [(4,13,40); see also (2,11) for reviews of the effects of intracerebral microinjections]. Comparable effects were obtained with reversible ChE inhibitors, whether regarding cortical EEG (5,14), waking and SWS (35,41), or PS (3,36,44). The results of the present experiment are consistent with this dual implication of cholinergic transmission in waking and PS processes.

Increase in waking and concomitant decrease in SWS were reliably observed in our two experimental series. Waking elevation was due to an increased number of waking episodes. As a consequence, SWS episodes were more numerous; however, as their duration was markedly shortened, SWS amount was reduced. This indicated an impaired ability of DFP-treated rats to maintain prolonged periods of sleep. These changes were much more pronounced than those displayed by control rats; thus, they principally resulted from DFP administration per se. It could be argued that they were the consequence of some side effects of DFP, such as discomfort or sickness, given that DFP administration can alter autonomic and motor functions (19,31). However, such effects were observed only with high doses of DFP, not with low doses (20). In addition, as in previous studies that used repeated administration of low DFP doses (6,7,32), we detected no behavioral symptoms of cholinergic toxicity and no weight loss at any time of the treatment period.

The effects of DFP administration on PS were less clear cut. Rats in the first experimental series exhibited increased PS time, which was due to an increase in the number of PS episodes and not in their mean duration, which rather tended to be reduced. On the other hand, rats in the second experimental series did not show PS decrease, unlike controls, they had a greater number of PS episodes than controls, but their PS amount did not increase above baseline. Yet, ChE inhibition was comparable in the two series (as reported in the companion article). Thus, reasons for the variability in the effects of DFP on PS are unclear. Anyhow, it is noteworthy that PS was never decreased, despite the marked reduction and fragmentation of SWS. As a consequence, the PS/SWS ratio was strongly enhanced, which was never observed in control rats. Results from other two studies bring support to the view that chronic exposure to low doses of DFP can facilitate PS initiation. First, using a protocol that reduced brain ChE activity to 30% of normal as early as the first injection, Gnadt et al. (18) failed to find any changes in PS during the course of dosing; however, by 2 days after cessation of treatment, they observed higher PS amounts in DFP-treated rats than in controls. Second, in a recent experiment we did not observe significant PS increase in rats exposed for 10 days to the same DFP regimen as that used here; however, when they were submitted to a 6-h PS deprivation, DFP-treated rats manifested larger numbers of attempts to enter in PS than did oil controls (12). Thus, the facilitatory effect of DFP on PS could not be fully expressed under basal conditions,

FIG. 3. Time course of the sleep–wake changes during 21-day DFP exposure. Data are from nine rats that received a daily injection of DFP for 21 consecutive days (D1–D21), and from seven rats that received a daily injection of oil vehicle. Six additional rats were given a daily injection of saline for 9 days. A baseline recording was performed on D0. Mean duration (in seconds) of waking, slow-wave sleep (SWS), and paradoxical sleep (PS), and mean percentage of time spent in PS relative to SWS (%PS/SWS), obtained over the 6-h recording period, are represented ±SEM. *Different from oil controls; all $p < 0.05$ or less.

perhaps because of antagonizing effects of waking elevation and SWS fragmentation caused by DFP, but it was revealed when PS pressure was increased as a result of PS deprivation.

Time Course of the Sleep–Wake Changes

It is obviously interesting to compare the time course of the sleep–wake alterations observed here with that of ChE activity changes described in the companion article. We paid particular attention to the changes occurring in the PMT, given the critical role that have PMT neurons in behavioral state regulation (as previously detailed in the Introduction).

In both experimental series, the sleep–wake changes progressively increased across the first days of DFP exposure before reaching an asymptotic level. The day from which they became maximal and stabilized was determined on the basis of the data obtained in the second experimental series (21-day exposure), which provided a greater number of time points than did the first one. It appeared that the sleep–wake changes exhibited by the DFP group ceased increasing after six to nine injections, according to whether they were considered by themselves or relatively to those displayed by oil controls. Then, the changes persisted with the same magnitude for as long as the injections were repeated, which indicates that no tolerance to the effects of DFP developed. This kinetic pattern substantially differed from the profile of ChE inhibition in the plasma, where ChE activity was dramatically reduced as early as the first DFP injection. In contrast, it showed several similarities with the profile of brain ChE inhibition. In particular, ChE activity in the mesopontine cholinergic nuclei (LDT/PPT) and the pontine reticular formation (PnO/PnC) progressively declined across the first days of DFP exposure, was maximally inhibited (74–82% inhibition) after 6 days and then maintained constant for as long as DFP treatment was continued. Thus, during dosing the sleep–wake changes gradually increased as a function of increasing ChE inhibition, to stabilize at the same time or slightly after ChE inhibition had reached its maximal level. In contrast, the profile of behavioral and neurochemical changes largely differed when DFP treatment was stopped. Whereas the sleep–wake changes rapidly abated, persisting no longer than 2–4 days after the end exposure, ChE activity in the LDT/PPT and PnO/PnC had not yet fully recovered to control levels by 20 days after the last DFP injection. To summarize, during DFP treatment there was a clear parallelism between the time course of behavioral state changes and the time course of ChE inhibition in the PMT, but after cessation of treatment there was a clear mismatch between behavioral state and ChE activity recovery.

Limitations, Potential Interpretations, and Perspectives

It is widely accepted that DFP acts primarily via the inhibition of AChE. Although AChE may have other actions than the hydrolysis of ACh at the synapse (1,10,21), and although DFP also affects noncholinergic systems [e.g., (16,22,45)], the physiological and behavioral effects of DFP are generally interpreted as resulting from increased cholinergic neurotransmission, due to prolonged action of ACh and/or to increased ACh levels. Supporting this notion, elevated levels of brain ACh were found after acute and repeated administration of DFP (28,33,38,50). This leads us to speculate that the sleep–wake changes observed in the present experiment were principally underlain by changes in brain cholinergic neurotransmission, and in particular by those taking place in the PMT. This assumption is consistent with a number of literature data (cited above), demonstrating that increased cholinergic activity, notably in the PMT, promotes the waking and PS states. It finds support in the present findings that during DFP exposure the alterations in the sleep–wake states paralleled the changes of brain ChE activity, and in particular those occurring in the LDT/PPT and the pontine reticular formation. Brain ACh release is known to vary as a function of vigilance states, being low during SWS and higher during waking and PS (26,27,29,52). It is, therefore, conceivable that, by augmenting ACh levels, DFP administration biased the wake–sleep cycle towards waking and PS, at the expense of SWS. By enhancing cholinergic transmission in the thalamocortical systems, it would promote cortical activation processes, characteristic of the waking and PS states. By enhancing cholinergic transmission in the pontine reticular formation, it would facilitate the occurrence of PS, increasing PS time or, at least, preventing its reduction despite the large increase in waking. The effects on waking would be more rapid and more consistent than those on PS because brain activating regions involved in waking regulation are much more widely distributed than those involved in PS generation.

We are aware that these proposals remain speculative. First, we focused on the pontomesencephalic cholinergic system, without considering the basal forebrain cholinergic system, which has also an important role in cortical activation [reviewed in (24)]. More generally, given the systemic route of DFP administration used and the widespread distribution of ChE in brain, and because behavioral state control involves complex interactions between numerous brain structures and neurochemical systems, it is impossible to determine the specific contribution of a given brain area, or of a given neurotransmitter, to the behavioral state changes caused by DFP. Second, given the complex relationships between ChE activity and ACh levels, another limitation is the incomplete understanding of how cholinergic transmission is modified when ChE activity is progressively inhibited over a long period of time. Nonetheless, as it has been shown that low inhibition of brain ChE is sufficient to increase brain ACh levels (17), and that the elevations in ACh levels are maintained during prolonged exposure (28,33,38), the hypothesis that central cholinergic transmission was increased all along our DFP treatment seems realistic. Of course, the question of regional variations in cholinergic transmission during DFP exposure remains open. Another unresolved question is why behavioral state alterations rapidly lessened after DFP withdrawal while ChE inhibition persisted, which again points to the need to further determine how cholinergic transmission relates to ChE inhibition. Future studies aiming to directly determine extracellular ACh levels in different brain regions and at different time points during and after DFP treatment, should provide valuable clues regarding these questions.

In conclusion, the longitudinal approach used in the present experiment gave us the possibility of following across days the dynamics of changes that occurred in the sleep–wake states when ChE activity was progressively changing. Chronic, low-level exposure to DFP was found to augment the proportion of activated states at the expense of SWS, and these changes gradually increased as a function of increasing brain ChE inhibition. Such results suggest that the dual waking- and PS-promoting effects of DFP resulted from increased cholinergic neurotransmission.

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