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Chronic, Low-Level Exposure to the Cholinesterase Inhibitor DFP. I. Time Course of Neurochemical Changes in the Rat Pontomesencephalic Tegmentum

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DEURVEILHER, S., I. S. DELAMANCHE, B. HARS, P. BRETON AND E. HENNEVIN. *Chronic, low-level exposure to the cholinesterase inhibitor DFP. I. Time course of neurochemical changes in the rat pontomesencephalic tegmentum.* PHARMACOL BIOCHEM BEHAV. **64**(1) 95–103, 1999.—Rats were repeatedly administered with a low dose of diisopropylfluorosphosphate (DFP; 0.2 mg/kg/day, SC, for 9 or 21 days), an irreversible cholinesterase (ChE) inhibitor. Control rats received a daily injection of oil vehicle. Neurochemical changes occurring in the pontomesencephalic tegmentum (PMT), a brain stem region critically involved in behavioral state control, were evaluated at various times of treatment and after DFP withdrawal. First, enzyme assay revealed a profile of ChE inhibition in the whole PMT which looked like that observed in the striatum; both the inhibition and recovery proceeded more slowly than they did in the plasma. Second, quantitative histochemistry indicated that ChE activity in the mesopontine cholinergic nuclei and the pontine reticular formation progressively decreased across the first days of DFP exposure, to reach an asymptotic level of inhibition after 6 days (74–82% inhibition). The inhibition was less pronounced in the locus coeruleus (49%). Third, [^{3}H]QNB autoradiography showed that muscarinic receptor density was unchanged in any of the PMT areas selected. These results are discussed regarding the question of regional variation in susceptibility to anti-ChE agents. To what extent behavioral state alterations occur concomitantly with ChE activity changes is assessed in the companion article. © 1999 Elsevier Science Inc.

DFP Irreversible cholinesterase inhibitor Chronic low-level exposure Rat
Pontomesencephalic tegmentum Striatum Plasma
Cholinesterase assay Quantitative cholinesterase histochemistry Quantitative [^{3}H]QNB autoradiography

IT is well known that the sleep–wake states are highly sensitive to acute changes in cholinergic activity induced by electrical stimulation of cholinergic nuclei or by acute injection of cholinergic agents [see for reviews, (25,41)]. What is less known is how the behavioral states reorganize when cholinergic activity is modified over a long period of time. The present experiment and that reported in the companion article were designed to address this issue: progressive changes in cholinergic transmission were induced across days, and the time course of concomitant alterations in the sleep–wake states were examined.

To induce gradual change in cholinergic activity, we repeatedly administered a low dose of diisopropylfluorophosphate (DFP), an organophosphorus (OP) anticholinesterase compound. OP agents covalently bind at the active site of cholinesterases (ChE), thereby inactivating the enzyme irreversibly. Their physiological and behavioral effects are generally attributed to the inhibition of acetylcholinesterase (AChE), the predominant class of ChEs in the central nervous system (24). By inhibiting the hydrolytic enzyme of acetylcholine (ACh), OP agents cause accumulation of ACh in the extracellular space, leading to increased cholinergic neurotransmis-

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sion (35). DFP is a prototypical OP, but it is much less toxic than other irreversible ChE inhibitors (39). In the present experiments, we took advantage of the fact that with chronic low-level exposure to DFP, brain ChE activity can be progressively lowered without manifestations of behavioral symptoms of cholinergic intoxication (3,6,28).

Rats were treated with DFP (0.2 mg/kg/day) for 9 or 21 consecutive days, and the effects induced by this treatment were examined at different time points during DFP exposure and after DFP withdrawal. Two separate studies were conducted in parallel. The first one was designed to determine the neurochemical changes occurring in brain stem areas known to be involved in behavioral state control. The second one was aimed at evaluating concomitant changes in the sleep–wake cycle. We report here the neurochemical results, and the data concerning the states of vigilance are presented in the companion article.

The present neurochemical study principally focused on the pontomesencephalic tegmentum (PMT), because several neuronal groups critically involved in the regulation of wakefulness and paradoxical sleep are located in this brain stem region. The PMT contains cholinergic neurons that are distributed in the laterodorsal (LDT) and pedunculopontine (PPT) tegmental nuclei, and that widely project both to the forebrain and to the brain stem. Converging lines of evidence [reviewed in (25,38)] indicate that 1) through their ascending projections to the thalamus, these cholinergic neurons have an important role in cortical activation; 2) through their descending projections to the oral (PnO) and caudal (PnC) parts of the pontine reticular formation, they are primarily implicated in the generation of paradoxical sleep; 3) they control behavioral states in close interaction with monoaminergic neurons of the PMT, i.e., neurons of the locus coeruleus (LC) and of the dorsal raphe.

How anticholinesterase agents alter ChE activity and cholinergic transmission in the PMT has never been studied. We assessed the changes produced by chronic DFP exposure in that region using three types of neurochemical analyses. First, ChE activity was measured in the entire PMT by classical biochemical assay. Second, quantitative histochemistry was performed to estimate and compare the changes in ChE staining occurring in discrete areas of the PMT selected owing to their role in behavioral state control, namely, the LC, the LDT, the PPT, the PnO, and the PnC. Third, [^{3}H]QNB autoradiography was used to determine whether muscarinic receptor density was modified in those same PMT areas. In addition, ChE activity was also measured in the plasma and in the striatum to compare the dynamics of DFP action at peripheral and central levels, as well as across brain regions.

METHOD

Animals

A total of 87 male Wistar rats (Iffa-Credo, France) weighing 330–380 g at the onset of drug treatment, served as subjects. 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. Because some of the animals were used in the behavioral state study reported in the companion article, all of the other rats were submitted to a surgery mimicking, in part, the surgical preparation for sleep–waking recordings: under pentobarbital anesthesia (60 mg/kg, IP), a stainless steel screw was inserted into the skull and covered with dental acrylic cement. All the experimental procedures used are in compliance with the European legislation (86/609/EEC) on animal experimentation.

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 was injected subcutaneously at a dose of 0.2 mg/kg/day. Control rats received a daily injection of oil. All injections were made at the upper level of the rat's back, and were given in a volume of 1 ml/kg.

One week after surgery, rats were given 3–4 days of familiarization to the injection conditions: they were weighed each day, then hand restrained for a sham injection (no needle insertion). After this adaptation period, all of the animals received an initial injection of oil (to replicate the conditions used in the vigilance state study). On the following days, rats received a daily injection of DFP or of oil for 1, 3, 6, 9, or 21 days (D1–D21). All injections were made between 9.50 and 10.20 h. On D1, D3, D6, and D9, animals were sacrificed 3–4 h after DFP or oil injection. Rats used in the behavioral state study were sacrificed 24 h (D9 + 1 day and D21 + 1 day) or 20 days (D9 + 20 days) after their last injection.

Two experimental series were carried out. In the first one (9-day exposure), biochemical ChE assay was done in the plasma and the striatum; quantitative ChE histochemistry was performed in the striatum and selected areas of the PMT; the density of muscarinic receptors was quantified in the same PMT areas, using [^{3}H]QNB autoradiography. In the second series (21-day exposure), biochemical ChE assay was done in the plasma, the striatum, and the PMT.

Tissue Collection and Preparation

Animals were sacrificed by decapitation, and two blood samples were immediately collected in heparinized tubes from the rat's trunk and centrifugated at 3000 rpm for 10 min. The plasma was removed and frozen at −80°C. Brains were rapidly removed, frozen immediately in dry ice-chilled isopentane and stored at −80°C until used. Later on, two brain regions were hand dissected out at −18°C. The first one included the entire striatum (caudate-putamen and accumbens nucleus). The second one included the entire PMT (from the end of the substantia nigra to the end of the locus coeruleus).

For ChE activity assay, the striatum and the PMT were weighed [striatum: 61 ± 24 mg (mean ± SD) and 65 ± 21 mg in the first and second experimental series, respectively; PMT: 71 ± 24 mg] and diluted at 20 mg of tissue per ml of extraction medium containing 0.5% (w/v) Triton X-100, 1 M NaCl in 0.1 M phosphate buffer (pH 8.0). The homogenization was performed in ice for 1 min with an Ultra-Turrax homogenizer.

For ChE histochemistry and [^{3}H]QNB autoradiography, coronal tissue sections (20 μm thick) were cut using a microtome cryostat. Through the striatum, four adjacent sections were taken for ChE staining (the remaining block was used for ChE activity assay). Through the PMT, six series of sections (400 μm apart) were collected from the end of the LC to the midpart of the PPT; in each series, the first four sections were taken for ChE histochemistry and the following nine for [^{3}H]QNB autoradiography. All sections were immediately thaw mounted onto gelatin-coated slides.

ChE Activity Assay

ChE activity was routinely estimated with a computer-controlled spectrophotometer (Cobas Fara; Roche, France), using a modification of the Ellman method (9). A 5-μl aliquot of

tissue homogenates or of plasma was mixed with a 250-μl solution containing 0.25 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 2.9 mM acetylthiocholine bromure in 10 mM Tris-HCl buffer (pH 7.5). Changes of absorbance were measured at 412 nm for 200 s. Blank consisted in the same reaction mixture except that plasma or brain samples were replaced by distilled water. Assays were run in duplicate at 30°C. The activity measured by the hydrolysis of acetylthiocholine was designated as total ChE activity. Specific activity of ChE was defined as nmol of acetylthiocholine hydrolyzed per min per ml of plasma or per mg of tissue.

Quantitative ChE Histochemistry

ChE staining was performed according to a modification of the method of Koelle-Friedenwald (17). Sections were first fixed for 5 min in 0.1 M phosphate buffer (pH 7.2) with 10% (v/v) neutral formalin. After washing, twice in the same buffer and once in 0.2 M acetate buffer (pH 6.0), sections were incubated at 37°C for 120 min in 0.1 M acetate buffer (pH 6.0) containing 20 mM glycine, 4 mM copper sulphate, and 4 mM acetylthiocholine iodide (for total ChE staining) or 4 mM butyrylthiocholine iodide (for BuChE staining). Reactions were stopped by rinsing the sections three times in acetate buffer. Sections were placed for 2 min in the same buffer with 5% (v/v) ammonium sulfide, then rinsed three times in acetate buffer. Last, they were dehydrated in alcohols and toluene before coverslipping with Eukitt.

ChE staining was quantified microdensitometrically with a computer-assisted imaging system (Visiolab 1000; Biocom, France). Delineation of the digitized region was drawn with reference to Paxinos and Watson's atlas (31). Measurements were performed from bregma between P 9.3–9.8 for the LC and PnC; P 8.7–9.1 for the LDT; P 8.0–8.3 for the PPT and the PnO; A 1.7–0.7 for the striatum. Mean gray level in each of the considered regions was converted to optical density units, using the background near each section as reference.

Quantitative [^{3}H]QNB Autoradiography

The protocol was essentially the same as that used by Quirion et al. (34). Sections were preincubated for 15 min at room temperature (RT) in Krebs' buffer containing 118 mM NaCl, 1.18 mM $MgSO_4$–$7H_2O$, 1.18 mM KH_2PO_4, 5.5 mM glucose, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 4.7 mM KCl (pH 7.4). They were then incubated for 60 min at RT in the same buffer with 1 nM l-[^{3}H]quinuclidinyl benzylate ([^{3}H]QNB; specific activity 47.3 Ci/mmol; Amersham, UK), a nonselective antagonist of muscarinic receptors. Adjacent sections were treated in the presence of 1 μM atropine sulphate to determine nonspecific binding. After incubation, sections were washed at 4°C, twice in Kreb's buffer, and once in distilled water (5 min each), before drying overnight under a stream of cold air. Tissue sections and calibrated tritium standards ([^{3}H]Microscales; Amersham, UK) were exposed to tritium-sensitive film (LKB-Ultrofilm) in light-proof cassettes at 20°C. After 3 weeks, the films were developed manually using Kodak D-19 developer (4 min) and fixed in Kodak Unifix DL4 (10 min).

Autoradiograms were quantified microdensitometrically using a computerized image-processing device (RAG 200; Biocom, France). The regions of interest (identified by comparison with adjacent ChE-stained sections) were digitized. For each sheet of film, the density of receptor binding sites in each of the PMT areas selected was calculated from the mean gray level of the area, using a calibration curve obtained by plotting the radioactivity values and the corresponding gray levels of the standards. Receptor density was expressed as femtomol of binding per milligram tissue equivalent (fmol/mg).

Statistical Analyses

For ChE activity assay in the plasma, four measurements were done per rat (two samples per rat and two measurements per sample). For ChE activity assay in the striatum and the PMT, one sample of each region was processed per rat, and each sample was assayed twice. For quantification of optical density of ChE staining, four measurements were done per rat for each region selected (a bilateral measurement on two adjacent sections). For quantification of [^{3}H]QNB binding sites, 8 or 10 measurements were done per rat for each PMT area studied (a bilateral measurement on four or five adjacent sections). For each type of measure, the values obtained in a given animal were averaged, and it was that mean value that was used for statistical analyses.

All statistical comparisons were carried out with factorial analysis of variance and Fisher's post hoc test. The effects of DFP and of oil were compared at a given day and across days using raw data. The effects of DFP were compared across compartments (plasma vs. brain tissue) or across brain areas using data expressed as percent of control: the value obtained in each DFP-treated rat was divided by the mean value obtained in day-matched oil controls (except in the following cases: for rats in group D6 of the first experimental series, the mean value obtained in oil groups D3 and D9 was taken as control; for rats in groups D3 and D9 of the second experimental series, the value obtained in oil group D1 was taken as control).

RESULTS

ChE Activity Assays in the Plasma, the Striatum and the Pontomesencephalic Tegmentum

First experimental series: 9-day exposure. The results are from 32 rats treated with DFP and 29 rats injected with oil. ChE activity was assayed in the plasma and the striatum after one, three, six, or nine DFP injections, after one, three, or nine oil injections, as well as 24 h and 20 days after the end of 9-day treatment with DFP or with oil.

Repeated oil injections did not affect ChE activity, whether in the plasma, $F(2, 12) = 1.043$, NS, or in the striatum, $F(2, 11) = 2.198$, NS. In contrast, as shown in Fig. 1, DFP administration caused strong ChE inhibition. A comparable degree of inhibition was achieved in the plasma and the striatum after six injections, when inhibition became maximal in each compartment. However, the time course of inhibition differed between the plasma and the striatum. It was more progressive in the striatum, as attested by the significant interaction between the factors compartment and day of injection, $F(3, 29) = 16.54$, $p < 0.0001$. In addition, after cessation of treatment, the recovery was less rapid and less complete in the striatum than in the plasma. At D9 + 1 day, the increase of ChE activity above D9 level was smaller in the striatum, $F(1, 16) = 23.993$, $p < 0.001$; at D9 + 20 days, ChE activity relative to control levels was lower in the striatum ($p < 0.001$).

Second experimental series: 21-day exposure. The results are from 17 rats treated with DFP and 9 rats injected with oil. ChE activity was measured in the plasma, the striatum, and the PMT after three or nine DFP injections, after one oil injection, as well as 24 h after the end of 21-day treatment with DFP or with oil.

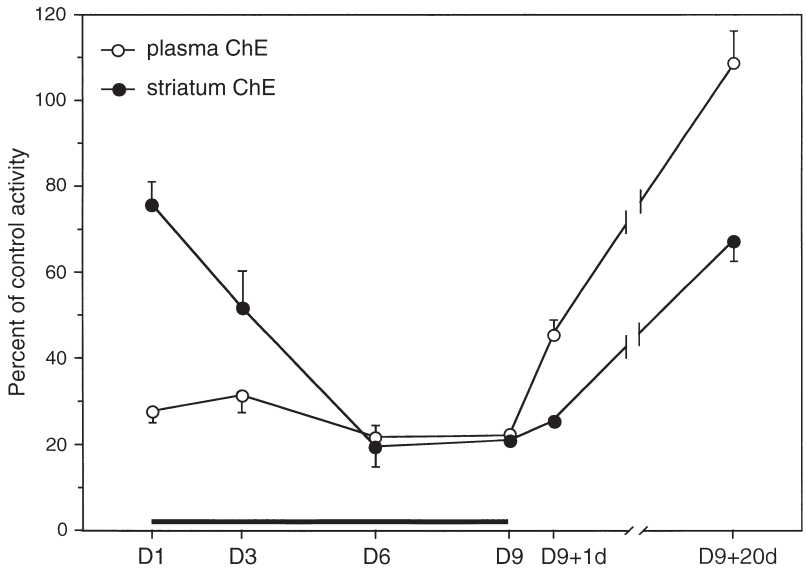

FIG. 1. Differential time course of ChE inhibition in the plasma and the striatum during and after DFP exposure. Data (mean ± SEM) are expressed as percent of control. The bar above the x-axis represents the period of DFP exposure. The raw values of ChE activity measured in DFP-treated rats differed from those measured in day-matched oil controls from D1 to D9 + 1 day for the plasma, and from D1 to D9 + 20 days for the striatum (all $p < 0.01$). The control values (mean ± SD) averaged over all time points were 335 ± 71 nmol of substrate hydrolyzed/min/ml for the plasma, and 31 ± 4 nmol of substrate hydrolyzed/min/mg for the striatum.

The degree of ChE inhibition observed in the plasma and the striatum after three and nine DFP injections was comparable with that observed on the same days in the first experimental series (for all comparisons, $p > 0.073$). In the PMT, basal ChE activity was low (about three times lower than that in the striatum), and DFP yielded 72% inhibition at D9, which was less than in the striatum ($p < 0.005$). Nonetheless, as shown in Fig. 2, the time course of inhibition in the PMT

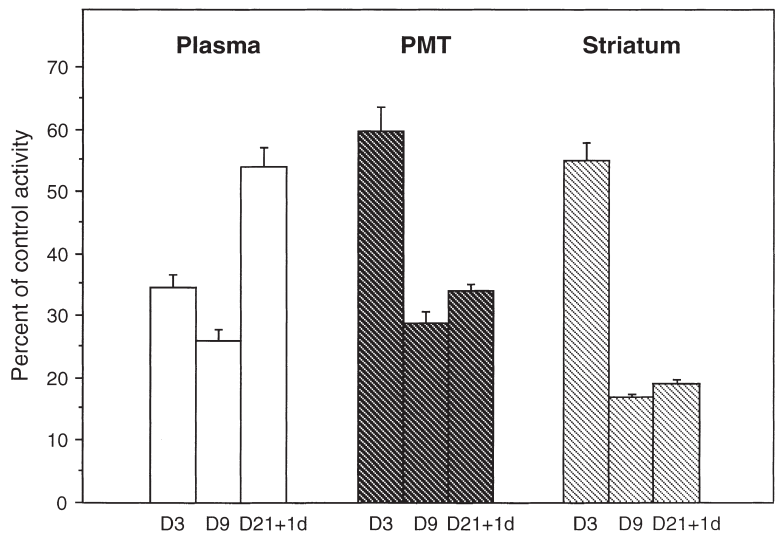

FIG. 2. Differential pattern of ChE inhibition in the plasma, the striatum, and the pontomesencephalic tegmentum (PMT) during and after DFP exposure. Data (mean + SEM) are expressed as percent of control. For the plasma, the striatum and the PMT, the raw values of ChE activity measured in DFP-treated rats at D3, D9, and D21 + 1 day differed from those measured in corresponding oil controls (all $p < 0.005$). The control values (mean ± SD) averaged over all time points were: 352 ± 53 nmol of substrate hydrolyzed/min/ml for the plasma; 33 ± 3 and 11 ± 1 nmol of substrate hydrolyzed/min/mg for the striatum and the PMT, respectively.

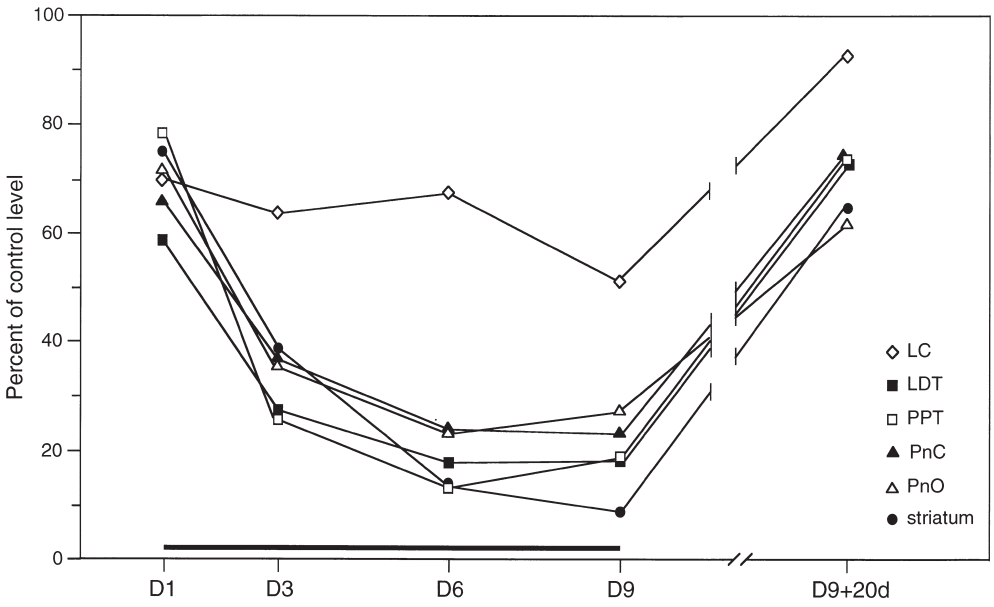

FIG. 3. Time course of changes in ChE staining in the striatum and different areas of the pontomesencephalic tegmentum during and after DFP exposure. Each data point represents the mean densitometric measure of ChE staining expressed as percent of control. Error bars were omitted for clarity. The bar above the x-axis represents the period of DFP exposure. The control values (mean ± SD, in optical density units × 100) averaged over all time points were: 26.2 ± 5.3 for the LC; 40.5 ± 9.8 for the LDT; 33.8 ± 9.5 for the PPT; 7.6 ± 2.1 for the PnC; 7.4 ± 1.6 for the PnO; 56.1 ± 6.9 for the striatum. Abbreviations: LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus; PnC, pontine reticular nucleus, caudalis; PnO, pontine reticular nucleus, oralis.

looked like that in the striatum, being more progressive than it was in the plasma. The increase of inhibition between D3 and D9 was comparable in the two brain regions ($p > 0.10$), whereas it differed between the PMT and the plasma ($p < 0.001$). In addition, by 24 h after the end of 21-day treatment, ChE inhibition in the PMT and the striatum remained equivalent to that observed at D9 ($p > 0.085$, in both cases), whereas it was much less marked in the plasma ($p < 0.0001$). This suggests that 24 h after cessation of 21-day treatment, ChE activity had begun to recover in the plasma but not yet in the two brain regions, which is consistent with the results obtained at D9 + 1 day in the first experimental series.

Last, it is noteworthy that the level of inhibition observed here at D21 + 1 day in the plasma and the striatum was comparable with that observed at D9 + 1 day in the preceding experimental series. Thus, prolonging DFP exposure by 12 days did not seem to change the degree of ChE inhibition relative to that at D9.

ChE Histochemistry in the Striatum and the Pontomesencephalic Tegmentum

The results are from 24 rats exposed to DFP and 17 rats injected with oil. ChE staining was quantified in the striatum and various PMT areas after one, three, six, or nine DFP injections, after one, three, or nine oil injections, as well as 20 days after withdrawal from 9-day exposure to DFP or to oil.

Basal ChE activity largely varied across the brain areas studied. The highest level was found in the striatum. Within the PMT, the LDT and the PPT exhibited the highest activity, followed by the LC; ChE activity was weak in the PnO and the PnC (see details in the legend of Fig. 3). Whatever the PMT area considered, BuChE staining was very low, and because it became nearly undetectable as early as the first DFP injection, it was not quantified.

Whereas oil injections induced no significant changes in any of the regions studied (all $p > 0.123$), decrease in ChE staining was apparent in each region after 1 DFP injection. This decrease was significant for the striatum and the LDT ($p < 0.05$, in both cases). From the third to the ninth injection, ChE staining was significantly reduced in each structure (all $p < 0.025$). Maximum inhibition was reached after only one injection for the LC ($p > 0.140$, for all comparisons between D1 and any other treatment days), while for the other regions it was reached after three (for the LDT) or six (for the striatum, PPT, PnO, and PnC) injections. As can be seen in Fig. 3, the magnitude of changes also differed between structures. Analysis of variance showed that the effects of brain area, day of injection, and the interaction between these two factors were all significant (all $p < 0.0001$). Post hoc comparisons indicated that ChE staining was less decreased in the LC than in any other regions (all $p < 0.0001$). By 20 days after the end of injections ChE staining had returned to near-control levels in each structure. Nonetheless, except for the LC, it tended to remain lower in DFP-treated rats than in controls, the difference being significant for the striatum and the PnO ($p < 0.05$, in both cases).

The highly significant correlation we found between the histochemical and biochemical measures obtained for the striatum, $r(24) = 0.934$, $p < 0.0001$, attested that our quantification of ChE staining was reliable for measuring changes in ChE activity.

TABLE 1

QUANTITATIVE AUTORADIOGRAPHY OF [^{3}H]QNB BINDING SITES IN DIFFERENT AREAS OF THE PONTOMESENCEPHALIC TEGMENTUM

Region	Treatment	D1	D3	D9	D9 + 20 days
LDT	DFP		255.3 (±63.2)	285.4 (±15)	284.8 (±52.7)
	Oil	252 (±37)	233.3 (±38.2)	261.8 (±1.2)	257.8 (±52.6)
PPT	DFP		137.4 (±18)	154.9 (±24.4)	145.6 (±49.2)
	Oil	147.6 (±34.7)	115.5 (±10.5)	139 (±14.1)	137.4 (±48.9)
LC	DFP		161.9 (±62.6)	171.7 (±36)	163.5 (±32.3)
	Oil	163.1 (±41.3)	158.7 (±32.4)	158.7 (±33.7)	151.4 (±71.3)
PnC	DFP		74.1 (±35.7)	80.8 (±23.4)	73.9 (±37.2)
	Oil	82.2 (±30.7)	59 (±15.7)	91 (±13.5)	60.8 (±35.1)
PnO	DFP		69.6 (±31.7)	76.4 (±20)	85 (±28)
	Oil	70.8 (±20.2)	59 (±6.9)	63.2 (±11.4)	61.7 (±35.2)

Data are from animals used for densitometric measures of ChE staining. For each rat, the density of [^{3}H]QNB binding sites was quantified in every structure. All values (mean ± SD) represent total binding in fmol/mg tissue equivalent; nonspecific binding was undetectable. Abbreviations are as in Fig. 3.

No significant differences were found for any regions between DFP and control groups (all $p > 0.10$).

After correction for differential absorption of tritium emissions by myelin (quenching), the quench-corrected measures of binding density (in fmol/mg of tissue) calculated in oil control group D1 were: 340.5 for the LDT, 295.2 for the PPT, 217.5 for the LC, 222.2 for the PnC, and 191.4 for the PnO. The correction factors used were those determined by Baghdoyan (2): LDT, 0.74; PPT, 0.50; LC, 0.75; PnC and PnO, 0.37.

[^{3}H]QNB Autoradiography in the Pontomesencephalic Tegmentum

Sections adjacent to those processed for ChE histochemistry were used for quantifying the density of muscarinic receptors in the PMT. The results are from 14 DFP-treated rats and 14 controls. The density of [^{3}H]QNB binding sites was quantified after three or nine injections of DFP, as well as 20 days after the end of 9-day exposure.

[^{3}H]QNB binding sites were found in all of the PMT areas selected, but their density differed between areas (see Table 1). The LDT exhibited the highest density, followed by the LC and the PPT; labeling was low in the PnC and PnO. After correction for differential absorption of tritium emissions by myelin (quenching), again binding was found highest in the LDT; it was lower in the PPT and much lower in the LC, PnC, and PnO (see details in the legend of Table 1).

Comparison of sites density across days failed to detect any significant changes in any PMT regions, whether in controls (all $p > 0.46$) or in DFP-treated rats (all $p > 0.58$). No significant differences were found between controls and DFP-treated rats in any areas (all $p > 0.10$). Thus, despite the marked ChE inhibition caused by DFP, no concomitant changes in muscarinic receptor density occurred in any of the PMT areas studied.

DISCUSSION

As a result of repeated exposure to low levels of DFP, ChE activity in the PMT was progressively reduced across days, falling to 28% of control after 9 days of treatment. The inhibition and recovery rate of enzymatic activity was comparable to that observed in the striatum; it was slower than in the plasma. Strong inhibition was found in the mesopontine cholinergic nuclei (LDT/PPT) and the pontine reticular formation (PnO/PnC); it was less pronounced in the LC. Given the very low level of BuChE activity, these changes essentially represented changes in AChE activity. Yet, the density of muscarinic receptors was unchanged in any of the PMT areas studied.

Changes of ChE Activity in the Plasma and the Striatum

Before discussing the effects of OP exposure in the PMT, we would like to point that the time course of ChE inhibition we observed in the plasma and the striatum fits nicely with that described in the literature. ChE activity was strongly depressed in the plasma as early as the first DFP injection. In contrast, as classically observed in brain structures during chronic exposure to low doses of DFP or other OPs (3,16,22,44), ChE inhibition in the striatum was progressive. This differential pattern of inhibition was likely due to the fact that, when DFP is administered peripherally, it may (a) be sequestered into fat tissues, (b) bind to various proteins in peripheral tissues and blood, and (c) be hydrolyzed by DFPases present in virtually all tissues and particularly the blood, which reduces the amount of DFP entering the brain (23,29,35). Maximal inhibition was achieved after six injections, and it maintained at that level until the end of treatment. Prolonging exposure did not change the extent of inhibition, which agrees with numerous results demonstrating that ChE inhibition by DFP does not develop tolerance as a result of repeated exposure (3,6,8,14, 42,44). The maximal inhibition observed in the plasma (76%) and in the striatum (81%) was in the same range as that previously described after chronic DFP treatment (8,20,40,42,43). Thus, even at the height of inhibition, the inhibition was not total. Such residual activity might be due to the partial recovery that occurs between daily injections (27,42,44), and/or to the existence of ChE isoenzymes that are less affected by DFP, because they are less sensitive or less accessible (26,29,42).

When the treatment was stopped, again ChE activity exhibited a differential pattern of changes in the plasma and the striatum. First, by 24 h after a 9- or 21-day treatment, the recovery was larger in the plasma than in the striatum, which agrees with previous results (27,42). Second, by 20 days after cessation of 9-day treatment, full recovery had occurred in the plasma but not in the striatum, which again agrees with previous reports (20,32,42) and with many studies that found prolonged inhibition in the brain after withdrawal from repeated

dosing with DFP (21,27,33). To account for the slower recovery in the brain than in the blood, several possibilities have been suggested, including (a) slower rate of ChE synthesis in the brain than in tissues that secrete ChE into the blood; (b) impaired brain ChE production caused by OP administration; (c) depot storage and subsequent slow release of OP compounds (5,13,30).

Changes of ChE Activity in the Pontomesencephalic Tegmentum

ChE activity changes in the PMT were determined both by direct enzyme assay made in the entire PMT, and by quantitative histochemistry performed in selected PMT areas. The biochemical results revealed a pattern of changes which looked like that observed in the striatum: as in the striatum, both the inhibition and recovery proceeded more slowly than they did in the plasma. However, the degree of inhibition was slightly less pronounced in the PMT (72% inhibition at D9) than in the striatum. The results from ChE histochemistry revealed a differential time course and degree of inhibition across the PMT areas studied. In the LDT/PPT and the PnO/PnC, the inhibition curves were comparable, and they paralleled that obtained in the striatum: ChE activity was progressively reduced over the first treatment days, and it was decreased by approximately 80% (from 74% in the PnO to 82% in the LDT) after nine injections. In contrast, the pattern of changes in the LC was quite different. First, the LC exhibited maximal inhibition as early as the first injection. Second, throughout the period of exposure, it was by far the less inhibited nucleus (49% inhibition at D9). Third, by 20 days after DFP withdrawal it had fully recovered, while all of the other areas were still weakly inhibited.

Thus, by showing an inhibition slightly less pronounced in the PMT than in the striatum and much less pronounced in the LC than in the other PMT areas, the present data complement previous studies that found a nonuniform degree of inhibition across brain regions both after acute (4,15,18,19,26,36,40) and chronic (5,11,12,14,16,40,43) OP exposure. It is worth noting that the striatum was generally found more inhibited than brain stem regions (11,12,14,19,36,43), which brings support to our results. Regional variations were also observed in the rate of inhibition during exposure (3,16,22), as well as in the rate of recovery after drug withdrawal (14,21,26,33,44). Three possibilities have been suggested to account for these regional variations. The first one implicates differences in ChE turnover, leading to differences in the rate of recovery from inhibition (15). To what extent this could account for the present results is difficult to evaluate, given that the enzyme lifetimes in the different areas of the PMT are unknown. The other two possibilities implicate regional variations either in basal ChE activity [inhibition would be stronger in regions with high activity than in regions with low activity; (18,26,40)] or in lipid abundance [which could lead to differences in the uptake and availability of ChE inhibitor; (15)]. However, the finding that the PnO/PnC (which have lower basal ChE activity and larger lipid abundance than any of the other areas studied) exhibited marked ChE inhibition clearly indicates that the degree of inhibition was not primarily determined by these two factors, which corroborates previous results (12,14,16,36,43).

Lack of Changes in Muscarinic Receptor Density in the Pontomesencephalic Tegmentum

The density of muscarinic receptors was quantified in the same PMT areas as those processed for ChE histochemistry, using [^{3}H]QNB autoradiography. As in recent results (2), the highest density was found in the LDT and the lowest in PnO/PnC. DFP exposure did not alter binding sites in any of these areas. It remains, of course, possible that other forms of receptor plasticity or selective changes in muscarinic receptor subtypes, not detectable by [^{3}H]QNB autoradiography, may have occurred. In any case, the present lack of changes contrasts with numerous results showing decreased [^{3}H]QNB binding after chronic DFP treatment [e.g., (1,7,8,20,32,40,43,44)]. It must be noted, however, that the doses used in all those studies were relatively high, causing strong brain ChE inhibition as early as the first injection, along with initial signs of poisoning in most cases.

Owing to that, the first question to be addressed is whether our failure to detect changes in muscarinic receptors was not merely due to the fact that the DFP dose used was too low and/or the duration of exposure too short to be able to induce receptor alterations. However, the treatment we used produced a level of ChE inhibition that was proved sufficient to be accompanied by a reduction in muscarinic receptor density (12,20,45). Moreover, decrements in muscarinic receptors were observed even with low DFP doses (3,28). For example, with the same regimen as that we used, Bushnell et al. (3) found that [^{3}H]QNB binding progressively declined in the hippocampus over the 15 days of treatment; they even observed reduced binding in the frontal cortex as early as the third injection. Thus, repeated low-level exposure to DFP is able to alter muscarinic receptor density, at least in certain brain regions.

This leads us to envisage another possibility: muscarinic receptors in the PMT areas studied might be less susceptible to express adaptive changes in response to DFP than those in other brain regions. Some data in the literature support this possibility. Most of the studies that simultaneously assessed receptor binding in the forebrain and the brain stem found the largest decreases in telencephalic structures, whereas small or no changes were detected in brain stem regions, which nevertheless exhibited pronounced ChE inhibition (10–12,37,43,46). For example, a chronic DFP treatment that markedly reduced binding in various telencephalic regions failed to alter binding in the brain stem, even when the dose was twice as high, even when the duration of exposure was twice as long (46). In this context, it is important to recall that the reductions in receptor density observed with low-level DFP exposure were all found in telencephalic structures (3,28). Thus, the present finding that muscarinic receptor density was unchanged in the PMT is in agreement with the view that, after OP treatment, brain stem regions could exhibit less receptor plasticity than telencephalic regions (10,11,46).

In conclusion, repeated low-level exposure to DFP allows fine manipulation of cholinergic transmission in the PMT, leading to a progressive decrease in ChE activity, then to a constant level of inhibition, and finally to a recovery of enzyme activity after drug withdrawal. Whether behavioral states are concomitantly changed is a question of particular relevance, given the critical role that cholinergic transmission in the PMT is supposed to play in behavioral state control. This question is addressed in the companion article.

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