Protractive effects of chronic treatment with an acutely sub-toxic regimen of diisopropylfluorophosphate on the expression of cholinergic receptor densities in rats

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Abstract

Individuals chronically exposed to low levels of organophosphate insecticides may present with subtle impairments in cognition. In addition, low level diisopropylfluorophosphate (DFP) exposure (0.25 mg/kg per day for 2 weeks) in rats resulted in protracted working memory impairment \cite{29}. The current studies attempt to show a temporal relationship between the DFP-induced impairment in performance of a spatial memory task and the protracted decrease in the expression of cholinergic receptors and acetylcholinesterase in specific brain regions. Cholinergic receptors labeled with the ligands $[^3]$H]epibatidine and $[^3]$H]AFDX-384 were affected to a much greater extent and for a longer period of time than were both acetylcholinesterase activities and cholinergic receptors labeled with $[^3]$H]QNB. Pre-testing administration of nicotine was shown to completely reverse this DFP-induced impairment in memory-related task performance. Additionally, prophylaxis with pyridostigmine bromide (PB) caused DFP-treated animals to exhibit near normal levels of memory-related task performance. These results are consistent with the development of a protracted phase of learning impairment to sub-acute DFP exposure, which may involve the loss of hippocampal nicotinic receptors, and may be prevented or reversed by PB or nicotine, respectively. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Disorders of the nervous system

Topic: Neurotoxicity

Keywords: DFP; Organophosphate; Acetylcholinesterase; Cholinergic receptor; Nicotine; Pyridostigmine bromide; Spatial learning; Memory

1. Introduction

Acute exposure to organophosphorus (OP) compounds such as diisopropylfluorophosphate (DFP) increases neural activity in CNS regions and peripheral organs innervated by acetylcholine-containing neurons. Severe toxicity and death may occur following acute exposure to high levels of OP insecticides; effects attributed largely to postsynaptic cholinergic receptor overstimulation. During chronic exposure, animals and humans may become tolerant to the acutely toxic effects of OP agents, such as OP-induced locomotor abnormalities or gastrointestinal disturbances \cite{4,10,32}. This behavioral and autonomic tolerance generally is considered to reflect the down regulation or adaptation of cholinergic muscarinic \cite{5,36,37,48} and nicotinic \cite{37} receptors in various brain regions. Although significant tolerance to OP agents has been documented,
tolerance develops to some but not all of the behavioral effects of DFP, and this tolerance may develop at different rates [33,44].

Despite the onset of tolerance in these situations, the adverse effects of OP compounds on higher brain functions, such as learning and memory may persist for quite some time after termination of toxicant exposure. The results from several studies have demonstrated the presence of OP-induced learning impairments several days after the behavioral signs of DFP, disulfoton, or soman toxicity have subsided [1,6,8,27]. Additionally, workers chronically exposed to OP agents present with a variety of psychiatric sequelae, including depression, apathy, irritability, and schizophreniform illness. One predominate set of symptoms includes loss of concentration, difficulty in thinking, and memory impairment [13,18,26]. Memory impairments induced by chronic OP administration appear to be most evident on novel learning tasks (i.e., those which require the greatest reliance on working memory) [16,42] and may persist for extended periods of time after DFP withdrawal. For example, we reported that spatial learning in rats is impaired for up to 21 days after withdrawal from a 14-day treatment regimen with DFP (250 μg/kg per day) [29]. This impaired learning was not temporally associated with DFP-induced reductions in brain acetylcholinesterase activity. However, a comparable DFP regimen did not impair performance of a well-learned delayed matching to sample task (in monkeys) or a previously experienced spatial navigation task (in rats), indicating that tasks dependent on reference memory were not significantly affected by DFP exposure [30]. These results support the possibility that chronic exposure to OP agents can result in specific long-term cognitive deficits even when overt symptoms of excessive cholinergic activity are not present.

Although the neuropathological basis for this protracted cognitive impairment is unknown, it is not likely that the insult represents a severe pathological event as may be observed in idiopathic neurodegenerative disorders such as Alzheimer’s disease. Rather, it is more likely that the behavioral changes, which are observed after accidental exposure to an OP agent, result from more subtle neurochemical alterations. Therefore, the purpose of the present study was to temporally relate the specific alterations in cholinergic neurochemical markers in specific brain areas with the time-course of behavioral changes observed previously [29] in the impairment of a spatial memory task. We also sought to determine whether nicotine (a memory enhancing agent) could reverse the deficits in cognitive function commonly observed after DFP exposure. Because of the modern use of the reversible cholinesterase inhibitor pyridostigmine bromide (PB) as a prophylactic protecting agent against nerve gas poisoning, we examined whether co-administration of PB with DFP could mitigate the behavioral or cognitive changes caused by the OP agent.

2. Materials and methods

2.1. Subjects

Male Wistar rats (Harlan Sprague–Dawley), approximately 4 months old (weighing 350–400 g) were used in these studies. Each rat was housed individually in a stainless steel mesh cage in a temperature controlled room (25°C) with free access to food (NIH-07 formula) and water, and maintained on a 12-h light/dark cycle (lights on at 18:00 h). All animal protocols were previously approved by the institutional Committee on Animal Use for Research and Education.

2.2. Drug administration

DFP (250 μg/kg; Sigma, St. Louis, MO) dissolved in saline was administered (s.c.) daily for 14 consecutive days. This is referred to in the present manuscript as the standard DFP regimen. All injections were given in a volume of 1 ml/kg body weight between 09:00 and 11:00 h.

2.3. Receptor autoradiography

Separate groups of rats (n=5–6/group) were examined at 1, 7, and 21 days after OP discontinuation. These tissues were derived from rats that had participated in earlier behavioral studies [29,30]. Immediately on completion of the behavioral studies, the brains were removed and flash frozen in dry ice/isopentane. The frozen tissues were stored at −70°C until use. Each frozen brain was sectioned coronally from the frontal pole through the level of the cerebellar peduncles. [3H]QNB, a non-selective muscarinic receptor ligand, and [3H]AFDX-384, a partially selective M2 muscarinic receptor ligand, were used to determine the numbers of muscarinic receptors in various brain regions. Brain sections were preincubated in Tris–phosphate buffer for 30 min at 25°C. The sections were then transferred to fresh Tris–phosphate buffer containing either [3H]QNB (800 pM) or [3H]AFDX-384 (5 nM) and incubated for 120 min at 25°C. Similarly, the number of nicotinic receptors was estimated using [3H]epibatidine as the radioactive ligand. These brain sections were preincubated in Tris–Hepes buffer for 30 min at 4°C, then incubated for an additional 120 min in fresh Tris–Hepes buffer containing [3H]epibatidine (200 pM) at 4°C. All sections were washed three times in their respective incubation buffers (minus radioactive ligands) for 4 min each at 4°C. Additionally, the sections were washed for 10 s in deionized water at 4°C. Unlabeled atropine or nicotine (10 μM) was added to the incubation buffer to determine non-specific binding for muscarinic and nicotinic receptors, respectively. After incubations and washings, all sections were air dried and stored in a dessicator at room temperature overnight. The sections were then exposed to tritium-sensitive Amersham
Hyperfilm for 2–6 weeks. Receptor binding in specific brain nuclei was quantified from autoradiographs using NIH Image software. In all cases, non-specific ligand binding was equal to background and receptor quantification was only performed for those brain regions that expressed a signal greater than background. Molar quantities of ligand bound were determined using values interpolated from the optical density versus a tissue (³H brain paste standards) radioactivity standard curve. ³H paste standards were applied to each autoradiographic film, and each structure was measured bilaterally in at least four sections for each animal. Binding analyses for each ligand were performed in consecutive sections from the same brain sample.

2.4. Brain acetylcholinesterase assay

Brain regions (frontal cortex and hippocampus) were isolated and assayed spectrophotometrically in phosphate buffer at pH 7.9 according to previously published methods [14]. Briefly, brain tissues were dissected and homogenized (25%, w/v, in buffer) for 1 min using a Bellco glass homogenizer with Teflon pestle. The homogenate was then centrifuged at 40,000×g for 30 min at 4°C. The supernatant (100 µl) was subsequently introduced into a cuvette containing the reaction mixture (7.5 µM acetylthiocholine iodide–substrate and 10.0 mM dithiobis-nitrobenzoic acid, DTNB). Absorbance at 412 nm was recorded for 25°C for 4 min. Protein concentrations were expressed as µM of substrate hydrolyzed/min per mg protein and the percentage of inhibition of enzyme activity for each OP dose (relative to control levels) was determined.

2.5. Water maze testing

Maze testing was performed in a circular pool (diameter, 180 cm; height, 76 cm) made of plastic (Bonar Plastics, Noonan, GA) with the inner surface painted black. The pool was filled to a depth of 35 cm of water (non-opaque, maintained at 25°C) which covered a black 10-cm square platform. The platform was submerged one cm below the surface of the water and placed in the center of the northeast quadrant on all trials. The pool was located in a large room with a number of visual extra-maze cues including highly reflective geometric images (squares, triangles, circles, etc.) mounted on the wall. Diffuse lighting and black curtains were used to hide the experimenter and the awaiting rats. Swimming activity of each rat was monitored via a ccTV camera mounted overhead which relayed information, including the latency to find the platform and the swim speed (latency/distance traveled), to a video tracking system (Poly-Track, San Diego Instruments, San Diego, CA).

2.6. Hidden platform test

Each rat was given four trials per day (session) for four consecutive days. On days 1–4, a trial began by placing the rat in the water facing the pool wall in one of the four quadrants. Rats were not allowed to acclimate to the pool prior to testing at any point during days 1–4. The daily order of entry into individual quadrants was randomized such that all four quadrants were used once every day. For each trial, the rat was allowed to swim a maximum of 90 s in order to find the hidden platform. When successful, the rat was allowed a 30-s rest period on the platform. If unsuccessful within the allotted time period, the rat was assigned a score of 90 s, and was physically placed on the platform and allowed the 30-s rest period. In either case, the rat was given the next trial (intertrial interval, 30 s) after the rest period.

2.7. Administration of nicotine in DFP-treated rats

In this experimental series, the standard 14-day saline (one group) or DFP (three groups) regimen was used. Two weeks after completion of the regimen all four groups (n=10–12 rats/group) were initiated in the standard water maze task (as described above). The saline regimen group received a subcutaneous injection of saline 15 min before the first trial of the first day’s session. Likewise, one of the groups that had received the DFP regimen was injected with saline before each day’s session. For the other two DFP groups, one group received 0.5 mg/kg of nicotine (s.c.), and the other received 1.0 mg/kg of nicotine (s.c.) before each day’s session.

2.8. Evaluation of olfactory behavior

Olfactory behavior (rearing and sniffing frequency) was measured immediately following daily administration of PB and/or DFP (see below) to assess for signs of overt cholinergic toxicity. Animals were placed in clear polypropylene containers (25×45×25 cm) for 35 min. After a 5-min acclimation period, rearing and sniffing behavior was recorded for 30 min. Following the 30-min observation period, animals were returned to their home cages.

2.9. Co-administration of pyridostigmine bromide (PB) and DFP

Rats (n=10–12/group) were administered either saline (0.9%; controls), PB (0.40 mg/kg, p.o., t.i.d.), DFP (250 µg/kg, s.c., once daily), or a combination of PB and DFP for 7 days. Immediately after treatment with the drugs, the animals were monitored for their olfactory reactivity (see
above) and the data recorded. Standard water maze testing was initiated 1 week after exposure.

2.10. Statistics

During maze acquisition, data were collapsed across trials for each day and averaged to obtain a mean latency for each animal. A two-way analysis of variance with post hoc Newman–Keuls comparisons were used to compare daily group latencies and swim speeds during days 1–4 of water maze testing. A one-way ANOVA and paired Student’s t-test (post hoc) were used to determine differences in receptor numbers between each experimental group and its respective control (saline) group. Significant differences were determined at both the P<0.05 and P<0.01 levels.

3. Results

3.1. Effect of the DFP regimen on brain cholinergic receptor subtype densities

3.1.1. Muscarinic (total) receptor binding

Table 1 presents the autoradiographic data derived from DFP-treated and control rats for which brain sections were labeled with the non-subtype selective muscarinic antagonist [3H]QNB. Forebrain regions of relatively high levels (reproductively greater than background) of binding are listed. Relatively high levels of expression of binding sites were located in cortical, striatal, and hippocampal regions. Although a non-subtype selective ligand was used, the expression pattern observed reflects primarily the M1 subtype of muscarinic receptor [46]. Despite the robust level of receptor expression in these areas, there was very little change in the number of [3H]QNB binding sites in rats treated with DFP. The primary exception occurred in layers three and four of the parietal cortex, which exhibited a time-dependent loss in binding sites up to 7 days after DFP withdrawal (Table 1). This loss in [3H]QNB binding sites, which amounted to 23% change from control levels, had returned almost to control levels within 21 days. The results from a typical autoradiographic experiment are shown in Fig. 1. Even at 1 day after DFP withdrawal, there is clear evidence of loss of [3H]QNB binding sites within the parietal cortex. This loss in binding sites was observ- able in all three sections representing rostral through caudal levels of the brain (Fig. 1).

3.1.2. Muscarinic (M2) receptor binding

The relative levels of muscarinic receptors labeled with AFDX-384 (primarily the M2 subtype) were also estimated in sections from the same control and DFP-treated rats. M2 receptor distribution did not always parallel [3H]QNB binding sites, suggesting that [3H]QNB labeled a different population of receptors than did [3H]AFDX-384. For example, M2 receptors were not as highly concentrated in cortical and hippocampal regions (except for the subiculum), rather there were high levels found in the basal ganglia and the superior colliculus (Fig. 1). In contrast to the results with [3H]QNB, there was a more generalized decrease in the expression of [3H]AFDX-384 binding sites in the regions examined (Table 2). In sections taken from brains harvested 1 day after the last the DFP exposure, the number of binding sites was significantly reduced in the striatum, dentate gyrus, CA1 hippocampal region, parietal cortex, and subiculum as compared with saline infused control rats (Table 2). As with the effect on nicotinic receptor density (see below), the maximal reduction in M2 receptor expression occurred on withdrawal day 7. By this time only the decreased receptor expression in the striatum, subiculum, and nucleus accumbens was evident. In fact, by withdrawal day 21 the receptor numbers in the striatum and subiculum continued to be significantly reduced. The most dramatic reduction in [3H]AFDX-384 binding site density was measured in the subiculum where levels declined on day 7 by 64%, with little recovery by withdrawal day 21 (Table 2).

3.1.3. Nicotinic (neuronal) receptor binding

Table 3 presents autoradiographic data derived from DFP-treated and control rats for which brain sections were labeled with [3H]epibatidine, a ligand that preferentially labels high affinity (neuronal) nicotinic receptors. Unlike the distribution of muscarinic receptors, [3H]epibatidine preferentially labeled sites within thalamic regions.

<table>
<thead>
<tr>
<th>Region</th>
<th>Control 1 day WD</th>
<th>7 days WD</th>
<th>21 days WD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1 hippocampus</td>
<td>100±2.5</td>
<td>94.0±3.7</td>
<td>90.4±4.7</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>100±2.0</td>
<td>92.7±3.2</td>
<td>90.1±5.0</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td>100±3.6</td>
<td>91.1±1.6</td>
<td>93.7±7.4</td>
</tr>
<tr>
<td>Parietal cortex (1–2)</td>
<td>100±3.4</td>
<td>93.5±1.7</td>
<td>82.7±5.7*</td>
</tr>
<tr>
<td>Parietal cortex (3–4)</td>
<td>100±4.2</td>
<td>87.5±1.7*</td>
<td>77.5±6.4**</td>
</tr>
<tr>
<td>Parietal cortex (5–6)</td>
<td>100±4.7</td>
<td>90.7±1.6</td>
<td>77.9±9.3*</td>
</tr>
<tr>
<td>Striatum</td>
<td>100±3.9</td>
<td>89.5±1.4</td>
<td>93.6±9.1</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>100±7.3</td>
<td>100.2±4.7</td>
<td>85.0±5.9</td>
</tr>
</tbody>
</table>

P<0.05; **P<0.01 compared with control means (saline-infused rats); n=6/group. Data are normalized with regard to their respective controls.
Fig. 1. Left panel: color-enhanced autoradiographs of $[^3]$H]QNB binding (primarily M1 subtype) and $[^3]$H]AFDX-384 binding (M2 subtype) to muscarinic cholinergic receptors in different levels of the brain from rostral through caudal (left to right) sections in saline (vehicle)-treated rats or in rats that received the standard DFP regimen. Brains were harvested 1 day after completion of the regimens. Relative levels of binding are depicted from high through low according to the color scheme shown. Note the lower level of QNB binding in the parietal cortex for the DFP-treated rat and the lower level of $[^3]$H]AFDX-384 binding in many regions like striatum, hippocampus and parietal cortex of the DFP-treated rats when compared with those of the saline-treated rats. Right panel: color-enhanced autoradiographs of $[^3]$H]epibatidine binding to high affinity nicotinic cholinergic receptors in different levels of the brain from rostral through caudal (left to right) sections in saline (vehicle)-treated rats or in rats that received the standard DFP regimen. Brains were harvested 1 day (upper sections) or 21 days (lower section) after completion of the regimens. Relative levels of binding are depicted from high through low according to the color scheme shown. Note the low level of binding in the cortical and hippocampal regions for the sections derived from the 1 day post-DFP rat. At 21 days after completion of the regimen binding was reduced in the dentate gyrus of the DFP-treated rats when compared with those of saline-treated rats.
Table 2

[14H]AFDX-384 binding to brain muscarinic cholinergic receptors at varying time intervals after withdrawal from chronic low-level DFP treatment

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>1 day WD</th>
<th>7 days WD</th>
<th>21 days WD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1 hippocampus</td>
<td>100±7.2</td>
<td>57.0±8.3**</td>
<td>74.3±13.5</td>
<td>94.6±10.8</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>100±10.6</td>
<td>69.9±10.1*</td>
<td>87.9±12.9</td>
<td>69.2±10.6</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td>100±7.9</td>
<td>62.0±9.5**</td>
<td>63.9±9.7*</td>
<td>105.0±12.4</td>
</tr>
<tr>
<td>Parietal cortex (layers 1–2)</td>
<td>100±9.3</td>
<td>83.1±7.9</td>
<td>75.3±10.1</td>
<td>90.1±5.4</td>
</tr>
<tr>
<td>Parietal cortex (layers 3–4)</td>
<td>100±12.4</td>
<td>73.0±9.9*</td>
<td>71.0±13.1</td>
<td>85.5±5.8</td>
</tr>
<tr>
<td>Parietal cortex (layers 5–6)</td>
<td>100±9.8</td>
<td>76.0±8.9*</td>
<td>69.9±10.7</td>
<td>82.7±12.0</td>
</tr>
<tr>
<td>Striatum</td>
<td>100±10.1</td>
<td>56.8±7.7**</td>
<td>61.1±11.0*</td>
<td>77.1±11.3*</td>
</tr>
<tr>
<td>Subiculum</td>
<td>100±14.6</td>
<td>49.6±14.6*</td>
<td>36.0±7.37**</td>
<td>42.3±2.8**</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>100±4.1</td>
<td>92.5±8.2</td>
<td>96.1±4.3</td>
<td>92.0±6.4</td>
</tr>
</tbody>
</table>

*P<0.05; **P<0.01 compared with control means (saline-infused rats); n=6/group. Data are normalized with regard to their respective controls.

striatum, subiculum, dentate gyrus of the hippocampus, medial habenula, interpeduncular nucleus, and superior colliculus (Fig. 1). Again, these results replicate earlier reports for high affinity nicotine binding in the rat brain [12]. As with the results from the [14H]epibatidine binding sites, in this case, the greatest effect was observed within the dentate gyrus of the hippocampus (Table 3). The loss of nicotinic receptor sites differed from that observed for the [14H]QNB-labeled sites, and it was similar to that for [14H]AFDX-384-labeled sites. The decrease in dentate nicotinic receptor expression (by almost 50% of control levels) was similar to that of [14H]epibatidine binding sites, whereas [14H]QNB binding was essentially unaltered in this brain region. Also, 21 days after DFP withdrawal, the density of [14H]AFDX-384 binding sites was unchanged, and there was only a partial recovery of dentate nicotinic receptors (the receptor levels were still significantly reduced by about 30% at this timepoint). This reduction in dentate nicotinic receptor expression 21 days after the DFP regimen is evident in the typical autoradiograph shown in Fig. 1.

In Fig. 2, the DFP-induced changes in cholinergic receptor binding sites and acetylcholinesterase levels in cortical and hippocampal regions from 1 to 21 days after DFP withdrawal are compared. One readily apparent feature of the profile of changes in these cholinergic receptors is that hippocampal (dentate and subiculum) changes were often greater in magnitude and reduced in recovery compared with cortical changes. Also, the muscarinic M2-subtype and nicotinic receptors were affected to a greater extent by the DFP treatment than were the muscarinic M1-subtype receptors and acetylcholinesterase.

3.2. Effect of pre-training injection of nicotine in DFP-treated rats

The DFP regimen resulted in impaired performance in the spatial memory task as compared with saline controls (Fig. 3). Even after 4 days of water maze sessions, the DFP-treated rats did not approach the proficiency of controls in terms of swim latencies. For those rats that received pre-session injections of nicotine, task acquisition was similar to saline controls and task performance was improved compared with the DFP group that received pre-session injections of saline (Fig. 3). The same relationship between the experimental groups was obtained when swimming distance was measured (Fig. 3). There was a significant main ‘drug’ effect by ANOVA, $F(3,227)=4.25, P=0.006$. The DFP/saline group was significantly different from control and nicotine-treated groups. Additionally, swim speed (the ratio of swim latency/swim distance) remained constant among all experimental groups.

3.3. Effect of co-administration of pyridostigmine bromide on DFP-induced impaired spatial memory

Pyridostigmine bromide (PB) was evaluated for its ability to mitigate the acute as well as the long-term (cognitive) effects of chronic DFP exposure. The data

Table 3

[14H]Epibatidine binding to brain nicotinic cholinergic receptors at varying time intervals after withdrawal from chronic low-level DFP treatment

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>1 day WD</th>
<th>7 days WD</th>
<th>21 days WD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dentate gyrus</td>
<td>100±9.4</td>
<td>69.2±8.6**</td>
<td>50.9±4.2**</td>
<td>70.3±3.2**</td>
</tr>
<tr>
<td>Medial geniculate nucleus</td>
<td>100±5.2</td>
<td>99.7±10.7</td>
<td>82.4±4.8</td>
<td>94.2±8.7</td>
</tr>
<tr>
<td>Parietal cortex (layers 4–6)</td>
<td>100±4.4</td>
<td>77.8±14.2</td>
<td>68.9±18.3</td>
<td>105.3±5.4</td>
</tr>
<tr>
<td>Parietal cortex (layer 3)</td>
<td>100±3.2</td>
<td>77.9±10.1*</td>
<td>82.9±10.5</td>
<td>105.2±5.4</td>
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<tr>
<td>Striatum</td>
<td>100±2.9</td>
<td>80.1±8.1</td>
<td>81.7±10.2*</td>
<td>109.1±8.4</td>
</tr>
<tr>
<td>Subiculum</td>
<td>100±5.0</td>
<td>89.9±7.9</td>
<td>88.2±7.0</td>
<td>90.7±6.5</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>100±3.9</td>
<td>103.2±9.2</td>
<td>90.8±4.7</td>
<td>97.8±3.9</td>
</tr>
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</table>

*P<0.05; **P<0.01 compared with control means (saline-infused rats); n=6/group. Data are normalized with regard to their respective controls.
Fig. 3. Effect of pre-session injection of nicotine (Nic, 0.5 or 1.0 mg/kg, s.c.) on water maze performance (as measured by swim latency and swim distance) of rats previously treated with either the standard low-level regimen of DFP (DFP/Sal; 0.25 mg/kg per day for 14 days) or control saline (Sal/Sal) regimen. Water maze testing was initiated 2 weeks after completion of the DFP or saline regimen. Both doses of nicotine significantly reversed the DFP-induced impairment in task acquisition as measured by swimming latencies (time required to locate the hidden platform) and swimming distance (distance traveled to locate the hidden platform). There was a significant treatment effect by ANOVA, $F(3,227)=4.25$, $P<0.006$. The DFP/SAL group was significantly different from the other three groups; $P<0.05$ (Student–Newman–Keuls multiple comparison test).

Fig. 2. Summary of the time-course for the changes in brain cerebral cortical or hippocampal levels of acetylcholinesterase (AChE) activity, neuronal nicotinic cholinergic ([$^3$H]epibatidine) receptors, and muscarinic cholinergic ([$^3$H]QNB and [$^3$H]AFDX) receptors after completion of the standard low-level regimen of DFP (0.25 mg/kg per day for 14 days). There was a delay in the recovery of hippocampal AChE levels relative to cortical levels. Also, nicotinic receptor expression in the hippocampus was significantly reduced for up to 3 weeks after DFP exposure. Changes in muscarinic receptors as measured by [$^3$H]QNB binding were of smaller magnitude than those for nicotinic receptors, and there was less of a regional difference between the time courses. However, [$^3$H]AFDX-384 binding, selective for M2 receptors, revealed a significant reduction in this subtype up to 3 weeks after DFP exposure similar to that of the epibatidine binding. The data for AChE activity was summarized from an earlier study [29] for comparison purposes. Each point represents the mean from five to six experiments and data are represented as percent control (rats that received 14 consecutive saline injections). See Tables 1–3 for statistical comparisons.

4. Discussion

Chronic, low-level DFP exposure has been shown to produce memory deficits in rats in the absence of overt signs of cholinergic toxicity [29,30]. These earlier results demonstrated that memory impairments were still evident 21 days after DFP withdrawal; although, decreases in acetylcholinesterase activity by DFP, which were initially observed, had returned to control levels within this time period [29]. Therefore, reduced cholinesterase activity per se did not appear to be associated with the memory deficits observed after DFP withdrawal. Similarly, the results from previous studies also have indicated that the degree of DFP-induced acetylcholinesterase inhibition does not correlate with the level of cognitive impairment demonstrated in OP-exposed rats [8] or monkeys [17].

In the current study, we sought to determine whether a relationship exists between specific alterations in the number of cholinergic receptor proteins in specific brain areas and the pattern of behavioral changes observed upon DFP exposure. First, the present data demonstrated that hippocampal cholinergic proteins were more sensitive to the effects of DFP than cortical proteins. The mechanism presented in Fig. 4 illustrate that each DFP regimen, particularly the combined PB/DFP regimen, produced reductions in rearing in the first 3 days of administration. Rearing in animals receiving PB or DFP alone began to recover to near-control levels after 5–6 days of exposure, whereas those receiving the combined PB/DFP regimen continued to display this motor abnormality throughout the observation period. The animals were subjected to water maze testing 1 week after the completion of each drug regimen. As demonstrated in Fig. 4, DFP-treated rats were significantly impaired in water maze performance even on the first day of testing as compared with the saline control group. The animals that received the combined PB/DFP regimen exhibited near saline levels of maze performance. The group that received PB alone exhibited a similar level of task performance except for the anomalously high mean latency recorded during the third session (day) of testing.
of selectivity for the effects of the DFP regimen on hippocampal neurons as compared with cortical neurons (and compared with other brain regions not illustrated here) is not yet apparent; however, protracted deficits in the hippocampus could be due to a higher percentage of cholinergic receptors located on axon terminals (as compared to dendrites and cell bodies) in the hippocampus compared to the cortex. This selectivity fits well with the deficits observed in water maze performance [29,30], a task known to require intact hippocampal processing. In fact, it would have been surprising if the neurochemical effects to the OP treatment were not anatomically selective in view of the fact that rats were not impaired in all types of memory tasks [30].

Previous studies have demonstrated decreases in \(^{[3}^H\)QNB [43] and \(^{[3}^H\)nicotine binding [11,38,43] in rats following chronic organophosphate exposure. Similarly, the current data also demonstrated that all cholinergic proteins measured (M1, M2, and nicotinic receptors, as well as acetylcholinesterase) were decreased following DFP exposure. However, the data showed that AChE and M1 receptors were either least affected (M1 receptors) or had more rapidly returned to control levels (AChE) than were the nicotinic and M2 receptors. One difference between the neuro-architecture of M1 muscarinic receptors located in the cerebral cortex and nicotinic receptors in the hippocampus is that M1 receptors are considered to exist primarily postsynaptically (located on cell bodies and dendrites), whereas nicotinic receptors [47] and M2 muscarinic receptors [20] are largely presynaptic (located on axon terminals).

One possible explanation for the different effects of DFP on these receptor subtypes involves their specific neuronal locations. Recently, it has been demonstrated that repeated low doses of DFP (250 \(\mu\)g/kg per day for 14 days) produced significant reductions in anterograde and retrograde fast axonal transport in peripheral nerve axons of rats at both 1 and 24 h after the last toxicant exposure [41]. These data demonstrate that a dosing regimen of DFP, which has been shown to produce protracted working memory impairment [29], as well as alterations in the numbers of cholinergic receptor subtypes in rat brain, also produces bi-directional changes in axonal transport. Although these transport studies were performed in peripheral nervous system axons, no evidence exists to support the notion that transport compromise would be different in central nervous system axons. In fact, other neurotoxicants have produced very similar reductions in fast transport in both PNS and CNS axons [39,40]. Thus, a compromise of receptor transport would produce more significant changes in receptor numbers in areas furthest from the site of exposure. This behavior in animals that received PB or DFP alone began to recover to near-control levels by 5–6 days after exposure. Water maze testing was initiated 1 week after exposure. Only the DFP group was significantly different from the saline group in water maze task performance. \(*P<0.05\) compared with SAL group.

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Another potential explanation for the decrease in nicotinic receptor density after DFP withdrawal is that the drug caused a form of receptor down-regulation. Previous studies have reported that OP exposure causes down regulation of rat brain nicotinic receptor binding [11,38,43]. This down regulation of nicotinic receptors is
presumably due to an increase in synaptic acetylcholine. However, it has been demonstrated that increased levels of the nicotinic agonists nicotine [25,37,48], anabasine, (+)-anatoxin-a, cytisine [9,31,38], and the acetylcholine analog methylcarbamylcholine [48], produced over-stimulation of nicotinic receptors generally resulting in receptor up-regulation. Additionally, receptor levels in the hippocampus and cortex were observed to decrease after DFP withdrawal, before initiating a return towards normal levels. Therefore, acetylcholine-induced receptor downregulation does not appear to be the cause of the reduction in nicotinic receptor numbers observed in the present experiments.

Central nicotinic receptors (shown here to be significantly affected by DFP exposure) have been studied as potential pharmacological targets to improve learning and memory in experimental animals [2,3,7,15,21±24] and humans [28,34,35,45]. DFP-treated rats (standard DFP regimen) exhibited a significantly reduced rate of learning compared with control rats; however, the rate of learning was similar to that for controls in the DFP groups that received one of two doses of nicotine each day prior to maze testing. Whereas nicotine is used clinically only for smoking cessation, several pharmaceutical companies are currently evaluating novel analogs that are purported to exhibit a reduced side-effect profile relative to nicotine. These compounds soon may be available for treating cognitive deficits associated with long-term OP exposure.

In the final experimental series, we sought to determine whether concomitant administration of pyridostigmine bromide could alter the responses to the standard DFP regimen. PB was administered concomitantly with DFP because PB is used by our armed forces as a prophylactic measure against chemical nerve agents. These experiments should be considered preliminary since only one dose of PB (0.4 mg/kg, p.o.) was tested, although this regimen was constructed to mimic field doses administered during the Persian Gulf War (30 mg oral PB every 8 h for 1–7 days) [19]. The mechanism for PB’s OP-protective action resides in its ability to prevent cholinesterase from permanent inactivation by DFP.

Both PB alone, and DFP alone caused a reversible inhibition of olfactory behavior in rats over the 1-week administration period. When the two agents were combined there was a significant enhancement of the inhibition of this behavior. This was perhaps not too surprising, since both compounds are cholinesterase inhibitors. Although PB does not produce irreversible inhibition of the enzyme, the 3-day dosing schedule was most likely as effective as DFP at maintaining cholinesterase inhibition. However, despite the initial additive toxic effects, PB/DFP-treated animals were almost as efficient as saline controls in learning the water maze task (although the PB regimen was not quite as effective as was the pre-testing nicotine regimen in reversing the effects of DFP on maze performance). Since PB’s actions are generally restricted to the peripheral nervous system (it is a quaternary amine), the mechanism for the drug’s beneficial action on water maze performance is unclear. It seems unlikely that the PB would directly alter the effect of DFP on brain cholinesterase inhibition or on brain cholinergic receptor expression unless the PB indirectly reduced the amount of DFP entering the CNS. Alternatively, the ability of PB to reduce the overall peripherally mediated toxicity of OP agents may have provided for a better overall post-DFP treatment outcome. Nevertheless, under the conditions of these experiments, prophylactic administration of PB appeared not only to provide protection against nerve agent exposure acutely, but it may also help mitigate some of the cognitive impairment observed after toxicant exposure.

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