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Enhanced mRNA Expression of Neurofilament Subunits in the Brain and Spinal Cord of Diisopropyl Phosphorofluoridate-Treated Hens

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ABSTRACT. Diisopropyl phosphorofluoridate (DFP) is an organophosphorus ester, and a single injection of this compound (1.7 mg/kg, s.c.) produces delayed neurotoxicity (OPIDN) in hens in 7–14 days. Clinically, the disease is marked by hindlimb ataxia followed by paralysis after some time. A characteristic feature of this neuropathy is axonal swelling in the initial stages and comparative dissolution of the accumulated material and degeneration of distal axons with disease progression. Axonal swelling consists of aggregated neurofilaments, microtubules, and proliferated smooth endoplasmic reticulum. We studied expression of neurofilament (NF) mRNAs in brain regions and spinal cord to elucidate their role in OPIDN. There was a 50–200% increase in NF transcripts in 24 hr after DFP administration. The NF-L mRNA level started falling after 1–5 days and came down to control level in susceptible brain regions (i.e. cerebellum and brainstem) and spinal cord, but not in cerebral cortex, which does not show degeneration of axons in OPIDN. Cerebral cortex exhibited elevated levels of both NF-L and NF-M transcripts in DFP-treated hens throughout the period of observation. The induction of NF messages is consistent with the previously reported effect on extension of neurites of human neuroblastoma cells in culture. The transient increase in NF messages in susceptible tissues either may be responsible for the delayed degeneration of axons in OPIDN or is the result of interruption of regulatory signal due to progressive degeneration of axons. BIOCHEM PHARMACOL **57**;11:1245–1251, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. DFP; neurofilament proteins; OPIDN; hen; mRNA; brain; spinal cord

Organophosphorus esters are chemicals used widely in agriculture and industry. Some of these chemicals produce distal axonopathy in the central and peripheral nervous systems of humans and other sensitive species, such as cows, hens, cats, dogs, and water buffaloes [1, 2]. Examples of such neurotoxic chemicals are TOCP†, *N,N′*-diisopropyl phosphofluoroamidate (mipafox), *O*-methyl *O*-4-bromo-2,5-dichlorophenyl phenylphosphonothioate (leptophos), and DFP. Hen is the animal of choice to study this disease, since this species is very sensitive to OPIDN. Chlorpyrifos [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate], an organophosphorus ester which produces OPIDN at lethal dose only, also produces delayed neurotoxicity at a lower dose when it is administered along with safrotin, (*E*)-1-methylethyl 3-[[(ethylamino)methoxyphosphinothioyl]oxy]-2-butenoate [3]. Neither of them alone produced OPIDN at the given doses. Thus, exposure of humans to a combination of chemicals that do not produce delayed neurotoxicity individually can result in OPIDN. Clinically, OPIDN in hens is characterized by ataxia in 7–14 days and paralysis as the disease progresses. Ultrastructural studies show axonal swellings containing aggregates of neurofilaments, microtubules, and proliferation of agranular endoplasmic reticulum immediately after the beginning of clinical signs. As the disease progresses, neurofilaments are partially matted and comparatively rarefied, but the microtubules are better preserved and visible for a longer time in OPIDN [4, 5].

NF are 10 nm in diameter and several micrometers in length, and are composed of three protein subunits, NF-H, NF-M, and NF-L, of 180–200, 130–170, and 60–70 kDa, respectively. These proteins are neuron-specific, but NF-M protein and NF-L mRNA also have been detected in Schwann cells under certain conditions [6]. Neurofilaments move with microtubules down the axon at a slow speed, and there is little degeneration until they reach the nerve terminal, where they are degraded rapidly [7]. The concentration of cytoskeletal proteins in the nerve terminal depends on their post-translational modification and the proteinases that cause their degradation. The inhibition of NF proteolysis results in their accumulation in the synaptic terminals [8].

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† *Abbreviations*: TOCP, tri-*o*-cresyl phosphate; DFP, diisopropyl phosphorofluoridate; OPIDN, organophosphorus ester-induced delayed neurotoxicity; NF, neurofilament(s); NF-H, neurofilament high molecular weight protein; NF-M, neurofilament middle molecular weight protein; NF-L, neurofilament low molecular weight protein; and NTE, neuropathy target esterase.

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Neurofilaments determine axonal caliber, which is dependent upon their number and state of phosphorylation [9], and conduction velocity, which is directly proportional to axonal caliber [10]. NF-H and NF-M are highly phosphorylated [11 and references therein], and their phosphorylated terminals form the side arms of neurofilaments and modulate the distance between adjacent neurofilaments. Although a variety of diseases and chemical intoxications show enhanced NF phosphorylation and slow axonal transport, it is probably the site of NF phosphorylation that determines the effect of phosphorylation on their transport [12] or accumulation. The axonal swellings in DFP-treated hens contain highly phosphorylated neurofilaments [13].

Various neurodegenerative diseases, toxic chemical-induced neuropathies, and heritable diseases show accumulation of neurofilaments in the cell body and proximal or distal axons, at least in the early stages of disease. The forced expression of NF-L [14], NF-M [15], and NF-H [16] in transgenic mice demonstrates accumulation of neurofilaments in cell bodies and proximal axons, and increased frequency of axonal degeneration. In contrast to other chemical-induced axonopathies and neurodegenerative disorders, OPIDN is associated with accumulation of neurofilaments in axonal swelling in the initial stages and their comparative rarefaction at later stages. The aim of this study was to determine the role of expression of NF mRNAs in this neuropathy.

MATERIALS AND METHODS

Materials

DFP, dithiothreitol, and eserine were obtained from the Sigma Chemical Co., and [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from New England Nuclear. Nytran membrane was purchased from Schleicher & Schuell, and Random Primer Labeling system was purchased from Life Technologies, Inc. Other chemicals were purchased from standard commercial sources.

Animal Treatment

White Leghorn laying hens (*Gallus gallus domesticus*) 18 months old and weighing ~1.6 kg were purchased from Featherdown Farms. Four hens in each group were administered a single dose of DFP (1.7 mg/kg in propylene glycol, s.c.) 15 min after prophylactic doses of atropine (1 mg/kg in normal saline, s.c.) and eserine (1 mg/kg in DMSO, s.c.). The control group was injected with atropine, eserine, and the vehicle propylene glycol. The birds were examined daily for ataxia and killed 1, 5, 10, and 20 days posttreatment. The cerebral cortex, cerebellum, brainstem, and spinal cord were removed quickly, frozen in liquid nitrogen, and kept at −70°. The experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of animals for experimental procedures.

Preparation of cDNA Probes

NF-H, NF-M, NF-L, β-actin, and 18S RNA cDNA probes were used for northern hybridization. The NF-H clone was provided by Dr. R. H. K. Liem (Columbia University), NF-M and NF-L clones were provided by Dr. J. P. Julien (McGill University), and β-actin (HFBCC49) and 18S RNA (HHCSA65) clones were purchased from the American Type Culture Collection. All the probes were amplified and DNA was purified by standard methods [17]. The insert of NF-L cDNA was obtained by digestion with *Pst*I, and those of other cDNA probes were obtained by digestion with *Eco*RI.

Northern Blot Hybridization

Total RNA was prepared from cerebral cortex, cerebellum, brainstem, and spinal cord of control and DFP-treated hens by the single-step isolation procedure [17] and 20, 30, 30, and 30 μg RNA, respectively, was used for electrophoresis. RNAs were transferred onto Nytran membrane and hybridized with radiolabeled probes [18]. cDNA probes were prepared by Random Primer Labeling system using [α-^{32}P]dCTP as the radioactive nucleotide. The blots were exposed to Phosphor-imaging plates, and the radioactivity in bands was quantified with Fujix Bio-imaging Analyzer System (BAS1000). Radioactivity (PSL values) of mRNA bands in total RNA samples prepared at different time points (1, 5, 10, and 20 days) from DFP-treated hen tissues was plotted as the percentage of the value obtained for mRNA in the same tissue from control hens (day 0). The radioactivity of mRNA bands in control samples was assigned as 100%.

Statistical Analysis

The hybridized northern blots were exposed to Phosphor-imaging plates at least two times, and spots were visualized and quantified by a Fujix Bio-imaging Analyzer system. The results obtained were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. $P < 0.05$ was considered significant.

RESULTS

Clinical Signs

Hens showed signs of acute toxicity on DFP (1.7 mg/kg, s.c.) administration in spite of pretreatment with the antidotes atropine and physostigmine. However, they were able to stand within a few hours after dosing and exhibited no sign of leg weakness before being killed on days 1 and 5. They developed mild ataxia in 10 days and severe ataxia or paralysis in 20 days after DFP administration.

Expression of NF Transcripts in the Cerebral Cortex of DFP-Treated Hens

A single dose of DFP (1.7 mg/kg) produced significant increase in the expression of NF-L mRNA in 1 day (57 ±

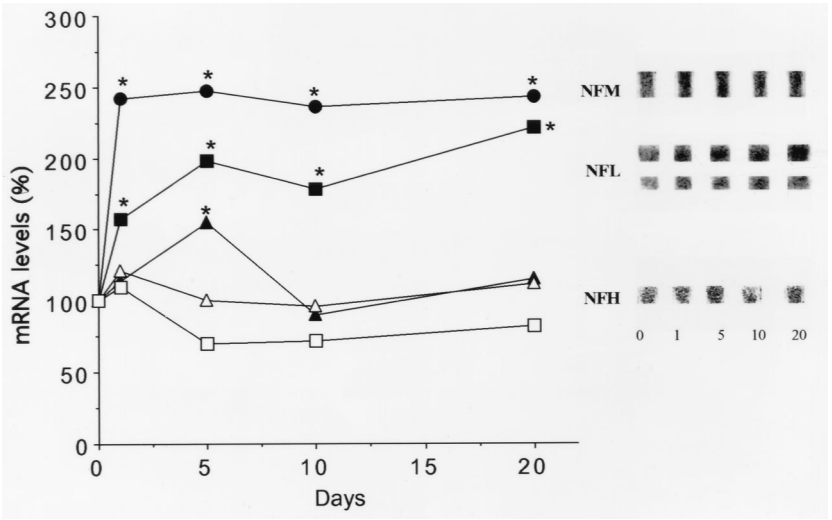

FIG. 1. Expression of NF-H, NF-M, NF-L, and β-actin mRNAs, and 18S RNA in the cerebral cortex of DFP-treated hens. Hens were treated with a single dose of DFP (1.7 mg/kg, s.c.) and killed after 1, 5, 10, and 20 days. Total RNA was purified from the cerebral cortex of each bird, and 20 μg was used for northern blotting and hybridization as described in Materials and Methods. NF-H, NF-M, NF-L, β-actin, and 18S RNA probes were labeled with [α^{32}P]dCTP by Random Primer Labeling system. Profiles of mRNA expression represent the means ± SEM of the percent of control values from untreated hens. The radioactivity of bands was measured in PSL units by exposing northern blots to Phosphor-imaging plates, followed by quantification with the Fujix Bio-imaging system. Four birds were used in each group, but the error bars are not shown for clarity. Significant difference ($P < 0.05$) from the control is marked with an asterisk. There was significant increase in NF-L and NF-M mRNA after 1 day. NF-H mRNA showed only a transient increase after 5 days. Symbols ▲, ●, ■, △, and □ represent the profiles of NF-H, NF-M, NF-L, β-actin, and 18S RNA, respectively. NFH, NFM, and NFL in the autoradiograms represent relevant portions of representative autoradiograms showing the mRNA expression of NFH, NFM, and NFL, respectively.

8%, N = 4), and then expression increased up to 121 ± 11% by 20 days post-treatment (Fig. 1). The expression of NF-M mRNA increased by 142 ± 13% in 1 day and stayed at that level up to 20 days. In the case of NF-H, expression increased by 55 ± 8% in 5 days, returned to control level in 10 days, and then stayed at control level up to 20 days after DFP treatment. β-Actin and 18S RNA probes were used as controls, and the expression of β-actin and 18S RNA messages did not change throughout the period of observation in the cerebral cortex of DFP-treated hens. Expression of neurofilament subunits as well as putative control messages (i.e. β-actin, 18S RNA) in all hens was studied on the same blot to reveal any error in purification, estimation, or loading of RNA samples. Alteration in the expression of neurofilament subunits was real and could not be ascribed to the above-mentioned errors, since neurofilament subunit mRNAs showed a time-course profile different from that shown by control messages. The expression of neurofilament subunits was not normalized to control message β-actin or 18S RNA message, since these putative control messages also showed some alteration in their expression at some time points in some other tissues. Hen NF-L mRNA existed as two bands of 4.3 and 2.4 kb, and NF-M and NF-H mRNA showed bands of 3.0 and 4.5 kb, respectively.

Expression of NF Transcripts in the Cerebellum of DFP-Treated Hens

Increased NF-L mRNA expression was observed on DFP post-treatment days 1 (115 ± 11%, N = 4), 5 (172 ± 14%), and 10 (141 ± 13%). Expression increased up to day 5 and then returned to control level by day 20 after DFP administration. NF-M mRNA exhibited an increase of 123 ± 14, 101 ± 16, and 189 ± 19% on post-treatment days 1, 5, and 10, respectively (Fig. 2). Thus, there was an increase in NF-M expression up to 10 days, followed by return to control level in the next 10 days (20 days post-treatment). NF-H expression peaked after 1 day (102 ± 9% increase), stayed at that level up to 10 days, and then returned to an increased level of 43 ± 9% in 20 days. Thus, NF-H mRNA level did not return to control level in 20 days after DFP treatment. β-Actin expression was higher after 1 and 5 days, but 18S RNA expression did not alter significantly at any time point after DFP administration.

Expression of NF mRNAs in the Brainstem of DFP-Treated Hens

There was a maximum increase in NF-L mRNA expression after 1 day (90.5 ± 7.5%, N = 4) of DFP administration and this returned to a lower level (49.6 ± 9.8% higher) in 5 days (Fig. 3). This was followed by a further decrease in NF-L mRNA expression in the next 5 days and stayed at that level till the end of the observation period (20 days post-administration). NF-M mRNA expression increased by 160 ± 17% in 1 day and then remained at that level through the period of observation, 20 days. Expression of NF-H mRNA increased by 52 ± 6% in 1 day, and then it came down to 124% of control level (24 ± 6% higher) after 5 days of DFP administration. Thereafter, the expression of NF-H mRNA was 41 ± 3 and 29 ± 1.4% less than the

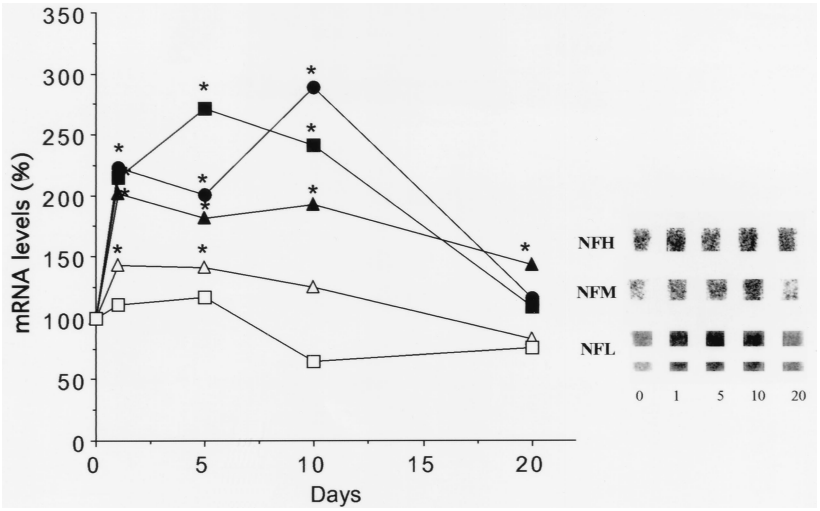

FIG. 2. Expression of NF-H, NF-M, NF-L, and β-actin mRNAs and 18S RNA in the cerebellum of DFP-treated hens. The hens were treated with a single dose of DFP (1.7 mg/kg, s.c.) and killed after 1, 5, 10, and 20 days. Total RNA was purified from the cerebellum of each bird, and 30 μg was used for northern blotting and hybridization as described in Materials and Methods. Profiles of mRNA expression represent the percent (mean ± SEM) of control values for untreated hens. Four birds were used in each group, but the error bars are not shown for clarity. Significant difference ($P < 0.05$) from control is marked with an asterisk. See legend to Fig. 1 for symbols.

control value after 10 and 20 days of DFP treatment, respectively. Thus, there was an initial increase in the NF-H mRNA level, which significantly decreased as the disease progressed. There was no significant alteration in β-actin mRNA and18S RNA expression at any time-point after DFP administration.

Expression of NF mRNAs in the Spinal Cord of DFP-Treated Hens

NF-L mRNA expression increased maximally (175 ± 17%, N = 4) on post-treatment day 1, but less so on post-treatment day 5 (152 ± 15%) (Fig. 4). On post-treatment day 10, NF-L mRNA level was close to the control level, and it did not increase significantly in the next 10 days (20 days post-treatment). NF-M mRNA expression was increased by 226 ± 20 and 190 ± 15% on post-treatment days 1 and 5, respectively. This was, however, followed by a 50 ± 2% decrease by the end of the observation period (post-treatment day 20) in the spinal cord. Thus, there was significant increase in NF-L and NF-M expression up to 5 days, followed by a decrease after that time. A similar pattern was observed in the case of NF-H mRNA expression. There was an increase in NF-H mRNA expression after 1 (118 ± 10%) and 5 (125 ± 10%) days of DFP administration, followed by a decrease to about 50% of control level after 10 and 20 days of treatment. There was no significant change in the level of β-actin mRNA and 18S RNA level at any time-point after DFP administration.

DISCUSSION

Alteration in expression of NF mRNAs has been observed in chemical-induced polyneuropathies, on nerve injury or transection, during regeneration of nerves or aging, and in some diseases such as diabetes, amyotrophic lateral sclerosis, and Alzheimer's disease [19–24]. However, to our knowledge, there is no report on alteration in the expression of NF mRNAs in organophosphorus ester-induced delayed neurotoxicity. We have found significant changes in the expression of NF mRNAs 1 through 20 days after the administration of DFP. The birds looked apparently normal without any clinical signs over the first 5 post-treatment

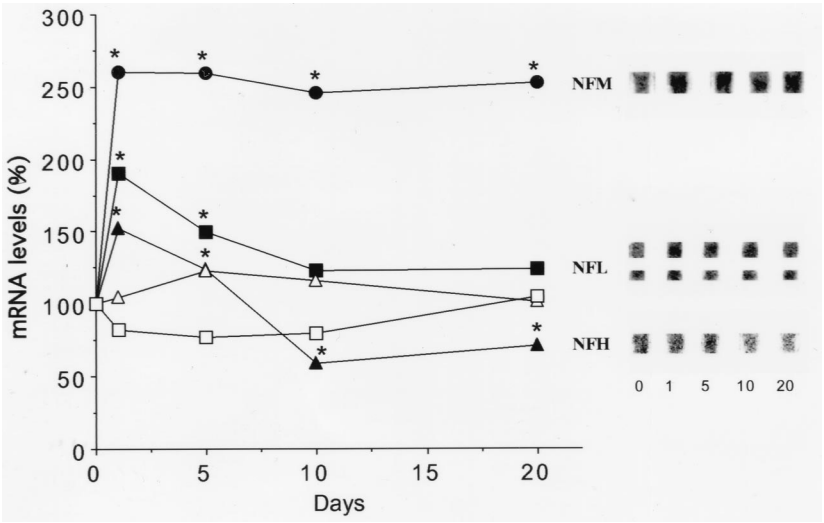

FIG. 3. Expression of NF-H, NF-M, NF-L, and β-actin mRNAs, and 18S RNA in the brainstem of DFP-treated hens. The hens were treated with a single dose of DFP (1.7 mg/kg, s.c.) and killed after 1, 5, 10, and 20 days. Total RNA was purified from the brainstem of each bird, and 30 μg was used for northern blotting and hybridization as described in Materials and Methods. The profiles of mRNA expression represent the percent (mean ± SEM) of control values from untreated hens. Four birds were used in each group, but the error bars are not shown for clarity. Significant difference ($P < 0.05$) from control is marked with an asterisk. See legend to Fig. 1 for symbols.

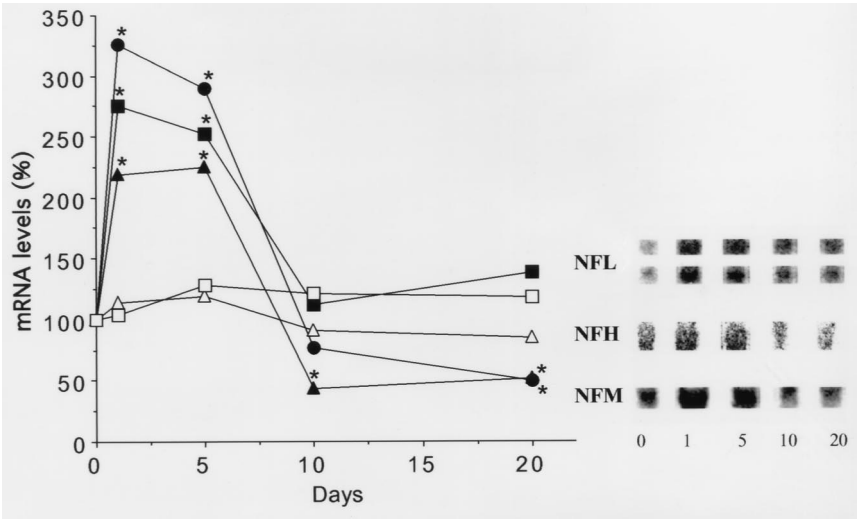

FIG. 4. Expression of NF-H, NF-M, NF-L, and β-actin mRNAs, and 18S RNA in the spinal cord of DFP-treated hens. The hens were treated with a single dose of DFP (1.7 mg/kg, s.c.) and killed after 1, 5, 10, and 20 days. Total RNA was purified from the spinal cord of each bird, and 30 μg was used for northern blotting and hybridization as described in Materials and Methods. Profiles of mRNA expression represent the percent (mean ± SEM) of control values from untreated hens. Four birds were used in each group, but the error bars are not shown for clarity. Significant difference ($P < 0.05$) from control is marked with an asterisk. See legend to Fig. 1 for symbols.

days. By post-treatment day 10, there was mild ataxia progressing to severe ataxia/paralysis by post-treatment day 20, when they were killed.

DFP treatment increased expression of NF transcripts (50–200%) in all the brain regions and the spinal cord in 1 day, except in cerebral cortex, which exhibited an increase in NF-H transcript after 5 days of DFP administration. Such an increase in expression of NF mRNAs has not been observed on treating animals with other chemical neurotoxicants such as aluminum [25, 26], β-iminodipropionitrile [27], or acrylamide [20]. Some of them even show decrease or no change in NF mRNA expression. Similarly, studies on NF mRNA expression during aging of animals [28] or regeneration of nerves [6], in injured or transected nerves [21, 24], and in several diseases (e.g. diabetes, Alzheimer's disease, and amyotrophic lateral sclerosis) [19, 22, 23] show an increase in the expression of NF transcripts only during development and maturation. On the other hand, most of the diseases with neuronal degeneration, which are marked by accumulation of NF in the body or proximal axon of neurons, are associated with the decreased expression of NF transcripts.

The increased (50–200%) expression of NF mRNAs in DFP-treated hens is surprising, since increased expression of NF transcripts has been observed only during development and maturation of nerves [6] and not in other chemical-induced neuropathies or nervous system disorders. However, this is consistent with some other effects observed on DFP treatment of animals and cells. (1) Very low concentrations of DFP cause greater proliferation of cultured neuroblastoma cells compared to control cells, and higher concentrations show fewer cells, with neurites having thread-like appearance and numerous swellings [29]. (2) The soluble extract of DFP-treated hen spinal cord (24 hr post-treatment) markedly enhances the extensions of neurites of human neuroblastoma cells in culture. The elongation of neurites is comparable to that obtained on treating the cells with 1 μg/mL nerve growth factor [30]. (3) Transient induction of β-adrenoceptors and muscarinic receptors has also been noticed in the cardiac atria of DFP-treated rats [31]. Furthermore, induction of NF mRNA expression by DFP treatment is not unique. We also have found temporal induction of calmodulin kinase II mRNA in the brain and spinal cord of DFP-treated hens [18]. The alterations observed in the mRNA levels of NF subunits in this study may be due to change in either the rate of transcription of DNA or the stability of NF mRNAs. Furthermore, induction or suppression of mRNA levels need not necessarily increase or decrease the concentration of respective NF subunits in tissues. Work is in progress to compare NF subunit levels in the spinal cord and cerebral cortex of control and DFP-treated hens by western blotting and immunohistochemistry.

At this time, it is difficult to correlate the enhanced level of NF subunit transcripts in brain and spinal cord with histopathological characteristics of OPIDN, since there is no accumulation of NF in the cell body, and even in axons the NF accumulation is morphologically different from that observed in some other nervous system diseases. However, expression of NF may be related to the susceptibility of nerves to degeneration. For example, the message of all three NF subunits falls to control level or even below it in 10 days after DFP treatment in the spinal cord, which is more susceptible to DFP-induced degeneration than cerebellum and brainstem [32, 33]. On the other hand, induction of both NF-L and NF-M mRNAs, 1 through 20 days, is observed only in cerebral cortex, which does not show degeneration of axons in OPIDN. Studies in transgenic mice show that, whereas increases in any single NF subunit inhibit axonal growth, increases in NF-L along with either NF-H or NF-M promote radial axonal growth [10]. Therefore, it is feasible that temporal induction of NF mRNAs may delay axonal degeneration (i.e. cerebellum, brainstem, spinal cord), and continuous combined induction of NF-L along with NF-M may protect tissue (i.e. cerebral cortex) from DFP-induced degeneration. Although neurofilaments traditionally are considered to be static, a number of studies suggest them to be highly dynamic structures. For example,

microinjection of biotin-labeled NF-L into DRG shows incorporation of labeled NF-L into the whole length of axons [6, 34].

Alternatively, enhanced expression of NF mRNAs for only short periods in susceptible brain regions (i.e. cerebellum and brainstem) and spinal cord may be ascribed to inhibition of retrograde axonal transport with the progression of OPIDN [35]. Inhibition of retrograde transport on transection or crushing of the sciatic nerve [21, 24] has been shown to decrease the expression of all three NF subunits. Thus, continuous increased expression of NF-L and NF-M mRNAs in the cerebral cortex, which does not show axonal degeneration in OPIDN, and down-regulation of all three NF subunits in spinal cord, which is more sensitive to degeneration in OPIDN [32, 33], may be ascribed to the progression of axonal degeneration in the respective tissues. This mechanism is, however, unable to explain the continuously higher level of NF-M mRNA in the brainstem, which also shows degeneration of axons in OPIDN. Schwann cells in the rat sciatic nerve have been reported to form NF-M when they differentiate into myelin-forming cells [36]. However, it is not yet known whether glial cells in some brain regions are able to synthesize the NF-M subunit. More work is required to determine whether induction of NF messages for short periods delays degeneration of axons in OPIDN or is the result of progressive degeneration of axons in OPIDN.

Previous studies have demonstrated that more than 70% inhibition of NTE activity accompanied by aging correlated well with the ability of an organophosphorus compound to produce OPIDN. DFP (1 mg/kg, s.c.) treatment inhibits >80% NTE activity in 4 hr in the brain and sciatic nerves of hen [37, 38], and it recovers to more than 50% of control activity before the development of ataxia or paralysis in 10–14 days. This transient inhibition of NTE activity is apparently consistent with the temporal induction of expression of NF mRNAs (particularly NF-L mRNA) in the degeneration-susceptible brain regions and spinal cord of DFP-treated hens. However, the biological functions of this enzyme are still unclear, and its role in the mechanism of OPIDN is yet to be defined. Thus, while inhibition of NTE activity above 70–80% by organophosphorus esters is essential to produce OPIDN in sensitive species such as hens, its inhibition above 80% does not produce OPIDN in rats and 1-month old chickens. Similarly, even though there is greater inhibition of NTE in brain compared with spinal cord by several organophosphorus esters, more degeneration is observed in the spinal cord [37, 38]. Organophosphorus compounds that do not inhibit NTE activity (e.g. parathion, paraoxon) or inhibit NTE activity (e.g. phenylmethylsulfonyl fluoride, *n*-butanesulfonyl fluoride) even more than 80% but do not result in the aging of enzyme also do not produce OPIDN.

In summary, the results showed transient enhanced expression of NF transcripts on DFP treatment. This profile of NF expression is different from that shown by non-organophosphorus neurotoxic chemicals or that observed in various neuropathologies. The enhanced expression of NF, particularly NF-L mRNA, came down to the base value in 10–20 days in DFP-susceptible tissues (i.e. spinal cord, cerebellum, and brainstem), but remained elevated in the resistant tissue (i.e. cerebral cortex) throughout the period of observation of 20 days. The transient elevation of NF mRNA levels in susceptible tissues either is responsible for the delayed degeneration of axons or is the result of progressive degeneration of axons in OPIDN.

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