0022-3565/93/2653-1492$00.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 265, No. 3
Printed in U.S.A.

Administration of 3,3′-Iminodipropionitrile to the Rat Results in Region-Dependent Damage to the Central Nervous System at Levels Above the Brain Stem[1]

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Accepted for publication February 16, 1993

ABSTRACT

Axonal swellings and neurofilamentous accumulations in the brain stem, spinal cord and peripheral nervous system are the most widely documented effects of exposure to 3,3′-iminodipropionitrile (IDPN). Evidence from morphological and functional studies, however, suggests that IDPN also may damage areas of the central nervous system above the level of the brain stem. To examine this possibility, we evaluated the astrocyte reaction to injury as an indirect means of detecting potential sites of IDPN-induced damage to the central nervous system. An immunoassay for the astrocyte intermediate filament protein, glial fibrillary acidic protein (GFAP), was used to quantify gliosis. Rats were given IDPN (0–600 mg/kg/day i.p.) for 3 days. The concentration of GFAP in discrete brain regions was examined at postdosing times ranging from 3 days to 3 weeks. IDPN caused time-, dose- and region-dependent increases in GFAP; elevations were observed in the pons-medulla, midbrain, cerebral cortex and olfactory bulbs, but not in cerebellum, hypothalamus, hippocampus and striatum. Of these areas, cortex and olfactory bulbs showed the largest increases. Dissection of cortex into four subregions showed that the IDPN-induced increase in cortical GFAP was relatively uniform across this brain region. Application of the de Olmos cupric-silver degeneration stain to IDPN-treated tissue revealed intense argyrophilia in the glomerular layer of the olfactory bulbs and diffuse staining of axons in several regions of the cortex. The data indicate that IDPN is neurotoxic to the olfactory bulbs and cortex of the rat.

Initial neuropathological assessments of rodents exposed to IDPN led to the discovery of giant swellings of the proximal axon (Hartmann *et al.*, 1958). Neurofilamentous accumulations were the hallmark of these lesions and they were found to be particularly prominent in neurons of the anterior horn of the spinal cord (Chou and Hartmann, 1964; Slagel and Hartmann, 1965). Subsequent to these observations, IDPN was found to impair axonal transport of neurofilaments in the extraspinal (PNS) portion of the affected axons without disrupting transport of other critical cytoskeletal elements, such as actin and tubulin (Griffin *et al.*, 1978, 1983; Yokoyama *et al.*, 1980). The action of IDPN on specific cytoskeletal elements of the peripheral axon has led to its use as a research tool for examining the molecular basis of axonal transport (Papasozomenos *et al.*, 1985) and for understanding the mechanisms underlying peripheral axonopathies (Griffin *et al.*, 1987).

Despite the emphasis on the PNS, spinal cord and brain stem as primary targets of the neurotoxic effects of IDPN, evidence exists which suggests that areas above the level of the brain stem also may be damaged. For example, although neuropathological assessments of IDPN-exposed rats failed to reveal evidence of neuronal degeneration (Chou and Hartmann, 1964; Schulze and Boysen, 1991), the giant axonal swellings prominent in the cord and periphery occasionally have been seen in the CNS at levels as high as the mesencephalon (Chou and Hartmann, 1964). Data obtained from studies of whole animal function also suggest an involvement of higher centers in the effects of IDPN. The primary observation to support this contention is the "waltzing syndrome," a behavioral manifestation of IDPN exposure characterized by repetitive head movements, retropulsion, circling, hyperactivity and inability to swim (Delay *et al.*, 1952; Thuillier and Burger, 1954). Cadet (1989) has reviewed the behavioral and biochemical pharmacology of the waltzing syndrome and concluded that several supraspinal areas are involved in this life-long condition. Recent work by Peele and co-workers (1990) provides further support for a role of higher levels of the CNS in the neurotoxic effects of IDPN. They demonstrated that exposure of the rat to IDPN disrupted a number of learning and memory tasks,

Received for publication September 11, 1992.
[1] This work was supported in part by Interagency Agreement RA-ND-89-4 from the National Institute on Drug Abuse.
[2] Supported by a Postdoctoral Fellowship from the Spanish Comisión Interministerial de Ciencia y Tecnología.

ABBREVIATIONS: IDPN, 3,3′-iminodipropionitrile; PNS, peripheral nervous system; CNS, central nervous system; GFAP, glial fibrillary acidic protein; SDS, sodium dodecyl sulfate; ANOVA, analysis of variance; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

findings that could not be attributed to the propensity of the compound to disrupt overall performance. Taken together, the permanent functional alterations induced by IDPN and the scattered reports suggestive of brain pathology raise the possibility that IDPN causes subtle damage to higher centers of the CNS that has escaped detection with traditional neuropathological techniques.

Given the evidence reviewed above, the purpose of the present study was to determine if brain areas above the level of the brain stem are damaged by exposure to IDPN. Astrogliosis was used as the main criteria for determining the existence and location of sites of IDPN-induced brain damage. Hypertrophy of astrocytes ("reactive gliosis," astrogliosis) occurs at sites of neural (*i.e.*, neuronal or glial in origin) damage following a variety of nervous system insults (Eng, 1988; Hatten *et al.*, 1991; O'Callaghan, 1991b; Norton *et al.*, 1992). The hallmark of this response is an accumulation of astrocyte intermediate filaments, of which GFAP is the major constituent (for a review see Eng, 1988). By using a radioimmunoassay for GFAP, we have quantified the astrocytic response to chemically induced injuries of the CNS (for reviews see O'Callaghan, 1988, 1991b, 1992). We have demonstrated that administration of a variety of prototype CNS toxicants results in dose-, time- and region-dependent increases in GFAP. Large increases in this protein result from exposure to toxicants at dosages below those that cause light microscopic evidence of neural cell loss or damage (Brock and O'Callaghan, 1987; Balaban *et al.*, 1988; O'Callaghan, 1991b). Thus, assaying the concentration of GFAP in discrete regions of the rat brain represents a potentially useful approach for detecting sites of IDPN-induced damage that may have escaped detection with traditional histological stains. In the present study we used assays of GFAP to reveal the degree and location of IDPN-induced damage of the CNS at levels above the brain stem. The cupric silver method for detection of degenerative changes in neurons (Switzer, 1991; Beltramino *et al.*, 1992; O'Callaghan and Jensen, 1992) also was used to reveal potential sites of neuronal damage that may have provided the stimulus for the observed increases in GFAP.

Methods

Materials. Rabbit antibovine GFAP was obtained from Dako Corporation (Carpenteria, CA). [^{125}I]Protein A (2–10 μCi/μg; 1 Ci = 37 GBq) was purchased from New England Nuclear (Boston, MA). Nitrocellulose paper (0.2-μm porosity) was purchased from Schleicher and Schuell (Keene, NH). Isopropanol (high-performance liquid chromatography grade), paraformaldehyde (purified grade) and sucrose were obtained from Fisher Scientific (Fair Lawn, NY). Gelatin of EIA purity, electrophoresis grade SDS and Triton X-100 were purchased from Bio Rad Laboratories (Richmond, CA). All other reagents used for GFAP immunoassays were of at least analytical grade and were obtained from a variety of commercial sources. BCA protein assay reagent and bovine serum albumin were obtained from Pierce Chemical Co. (Rockford, IL) and Sigma Chemical Co. (St. Louis, MO), respectively. Sodium cacodylate trihydrate was purchased from EM Sciences (Fort Washington, PA). IDPN (base, 99% purity) was purchased from Kodak (Rochester, NY).

Animals. Male Long Evans rats (Charles River, Inc.) were obtained at 60 days of age and were maintained in a colony certified by the American Association for Accreditation of Laboratory Animal Care. Animals were housed two per cage in plastic hanging cages and maintained on a 12 hr light:12 hr dark photoperiod (light:dark = 6:00 A.M.:6:00 P.M.). Food (Purina Lab Chow) and water were provided *ad libitum*. Temperature was maintained at 21.0 ± 2°C and relative humidity at 50 ± 10%. Animals were acclimated to the environment of the colony room for 7 days before dosing.

Dosing, brain dissection and tissue preparation. IDPN was diluted in 0.9% saline for injection. Saline alone served as the vehicle control. Rats were administered IDPN (0, 200, 400, 500 or 600 mg/kg i.p.) in a volume of 1 ml/kg for 3 consecutive days. This regimen has been shown previously to produce learning and memory deficits in the rat (Peele *et al.*, 1990). At various postdosing intervals, rats were sacrificed by decapitation and their brains were removed immediately from the skull. Brain regions were dissected free-hand on a cold plate, weighed, frozen on dry ice and stored at −70°C. Cerebellum, pons-medulla, hypothalamus, hippocampus, midbrain and cortex were dissected according to Glowinski and Iversen (1966). In one experiment cortex was divided into four parts to approximate the cingulate, frontal, parietal and occipital-temporal cortical areas (Krieg, 1947; Paxinos and Watson, 1982). Olfactory bulbs also were obtained in these animals by transection of the olfactory peduncle. The frozen regions then were homogenized with the aid of a sonic probe (Kontes Cell Disrupter, Vineland, NJ) in 10 volumes of hot (90–95°C) 1% (w/v) SDS and stored frozen at −70°C before radioimmunoassay. Day-to-day variations in control GFAP values arose from day-to-day variations in dissection accuracy. Therefore, samples from control and treated animals for each experimental condition (*e.g.*, each postdosing time point in the time course study) were processed simultaneously in a semirandom order.

Protein assay. Total protein in the SDS homogenates was assayed by the method of Smith *et al.* (1985). Bovine serum albumin was used as the standard.

GFAP radioimmunoassay. GFAP was measured by solid-phase radioimmunoassay according to modifications (Brock and O'Callaghan, 1987;) of the dot-immunobinding procedure of Jahn *et al.* (1984). A detailed protocol has been published recently (O'Callaghan, 1991a). Briefly, samples were assayed for total protein, adjusted to equal protein concentration (0.25 mg/ml) in dot-immunobinding buffer (120 mM KCl; 20 mM NaCl; 2 mM $NaHCO_3$; 2 mM $MgCl_2$; 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4; 0.7% Triton X-100; and 0.2% NaN_3) and applied to dry nitrocellulose sheets that were free of wetting agent. A slot-blot manifold (Minifold II, Schleicher and Schuell) was used as a template for sample application, and 20-μl aliquots of sample homogenate were loaded into each slot. The spotted sheets were fixed, blocked with gelatin, washed, incubated with primary antibody and then with [^{125}I]Protein A as described by Jahn *et al.* (1984). The primary anti-GFAP antibody stock was used at a dilution of 1:500. The concentration of GFAP in each sample was determined by comparison to a standard curve constructed from dilutions of a rat hippocampal homogenate of known GFAP concentration (see O'Callaghan, 1991a).

Silver degeneration stain. A separate group of rats was administered saline (0.9%) or IDPN (400 mg/kg) daily for 3 days. These rats were anesthetized with pentobarbital (75 mg/kg i.p.) and sacrificed by transcardial perfusion 4 or 10 days after the last injection of saline or IDPN. The perfusate consisted of 4% paraformaldehyde (w/v), 1.43% sodium cacodylate (w/v) and 4% sucrose (w/v). The initial 5 to 10 ml of perfusate was administered at a dilution of 1:10 and then followed by undiluted fixative for a total of 150 ml/rat. Frozen sections (40-μm thick) were prepared at 250-μm intervals throughout the rostral caudal axis of the brain. The cupric silver degeneration stain of Carlsen and de Olmos (1981) was used to stain each section. Adjacent sections were stained with thionine to reveal neural perikarya. The perfused brains were assigned code numbers and they were then processed and stained through the facilities of Neuroscience Associates (Knoxville, TN). Control and experimental tissues were processed in parallel. The stained slides were evaluated by an observer unaware of the treatment conditions. Sample codes were not broken until all slides had been examined and scored for the presence of degenerating neurons (silver staining) or damaged perikarya (thionine staining).

Statistics. All data were analyzed by using multivariate (figs. 1 and 3) or univariate (figs. 2 and 4) ANOVA procedures. For figures 1 and 3

a significant interaction of region and treatment was followed by one-way ANOVAs for each region to test for treatment effects. For figure 2 a significant overall effect of treatment was followed by Duncan's Multiple Range Test for mean comparisons between dose groups. For figure 4 a significant time by treatment interaction was followed by one-way ANOVAs at each time point to test for treatment effects. The α-level used to determine significance was .05 in all cases. All statistical analyses were performed by using SAS User's Guide (SAS, 1985).

Results

The animals treated with IDPN developed the characteristic IDPN behavioral syndrome and lost body weight. No mortality was associated with the 3-day exposure to the 0-, 200- or 400-mg/kg doses. Mortality induced by higher doses during a 10-day postdosing period was one of nine animals after three 500-mg/kg doses and 8 of 10 after three 600-mg/kg doses. Chemical-induced neurotoxicity often results in loss of tissue wet weight and total protein at sites of damage (*e.g.*, see Brock and O'Callaghan, 1987; O'Callaghan and Miller, 1985). IDPN exposure, however, did not affect wet weight or total protein in any brain region examined (data not shown).

The administration of the 400 mg/kg regimen of IDPN resulted in region-dependent increases in the concentration of GFAP (fig. 1). At 3 days postdosing only the midbrain showed an increase (24%). By 7 days postdosing, however, GFAP was increased in the pons-medulla (12%), midbrain (44%) and cortex (68%). Three weeks after dosing with IDPN the concentration of GFAP in the affected regions had returned to control levels.

The cortex and cerebellum were examined for dose-dependent effects of IDPN on GFAP (fig. 2). The data obtained confirmed the regional specificity of the IDPN-induced increase in GFAP. Whereas IDPN caused a dose-dependent increase in the GFAP concentration in cortical tissue (44–65%), no increase in GFAP was observed in the cerebellum, even at doses inducing mortality in 8 of 10 animals (*i.e.*, 600 mg/kg for 3 days).

To examine the possibility that the IDPN-induced increase in cortical GFAP might be localized to a specific area within this region, the cortex was dissected into four subdivisions. At the time this experiment was conducted we observed that IDPN-treated rats performed poorly on an olfactory discrimination task (Peele *et al.*, 1990). Together with preliminary evidence of damage to olfactory epithelial cells (St. Clair *et al.*, 1991), these findings were suggestive of olfactory damage. To examine the latter possibility we included the olfactory bulbs when we assayed the concentration of GFAP in specific subdivisions of cerebral cortex. The effect of IDPN on the concentration of GFAP in cerebral cortex was fairly uniform (fig. 3). Ten days after three 400-mg/kg doses, the increase in GFAP was not restricted to any particular cortical area. Increases in GFAP were seen in cingulate (63%), frontal (40%), parietal

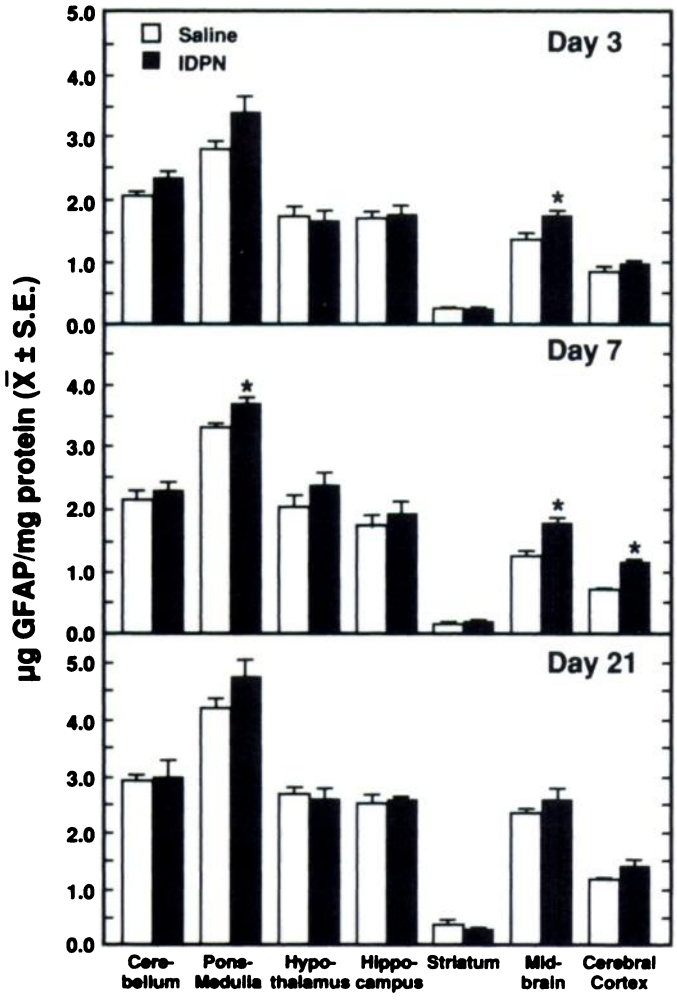

Fig. 1. Time course of the effects of IDPN (400 mg/kg/day for 3 days) on the concentration of GFAP in homogenates of seven brain regions; n = 5. *Significantly different from corresponding saline control, $P < .05$.

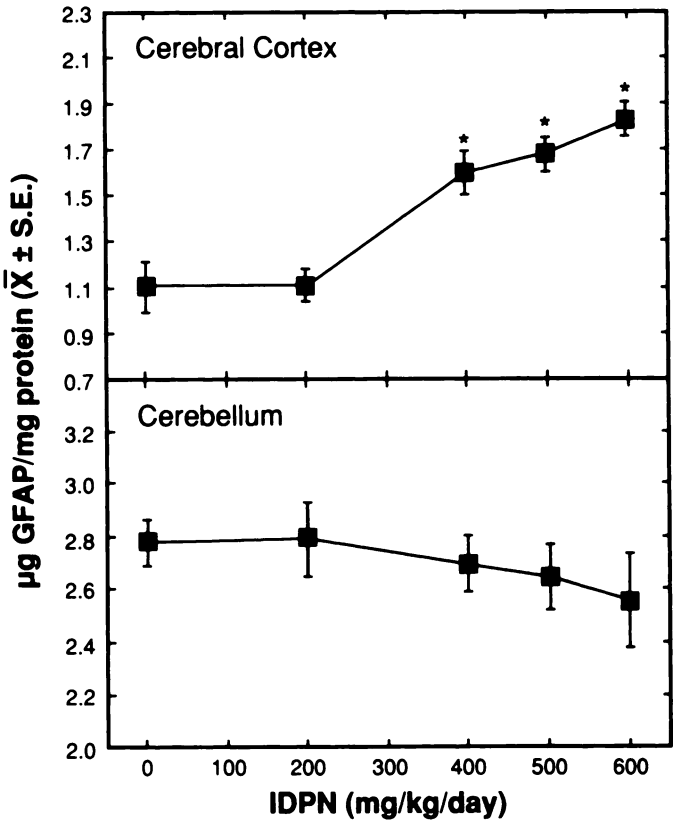

Fig. 2. Dose-response curves for the effects of IDPN on the concentration of GFAP in homogenates of the cortex and cerebellum. Dosages were administered for 3 consecutive days and rats were sacrificed 10 days after the last dose; n = 5 for the 0-, 200- and 400-mg/kg groups; n = survivors in the 500-mg/kg (eight of nine) and 600-mg/kg (2 of 10) dose groups. *Significantly different from saline controls, $P < .05$.

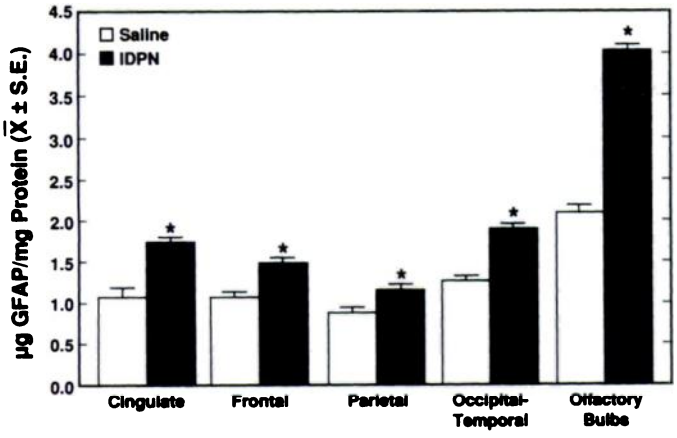

Fig. 3. Effect of IDPN (400 mg/kg/day for 3 days) on the concentration of GFAP in homogenates prepared from subdivisions of the cortex and olfactory bulbs. Rats were sacrificed 10 days after the last dose; $n = 6$. *Significantly different from saline control, $P < .05$).

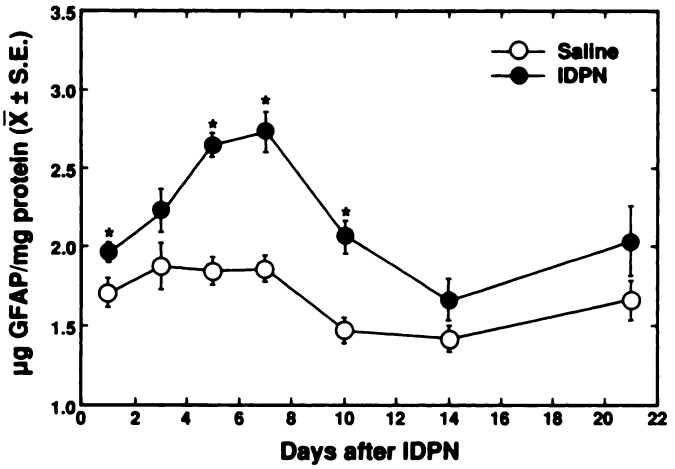

Fig. 4. Time course of the effects of IDPN (400/kg/day for 3 days) on the concentration of GFAP in homogenates of cingulate cortex; $n = 6$. *Significantly different from corresponding saline control, $P < .05$).

(32%) and occipital-temporal (49%) cortical areas. IDPN exposure also resulted in an increase in the concentration of GFAP in the olfactory bulbs (fig. 3). Indeed, the percentage of increase in olfactory bulb GFAP (84%) was larger than any percentage of increase seen in the cortex after any IDPN exposure regimen.

In the cortex, the largest increase in GFAP (as a percentage of control) due to IDPN was in the cingulate region (see fig. 3). To determine if the temporal increase in GFAP in this subdivision of the cortex approximated the time course of the increase in GFAP in the cortex as a whole, a detailed time course analysis was performed (fig. 4). A significant increase in the concentration of GFAP (15%) was present as early as 1 day after three daily 400-mg/kg doses of IDPN. The effect peaked around 1-week postexposure, but similar increases in GFAP were obtained at 5, 7 and 10 days postexposure (43, 47 and 40%, respectively). Increases in GFAP concentration were no longer evident at later time points (*i.e.*, 14 and 21 days after IDPN).

When tissue from IDPN-treated rats was reacted with the cupric silver degeneration stain, argyrophilic fibers and axons were observed in the olfactory bulbs and the cortex (fig. 5). Four days after the 400-mg/kg IDPN exposure regimen, an intense argyrophilic reaction was observed in the glomerular layer of the olfactory bulbs (fig. 5B). Corresponding controls (fig. 5A) showed, at best, weak staining of the glomeruli, a finding that may be related to naturally occurring turnover of incoming neuronal fibers that synapse in this region (Graziadei and Monti-Graziadei, 1979; Switzer *et al.*, 1985). In the cortex, argyrophilic axons were observed, in sparse numbers, throughout this brain region (fig. 5D). Many of the stained profiles exhibited the beaded appearance characteristic of degenerating axons (*e.g.*, fig. 5D) (de Olmos *et al.*, 1981). Within the cortex, the argyrophilic fibers were not confined to any particular cortical layer. Staining was negative in samples of cortex obtained from control rats (fig. 5C). Brain sections obtained from rats 10 days after the 400-mg/kg IDPN exposure regimen also exhibited argyrophilia in the glomerular layer of the olfactory bulbs, but the intensity of the staining was reduced markedly in comparison to that observed at the earlier time point (data not shown). The number of argyrophilic axons in the cortex also was reduced markedly at the 10-day time point compared to the number observed 4-days postexposure to IDPN (data not shown). In bulbar and cortical sections showing argyrophilia, Nissl staining of adjacent sections did not reveal differences in cell structure between control and IDPN-treated rats (data not shown). Pons-medulla and midbrain, brain areas that showed much smaller IDPN-induced increases in GFAP than were observed in the olfactory bulbs and the cortex, showed only a very few argyrophilic fibers (data not shown); in these regions, Nissl-stained material from adjacent sections were unremarkable (data not shown).

Discussion

Trauma to the CNS results in an astrogliotic reaction that can spread far from the site of injury (Bignami and Dahl, 1976; Mathewson and Berry, 1985) and that can result in a persistent GFAP-positive scar (Reier, 1986). In contrast, chemical-induced damage to the CNS evokes an astrocyte reaction (increase in GFAP) that is restricted to the sites of damage and that resolves over time to base-line levels (O'Callaghan *et al.*, 1990; O'Callaghan, 1991b; Norton *et al.*, 1992). Because localized damage results in localized astrogliosis after chemical-induced injury of the CNS, we assayed the concentration of GFAP in several brain regions as an indirect means of locating and quantifying potential sites of IDPN-induced brain damage. Given the time-dependent nature of chemical-induced astrogliosis, we also measured the concentration of GFAP at several time points after exposure to IDPN to minimize the possibility for obtaining false-negative results. By using this strategy we found that IDPN elevated the concentration of GFAP in the medulla-pons, midbrain and cortex at 1-week postdosing, findings suggestive of underlying damage in these brain areas.

The increases in GFAP observed in the medulla-pons and midbrain were not entirely unexpected because previous reports described IDPN-induced axonal swelling in these areas (Chou and Hartmann, 1964; Slagel and Hartmann, 1965). We had no *a priori* evidence to predict the observed increase in cortical GFAP, however, because damage to this region had not been observed following the application of a variety of traditional histological stains (*e.g.*, hematoxylin and eosin, thionine, cresyl violet, toluidine blue and luxol fast blue) (see Chou and Hartmann, 1964; Schulze and Boysen, 1991). Exposure to neurotoxicants can result in brain damage that remains undetectable by

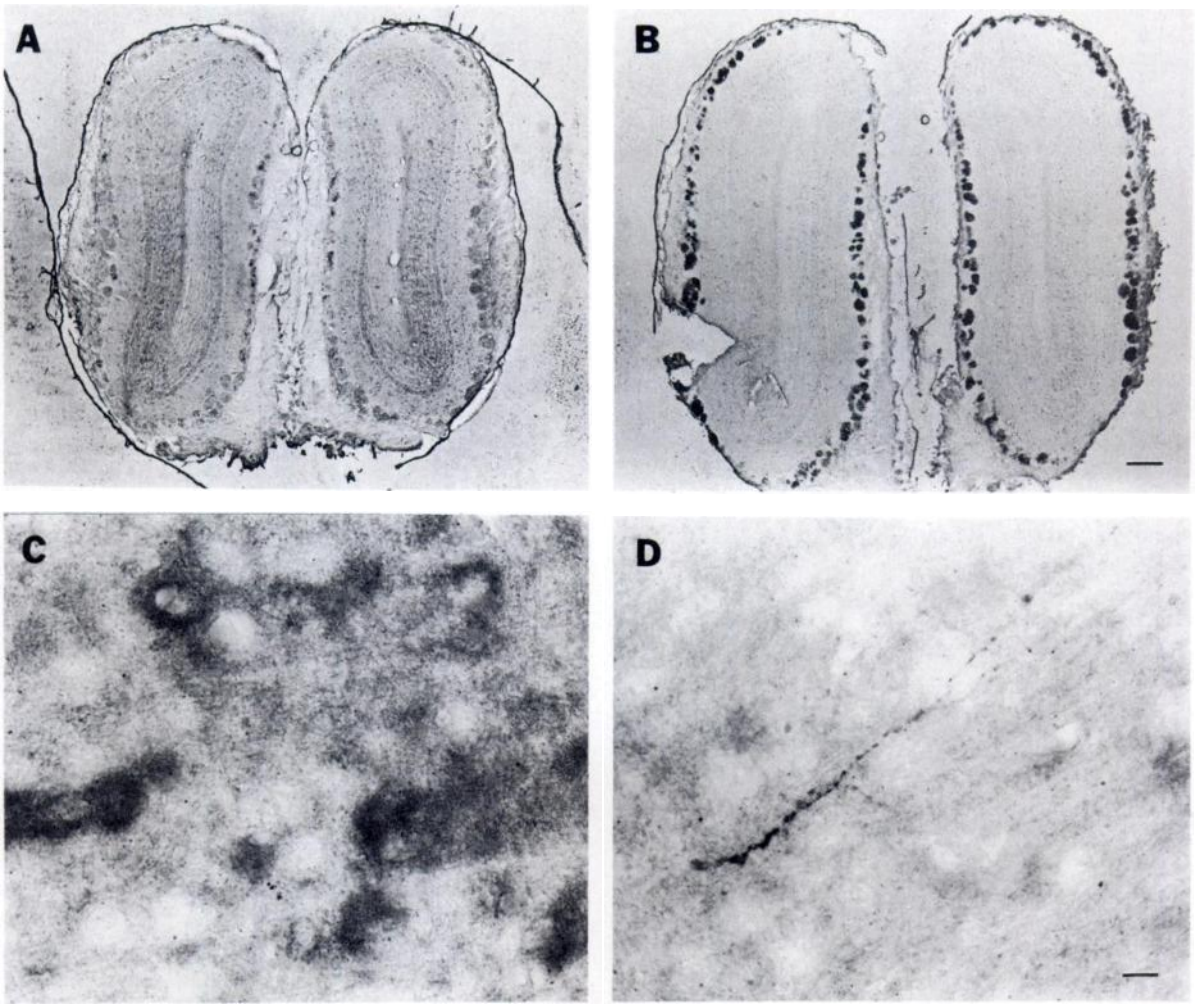

Fig. 5. Cupric silver degeneration stain of coronal sections of the olfactory bulbs (A and B) and the frontal cortex (C and D) prepared from rats sacrificed 4 days after the administration of the last of three daily dosages of saline (A and C) or IDPN (400 mg/kg/day) (B and D). Calibration bars = 250 μm and 6 μm for the olfactory bulbs and cortex, respectively.

classical histological stains [*e.g.*, dopaminergic terminal damage in striatum after MPTP (see Hallman *et al.*, 1985; O'Callaghan, 1991b); neuronal perikaryal damage in cortex after trimethyltin (see Brock and O'Callaghan, 1987; Balaban *et al.*, 1988)], yet such damage often is accompanied by a robust gliosis as evidenced by a large increase in GFAP (reviewed in O'Callaghan, 1991b). Therefore, we viewed the IDPN-induced increment in cortical GFAP as being a strong indicator of occult damage to this region. No evidence for a damage "hot spot" was found by analyzing GFAP levels in roughly defined divisions of the cortex; however, the possibility of selective damage (*e.g.*, to a specific cortical cell type or to a group of subcortical neurons with widespread synaptic fields in the cortex) cannot be ruled out. The possibility that the increase in cortical GFAP resulted from a direct action of IDPN on astrocytes, rather than as a consequence of damage-induced astrogliosis, seems unlikely in view of the discovery of argyrophilic neurons in this region (discussed below).

The dose-dependent nature of the GFAP response to IDPN suggests that chemically induced gliosis is not an all-or-none response but, instead, is graded with respect to the degree of damage. This finding is consistent with our experience with a variety of other neurotoxicants (for a review see O'Callaghan, 1991b). Although significant increments in cortical GFAP were not observed unless dosages of 400 mg/kg/3 days or higher were used, these exposure levels (1.2 g/kg over 3 days) were comparable, if not lower, than the doses used by Chou and Hartmann (1964) (2 g/kg in a single dose, or as four 0.5-g/kg doses over 10 days) and by Schultze and Boysen (1991) (4.4 g/kg, as 35 125-mg/kg doses over a 42-day period). Thus, our evidence for IDPN-induced cortical damage, despite the failure of previous studies to reveal significant pathology in this region, was not due to the fact that we used a higher dosage regimen. Finally, the fact that the cerebellum was not affected even at the highest IDPN exposure regimen not only confirms the regional specificity of the effects of IDPN, but also shows that widespread brain damage and gliosis is not the inevitable consequence of high (lethal) dosages of this compound.

Previous investigations using a variety of CNS injury models have shown that astrogliosis may follow markedly different time courses depending on the nature of the specific insult used (for a review, see O'Callaghan, 1991b; Norton *et al.*, 1992). This observation holds for chemical- as well as trauma- or disease-induced injuries of the CNS (Norton *et al.*, 1992). For example, the increase in GFAP caused by the dopaminergic neurotoxicant, MPTP, is rapid in onset but lasts only 2 weeks (O'Callaghan *et al.*, 1990). In contrast, the increase due to the organometal, trimethyltin, is slow in onset and lasts at least 12 weeks

(Brock and O'Callaghan, 1987), whereas hyperbilirubinemia induces an astrocyte reaction that lasts at least 1 year (O'Callaghan and Miller, 1985). In each case, there is a link between the time course of the GFAP response and the presence of the damage-inducing insult, suggesting that sustained cellular damage is required for maintaining the signal responsible for the continued increase in GFAP (see O'Callaghan *et al.*, 1990, for a more detailed discussion of this point). The increase in GFAP induced by IDPN in the cortex peaks around 1-week postexposure and declines thereafter (fig. 4). Although pharmacokinetic and metabolic data for IDPN have yet to be described in detail, there is evidence indicating that the presence of the chemical in the body is restricted to a period of a few days (Vivanco *et al.*, 1972; Williams *et al.*, 1970). These data suggest that the increase in GFAP due to IDPN follows a time course linked to a toxic action exerted by the acute presence of the toxicant, in agreement with what has been seen for other toxic exposures of the CNS (see O'Callaghan *et al.*, 1990).

Our observation of a large IDPN-induced increase in the olfactory bulb GFAP was consistent with our recent evidence of an IDPN-induced degeneration of olfactory epithelium (Genter *et al.*, 1992). Thus, degeneration of incoming axonal processes in the olfactory bulbs may account for the large increase in bulbar GFAP. The bipolar sensory neurons in the olfactory epithelium project into the olfactory bulb (see, *e.g.*, Switzer *et al.*, 1985), and it is known that axotomy of these neurons results in increased GFAP immunoreactivity in the olfactory bulb (Anders and Johnson, 1990). As with the cortical increases in GFAP, the effects of IDPN on GFAP in the olfactory bulb were not accompanied by evidence of obvious neural degeneration based on Nissl staining. These findings would be expected if, as we contend (Genter *et al.*, 1992), exposure to IDPN damages olfactory sensory afferents, the perikarya of which lies outside of the olfactory bulb proper (Switzer *et al.*, 1985; Anders and Johnson, 1990).

Under conditions in which traditional stains used in neuropathology fail to reveal evidence of neuronal damage, the application of sensitive silver methods often provides such information (Balaban *et al.*, 1988; Switzer, 1991; O'Callaghan and Jensen, 1992; Beltramino *et al.*, 1992). Moreover, enhanced expression of GFAP and the silver degeneration reaction (argyrophilia) appear to go hand-in-hand following the administration of a variety of neurotoxic agents (O'Callaghan and Jensen, 1992). The present data obtained for IDPN are consistent with these previous observations. Thus, a marked argyrophilic reaction in olfactory glomeruli and beaded argyrophilic fibers in the cortex were observed in conjunction with the increases in GFAP concentration found in these regions. In both the olfactory bulbs and the cortex, evidence of axonal degeneration was obtained with the de Olmos (1981) silver degeneration stain at a time point (4 days) that preceded the time point for the peak increase in GFAP (7–10 days) (Genter *et al.*, 1992). These data imply that the onset of axonal degeneration precedes the onset of gliosis as would be expected if the degenerating fibers provide the stimulus for the observed reactive gliosis. Thus, it appears likely that degeneration of sensory afferents in the olfactory glomeruli and degeneration of widely distributed axons in the cortex activate resident astrocytes and enhance the synthesis of GFAP.

The silver reaction for impregnation of degenerating neurons, like the astrocyte reaction to neuronal and glial injury, is a time-dependent process (Balaban *et al.*, 1988; de Olmos *et al.*, 1981). We used the relatively complete time course of the GFAP increase after IDPN to guide our choice of two time points for silver staining, *i.e.*, one before and one at the time of peak increments in GFAP. By using this strategy we were able to demonstrate a remarkable regional concordance between the presence of argyrophilic axons (4-days post-IDPN) and the subsequent increase in GFAP (7- to 10-days post-IDPN). At either the 4- or 10-day survival point, however, we did not observe fine argyrophilic debris in the cortex, an often characteristic feature of synaptic degeneration (de Olmos *et al.*, 1981). Given that the optimal time for impregnation of degenerating synaptic end structures occurs within 2 to 3 days postlesion (de Olmos *et al.*, 1981); if such damage did result from exposure to IDPN, our failure to see synaptic debris on postdosing days 4 or 10 would be expected. If our choice of survival times for silver staining were less than optimal, in general, this also may explain why we did not observe substantial evidence for neuronal degeneration in the pons medulla and midbrain. Based on our past experience (Balaban *et al.*, 1988), it is likely that a comprehensive silver staining time course analysis might be required to document the presence of degenerating neurons in all brain regions showing increments in GFAP.

Although it appears likely that axonal degeneration is responsible for IDPN-induced increments in GFAP, damage to non-neuronal cell types as a contributing factor cannot be ruled out. For example, the astrocyte (GFAP) response to IDPN in areas exhibiting the least amount of argyrophilic axons may be triggered by demyelination in the absence of substantial neuronal degeneration. The propensity of IDPN to cause secondary demyelination at lower levels of the CNS (Griffin and Price, 1981) gives support to this view. Moreover, demyelination is a potent stimulus for astrogliosis (Norton *et al.*, 1992) whereas silver staining appears to reveal primarily degenerating neurons (*e.g.*, see Carlsen and de Olmos, 1981; de Olmos *et al.*, 1981; Switzer 1991; Beltramino *et al.*, 1992). Quantification of myelin basic protein or an electron microscopic analysis of cortical myelin may provide a resolution to this question.

In summary, our findings provide strong evidence that IDPN damages areas of the rat CNS above the level of the brain stem that have escaped detection by the application of traditional histological stains. Our data may constitute the first evidence for neuropathological effects that underlie or contribute to the functional deficits resulting from exposure to IDPN. At the least, our findings underscore the need for further evaluation of morphological, biochemical and functional effects of IDPN in relation to damage of neural substrates above the level of the brain stem.

Acknowledgments

The authors thank Drs. S. Barone, Jr. and D. B. Miller for a critical review of the manuscript and Dr. R.C. Switzer III for productive discussions.

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