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BRAIN GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP) AS A MARKER OF NEUROTOXICITY DURING INHALATION EXPOSURE TO TOLUENE

Health and Environmental Sciences Department
Publication Number 4647
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Brain Glial Fibrillary Acidic Protein (GFAP) as a Marker of Neurotoxicity During Inhalation Exposure to Toluene

Health and Environmental Sciences Department

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PREPARED UNDER CONTRACT BY:

HUGH L. EVANS, PH.D.
NEW YORK UNIVERSITY MEDICAL CENTER
NELSON INSTITUTE OF ENVIRONMENTAL MEDICINE
TUXEDO, NEW YORK 10987

JUNE 1997

American Petroleum Institute

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API STAFF CONTACTS

Dr. Robert Drew, Health and Environmental Sciences Department
David Mongillo, Health and Environmental Sciences Department

MEMBERS OF THE NEUROTOXICOLOGY TASK FORCE

Wayne Daughtrey, Exxon Biomedical Sciences, Inc

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ABSTRACT

Glial fibrillary acidic protein (GFAP) was measured during and after sub-chronic exposure to toluene. Rats received inhalation exposure to air or 100 - 3,000 ppm toluene, 6 hr/day, 5 days/wk for up to 42 days. Toluene, in concentrations that are low for the rat (100 to 1,000 ppm), altered GFAP and motor behavior without affecting body weight or producing overt signs of neurotoxicity. However, the declines in GFAP concentration during toluene exposure differ from the more commonly reported toxicant-induced pattern of increased GFAP. At a higher concentration (3,000 ppm), toluene produced increased GFAP concentrations, observable neurological signs and weight loss. These results are discussed in relation to methodological issues and the relevant scientific literature. GFAP can provide an index of toxicity, even with exposures below the level which produce overt signs of toxicity. For toxicity screening with animals, a battery including GFAP as well as behavioral and neurochemical measures would be useful. Implications for future research are discussed.

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EXECUTIVE SUMMARY

Measures of brain cell-specific proteins show promise as markers of neurotoxicity in animals, particularly after exposure to heavy metals. One such marker is glial fibrillary acidic protein (GFAP). Increased GFAP indicates reactive gliosis following neuronal injury from toxic exposures. Modern biochemical techniques for measurement of GFAP may prove to be faster, less expensive and more quantitative than classical neuropathological examination, and thus may be useful for evaluating potential neurotoxins. The purpose of this study was to determine whether an immuno-assay for GFAP in the rat's brain can provide practical evidence of toluene-induced neurotoxicity. The U.S. Environmental Protection Agency (USEPA, 1994, 1995) has suggested that a Radio-Immune-Assay (RIA) of brain GFAP be used in the screening for neurotoxicity of chemicals. Previous findings reported to API that an Enzyme-Linked-Immuno-Sorbant Assay (ELISA) of GFAP yielded results similar to results from the older RIA method and that the ELISA was sensitive to repeated oral exposure to lead (Pb) at exposure levels which produced behavioral and histological evidence of neurotoxicity (Evans, 1994a). The ELISA method has two advantages over the RIA method: freedom from radioactive materials, and simplicity. Although GFAP was a useful marker of Pb-induced neurotoxicity, GFAP was a less useful marker of Pb exposure than traditional indices such as blood lead concentration (Evans, 1994a).

Toluene was chosen as a model neurotoxicant for these studies because its neurotoxicity in the rat has been characterized. The present studies documented changes in GFAP concentration during subacute inhalation exposure to toluene. Adult male F344 rats, at approximately 47 days of age, received inhalation exposure to room air or 100, 300, 1,000 or 3,000 ppm toluene, 6 hr/day, 5 days/wk for up to 42 days. These exposures approximate an occupational exposure schedule. During and after exposure, the concentration of GFAP was determined in four brain regions. These changes in GFAP were compared with standard neurotoxicity criteria: behavioral or neuropathological changes. Body weight was monitored as a sign of general toxicity.

The toluene concentration-effect data for GFAP concentration suggest that 50% of brain samples are affected by an exposure of at least 3 days to 1,000 ppm toluene. At concentrations that are quite low with respect to the literature on the laboratory rat (100 to 1,000 ppm), toluene altered GFAP concentration without affecting body weight, brain pathology or producing overt signs of neurotoxicity. Changes in GFAP were seen as early as the third day of exposure; however, the declines in GFAP concentration differ from the more commonly reported toxicant-induced pattern of increased GFAP. GFAP was affected by toluene concentrations as low as 100 ppm, within the range of occupational exposures for humans. In contrast, a much higher concentration (3,000 ppm) of toluene impeded growth and caused observable neurological signs in the rats, confirming previous reports of toluene's toxicity at high concentrations. Increased GFAP after 7 days exposure to 3,000 ppm is suggestive of reactive gliosis, but cellular damage was not investigated at 3,000 ppm. At 1,000 ppm, cellular damage could not be seen at the light microscopic level.

The time-effect data suggest that, as toluene exposure continued, significant changes in GFAP appeared, then reversed as exposure duration continued. There was no evidence of permanent nervous system damage or functional impairment. For example, significant increases in GFAP at 42 days of exposure to 1,000 ppm toluene had returned to control levels by 14 days after exposure. No behavioral changes could be detected in the home cage in the 24 hours after the most recent exposure.

The information provided by GFAP is partly correlated with, but not redundant to, that available from standard assays of behavior and general signs of toxicity such as body weight. GFAP was clearly more sensitive to toluene than histopathology indicates at the light microscopic level. GFAP was nearly equal to the sensitivity of behavioral measures, keeping in mind that the most sensitive behavioral index was recorded during toluene inhalation, whereas GFAP was measured 24 hours or more after the last exposure, at a time when behavior in the home cage and neuropathological indices were unaffected. GFAP was of similar sensitivity to physiological

indices of inhaled toluene, as reported in the literature. The most sensitive indices at present are those reflecting changes in brain neurotransmitter function.

Because the direction of changes in GFAP concentration was inconsistent as repeated toluene exposure continued, GFAP alone may not provide a practical marker of the effects of short term occupational exposure to toluene. Measurement of GFAP concentration with an ELISA should be an element in toxicity screening batteries, along with behavior and indices of neurotransmitter function. An inter-laboratory workshop would be useful to advance the standardization of the GFAP ELISA. Further research is needed to identify how toxicant-induced changes in GFAP concentration are influenced by sub-types of astrocytes, changes in expression of the GFAP gene, adrenal cortical steroid production, or neurotransmitter function.

Section 1
INTRODUCTION

The nervous system is a target organ for inhaled toluene (ATSDR, 1994; Morata *et al.*, 1995) and for many other organic solvents (Arlien-Soborg, 1992). Exposure to solvents has been alleged in neurobehavioral disorders. The cellular and molecular mechanisms by which inhaled toluene causes changes in function of the central nervous system are not well understood (ATSDR, 1994). This is not surprising, since little more is known about the cellular and molecular mechanisms of action of inhaled volatile anesthetics, despite the routine use of those chemicals in human surgery (Pocok and Richards, 1993; Snyder and Andrews, 1996).

The detection of the effects of inhaled toluene, and an understanding of the mechanisms of neurotoxicity, may be indicated by molecular markers. Solvents and their metabolites are cleared rapidly from the body (Brugnone *et al.*, 1995) and markers of solvent neurotoxicity have not been validated for peripheral media, e.g., blood, urine (ATSDR, 1994; Tardif *et al.*, 1991).

Most promising as markers of neurotoxicity in animals are measures of brain cell-specific proteins (Evans, 1995; Aschner and Kimelberg, 1996). One such marker is GFAP, the major intermediate filament of astrocytes. When reactive gliosis accompanies brain injury, GFAP is increased. Gliosis is observed in the brains of humans who died of solvent inhalant abuse (Kornfeld *et al.*, 1994). GFAP has been suggested as a marker for solvent-induced neurotoxicity in animal studies (Arlien-Sorborg *et al.*, 1992). Two reports indicate that inhalation of organic solvents may affect brain GFAP content in rats (Rosengren *et al.*, 1986; Rosengren and Haglid, 1989). Inhalation of toluene was observed to increase or decrease the concentration of several protein markers of astrocytes in the rat's brain, although GFAP was not studied (Huang *et al.*, 1990 and 1992).

Modern biochemical techniques for measurement of GFAP may prove to be faster, less expensive and more quantitative than classical neuropathological examination, and thus may be

useful for toxicity testing. GFAP measurement is now included in Neurotoxicity Test Battery guidelines (USEPA, 1994, 1995). The present studies evaluated the concentration of GFAP in the rat's brain as a marker of toluene exposure and of toluene's neurotoxicity. This permits the evaluation of a simplified ELISA assay for GFAP (O'Callaghan, 1991) for studies in toxicology.

The effects of inhaled toluene upon GFAP were compared with effects of toluene on locomotor behavior and brain histopathology. Behavior is one of the more sensitive indices of toluene's effects (Baker, 1994; Burbacher, 1993; Wood, 1994; Snyder and Andrews, 1996). Neuropathological change has been described with the extremely high concentrations which occur in solvent abuse (Kornfeld *et al.*, 1994; Rodvall *et al.*, 1996) and in experiments with animals (Korbo *et al.*, 1996; Pryor and Rebert, 1993). Together, behavioral and histopathological measures provide two standard indices of neurotoxicity for the evaluation of the results from GFAP assays.

Section 2
METHODS

ANIMALS

Male F344 rats (Taconic Farms, Germantown, NY), weighing 76 to 100 g (age 40 days) upon arrival in the lab, were housed in pairs in plastic shoe box cages (25.9 cm W x 46.9 L x 20.8 H) with wood chips as bedding and *ad lib* access to tap water and to food (Rodent Laboratory Chow 5001®, Ralston Purina Corp., St. Louis, MO). Before toluene exposure began, rats were quarantined for 7 days, weighed and observed to ensure health and to determine baselines. Prior to each toluene exposure session, animals are transferred from home cages to stainless steel mesh exposure cages. Food and water are not available during inhalation exposures so as to prevent uncontrolled exposure by the oral route. This research was approved by the institutional animal care and use committee and conformed to the current animal care guidelines of state and federal agencies.

EXPOSURE TO TOLUENE

Groups of up to 32 rats were exposed to 0 ppm (conditioned air) or toluene (100, 300, 1,000 and 3,000 ppm) in dynamic exposure chambers, 6 hr daily, Monday through Friday. This exposure schedule was planned to simulate an occupational exposure. Inhalation exposures are conducted in either 1.3 or 0.13 m^3 stainless steel chambers. Control rats were exposed in separate chambers to filtered, conditioned air concurrently with the toluene groups. Control animals are housed and transported separately from test animals, to prevent unplanned exposure of control animals to experimental compounds which may linger in the fur or other body constituents of test animals.

Chamber atmospheres were maintained as described in published work (Dempster *et al.*, 1984). In brief, high concentrations of solvent atmospheres are generated by first producing an aerosol of the solvent by means of a Laskin nebulizer, and then feeding the aerosol into a heated vessel to vaporize the solvent droplets. Low concentrations of solvent are generated by passing an air

stream over the surface of the liquid solvent and feeding the resultant solvent-laden air into the chamber. Reagent grade toluene was provided by the API.

Concentration of toluene in the test chamber, determined by infrared analyzer (MIRAN/IACVF®, Foxboro Analytical, So. Norwalk, CT), using a 9.8 micron wavelength, was compared to the nominal chamber concentration determined from the total volume of toluene used each day for each chamber. Chamber atmospheres, temperature and relative humidity measurements are taken at 30 min intervals during the daily exposures. Mean toluene exposures were kept within ±10% of the nominal concentration.

BODY WEIGHT

Body weight was determined in the afternoon, when the rats were removed from the inhalation exposure, using a digital integrating balance (Sartorius® # 1403-MPZ, Sybron/Brinkmann Co., Westbury, NY) with an accuracy of ± 0.1 gas described by Evans *et al.*, 1986.

THYMUS AND ADRENAL GLAND WEIGHT

Rats exposed to 1,000 ppm toluene had their adrenal and thymus glands removed when decapitated after 3 or 7 days exposure. The wet weight was recorded, then expressed as a ratio to the body weight.

LOCOMOTOR BEHAVIOR

Behavior of pairs of rats was measured in their home cage, after the conclusion of a 5-day week of daily toluene inhalation exposures, and inside the inhalation chamber, during selected exposures to toluene or filtered air. Behavior was automatically measured by a computer at regular intervals using a system of photocells surrounding the cage (Evans *et al.*, 1986; Evans, 1989). The post-exposure studies used a stainless steel mesh home cage (17.8 cm W x 30.0 L x 20.3 H; Evans *et al.*, 1986) and recorded locomotion and rearing separately. During inhalation exposure, it was possible to record only a single index, a composite of total locomotor behavior, because less equipment could be fitted around the inhalation holding cage (stainless mesh

21.6 cm W x 27.9 L x 20.8 H). Measurement of behavior during inhalation exposure was done only for 0, 100 and 300 ppm exposures because the behavioral effects of ≥ 1,000 ppm toluene have already been reported (Wood, 1994). These behavioral measurements are quite similar to those of the EPA Neurotoxicity Guidelines for motor activity (USEPA, 1991), except that this study's data consisted of the total activity produced by each pair of rats inside each cage.

NEUROPATHOLOGY

Brains were perfused before being removed for histology, while fresh brains were used for GFAP protein assay (see below). When significant GFAP results had been determined, a sample of 3-4 brains from rats having the same level and duration of exposure were taken for histopathology. The numbers of rats were as follows: Control (N = 8), 100 ppm for 3 days (N = 3), 1,000 ppm for 3 days (N = 5), 1,000 ppm for 39 days (N = 8).

Rats were anesthetized with sodium pentobarbital, then perfused transcardially with 10% neutral buffered formalin. The brains were then removed from the skull, kept in formalin at 4° C for 24 hr, and sectioned coronally at 3 levels (frontal region: usually at or rostral to the optic chiasm; parietal region: level of the pyriform lobe; cerebellum/pons), so that the histology slides demonstrate the same brain regions that had been assayed for GFAP. The tissue blocks were processed through graded alcohols, cleared in xylene and embedded in EM 400® paraffin (Surgipath). Sections were cut from these blocks at 8 μm thickness for hematoxylin and eosin (H & E) staining and 5 μm for GFAP immunohistochemistry. The slides were evaluated, in a blinded fashion, for qualitative and semi-quantitative observations. Following this, the slides were decoded, and re-examined.

TOTAL PROTEIN IN THE BRAIN

Total protein in each brain specimen was determined using the method of Smith *et al.*, (1985) with the BCA Total Protein Assay Kit® (Pierce, Rockford, IL). Data from the GFAP assay were normalized for total protein of the same sample.

GFAP

Fresh brains were used for GFAP determinations. Groups of 8 rats were sacrificed by decapitation at each duration of exposure, and for each toluene concentration. Brains were immediately removed, placed upon a cold plate and dissected into 4 regions using a stereotaxic atlas as a guide (Paxinos and Watson, 1986). The regions were the cerebellum, hippocampus, thalamus and cerebral cortex. The initial study of this series also examined spinal cord, olfactory bulbs and striatum; these did not contain significant toluene-related changes and thus were not included in the followup studies. Specimens were weighed (Mettler® #AJ100 analytical balance, ± 0.1 mg), snap-frozen and stored at -80°C.

GFAP was assayed by an ELISA (sandwich format, microtiter plate-based Enzyme Linked ImmunoSorbent Assay) following the method of O'Callaghan (1991). Flat-welled Immulon® microtiter plates (Dynatech, Chantilly, VA) were coated (1.0µg/100µl /well) with a capture antibody, polyclonal anti-GFAP (Dako, Carpenteria, CA) for 1 hr at 37°C. Microtiter plates were washed with pH 7.4 phosphate-buffered saline (PBS), incubated for 1 hr with 5% nonfat dry milk (in PBS) to block nonspecific binding, then incubated with 100µl of sample or standard for 1 hr. Plates were washed with PBS containing 0.5% Triton X-100, then loaded with 100µl of monoclonal anti-GFAP (Boehringer Mannheim, Indianapolis, IN) for 1 hr at a dilution of 1:500, thus sandwiching the sample GFAP between the two antibodies. Plates were washed again with 0.5% TX-100 in PBS and coated with alkaline phosphatase conjugated anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) for 30 min to tag the monoclonal anti-GFAP. The wash was repeated and the p-nitrophenylphosphate substrate was added which generated a colorimetric reaction. The reaction was stopped by adding 0.4M NaOH (100 ml/well) when the standards showed a broad range of color change (10-30 min). Absorbance was read at 405nm in a microtiter plate reader (Anthos/Denley 2001®, Denley Instruments, Durham, NC).

GFAP was measured against a standard curve composed of serial dilutions from a homogenate of hippocampus from control rats. The homogenate was calibrated using pure GFAP by the method of additions. Standard curves were generated by a logit transformation of the absorbance data

and log of total protein. Asymptotic data points at the extreme lower or upper limits of the standard curve were excluded, taking care that there were sufficient points in the middle portion of the curve so that the least squares linear regression of the log of the GFAP concentration upon the logit of O. D. value yielded a correlation coefficient of > 0.9. Concentrations of GFAP in brain were then calculated from this regression equation.

Assays were performed in batches by exposure-duration, so that all results for a given exposure-duration could be compared directly to age-matched controls. To minimize variability due to different assays performed at different times during the course of these studies, each assay batch included specimens from an appropriate control group for comparison with data from toluene-exposed specimens in the same batch of assays. The literature reports minimal age-related changes in GFAP over periods of several weeks in the young adult rat (O'Callaghan and Miller, 1991; Wagner *et al.*, 1993).

When potentially significant changes in GFAP were observed, the same brain specimens were subject to a replicate assay (Figure 1, p.2-6). If the results from the replicate assay confirmed the original data, then the results of both replications were subjected to analyses of variance (ANOVA), with replications as one factor. (See the discussion in STATISTICS, p. 2-7.) GFAP was reduced in the hippocampus of rats after 21 days of exposure to 100 or 1,000 ppm toluene. The specimens were removed from the freezer 3 times and assayed 3 times to give the results shown in Figure 1. Replications were used as a grouping factor in the ANOVA statistical tests. The mean of the 3 replications (shown on the right) is used as the final result in the remaining figures of this report.

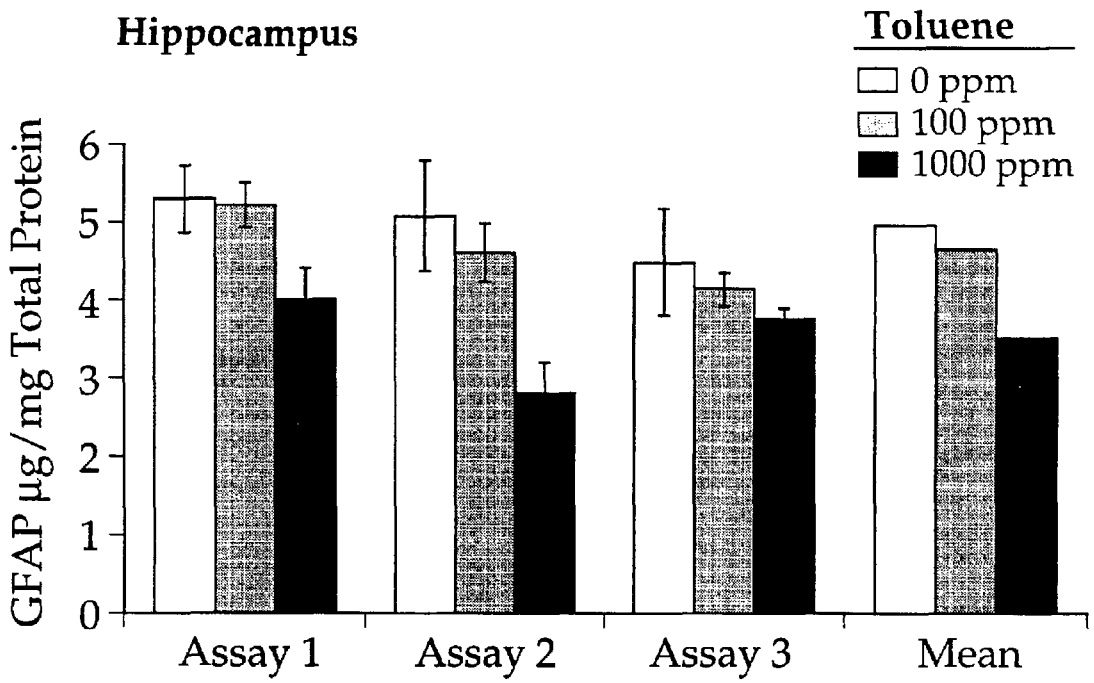

Figure 1. The Replication of GFAP Assay Results.

CORTICOSTERONE

The role of corticosterone (cort) in toluene-induced changes in GFAP was investigated in study 4 (Table 2, p. 3-10). Quantification of corticosterone in serum was done by High Performance Liquid Chromatography (HPLC) based on the method of Woodward and Emery (1987). Briefly, cort was extracted from serum collected at the time of sacrifice, between 400-1,000µl. Each sample was transferred to 13X100mm glass test tubes and 5.0µg/ml 19-Nortestosterone (19-NT, Steraloids, Wilton, NH) was added (for 1ml sample, 100µl 19-NT). Next, (for 1ml sample) 150µl of 0.3M NaOH was added to prevent extraction of phenolic contaminants. Cort was then extracted into 5ml (60:40v/v) diethyl ether-dichloromethane (Fisher Scientific). Samples were vortexed 30 sec each and immediately centrifuged at 2,000 RPMs for 10 min. The supernatent (4ml) was transferred to another glass test tube and 1ml of HPLC-grade water was added to each and vortexed for 30 sec. The samples were centrifuged as before and supernatent (3ml) was transferred to a glass test tube and evaporated under argon at room temperature. Samples were covered and stored at -20°C until assay when they were dissolved in 100µl methanol.

For chromatographic analysis, HPLC-grade water and methanol were degassed and filtered before use and delivered separately with a dual pump system. A Beckman Ultrasphere® ODS analytical column (4.9mmX250mm; particle size 5µm; Waters) was equilibrated using methanol-water (70:30v/v; Fisher Scientific). The flow rate was 1ml/min. Injection volume was 20µl. Separations were made at ambient temperature (23-25°C) and eluate was monitored at 250nm. The following standards were made in methanol: 2.3µg/ml 19-NT; cort (Steraloids, Wilton, NH) at 0.50, 1.25, 2.50, and 5.00µg/ml. This produced a linear standard curve from which the cort in the samples could be calculated based on the area under the curve. Areas of peaks were analyzed using Waters 840 Chromatography Data System software. Samples and standards were injected manually using a 20µl loop. R_t values were 6.6 and 8.9 min for cort and 19-NT, respectively.

STATISTICS

Statistical significance was tested by multi-factorial analyses of variance (ANOVA) for GFAP data or repeated-measures analyses of covariance (ANCOVA) for behavior or body weight from the BMDP library (Dixon, 1990; Dixon and Meridan, 1992). The covariate was the baseline score of each rat, as determined prior to exposure. The ANCOVA main effects (dose and duration of exposure) were analyzed by the BMDP program 5V using the Wald Chi-Square test, which can be used when some data are missing (Dixon, 1990; Dixon and Meridan, 1992).

Significant results were subsequently analyzed with the Student's t-test, one-way ANOVA or one-way ANCOVA (BMDP 2V) to determine the earliest significant time point. The criterion of significance was $p \leq 0.05$.

Section 3
RESULTS

This research was done in four studies, because there were not enough exposure chambers to provide for all toluene concentrations, and because the results of the initial studies were needed to plan the later studies. In each study, a group of young rats was purchased and prepared for inhalation exposure.

BODY WEIGHT

Table 1 shows that after 42 days of exposure up to 1,000 ppm toluene, there were no significant differences in body weight related to toluene exposure. Seven days exposure to 3,000 ppm was sufficient to retard the growth of body weight to 84% of the mean weight of control rats given 7 days of sham exposures ($F = 36.50$, $df = 1,17$).

Table 1. Body Weight during Exposure to Toluene

Toluene (ppm)	Pre-exposure	After 42 days exposure
0	117(7.6), 32*	253(15.3), 8
100	120(6.1), 32	261(14.4), 7
300	119(5.7), 32	258(9.4), 8
1,000	120(4.8), 32	256(10.3), 8

* = Mean body weight in grams (SD), Number of rats

THYMUS AND ADRENAL WEIGHT

There were no significant differences in gland weights between the control group and rats exposed to 1,000 ppm toluene after 3 or 7 days exposure.

BEHAVIOR DURING TOLUENE INHALATION

Locomotor behavior was less frequent during exposure to 100 and 300 ppm toluene when compared to the pre-exposure baseline or to the sham-exposed control group. Figure 2 (p. 3-3) illustrates the habituation pattern in which the total amount of behavior declined over the first

two test sessions, as the rats become acclimated to the handling and stimuli of the exposure chamber. Exposure to 300 ppm significantly depressed behavior compared to the matched control group (main effect for toluene $X^2 = 4.5$, df = 1), and this effect increased with duration of exposure (toluene x exposure duration interaction $X^2 = 20.0$, df = 4). The difference between 0 ppm and 300 ppm was not significant on the first test (Day 7), but was significant thereafter.

Exposure to 100 ppm significantly reduced behavior compared to the matched control group ($X^2 = 6.66$, df = 1). The interaction of toluene x duration of exposure was not significant for 100 ppm, indicating that the effect of toluene neither increased nor decreased with exposure duration. The significance of the effect of 100 ppm toluene is further indicated by the return to control values following the end of exposure to 100 ppm (weeks 2 and 4 after exposure in Figure 2, p. 3-3).

Behavior inside the chamber was not studied with 1,000 or 2,000 ppm, because of ample evidence in the literature of behavioral effects of toluene at 1,000 ppm and above. However, rats exposed to 3,000 ppm were observed to be inactive and ataxic when they were removed from the inhalation chamber after 6 hours exposure. This was noticeably different from the appearance of control rats who were active and agile.

BEHAVIOR AFTER TOLUENE INHALATION

Rearing and locomotor behavior were recorded in the home cage over the weekend after 5 days of exposure to either 0 ppm, 100 ppm or 300 ppm toluene. These observations began 1 hr after the most recent toluene exposure and concluded 48 hr later. Data were obtained from 4 to 8 cages with 2 rats in each cage. Toluene-exposed rats were not significantly different from sham-exposed controls in total rearing, total locomotion, nor in the diurnal pattern of either behavior (data not shown).

Behavior inside the inhalation exposure chamber was recorded every Friday, during the fifth consecutive exposure of the week. During exposure to 100 ppm toluene, locomotor behavior in

the inhalation chamber was significantly below the control group (0 ppm) for the 7 weeks of exposure to toluene. Weekly tests continued for 4 more weeks inside the exposure chamber, but without toluene exposure, during which time the behavior of rats previously exposed to 100 ppm toluene recovered to levels approximating those of the control group. Baseline data reflect the first introduction of the rats into the exposure chamber, without toluene.

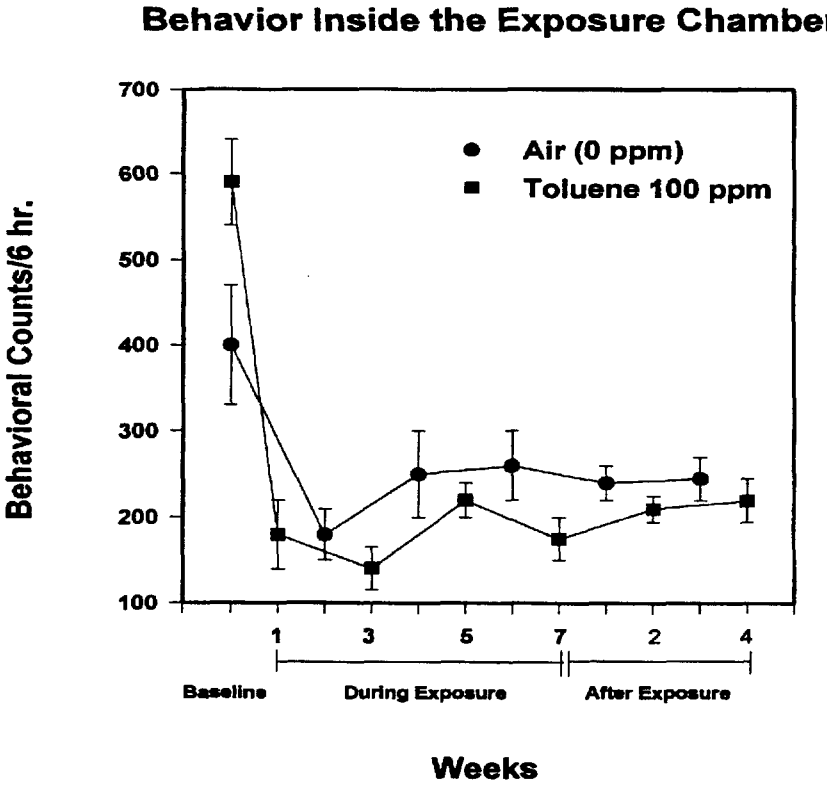

Figure 2. Behavior during Toluene Inhalation and Post-exposure.
Each point shows the mean ± SEM, N = 10 to 12.

NEUROPATHOLOGY

No neuronal damage was observed with H & E stain at the light microscopic level. Toluene exposures which significantly affected GFAP protein concentration caused similar alterations in brain specimens stained for GFAP immuno-reactivity.

QUALITY CONTROL: VARIABILITY IN PROTEIN DATA

Because an aim of this project was to appraise the GFAP assay for application to toxicity testing, several factors have been identified which may help to minimize unwanted variability in the

biochemical assays. Although most of these points will be familiar to experienced researchers, these points would be especially valuable to laboratories setting up to run this assay.

Tissue preparation. The dissection criteria are important. A training session and inter-lab comparisons should be conducted periodically. Unusual tissue weights may reveal dissection error.

Total protein. Glassware cleaning must receive normal degree of care. Glassware should be soaked in soap overnight then carefully washed to eliminate all traces of protein. The microtiter plates must be handled carefully to avoid contamination with extraneous protein. Incomplete sonification of brain tissue can be a problem, causing minute specks of intact tissue to be suspended, potentially overloading a well. Total protein results should be scrutinized to confirm they are within the range of values which are typical for each brain region, both from the lab's own historical data and from the published literature, then subject to statistical analyses to determine if there are significant differences between batches of control specimens, and if there are significant effects related to toxicant dose.

GFAP. It is preferable to prepare multiple aliquots of each brain homogenate immediately at the time the animal is sacrificed, so as to allow for the assay to be repeated as a replication and quality control without repeated thawing, as would be the case if only one aliquot were prepared. However, the multiple aliquots are costly, both in staff time and in the greater amount of freezer space required. The GFAP assay should be set up so a number of samples can be completely assayed in one long working day; an experienced technician can complete 8 plates (each with 40 samples in duplicate). In order to minimize staff scheduling problems, sample dilutions may be prepared and total protein determined in brain specimens the day before the GFAP assay; sample dilutions for GFAP assay may be kept overnight at -80°C, then thawed for the GFAP assay the next morning. Other schemes for breaking up the assay into 2 days' work have been less successful, inducing variability. Selection of the standard data is important; an internal standard

can be placed on each microtiter plate. Plate-to-plate variability of standard data should be <10%, ideally ≤5%.

BRAIN TOTAL PROTEIN

There were no significant changes in total protein related to toluene exposure.

BRAIN GFAP

Control rats had the highest GFAP concentrations in the hippocampus and cerebellum, confirming previous findings (Evans, 1994a; Evans *et al.*, 1993; Gong *et al.*, 1995; O'Callaghan, 1991).

When significant (or near-significant) changes in GFAP were observed, the brain specimen was assayed again to replicate the original finding. In a few instances, a specimen was assayed 3 times, e.g., Figure 1, p. 2-6.

The results are presented in terms of time-effect, from the shortest duration of exposure to the longest in this series of studies. No significant changes were observed in rats sacrificed after 1 day exposure to toluene.

Day 3 of toluene exposure was the shortest exposure duration to affect GFAP. The thalamus and cerebellum were the brain regions showing the earliest change in GFAP. GFAP concentration increased in the cerebellum after exposure to 100 or 1,000 ppm (Figure 3, p. 3-6). The main effect for toluene doses was significant ($F = 4.18$, $df = 2,21$). Both 100 ppm ($F = 35.30$, $df = 1,14$) and 1,000 ppm ($F = 5.42$, $df = 1,14$) were significant. Results for 300 ppm were not significant. There also was a slight increase ($p = 0.13$) in the hippocampus (Figure 4, p. 3-6). GFAP in the thalamus declined on Day 3 after 100, 300 and 1,000 ppm (Figure 3, $F = 9.46$, $df = 2,20$). The decline after 300 ppm was significant ($F = 219.42$, $df = 1,13$) and the decline after 1,000 ppm was significant ($F = 33.63$, $df = 1,14$).

These were the earliest changes in GFAP in the present studies. The lowest toluene concentration to affect GFAP was 100 ppm, shown in the data for cerebellum (left side of Figure 3, below). Data are means (± SEM) of replicate assays of 6-8 specimens each.

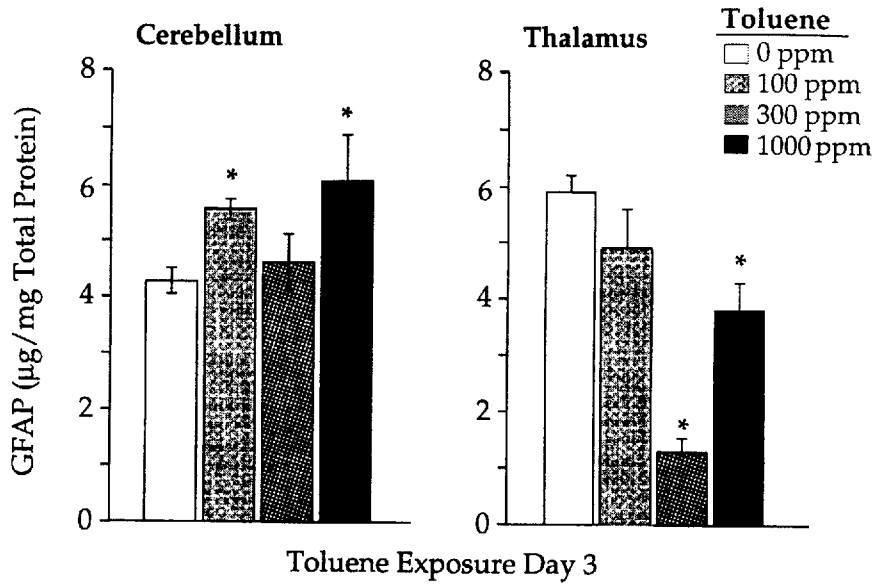

Figure 3. Significant Changes in Brain GFAP after the Third Day of Exposure to Toluene. Asterisks indicate individual means which differed significantly from control (0 ppm).

On Day 7, GFAP was increased in the hippocampus of rats exposed to 3,000 ppm toluene (Figure 4). The difference between 0 ppm and 3,000 ppm was significant ($F = 5.22$, $df = 1,18$). The increase on day 3 was nearly significant ($p = 0.13$)

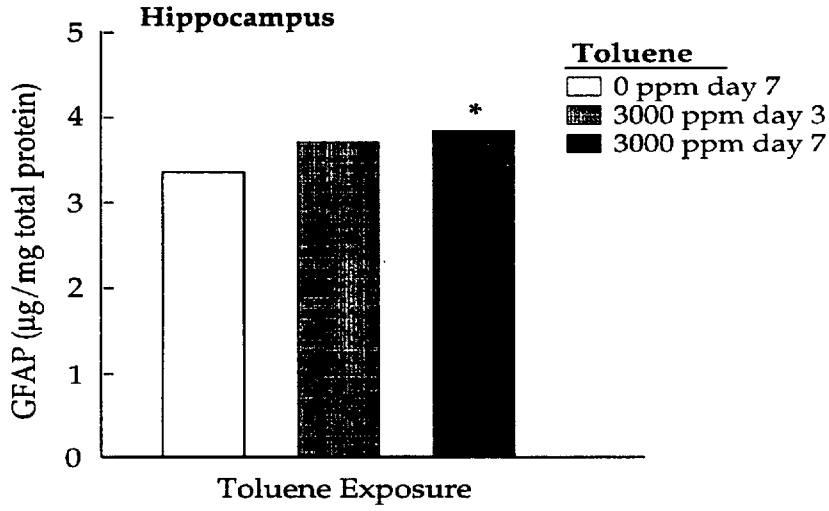

Figure 4. Increased GFAP in the Hippocampus of Rats Exposed for 3 and 7 Days to 3,000 ppm Toluene. N = 10 rats for each point. (Data as in Figure 3.)

On Day 21, exposure to 1,000 ppm toluene resulted in *decreased* GFAP concentrations in the hippocampus (Figure 1, p. 2-6, and Figure 5, below). The 3-dose (0, 100, 1,000 ppm) x 3-replications ANOVA was significant for the toluene main effect ($F = 4.20$, $df = 2,21$). This decline was significant for 1,000 ppm ($F = 5.74$, $df = 1,14$) but not for 100 ppm.

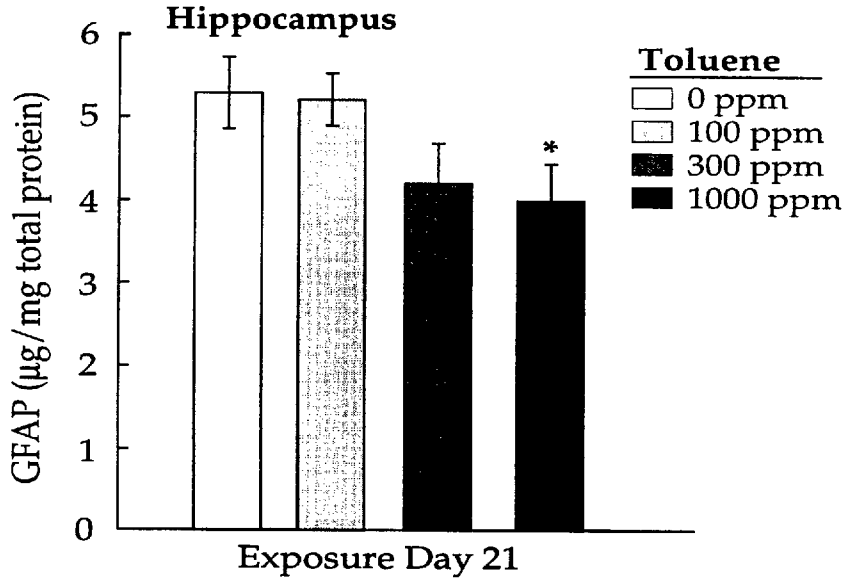

Figure 5. Effects of 21 Days Exposure to 100, 300 or 1,000 ppm Toluene on GFAP in the Hippocampus. Each point is the mean of 8 rats.

After 42 days of exposure, the longest exposure duration studied here, GFAP was significantly increased in the cerebellum with 300 ppm (Figure 6; $F = 11.32$, $df = 1,14$) and with 1,000 ppm (Figure 7; $F = 4.62$, $df = 1,13$). GFAP remained elevated in the cerebellum 7 days after the end of the 42-day exposure to 1,000 ppm ($F = 4.43$, $df = 1,14$), but was not different from control at 14 days post-exposure. GFAP of the 300 ppm group was unchanged at 7 and 14 days post-exposure (Figure 7, p. 3-8).

GFAP was increased above the concentration of age-matched control rats at each time sample. Evaluation of individual days indicated that the increase was significant only at Day 42. GFAP was significantly elevated on the last day of exposure (Day 0 of recovery) and on Day 7 after the last exposure. GFAP also was elevated on Day 42 of exposure to 300 ppm (Figure 6, p. 3-8) but not after exposure to 100 ppm.

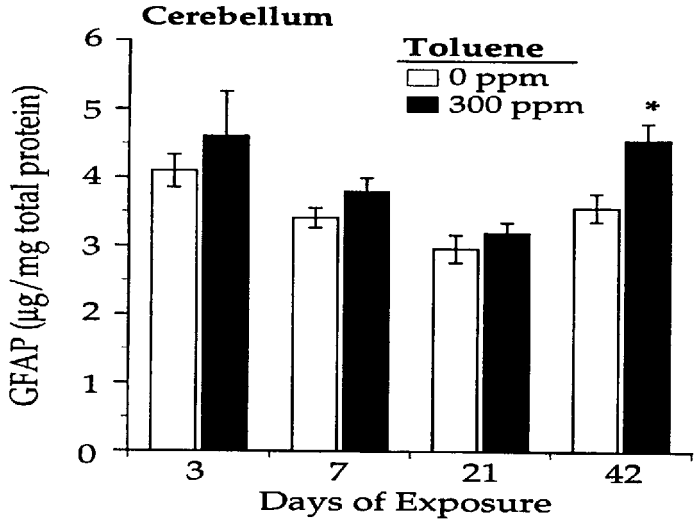

Figure 6. GFAP in the Cerebellum during 42 Days Exposure to 300 ppm Toluene.

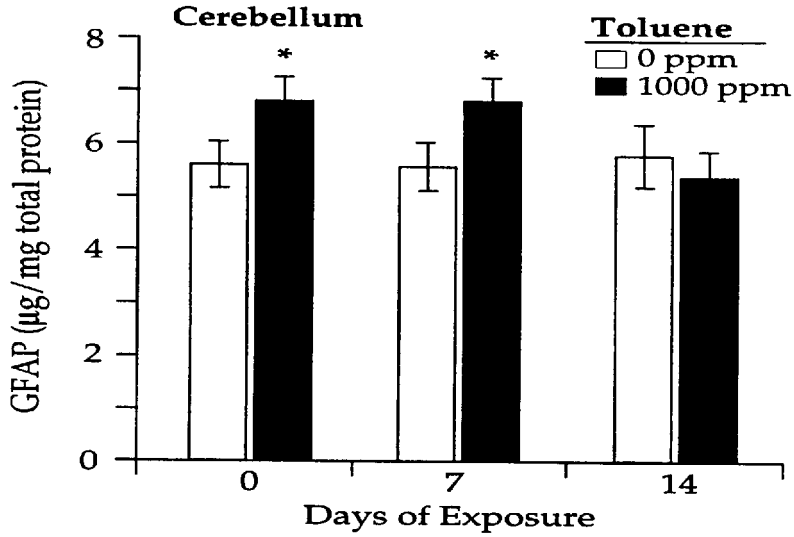

Figure 7. GFAP in the Cerebellum Returned to Baseline after 42 Days of Exposure to 1,000 ppm Toluene.

CORTICOSTERONE

In order to investigate the role of serum corticosterone in the toluene-induced decreases of GFAP in the thalamus (Figure 3, p. 3-6), a new group of rats was exposed to 0 or 1,000 ppm toluene for 7 days. Figure 8 (p. 3-9) shows that the concentration of GFAP in the thalamus was decreased and there were significant increases in serum cort of the same rats, after 3 and 7 days of exposure to 1,000 ppm. Cort was higher than the sham-exposed control group after 3 days ($F = 27.17$, $df = 1,9$; $p < 0.001$) and after 7 days ($F = 33.87$, $df = 1,10$; $p < 0.001$).

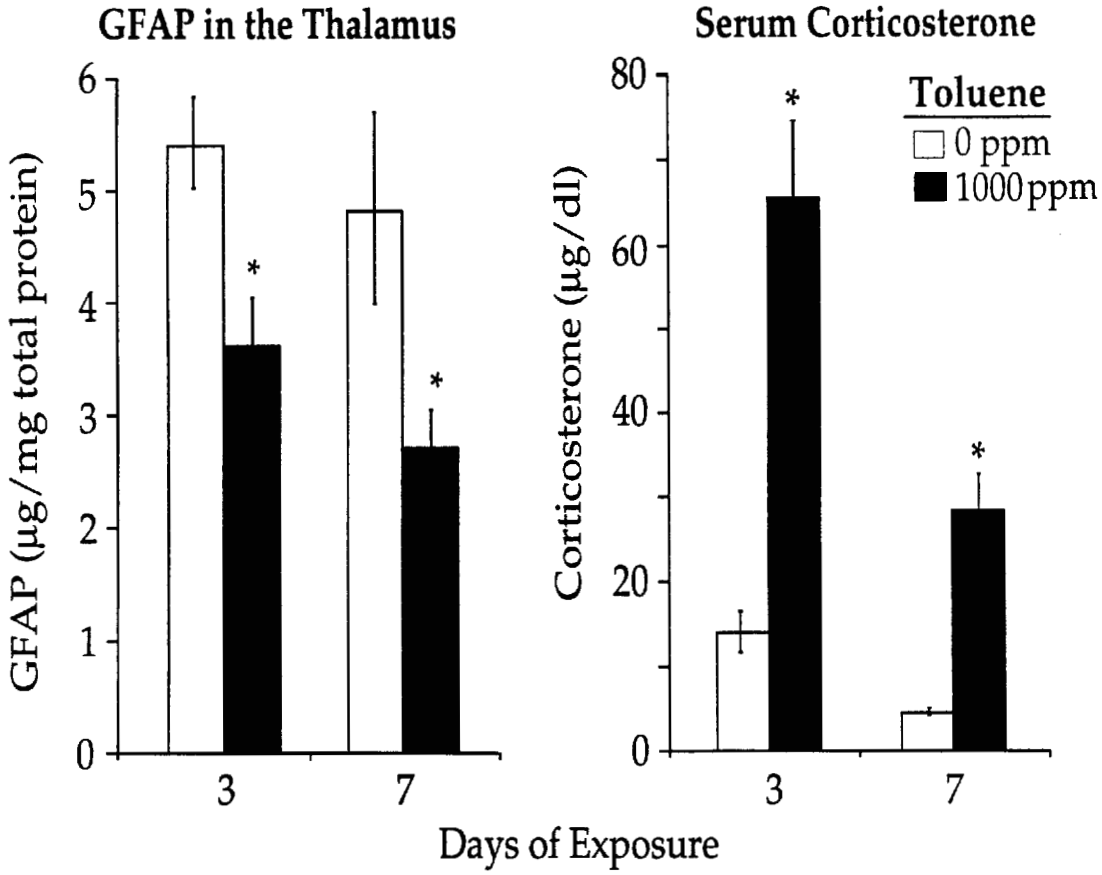

Figure 8. Reduction in Thalamic GFAP on Days 3 and 7 of Exposure to 1,000 ppm Toluene (left side). Serum Corticosterone after 3 and 7 Days Exposure to 1,000 ppm Toluene (right side). Control represents age-matched rats given sham exposures to filtered air.

Table 2 (p. 3-10) shows a summary of the dose-effect and the time-effect results for brain GFAP. It reports the brain regions which had significant changes in GFAP concentration for all exposure durations studied at a given concentration of toluene. Table 2 shows that, of the three brain regions affected by toluene, the cerebellum and the thalamus were significantly affected in 3 concentration-duration conditions, the hippocampus was affected in 2. A dose-response function is shown in Table 2 in which the number of significant changes in GFAP concentration increased with toluene concentration from 100 ppm to 1,000 ppm. It is difficult to compare the number of changes at 3,000 ppm because only 2 time points (Days 3 and 7) were studied at 3,000 ppm.

Table 2. Summary of Effects on GFAP

	Days of Exposure				
Toluene (ppm)	1	3	7	21	42
100	-	C	-	-	-
300		T	-	-	C
1,000		C,T	T	H	-
3,000		-	H		

- = no significant changes in any brain region
C = significant effect in cerebellum
T = significant effect in thalamus
H = significant effect in hippocampus
Blank cells indicate conditions that were not studied

Section 4
DISCUSSION

Toluene inhalation, using an occupational model of 6 hr/day, 5 days/week, for up to 6 weeks, caused significant dosage-dependent changes in brain GFAP concentration (Table 2, p. 3-10). Toluene altered GFAP concentration in 13% of the specimens exposed to the lowest concentration studied, 100 ppm. The average incidence of toluene-induced change in GFAP increased to 40% at 1,000 ppm, suggesting an EC_{50} for GFAP to be in the range of 1,000 ppm, with a minimum exposure duration of three 6-hr "days." The hippocampus, the most sensitive brain region, had significant changes in GFAP in 60% of the specimens at 1,000 ppm (Table 2). Few of these changes approached the magnitude of the large (200%) changes in GFAP seen with acute exposure to neurotoxic metals (O'Callaghan, 1988). However, effects of very low level exposures are likely to be small (e.g., Echeverria *et al.*, 1991). Toluene-induced changes in GFAP disappeared within 7 to 14 days after the end of toluene exposure (Figure 7, p. 3-8).

Many of the toluene-induced changes resulted in increased concentration of GFAP in the cerebellum (Figure 3, p. 3-6; Figure 6, p. 3-8; Figure 7, p. 3-8) and hippocampus (Figure 4, p. 3-6). Other investigators found the cerebellum was the brain region most often showing toluene-induced increases in other glial cell markers (Huang *et al.*, 1992). Increased GFAP was not observed in the thalamus nor in the cerebral cortex. Chemically-induced increases in GFAP concentration are usually interpreted as astro-gliosis (Aschner and Kimelberg, 1996; Balaban *et al.*, 1988; Sivron and Schwartz, 1995). Histological examination of a small number of samples from the present studies produced a few examples of astrocytic hypertrophy as reported (Fukui *et al.*, 1996) but not evidence of neuronal damage. More extensive exposure, involving toluene inhalation of more hours/day or more days/week, can significantly increase GFAP immuno-staining (Pryor, 1994) or alter glial markers other than GFAP (Huang *et al.*, 1990 and 1992).

Toluene-induced reduction in GFAP concentration was observed in the hippocampus (Figure 1, p. 2-6; Figure 5, p. 3-7) and thalamus (Figure 3, p. 3-6; Figure 8, p. 3-9). Decreased GFAP was

not observed in the cortex nor in the cerebellum. The declines in GFAP concentration during toluene exposure differ from the more commonly reported toxicant-induced pattern of increased GFAP (O'Callaghan, 1988), but are reminiscent of decreases in GFAP (Evans, 1994a; Gong *et al.*, 1995; Little *et al.*, 1994) and in GFAP mRNA (Harry *et al.*, 1996) induced by exposure to Pb, and the reduction in GFAP concentration (El-Fawal *et al.*, 1996) and in the number of thalamic astrocytes after exposure to methylmercury (Charleston *et al.*, 1996). Inhalation of toluene can both decrease and increase other brain markers (Huang *et al.*, 1990 and 1992). The generality of the decreased GFAP as a consequence of toxic exposure is suggested by the observation of decreased GFAP in fish exposed to PCBs (Evans *et al.*, 1993). Several possible mechanisms for this effect are discussed in IMPLICATIONS FOR FURTHER RESEARCH, p.4-5.

Toluene-induced changes in the adrenal-pituitary axis, exemplified by a 5-fold elevation in serum cort (Figure 8, p. 3-9) which accompanied the decline in thalamic GFAP (Figure 3, p. 3-6) may be a mechanism which results in reduced concentration of GFAP (O'Callaghan *et al.*, 1989 and 1991). Figure 8 confirms reports of increased serum cort (Andersson *et al.*, 1980; Svensson *et al.*, 1992) and prolactin (von Euler *et al.*, 1994) after toluene exposure. The cort concentrations in the present rats are in the range of F344 rats sacrificed after restraint stress (approx. 60 μg/dl, Dhabhar *et al.*, 1993) or decapitation (approx. 38μg/dl, Urbansky and Kelly, 1993). Toluene may exert a reversible effect on the neuroendocrine axis in the adult rat, with serious long-lasting or even lethal effects in the developing animal. This is consistent with reports of toluene's teratogenicity (Donald *et al.*, 1991) and increased risk of spontaneous abortion in women exposed occupationally to low levels of toluene (Ng *et al.*, 1992).

Changes in locomotor behavior (Figure 2, p. 3-3) confirm that exposure to as little as 100 ppm toluene was sufficient to affect this very sensitive endpoint and also demonstrate very consistent effects of toluene inhalation for exposure durations of up to 6 weeks. Changes in behavior are a primary concern with occupational exposures to solvents (Baker, 1994). The depression of behavioral activity during exposure to 100 ppm or 300 ppm toluene could be considered

neurotoxic, and similar to the finding of an increased tendency of humans to sleep during inhalation of similar concentrations of toluene (Echeverria *et al.*, 1991). The behavioral effects in the present studies occurred at toluene concentrations which may be the lowest yet reported to affect the behavior of rodents (Wood and Cox, 1995). These behavioral effects in rats are credible because they occurred within the range of concentrations at which humans first report subjective experiences of toluene exposure and at which neuro-behavioral dysfunction has been documented (Echeverria *et al.*, 1991; Baker, 1994). In the present studies, the locomotor behavior of pairs of rats provided a very sensitive marker of exposure and of immediate effects of toluene as low as 100 ppm. However, behavior of the present rats was not affected when measured in the home cage on the days following exposure.

Whether the present toluene exposures produced neuronal damage is doubtful. The elevation of GFAP after repeated, low level exposures (Figure 3, p. 3-6; Figure 6, p. 3-8; Figure 7, p. 3-8) or brief exposure to high levels (Figure 4, p. 3-6) is compatible with reactive gliosis which is known to accompany chemically-induced neuronal injury (Aschner and Kimelberg, 1996; Balaban *et al.*, 1988; O'Callaghan, 1988). However, no neuropathology was seen in a sample of the present brains examined by light microscopy, confirming the reported absence of damaged neurons in rats exposed to 2,000 ppm toluene, 4 hr/day for 1 month (Fukui *et al.*, 1996). Histopathology was observed in the hippocampus after 500 ppm toluene for 12 hr/day (Slomianka *et al.*, 1990) or 2,000 ppm for 8 hr/day (Pryor and Rebert, 1993) and a 16% neuronal loss after 1,500 ppm toluene, 5 day/wk for 180 days (Korbo *et al.*, 1996). Sensitive methods (electron microscopy or morphometry) can document subtle effects of toluene on the morphology of astrocytes (Fukui *et al.*, 1996) which could help to understand the changes in GFAP reported here. However, the literature summarized below clearly indicates effects of toluene which are not readily demonstrated morphologically.

The effects of toluene were reversible. Changes in behavior (Figure 2, p. 3-3) and in GFAP (Figure 7, p. 3-8) disappeared within 7 to 14 days of the end of toluene exposure. However, behavioral effects were studied only with very low exposures ($\leq$ 300 ppm). In humans, signs of

solvent intoxication recede after the end of exposure (Baker, 1994). Similar levels of toluene exposure have been shown to induce reversible changes (Bushnell *et al.*, 1985; Korbo *et al.*, 1996; Miyagawa *et al.*, 1995; Slomianka *et al.*, 1992; Taylor and Evans, 1985). Noteworthy is the report of persistent changes in dopamine binding (Hillefors-Berglund *et al.*, 1995).

The sensitivity and specificity of toluene-induced changes in GFAP can be gauged by comparison to other end points. Changes at 80 ppm toluene occur in several aspects of neurotransmitter function (Hillefors-Berglund *et al.*, 1995; von Euler *et al.*, 1993) or in serum prolactin (von Euler *et al.*, 1994). At 100 ppm, changes appear in locomotor behavior (Figure 2, p. 3-3), in expired CO_2 (Taylor and Evans, 1985), and in human psychological indices (Brubacher, 1993; Echeverria *et al.*, 1991). Body weight of rats is not affected by inhalation of up to 1,000 ppm (Table 2; Huang *et al.*, 1992; Huff, 1990; Miyagawa *et al.*, 1995). At concentrations above 1,000 ppm, toluene causes histopathology in the brain (Pryor and Rebert, 1993), affects measures of airway sensitivity (Alarie, 1995; Dudek *et al.*, 1992), alters the performance of learned behavior in lab animals (Bushnell *et al.*, 1994; Forkman *et al.*, 1991; Saito and Wada, 1993; Wood *et al.*, 1983), and affects auditory and optical sensory function (Johnson, 1994; Morata *et al.*, 1995; Niklasson *et al.*, 1993) in the rat. The latter end-points may not be inherently insensitive to toluene, but rather present logistical difficulty of testing a sufficient sample size within toluene's short wash-out time, as discussed by Evans (1994b) and Wood (1994).

The evidence obtained thus far suggests that measurement of GFAP concentration, by itself, provides few advantages as a marker of exposure to toluene over measures of toluene in breath or blood, or of hippuric acid in urine as reviewed by ATSDR (1994). Although toluene-induced changes in brain GFAP lasted longer than those peripheral exposure markers, the present data show that GFAP concentration was not linearly related to toluene exposure over a wide range of concentrations. Thus, a satisfactory marker of past exposure to toluene has yet to be identified.

Changes in GFAP concentration in the brain may indicate neurotoxicity in some circumstances, but the mechanisms determining whether GFAP will increase or decrease are not well understood. Because the direction of changes in GFAP concentration was inconsistent as repeated exposure continued, GFAP alone may not provide a practical marker of the effects of short-term occupational exposure to toluene. However, GFAP, when combined with evaluation of behavior and brain neurotransmitter function, may provide a useful battery for monitoring neurotoxicity of inhaled solvents.

IMPLICATIONS FOR FURTHER RESEARCH

The finding that GFAP concentration can increase under some dosages and decrease under others, may be an important advance in understanding the brain's reaction to toxic injury. This suggests two different types of astrocytic reaction to toxicants, possibly reflecting different sub-types of astrocytes or different stages of a temporal sequence beginning with defenses against toxicants and ending with neuronal death (Gong *et al.*, 1995; Aschner and Kimelberg, 1996). Closer scrutiny should be directed towards the differences between cerebellum and hippocampus in terms of changes in gene expression, cyto-architecture, neurotransmitter function, and/or adrenal steroid production. Although both of these regions were among the most sensitive brain regions in reflecting the effects of inhaled toluene, only the hippocampus had instances of decreased GFAP concentration. More research is needed to clarify these hypotheses.

The present data suggest that GFAP should be combined with other indices into a battery for testing neurotoxicity for the reasons proposed by the USEPA (1995). Although classical neuropathology remains a "gold standard," continued efforts should be directed at developing alternatives for toxicity testing. Biochemical assays, such as GFAP, provide increased sensitivity, economy, quantification and new sources of information not available with traditional neuropathology assays.

It would be useful to have workshops to plan and to interpret inter-lab comparisons of the results of the ELISA for GFAP. Shared samples could be distributed among several labs in order to determine sources of variability in the assay outcome, statistical power calculations and methodological refinement through the sharing of technical know-how.

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