

American Petroleum Institute  
1220 L Street, Northwest  
Washington, D.C. 20005



Robert T. Drew, Ph.D.  
Director, Health and  
Environmental Sciences  
(202) 682-9308  
(202) 682-9270 (FAX)

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**(Identification no: FYI-not assigned)** Draft Report, Brain GFAP as a Marker of Neurotoxicity During Inhalation Exposure to Toluene, New York University Medical Center; Nelson Institute of Environmental Medicine.

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Sincerely,

Robert T. Drew, Ph.D.



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## **Brain GFAP as a Marker of Neurotoxicity During Inhalation Exposure to Toluene**

Principal Investigator: Hugh L. Evans, Ph.D.,  
New York University Medical Center  
Nelson Institute of Environmental Medicine, Tuxedo, NY 10987

Co-investigators: Hassan El-Fawal, Ph.D.  
Bernard Jortner, V.M.D.  
Carroll Snyder, Ph.D.

A Report to the American Petroleum Institute

April 25, 1995

**DRAFT**

## ABSTRACT

**BACKGROUND** This is the second report of the performance of brain glial fibrillary acidic protein (GFAP) during sub-chronic exposure to a neurotoxicant (the first concerned oral exposure to Pb). The purpose of this study was to determine whether GFAP is a practical biomarker of toluene-induced neurotoxicity in the laboratory rat. Toluene was chosen as a model solvent for which there is evidence of neurotoxicity in the form of behavioral and neurophysiological effects.

**HOW** Rats received inhalation exposure to air or 100 - 3000 ppm toluene, 6 hr/day, 5 days/wk for up to 42 days. These exposures resemble an occupational exposure schedule. During and after exposure, the concentration of GFAP was determined in the brain. These changes in GFAP were compared with standard criteria of neurotoxicity: behavioral or neuropathological changes.

**RESULTS** Toluene, in concentrations  $\leq$  1,000 ppm, altered GFAP and motor behavior without affecting body weight, brain histology or producing overt signs of neurotoxicity. These results suggest that GFAP can provide an index of subtle degrees of toxicity. Changes in GFAP were seen as early as the third day of exposure. GFAP was affected by toluene as low as 100 ppm, a concentration quite low for experiments with rats, and within the range of occupational exposures for humans. The maximum concentration, 3,000 ppm for 7 days, caused increased GFAP suggestive of reactive gliosis, but no cellular damage could be seen at the light microscopic level.

**CONCLUSIONS** GFAP is a sensitive index of neurotoxicity, but not of exposure, to toluene. GFAP provides information which is partly correlated with, but

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not redundant to that available from standard assays of behavior. We do not yet understand the factors which determine whether a toxicant causes an increase or a decrease in GFAP concentration.

## ACKNOWLEDGMENTS

THE FOLLOWING PEOPLE ARE RECOGNIZED FOR CONTRIBUTIONS OF THEIR TIME AND EXPERTISE DURING THIS STUDY AND IN THE PREPARATION OF THIS REPORT:

### API STAFF CONTACTS

Dr. Robert Drew

Mr. David Mongillo

### MEMBERS OF THE NEW YORK UNIVERSITY MEDICAL CENTER WORK GROUP

We thank Alvin Little, Dr. Cheng Wang, Zhaolong Gong, and Kenneth Magar for expert technical assistance at New York University.

Dr. Hassan El-Fawal contributed to the planning and interpretation of the GFAP assay.

Dr. Carroll Snyder and Kenneth Magar contributed to the inhalation exposure methods.

Dr. Bernard Jortner provided neuropathology studies of our specimens in his laboratory at Virginia Polytechnical University.

Dr. J. P. O'Callaghan of United States Environmental Protection Agency provided helpful suggestions on the GFAP method.

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## EXECUTIVE SUMMARY

The purpose of this study was to determine whether an immuno-assay for glial fibrillary acidic protein (GFAP) in the rat's brain can provide practical evidence of solvent-induced neurotoxicity. The U.S. E.P.A. has suggested that GFAP be used in screening of new chemical compounds. We previously reported to API our findings that GFAP was sensitive to repeated oral exposure to Pb at exposure levels which produced behavioral and histological evidence of neurotoxicity. Although GFAP was a useful marker of Pb-induced neurotoxicity, GFAP was not a useful marker of Pb exposure. Toluene was chosen as a model neurotoxicant for these studies, because toluene's neurotoxicity in the rat has been characterized. However, there are currently no useful biomarkers of toluene's neurotoxicity in humans.

The present studies documented changes in GFAP concentration during subacute inhalation exposure to toluene and compared these results to changes in standard indices of neurotoxicity. Adult male F344 rats, at approximately 47 days of age, received inhalation exposure to room air or 100, 300, 1000 or 3000 ppm toluene, 6 hr/day, 5 days/wk for up to 42 days. These exposures resemble 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 criteria of neurotoxicity: behavioral or neuropathological changes. Body weight was monitored as a sign of general toxicity, or intercurrent disease.

Exposure to 3,000 ppm toluene caused reduced body weight gain and observable signs of sedation in the rats, thus confirming previous reports of toluene's toxicity at high concentrations. Toluene, in concentrations  $\leq$  1,000 ppm, altered GFAP without affecting body weight, brain histology or producing overt signs of neurotoxicity. These results suggest that GFAP can provide an index of subtle degrees of toxicity. Changes in GFAP were seen as early as the third day of exposure. GFAP was affected by toluene as low as 100 ppm, a concentration quite low for experiments with rats, and within the range of occupational

exposures for humans. Increased GFAP after 7 days exposure to 3,000 ppm is suggestive of reactive gliosis, but no cellular damage could be seen at the light microscopic level.

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 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 behavioral indices were unaffected.

As toluene exposure continued, significant changes in GFAP appeared, then were reversed as exposure duration continued. There was no evidence of neuronal death nor signs of permanent nervous system impairment. For example, significant increases in GFAP at 42 days of exposure to 1,000 ppm toluene had returned to control levels after 7 to 14 days.

The results indicated that measurement of GFAP concentration does not provide a good marker of exposure, but may indicate neurotoxicity in some circumstances which are not presently well understood. Because changes in GFAP concentration were irregular 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 behavioral evaluation, may provide a useful battery for monitoring neurotoxicity.

## Section 1. INTRODUCTION

There is need for a sensitive biomarker for inhalation exposure to organic solvents (e.g., Tardif et al., 1991), because both solvents and their metabolites are cleared rapidly from the body (Brugnone et al., 1995). A recent workshop suggested GFAP as a marker for neurotoxic solvents in animal studies (Arlie-Sorborg et al., 1992). Two reports indicate that inhalation of organic solvents may affect brain GFAP content in rats (Rosengren et al., 1986; Rosengren & 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). A practical goal of this project was to verify and extend these findings, and to evaluate the utility of a new, simplified ELISA assay for GFAP (O'Callaghan, 1991) for studies of neurotoxicity.

The cellular and molecular mechanisms by which inhaled toluene causes changes in function of the central nervous system are not known (U.S.A.T.S.D.R., 1994). This is not surprising, since little more is known about the mechanisms of action of inhaled volatile anesthetics which are used routinely in human surgery (Pocok and Richards, 1993). Toluene affects respiratory function at 4,900 ppm (Dudek et al., 1992) but our laboratory has shown toluene alters behavior at concentrations an order of magnitude lower. Behavioral change is an important indicator of toluene inhalation in rodents (Wood & Colotla, 1990; Wood et al., 1983; Bushnell et al., 1985) and humans. Histopathological change has perhaps been a less consistent indicator of toluene neurotoxicity, except with the extremely high concentrations which occur in solvent abuse (glue-sniffing). Together, behavioral and histopathological measures provide two standard indices of neurotoxicity against which to evaluate 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 food (Rodent Laboratory Chow 5001, Ralston Purina Corp., St. Louis, MO) and water available *ad lib*. Before toluene exposure began, rats were quarantined for 7 days, weighed and observed to ensure health and to determine baselines. This research was approved by the institutional animal care and use committee and conformed to animal care guidelines of the U.S.N.I.H.

### EXPOSURE TO TOLUENE:

Groups of up to 32 rats were exposed to 0 ppm (conditioned air) or toluene (100, 300, 1000 and 3000 ppm) in dynamic exposure chambers, 6 hr daily, Monday through Friday. Reagent grade toluene was provided by the A.P.I. Chamber atmospheres were maintained as described in our published work (Dempster, Evans and Snyder, 1983). Inhalation exposures are conducted in either 1.3 or 0.13 m<sup>3</sup> stainless steel chambers of the type designed by Drew and Laskin. Control rats are exposed concurrently with test rats in separate chambers to filtered, conditioned air. Control animals are housed and transported separately from test animals, so as to prevent unplanned exposure of control animals to experimental compounds which may linger in the fur or other body constituents of test animals. Prior to exposure, animals are transferred from home cages to stainless-steel mesh exposure cages. Food and water are not available during inhalation exposures. 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.

Exposures are conducted for 6 hr/day, up to 5 days/week, to simulate an occupational exposure. Concentration of toluene in 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  $\pm 10\%$  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  $\pm 0.1$  g as described by Evans et al. (1986).

#### **LOCOMOTOR BEHAVIOR**

Behavior was measured in the home cage, after the conclusion of a 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 mesh 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 a single index, a composite of total locomotor behavior because of the smaller size of 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 only one inhalation chamber was equipped for behavioral measures and because the behavioral effects of  $\geq 1,000$  ppm toluene had already been

reported in the literature. These behavioral measurements are quite similar to those of the E.P.A. Neurotoxicity Guidelines for motor activity (U.S. E.P.A., 1991).

## 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), 1000 ppm for 3 days (N=5), 1000 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 12 to 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  $\mu$ m thickness for hematoxylin and eosin (H & E) staining and 5  $\mu$ m 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 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). Samples used in the GFAP assay were normalized for total protein.

## 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 (Paxinos and Watson, 1986) as a guide. 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 changes and were not including in the follow-on studies. Specimens were weighed (Mettler #AJ100 analytical balance,  $\pm 0.1$  mg), snap-frozen and stored at  $-80^{\circ}\text{C}$ .

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 ( $1.0\mu\text{g}/100\mu\text{l}$  /well) with a capture antibody, polyclonal anti-GFAP (Dako, Carpinteria, CA) for 1 hr at  $37^{\circ}\text{C}$ . Microtiter plates were washed with phosphate-buffered saline (PBS, pH 7.4), incubated for 1 hr with 5% non-fat dry milk (in PBS) to block non-specific binding, then incubated with  $100\mu\text{l}$  of sample or standard for 1 hr. Plates were washed with PBS containing 0.5% Triton X-100, then loaded with  $100\mu\text{l}$  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 ul/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. Standard curves were generated by a logit transformation of the absorbance data and log of total protein, excluding asymptotic data points at the extreme lower or upper limits of the standard curve, taking care that there were sufficient points in the middle portion of the curve so that the least squares linear regression

yielded a correlation coefficient of  $> 0.9$ . Concentrations of GFAP in brain regions 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 (Fig. 1). If the results from the replicate assay confirmed the original data, then the results of both replications were subjected to ANOVA, with replications as one factor. See STATISTICS, below.

**Results of 3 Assays: Dose Response of Rat Hippocampal GFAP in Response to Toluene Inhalation**

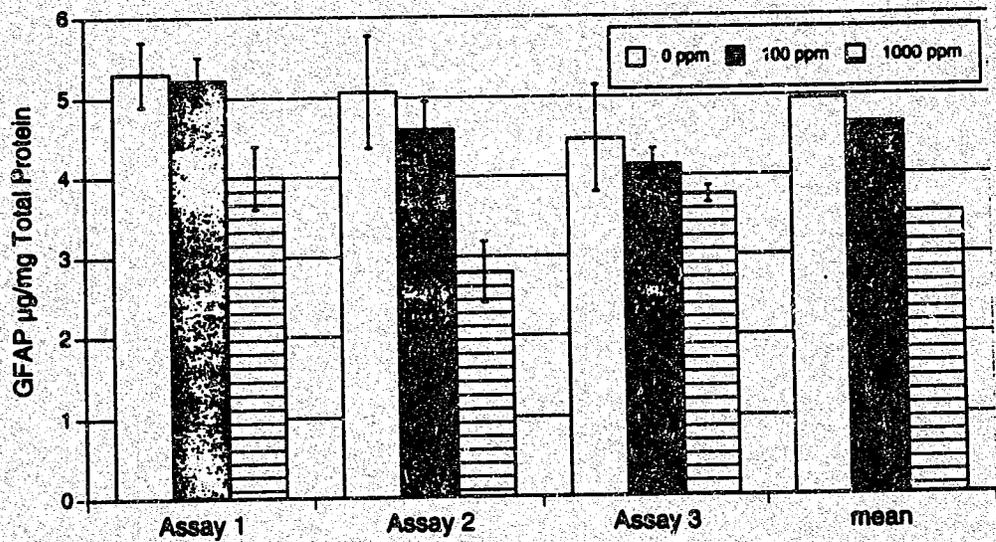


Fig. 1. GFAP was reduced in the hippocampus of rats after 21 days exposure to 100 or 1,000 ppm toluene. The specimens were removed from the freezer and assayed 3 times to give the results shown in the Figure. Replications were used as a grouping factor in the ANOVA statistical tests. The mean of the replications is shown as the final result in the remaining figures of this report.

## STATISTICS

Statistical significance was tested by multi-factorial analyses of variance (ANOVA, for GFAP data) or analyses of covariance (ANCOVA, for behavior or body weight) from the BMDP library (Dixon, 1990; Dixon & Meridan, 1992). ANCOVA was assessed using BMDP program 5V for repeated measures (Dixon & 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 using the Wald Chi-Square test, which can be used when some data are missing (Dixon, 1990; Dixon & 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**

**BODY WEIGHT**

Table 1 shows that after 42 days of exposure up to 1,000 ppm toluene, there was no significant difference 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 of Rats Exposed to Toluene**

Toluene (ppm)	Pre-exposure	After 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
1000	120(4.8), 32	256(10.3), 8

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

**BEHAVIOR DURING TOLUENE INHALATION.**

Locomotor behavior was less frequent during exposure to 100 and 300 ppm toluene (Fig. 2). The data illustrate the habituation pattern in which the total amount of behavior declines over the first 2 test sessions, as the rats become acclimated to the handling and stimuli of the exposure chamber. Behavior inside the chamber was not studied with higher concentrations, because of ample evidence in the literature of behavioral effects of toluene at 1,000 ppm and above.

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 Fig. 2).

No measurements were made of the behavior of rats exposed to 3,000 ppm, but these rats were observed to be inactive and ataxic when they were removed from the inhalation chamber after 6 hours exposure to 3,000 ppm. This was noticeably different from control rats.

#### BEHAVIOR AFTER TOLUENE INHALATION.

Rearing and locomotor behavior were recorded in the home cage every weekend after 5 days of exposure to either 0 ppm, 100 ppm or 300 ppm toluene. These observations represent a time period of from 1 to 48 hours after the most recent toluene exposure. Data were obtained from 4 to 8 cages with 2 rats in each cage. No significant differences were seen in total rearing, total locomotion, nor in the diurnal pattern of either behavior (data not shown).

### Behavior Inside the Exposure Chamber

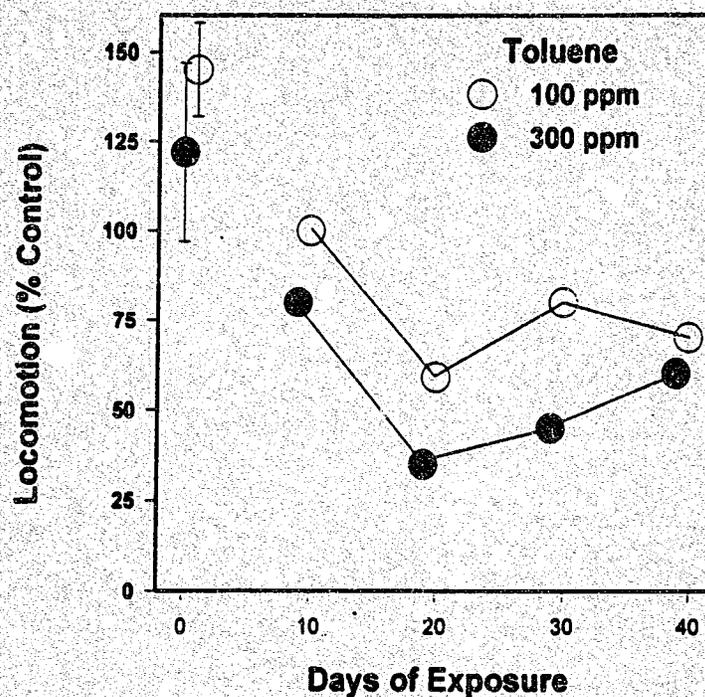


Fig. 2. Effects of 100 or 300 ppm toluene on behavior during the inhalation exposure. Data are expressed as the percentage of the behavioral activity of an age-matched group of rats while receiving sham exposure to filtered air (0 ppm). Day 0 represents the baseline, when both groups of rats were placed into the inhalation chamber for the first time, without toluene exposure. Behavioral measures were recorded every Friday, during the fifth consecutive exposure of the week. Each point is the mean  $\pm$  SEM, N = 9 to 12.

### Behavior Inside the Exposure Chamber

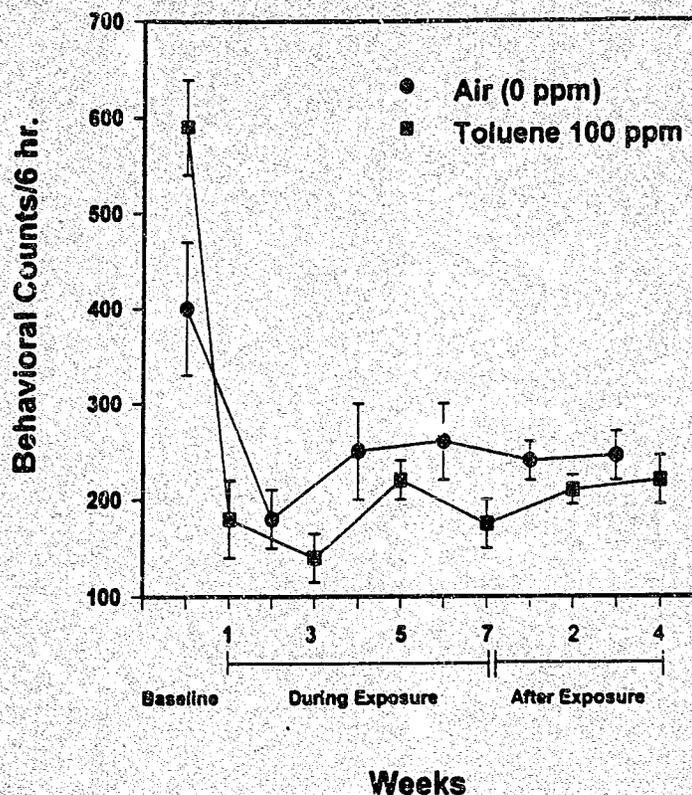


Fig. 3. Recovery of behavioral effects of 100 ppm toluene in the inhalation chamber. During exposure to 100 ppm toluene, locomotor behavior was significantly below the control group (0 ppm) for the 7 weeks of exposure to toluene. Testing continued for 4 more weeks inside the exposure chamber, but without toluene exposure, during which time the behavior of rats previously exposed to 100 ppm toluene recovered to activity levels approximating those of the 0 ppm control group. Baseline data reflect the first introduction of the rats into the exposure chamber, without toluene. Each point shows the mean  $\pm$  SEM, N = 10 to 12.

## NEUROPATHOLOGY

No lesions were observed with H & E stain at the light microscopic level. Sections will be stained for GFAP immunoreactivity, based upon the results with GFAP protein concentration determined in the present studies.

## FACTORS INFLUENCING VARIABILITY IN PROTEIN DATA

Because an aim of this project was to appraise the GFAP assay for application to toxicity testing, we have identified several factors which may help to minimize unwanted variability in the biochemical assays. Although most of these points will be familiar to experienced researchers, we found these points to be especially valuable in our work.

Total protein. Glassware must 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 and potentially over-loading 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; samples for GFAP assay may be kept overnight

at  $-80^{\circ}\text{C}$ , then thawed for the GFAP assay the next morning. Other schemes for breaking up the assay into 2 days' work have been less successful in our experience. Selection of the standard data is important; plate-to-plate variability of standard data should be  $<10\%$ , hopefully  $\leq 5\%$ .

### **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, 1994; Evans et al., 1993; 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.*, Fig. 1.

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 1000 ppm (Fig. 4). The main effect for both toluene doses was significant ( $F = 4.18$ ,  $df = 2,21$ ). Both 100 ppm ( $F = 35.30$ ,  $df = 1,14$ ) and 1000 ppm ( $F = 5.42$ ,  $df = 1,14$ ) were significant. GFAP in the thalamus declined on day 3 after 100 and 1000 ppm ( $F = 9.46$ ,  $df = 2, 20$ ). The decline after 1000 ppm was significant ( $F = 33.63$ ,  $df = 1,14$ ). There also was a slight increase in the hippocampus ( $p = 0.13$ ).

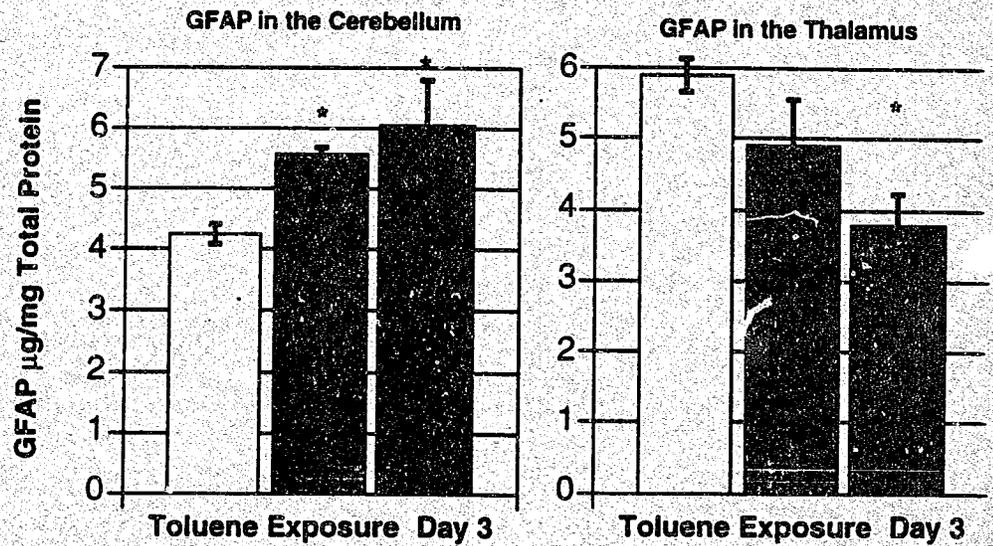
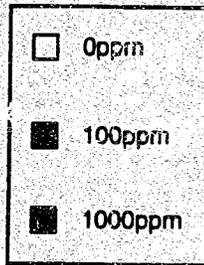


Fig. 4. Significant changes in brain GFAP on the third day of exposure to toluene. These were the earliest durations studied. The lowest toluene concentration to affect GFAP was 100 ppm, shown in the data for cerebellum (left side of Fig. 4).

On day 7, GFAP was increased in the hippocampus of rats exposed to 3,000 ppm toluene (Fig. 5). 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 ( $P = 0.13$ )

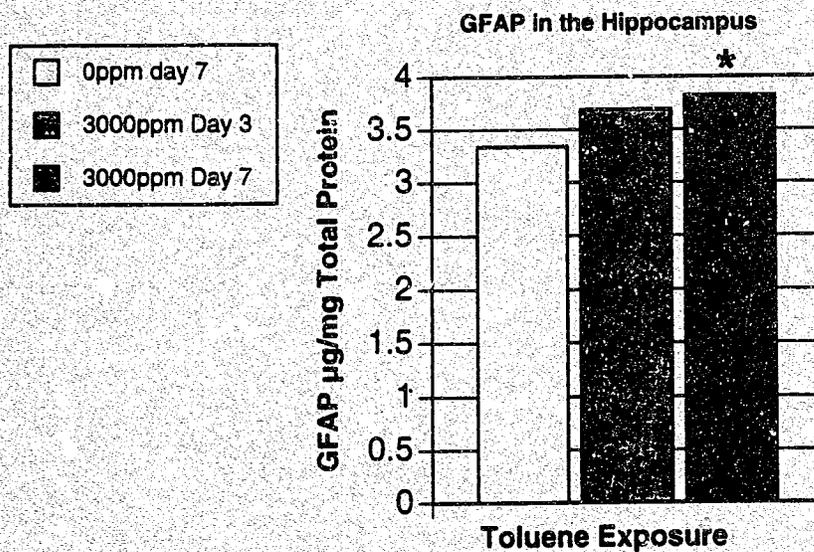


Figure 5. Increased GFAP in the hippocampus of rats exposed for 3 and 7 days to 3,000 ppm toluene.  $N = 10$  rats for each point.

On Day 21, exposure to 1,000 ppm toluene resulted in *decreased* GFAP concentrations in the hippocampus (Figs. 1 and 6). The 3-dose (0, 100, 1000 ppm) x 3-replications ANOVA was significant for the toluene main effect ( $F = 4.20$ ,  $df = 2,21$ ). This decline was significant for 1000 ppm ( $F = 5.74$ ,  $df = 1,14$ ) but not for 100 ppm.

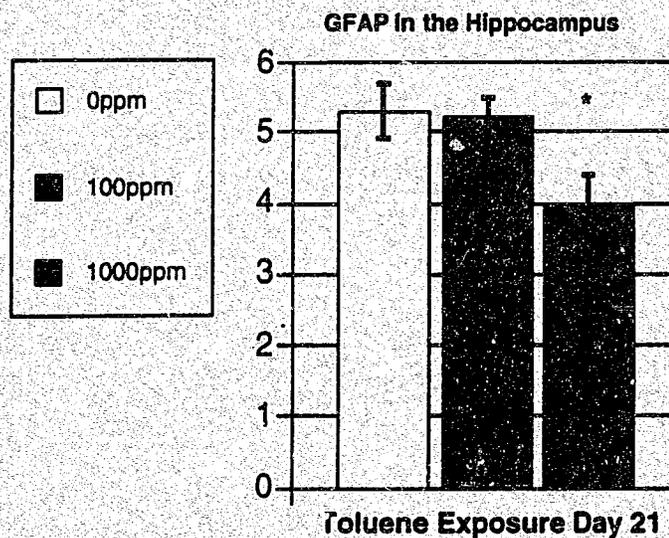


Fig. 6. Effects of 21 days exposure to 100 or 1,000 ppm toluene on GFAP in the hippocampus. Each point is the mean of 8 rats.

After 42 days of exposure, the longest exposure duration studied here, GFAP was significantly increased in the cerebellum ( $F = 4.62$ ,  $df = 1,13$ ). GFAP remained elevated in the cerebellum 7 days after the end of the 42-day exposure ( $F = 9.02$ ,  $df = 1, 27$ ), but was not different from control at 14 days post-exposure.

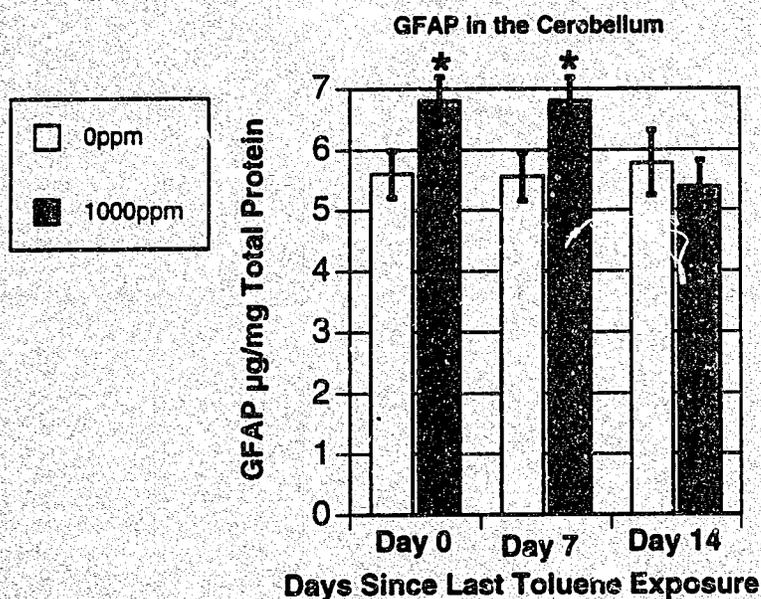


Fig. 7. GFAP in the cerebellum at the conclusion of 42 days exposure to 1,000 ppm toluene, and at 7 and 14 days after the end of this exposure.

Table 2 shows a summary of the dose-effect results for brain GFAP. It reports the number of significant changes in GFAP concentration for all the time points studied at a given concentration of toluene. For example, at 100 ppm, GFAP was significantly changed in the cerebellum at 1 of 5 durations of exposure (this was on day 3, Fig. 4). Exposure to 3,000 ppm was studied only at 3 and 7 days, thus the limited number of entries under 3,000 ppm in Table 2.

Table 2  
Significant GFAP Change: Toluene Dose-Effect

Brain Region	100 ppm	1000 ppm	3000 ppm
cerebellum	1/5	2/4	0/2
hippocampus	1/5	3/5	1/2
thalamus	0/5	1/5	N/A

#### Section 4

### DISCUSSION

Toluene inhalation, using an occupational model of 6hr/day, 5 days/week, for up to 6 weeks, caused changes of up to 30% in GFAP concentration. These exposures produced no evidence of neuronal injury of the type which triggers the astrocyte response (reviewed by Sivron and Schwartz, 1995). Toluene inhalation of more hours/day or more days/week can significantly increase GFAP immunostaining (Pryor, 1994) or alter glial markers other than GFAP (Huang et al., 1990 and 1991). In the present studies, toluene inhalation did not produce the large (200%) changes in GFAP as were seen with acute exposure to neurotoxic metals (O'Callaghan, 1988).

The most sensitive and consistent marker of toluene's effects in lab animals has been behavior (U.S. A.T.S.D.R., 1994; Saito & Wada, 1993; Wood & Colotla, 1990) or the metabolic product of muscular activity (Bushnell et al., 1985; Taylor & Evans, 1985). Changes in neurotransmitters in the brain also have been documented at very low concentrations of toluene (Von Euler et al., 1993). In the present studies, behavioral changes during exposure to 100 ppm or 300 ppm toluene could be considered neurotoxic and are within the range of concentrations at which humans first report subjective experiences of toluene exposure and at which nervous system dysfunction has been documented (U.S. A.T.S.D.R., 1994). In the present studies, the locomotor behavior of pairs of rats provided a very sensitive marker of the acute, reversible effects of toluene as low as 100 ppm. The rodent's locomotor behavior is sensitive to inhaled solvents, and our strategy of recording the behavior of rats in a social situation (pairs together during exposure) has the dual advantages of increasing the assay's sensitivity and its relevance to the human exposure setting (people seldom work with solvents in social isolation). The significant changes in behavior confirm that our studies delivered toluene to the rats and produced biological effects. The present behavioral results (Figs. 2 and 3) also demonstrate an indicator of toluene's effect that was

very consistent for exposure durations of up to 6 weeks. Behavioral changes were not detected after exposure, even though rats were tested with a sensitive technique.

Whether the present toluene exposures produced permanent neuronal damage is undecided. The elevation of GFAP after long term, low level exposures (Figs. 4 and 7) or brief exposure to high levels (Fig. 5) is compatible with reactive gliosis which is known to accompany chemically-induced neuronal injury (Balaban et al., 1988; O'Callaghan, 1988). However, no neuropathology was seen in a sample of brains examined by light microscopy. More sensitive electron microscopy or morphometry techniques may be capable of documenting subtle cellular changes in the brain which would help to understand the changes in behavior and GFAP. Another finding to suggest that toluene produced no permanent neuronal damage is that the toluene-induced changes in GFAP disappeared within 7 to 14 days (e.g., Fig. 7). Finally, the lack of permanent neuronal damage is supported by the finding that behavioral changes were reversible within 24 hours after the most recent exposure. However, behavioral effects were studied only with very low exposures ( $\leq 300$  ppm).

The declines in GFAP concentration during toluene exposure are a novel finding and are reminiscent of Pb-induced decreases in GFAP (Evans, 1994). The toluene-induced reductions in GFAP concentration (Figs. 1, 4, 6, 7) depart from the more commonly seen toxicant-induced pattern of increased GFAP. Our earlier studies in this series indicated that both Pb and toluene, two very different chemicals, can also cause reduced GFAP concentration (Evans, 1994; Little et al., 1994; Gong et al., 1994). The mechanisms and interpretation of the decline in GFAP is the subject of on-going research in this lab, which currently focuses upon the roles of GFAP gene expression and adrenal steroids in order to clarify the circumstances under which GFAP levels may be decreased. Several alternative mechanisms may explain the neurotoxicity of toluene at lower levels of exposure, such as those in the present studies. Perhaps only one of the several types of astrocyte is responsive in the present toluene exposures, or perhaps two types of astrocyte respond in different, counterbalancing ways, so that the total GFAP in a brain homogenate might not document

these changes. The neurotoxic effects of toluene seen in the present studies may involve alterations in release of neurotransmitters (Von Euler et al., 1993) or adrenal cortical hormones (O'Callaghan et al., 1989 and 1991).

The evidence obtained thus far suggests that measuring GFAP, alone, does not provide a sufficient index of neurotoxicity, but that GFAP may be a valuable component, along with behavior, in a battery of tests for screening for neurotoxicity of solvents such as toluene. Continued efforts should be directed at developing alternatives to classical neuropathology in testing. Biochemical assays, such as GFAP, provide increased sensitivity, economy and new sources of information not available with traditional assays of neurotoxicity.

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