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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

API PUBLICATION NUMBER 4647

PREPARED UNDER CONTRACT BY:

HUGH L. EVANS, PH.D.
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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
Charles Ross, Shell Oil Company
Ceinwen Schreiner, Mobil Business Resources Corporation
Christopher Skisak, Pennzoil Company

MEMBERS OF THE NEW YORK UNIVERSITY MEDICAL CENTER WORK GROUP

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

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

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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.

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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 $\pm 10\%$ 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 ± 0.1 g as 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 $\geq 1,000$ 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 μ m thickness for hematoxylin and eosin (H & E) staining and 5 μ 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 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.

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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.

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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 5 μ m; Waters) was equilibrated using methanol-water (70:30v/v; Fisher Scientific). The flow rate was 1ml/min. Injection volume was 20 μ l. Separations were made at ambient temperature (23-25°C) and eluate was monitored at 250nm. The following standards were made in methanol: 2.3 μ g/ml 19-NT; cort (Steraloids, Wilton, NH) at 0.50, 1.25, 2.50, and 5.00 μ g/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 20 μ l 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 $p \leq 0.05$.

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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.

Behavior Inside the Exposure Chamber

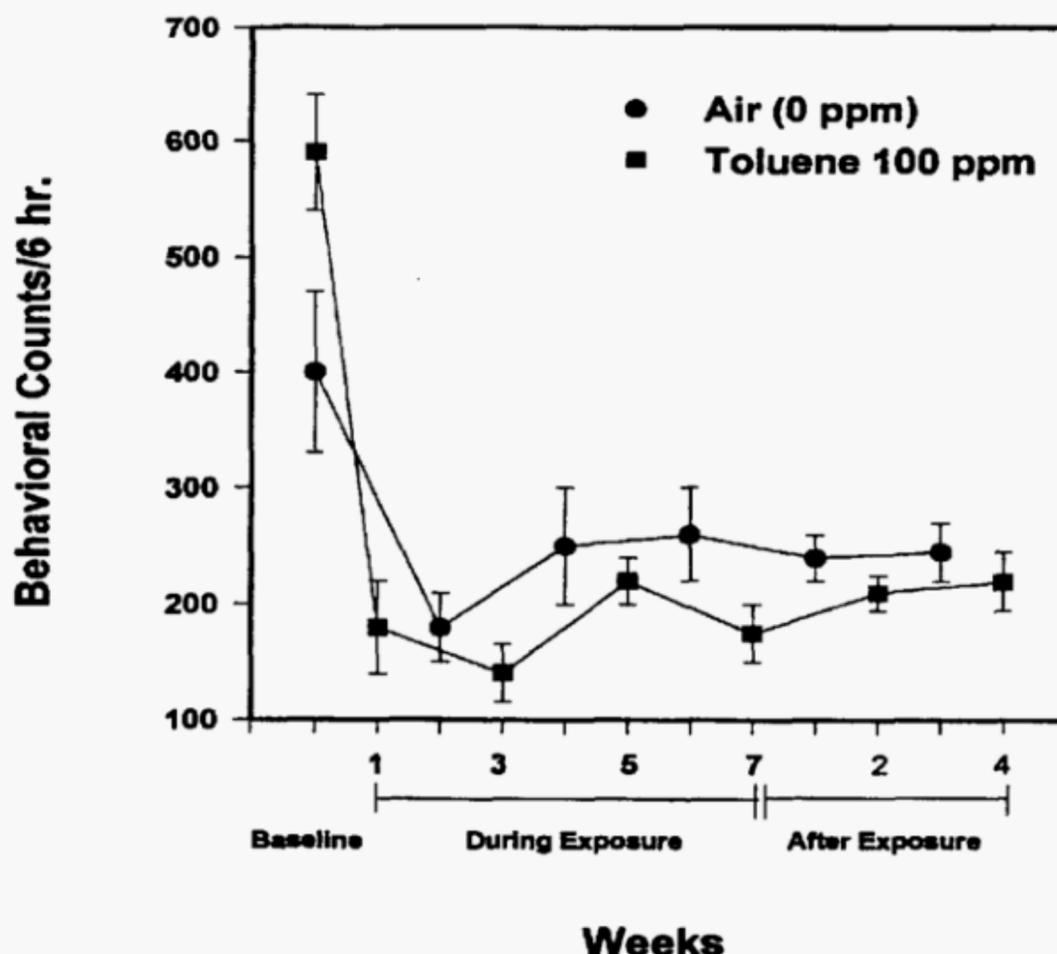


Figure 2. Behavior during Toluene Inhalation and Post-exposure. Each point shows the mean \pm SEM, N = 10 to 12.

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.

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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

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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.

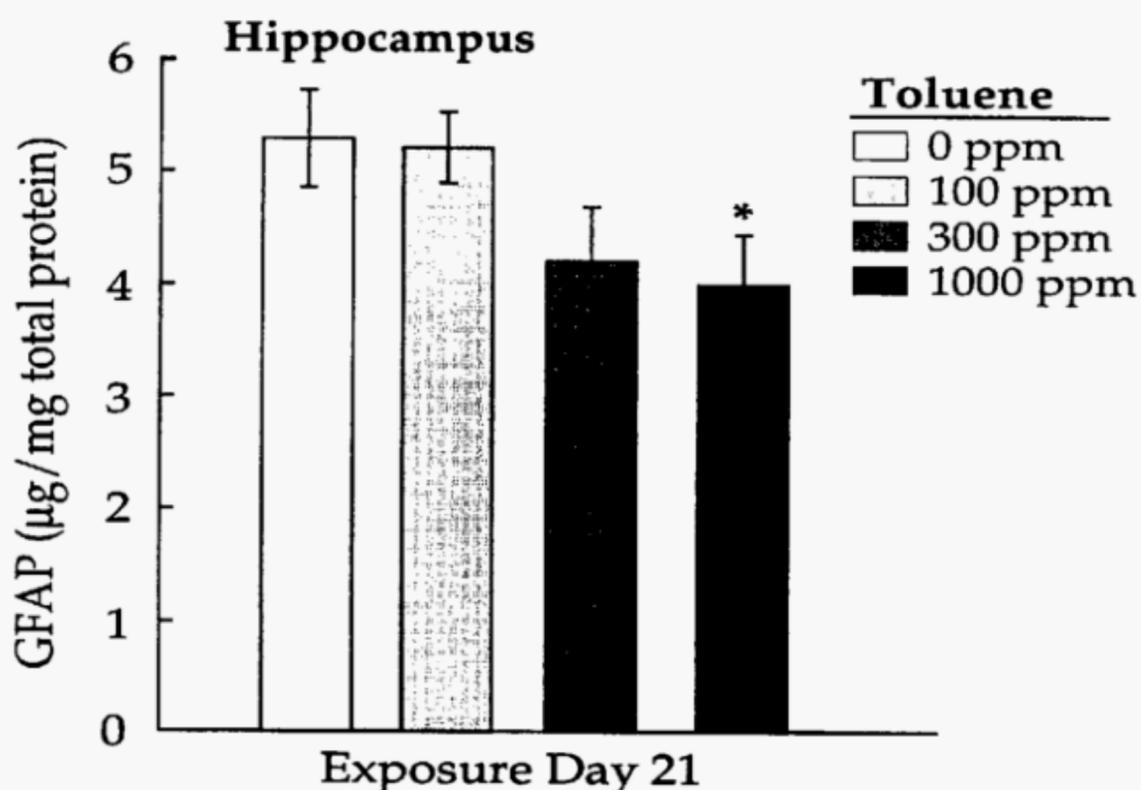


Figure 5. Effects of 21 Days Exposure to 100, 300 or 1,000 ppm Toluene on GFAP in the Hippocampus. Each point is the mean of 8 rats.

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.

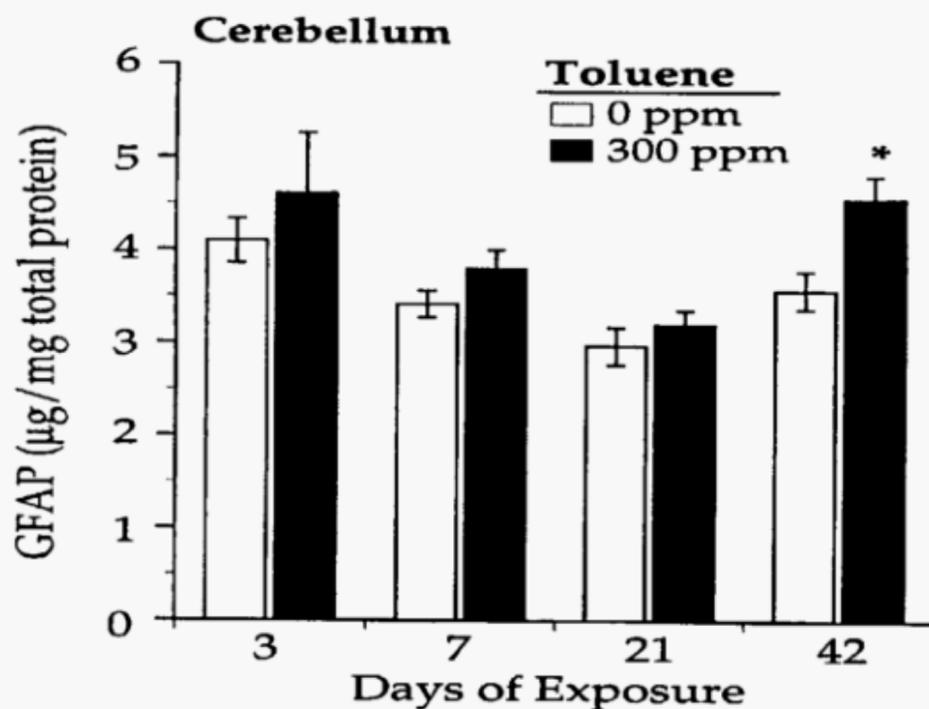


Figure 6. GFAP in the Cerebellum during 42 Days Exposure to 300 ppm Toluene.

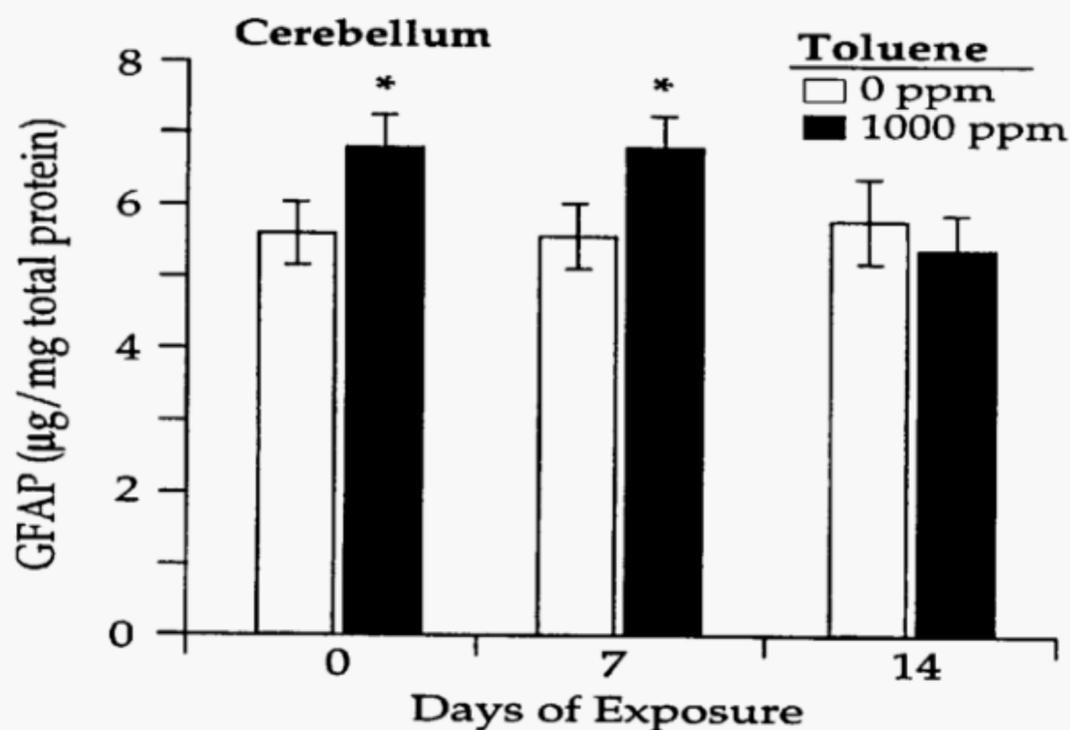


Figure 7. GFAP in the Cerebellum Returned to Baseline after 42 Days of Exposure to 1,000 ppm Toluene.

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 ($F = 33.87$, $df = 1,10$; $p < 0.001$).

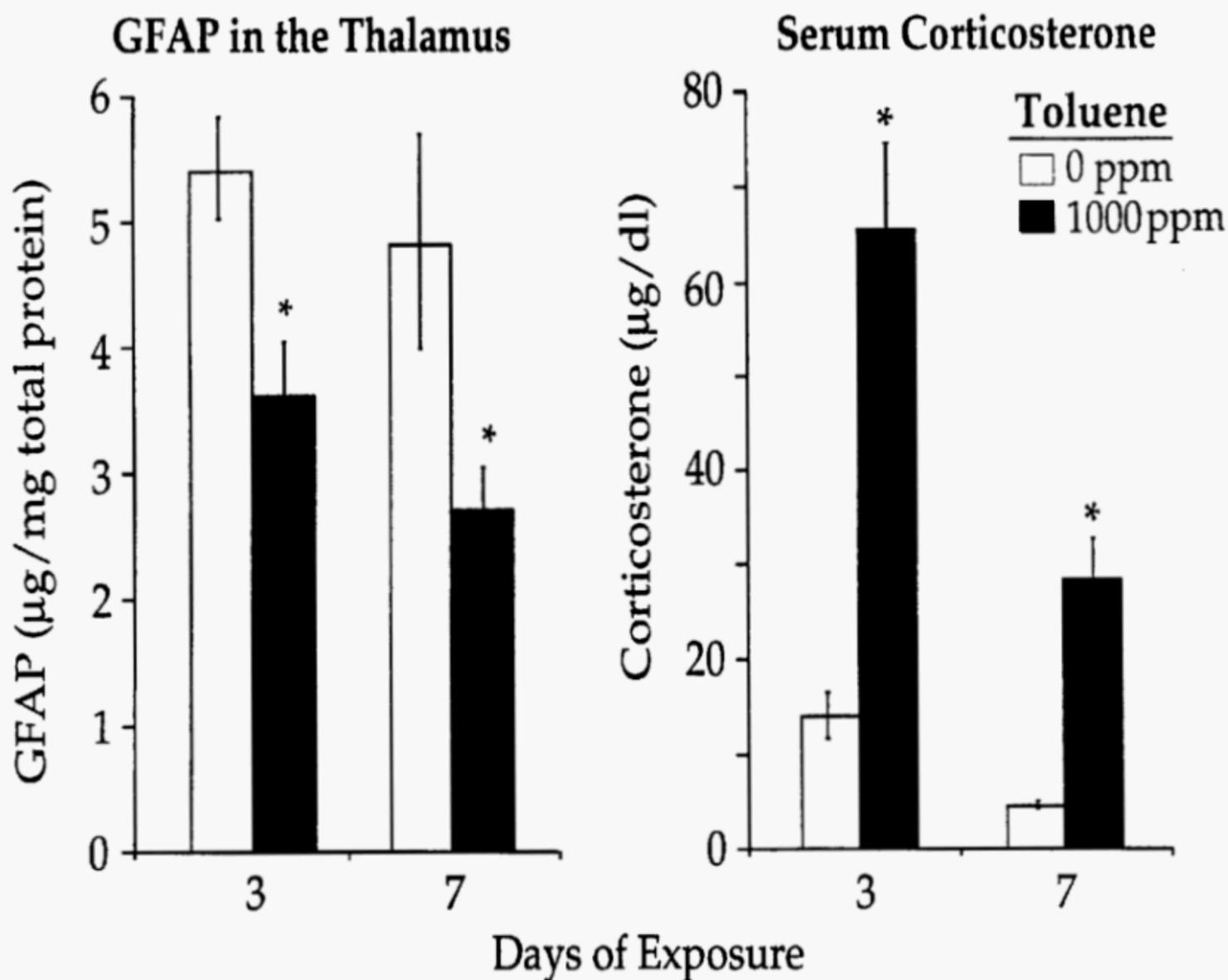


Figure 8. Reduction in Thalamic GFAP on Days 3 and 7 of Exposure to 1,000 ppm Toluene (left side). Serum Corticosterone after 3 and 7 Days Exposure to 1,000 ppm Toluene (right side). Control represents age-matched rats given sham exposures to filtered air.

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.

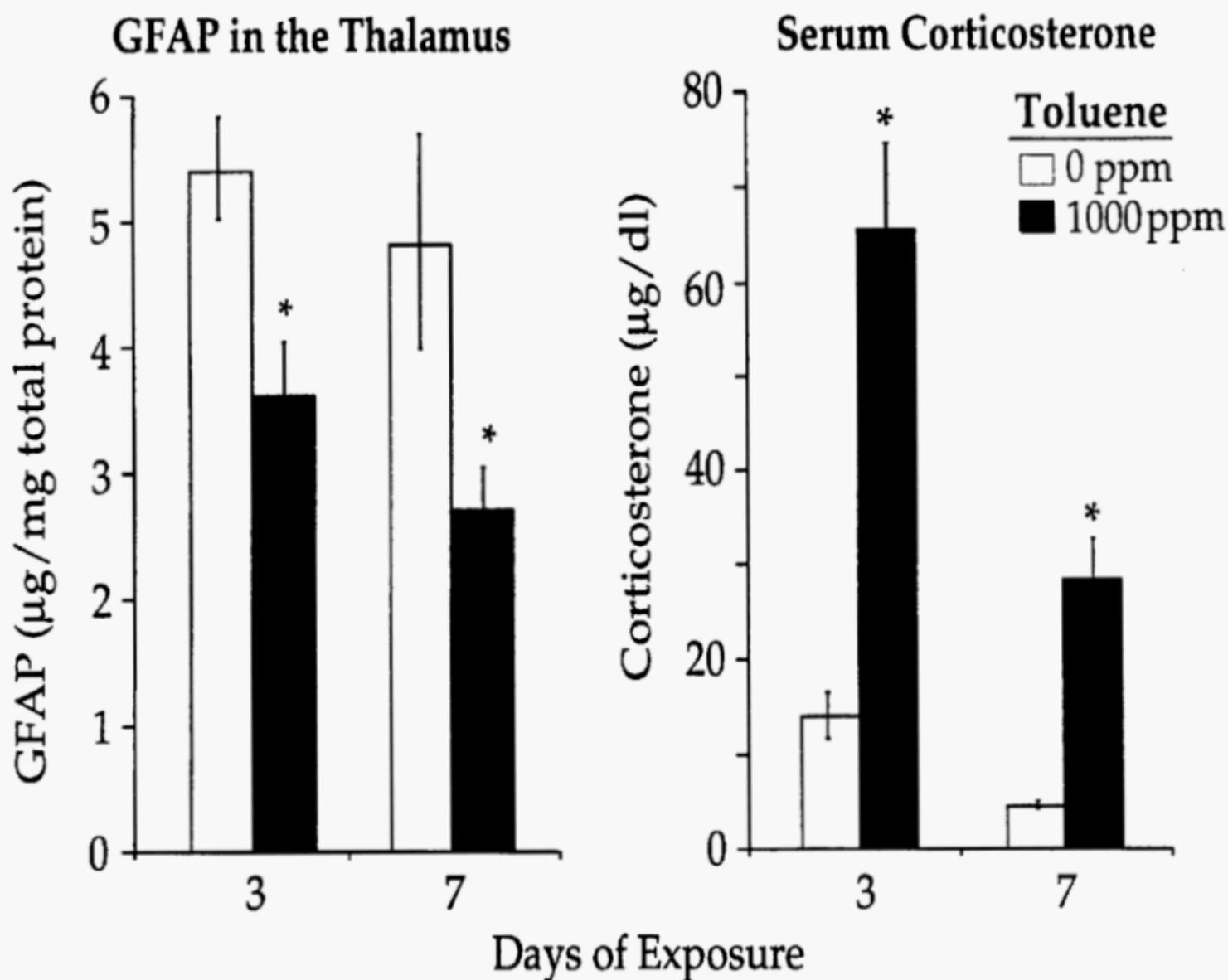


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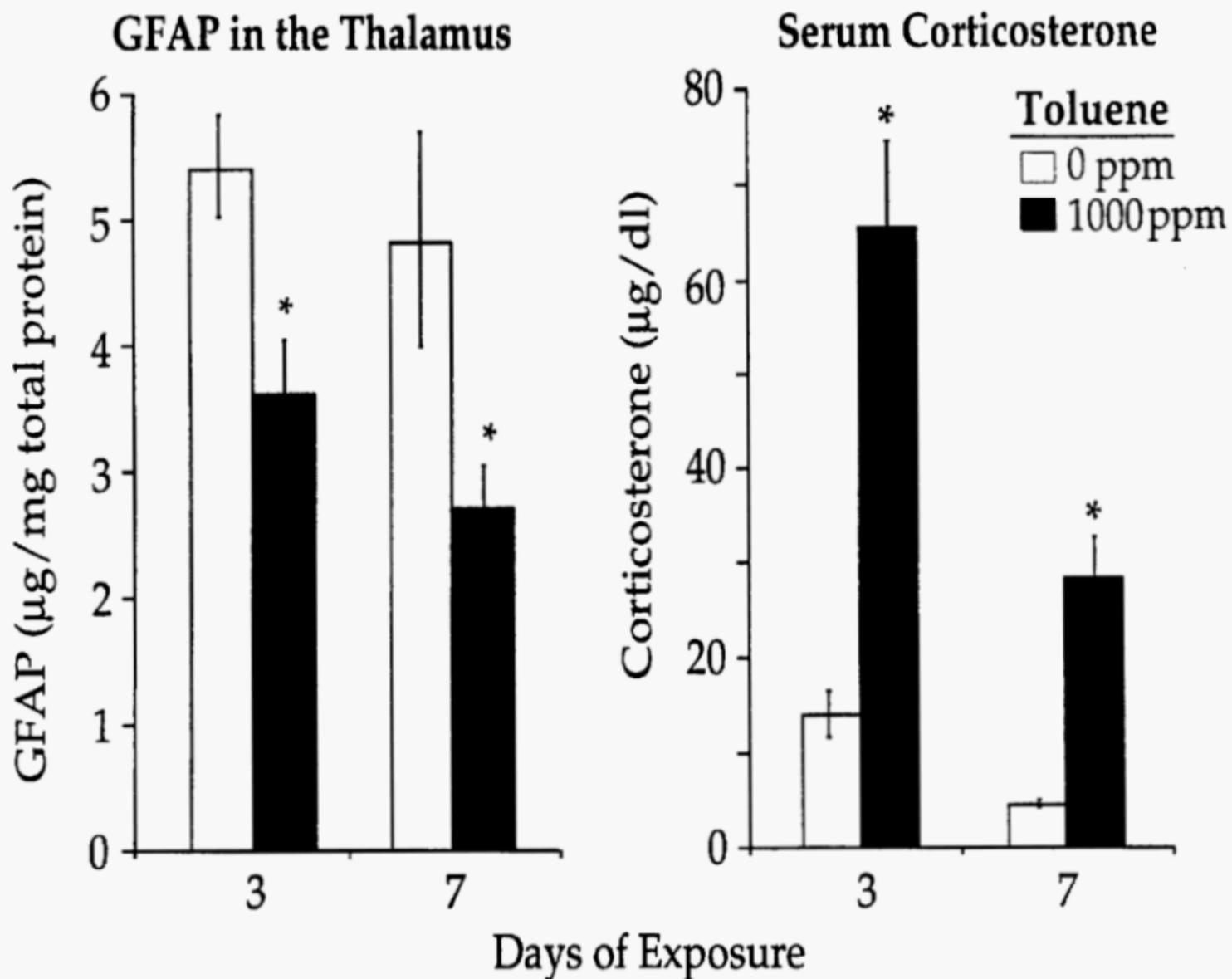


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not observed in the cortex nor in the cerebellum. The declines in GFAP concentration during toluene exposure differ from the more commonly reported toxicant-induced pattern of increased GFAP (O'Callaghan, 1988), but are reminiscent of decreases in 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 (≤ 300 ppm). In humans, signs of

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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 subtypes 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.

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