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Subchronic Dermal Application of *N,N*-Diethyl *m*-Toluamide (DEET) and Permethrin to Adult Rats, Alone or in Combination, Causes Diffuse Neuronal Cell Death and Cytoskeletal Abnormalities in the Cerebral Cortex and the Hippocampus, and Purkinje Neuron Loss in the Cerebellum

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N,N-Diethyl *m*-toluamide (DEET) and permethrin have been implicated as potential neurotoxic agents that may have played an important role in the development of illnesses in some veterans of the Persian Gulf War. To determine the effect of subchronic dermal application of these chemicals on the adult brain, we evaluated histopathological alterations in the brain of adult male rats following a daily dermal dose of DEET (40 mg/kg in 70% ethanol) or permethrin (0.13 mg/kg in 70% ethanol) or a combination of the two for 60 days. Control rats received a daily dermal dose of 70% ethanol for 60 days. Animals were perfused and brains were processed for morphological and histopathological analyses following the above regimen. Quantification of the density of healthy (or surviving) neurons in the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampus, and the cerebellum revealed significant reductions in all three treated groups compared with the control group. Further, animals receiving either DEET or permethrin exhibited a significant number of degenerating (eosinophilic) neurons in the above brain regions. However, degenerating neurons were infrequent in animals receiving both DEET and permethrin, suggesting that neuronal cell death occurs earlier in animals receiving combined DEET and permethrin than in animals receiving either DEET or permethrin alone. The extent of neuron loss in different brain regions was similar among the three treatment groups except the dentate gyrus, where neurodegeneration was significantly greater with exposure to DEET alone. The neuron loss in the motor cerebral cortex and the CA1 subfield of all treated groups was also corroborated by a significant decrease in microtubule associated protein 2-immunoreactive elements (15–52% reduction), with maximal reductions occurring in rats receiving DEET alone; further, the surviving neurons in animals receiving both DEET and permethrin exhibited wavy and beaded dendrites. Analysis of glial fibrillary acidic protein immunoreactivity revealed significant hypertrophy of astrocytes in the hippocampus and the cerebellum of all treated groups (24–106% increase). Thus, subchronic dermal application of DEET and permethrin to adult rats, alone or in combination, leads to a diffuse neuronal cell death in the cerebral cortex, the hippocampal formation, and the cerebellum. Collectively, the above alterations can lead to many physiological, pharmacological, and behavioral abnormalities, particularly motor deficits and learning and memory dysfunction.

Key Words: **brain injury; Gulf War syndrome; glial hypertrophy; glial fibrillary acidic protein; microtubule associated protein 2; neuron degeneration.**

INTRODUCTION

During the Persian Gulf War (PGW) in 1991, many service personnel were exposed to a variety of chemicals, including *N,N*-diethyl *m*-toluamide (DEET) and permethrin (2, 18). DEET and permethrin, in particular, were used extensively by service personnel as a protection against insectborne diseases (18). In the last decade, many veterans have complained of chronic symptoms including headache, loss of memory, fatigue, muscle and joint pain, and ataxia. DEET and permethrin have been implicated as two of the likely neurotoxic agents that may have played a significant role

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in the occurrence of neurological disorders in some veterans of the PGW.

The insect repellent DEET and the pyrethroid insecticide permethrin (3-phenoxybenzly [±]-*cis, trans*-3-[2,2-dichlorovinyl]-2,dimethylcyclopropane-1-carboxylate) have been used extensively by humans since their introduction. DEET is used as an effective repellent against mosquitoes, flies, ticks, and other insects in the form of lotion, stick, or spray (21, 28). Extensive and repeated topical application of DEET can result in human and animal poisoning including death (10, 15, 20, 29). The symptoms associated with DEET poisoning are characterized by tremors, restlessness, difficulty with speech, seizures, impairment of cognitive function, and coma (21). High levels of DEET exposure have been reported to cause spongiform myelinopathy (38). DEET efficiently crosses the dermal barrier (17, 34, 40,) and localizes in dermal fat deposits (5, 32). Permethrin is a type I synthetic pyrethroid insecticide that exists in four different stereoisomers (8). It provides insecticidal activity for several weeks following a single application. Permethrin toxicity is due to prolonged opening of the sodium channels, leading to repetitive discharges after a single stimulus (23). This repetitive nerve action is associated with tremors, hyperactivity, ataxia, convulsions, and, in some cases, paralysis (30).

The majority of the symptoms reported by the affected veterans of the PGW involve abnormal regulation of functions in either the central or peripheral nervous system or both. Recent studies in our laboratory have suggested significant sensorimotor deficits and blood–brain barrier disruption following exposure to DEET and permethrin (1). In this study, we evaluated the extent of neurodegeneration within the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampus, and the cerebellum of adult rats after daily dermal application of DEET and permethrin for 60 days, alone or in combination. Dermal doses of 40 mg/kg DEET and 1.3 mg/kg permethrin were applied daily, because they were determined to be the doses that military personnel were exposed to during the Persian Gulf War (Dr. W. C. McCain, U.S. Army Center for Health, Promotion, and Prevention Medicine, Aberdeen Proving Ground, MD, personal communication).

Following exposure of animals to DEET and permethrin for 60 days, alone or in combination, we rigorously quantified neurodegeneration in the above brain regions by several indices. These include: (i) measurement of the density of both healthy (surviving) and dying neurons; (ii) quantification of the reductions in the microtubule-associated protein 2 (MAP-2)-immunoreactive elements; and (iii) measurement of the extent of upregulation in the glial fibrillary acidic protein (GFAP)-immunopositive structures. In addition, we investigated the histopathological alterations in surviving neurons, particularly the orientation and cytoarchitecture of dendrites using MAP-2 immunostaining.

MATERIALS AND METHODS

Chemicals and Antibodies

DEET (97.7%, *N,N*-diethyl *m*-toluamide) was purchased from Sigma Chemical Company (St. Louis, MO). Technical-grade permethrin, (±)-*cis/trans*-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester (93.6%), was obtained from Roussel Uelaf Corporation (Pasadena, TX). The monoclonal antibody (SMI 52) against MAP-2 was from Sternberger Monoclonals (Lutherville, MD), the polyclonal antibody against GFAP was from Dako Laboratories (Carpinteria, CA), and the avidin–biotin complex (ABC) detection kits were purchased from Vector Labs (Burlington, CA). All other chemicals and reagents were of highest purity available from commercial sources.

Animals

Male Sprague–Dawley rats (200–250 g) obtained from Zivic Miller (Allison Park, PA) were used. Animals were randomly assigned to control and treatment groups of five rats ($n = 5$) and housed at 21–23°C with a 12-h light/dark cycle. They were supplied with Purina Certified Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. The rats were allowed to adjust to their environment for a week before treatment. Animal care was in accordance with the Army Guidelines and Duke University Animals Care and Use Committee.

Dermal Application of DEET and Permethrin

The chemicals (DEET and permethrin) were applied directly to the skin of preclipped areas (2.5 cm^2) in the back of the neck to give the desired concentration of test compounds in 1 ml of the vehicle solution. Groups of five rats received a daily dermal dose of 40 mg/kg DEET in 70% ethanol, or 0.13 mg/kg permethrin in 70% ethanol, or the combination of DEET and permethrin. Control animals received an equal volume of the vehicle. The treatment was carried out daily, 7 days a week, for 60 days. The doses of DEET and permethrin are based on an estimate of the exposure that may have occurred to army personnel during the PGW (1). For combined exposure, both chemicals (at the single dose level) were applied simultaneously on adjacent areas of the skin in the back of the neck.

Histopathological Assessment

Twenty-four hours after the last dose, animals belonging to each group ($n = 5$ per group) were anesthetized with pentobarbital (100 mg/kg) and perfused through the heart with saline followed by 4% parafor-

maldehyde and 0.1% gluteraldehyde in Tris buffer. The brains were removed, postfixed, and embedded in paraffin. Four-micrometer-thick coronal sections were cut through different brain regions. In every brain, representative sections ($n = 5$) through the motor and sensory cortex, the septal hippocampus, and the cerebellum were processed and stained with hematoxylin and eosin (H&E) for light microscopy.

MAP-2 and GFAP Immunohistochemistry

Sections were deparaffinized and blocked with 10% normal serum (normal horse serum for MAP-2, normal goat serum for GFAP) in 0.05 M TBS for 30 min. Sections were incubated overnight at room temperature in primary antisera diluted at 1:1000 for MAP-2 in 0.05 M Tris-buffered saline (TBS) containing 1% normal horse serum, and 1:10,000 for GFAP with 0.05 M TBS containing 1% normal goat serum. Following a thorough rinse in 0.05% TBS, sections were incubated for 1 h at room temperature in appropriate biotinylated secondary antibody (i.e., horse anti-mouse IgG for MAP-2, goat anti-rabbit IgG for GFAP, diluted 1:200) containing 1% normal serum (horse serum for MAP-2 staining, goat serum for GFAP staining).

Sections were rinsed with several changes of 0.05 M TBS and incubated for 1 h in the avidin–biotin peroxidase complex solution diluted 1:25 in 0.05 M TBS. Following this, the sections were rinsed with several changes of 0.05 M TBS and incubated with 3,3-diaminobenzene tetrahydrochloride (DAB) for 10 min. The reaction was stopped by several rinses in 0.05 M TBS. The sections were then dehydrated in alcohol, cleared in xylene, and coverslipped with Permount.

Quantitative Evaluation of the Number of Healthy and Dying Neurons in Different Brain Regions

The numerical density of healthy (surviving) and dying neurons per square millimeter of tissue area in H&E-stained sections was measured for layers III and V of the motor cortex, granule cell layer of the dentate gyrus, pyramidal cell layer of CA1 and CA3 subfields of the hippocampus, and Purkinje cell layer of the cerebellum in lobule 2 of the cerebellar vermis and crus 2 ansiform lobule of the cerebellar hemisphere. Five sections through each of the above brain regions were employed for measurements in each animal belonging to the following four groups: (a) control animals ($n = 5$); (b) animals treated with DEET ($n = 5$); (c) animals treated with permethrin ($n = 5$); (d) animals treated with both DEET and permethrin ($n = 5$). Measurements in sections from various groups were performed in a blinded fashion using experimental codes. The coding was such that animal treatments were not known during measuring; however, sections that came from the same animal were identified. All measurements were performed using a Nikon E600 microscope equipped with eyepiece grid. At a magnification of 400× (using 40× objective lens and 10× eyepieces), both dying and healthy neurons, within a unit area of each section, were counted.

The unit area selected for measurements varied for different regions of the brain, depending on the availability of the overall area for different layers. The area measured was 0.019 mm^2 for layer III of the motor cortex, 0.063 mm^2 for layer V of the motor cortex, 0.013 mm^2 for the dentate granule cell layer, 0.0063 mm^2 for the CA1 pyramidal cell layer, 0.013 mm^2 for the CA3 pyramidal cell layer, and 0.0063 mm^2 for Purkinje cell layer of the cerebellum. For measurement of the surviving neurons, only those that exhibited a hematoxylin-stained nucleus with a clear nucleolus were counted. For measurement of dying neurons, only those neurons that exhibited dense eosinophilic staining in both soma and proximal dendrites were counted. Finally, the density of neurons per unit area was transformed to the numerical density per square millimeter of respective brain region.

The mean value for each of the six brain regions (layers III and V of the motor cortex, granule cell layer of the dentate gyrus, CA1 and CA3 pyramidal cell layers of the hippocampus, and Purkinje cell layer of the cerebellum) was calculated separately for each animal by using data from five sections before the means and standard errors were determined for the total number of animals included per group. Mean values between different groups of animals were compared separately for each of the above brain regions using one-way ANOVA with Student Newman–Keuls multiple comparison post hoc test.

Morphometric Analyses of MAP-2-Positive and GFAP-Positive Immunoreactivity in Different Brain Regions

Morphometric analyses of MAP-2-positive and GFAP-positive immunoreactive structures in different regions were performed by using Scion Image for Windows, based on NIH Image for Macintosh (Scion Corporation, Frederick, MD). For every brain region, two sections were measured in each animal. All data were collected blind to experimental codes and means were calculated for each animal individually before the means were determined for the five animals per group. Statistical comparisons on MAP-2 and GFAP measurements in different brain regions between control and treated groups used ANOVA with Student Newman–Keuls multiple comparison post hoc test.

The area occupied by MAP-2-positive immunoreactive structures per unit area of tissue (0.044 mm^2) was determined for layers III and V of the motor cerebral cortex and the CA1 subfield of the hippocampus. The area occupied by GFAP-positive immunoreactive structures per unit area of tissue (0.0176 mm^2) was deter-

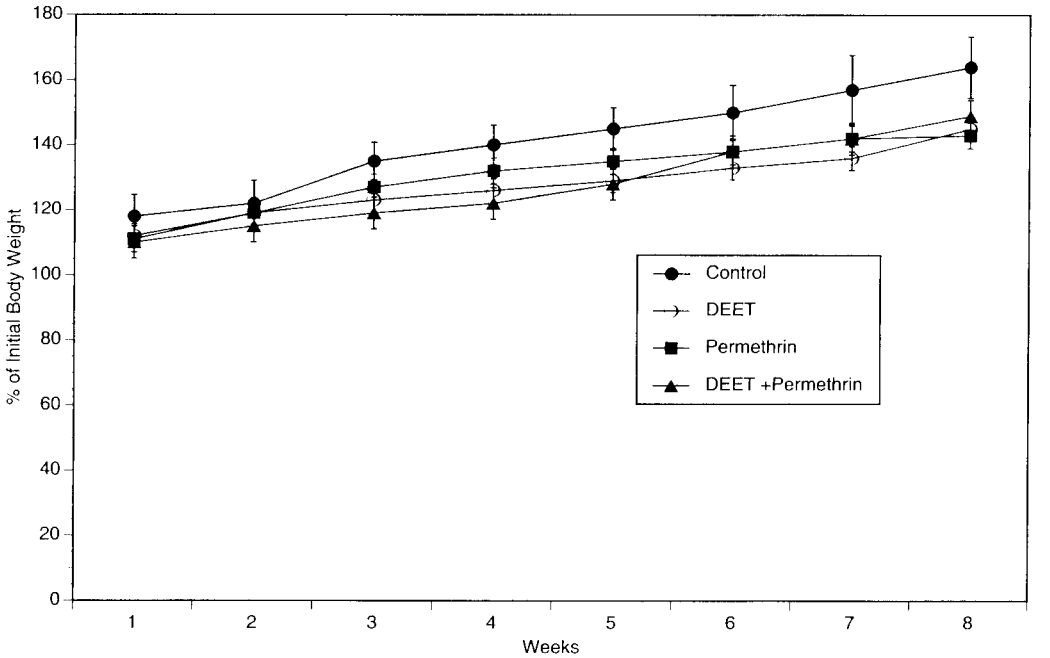

FIG. 1. Effect of daily administration of DEET (40 mg/kg/day, dermal) and permethrin (0.1340 mg/kg/day, dermal) on body weight of rats. Animals were assessed for body weight each week. The percentages of initial body weight for control (mean ± SEM) are 118 ± 6.7 (Week 1), 122 ± 7.1 (Week 2), 135 ± 5.8 (Week 3), 140 ± 6.1 (Week 4), 145 ± 6.5 (Week 5), 155 ± 8.5 (Week 6), 157 ± 10.7 (Week 7), and 164 ± 9.4 (Week 8). Analysis with one-way ANOVA with Student Newman–Keuls multiple comparisons test revealed no significant differences between groups at all time points during the exposure regimen ($P > 0.1$).

mined in layer V of the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampal formation, and the white matter of the cerebellum. For every region, the microscopic image using 20× objective lens was transferred to the computer screen by focusing the appropriate area of the immunostained section with a Nikon E600 microscope equipped with a digital camera (DAGE-MTI, CCD100) connected to an IBM computer. The same intensity of light in the microscope and the same parameters in the digital camera were used to digitize all the samples from different brain regions. Images in the Scion Image are two-dimensional arrays of pixels (picture elements). Pixels are represented by 8-bit unsigned integers, ranging in value from 0 to 255. Scion Image displays zero pixels as white and those with a value of 255 as black. The background and target values were set at 145 and 255, respectively, following digitization of the original gray value image in the computer screen. These values were determined by selecting the background and target areas in several sections from both control and treated animals before commencing the measurements on coded slides for statistical analysis. This scale eliminated the background staining completely and retained all the target (MAP-2- or GFAP-immunopositive) structures in the range (145–255). The binary image of MAP-2- or GFAP-positive elements was then generated by selecting a suitable threshold value (which varied from 155 to 165) to include all the MAP-2- or GFAP-positive structures without any background. The final binary image was cross-checked with the original gray value image by alternating the two images on the computer screen.

Finally, the image was frozen and the area occupied by the MAP-2- or GFAP-positive structures in the field was measured by selecting the "Analyze Particles" command of the Scion Image program. In this way, the area of individual particles (i.e., MAP-2- or GFAP-immunoreactive structures) in the selected field was measured, and the sum area of all particles was stored for further calculations and statistical analysis. Since spatial calibration of the image was performed in micrometers using the "Set Scale" function of the program prior to measurements, the results from area measurements were obtained in square micrometers and converted later into square millimeters.

RESULTS

General Observations

The clinical condition of animals treated with daily dermal application of DEET, permethrin, or the combination of DEET and permethrin was not different from that of control animals. In addition, statistically, no differences were observed in the weights of animals between the control and treated groups (Fig. 1).

Histopathological Changes

Evaluation of brain sections stained with hematoxylin and eosin (H&E) clearly revealed neuronal degeneration in rats treated with DEET, permethrin, or the combination of DEET and permethrin, in comparison to vehicle-treated rats. Degenerating neurons were characterized by eosinophilic staining of both cell body and proximal dendrites. In contrast, the healthy neurons in the same section exhibited hematoxylin-stained nuclei (with clear nucleoli) and eosin-stained perinuclear cytoplasm. The brain regions where neuronal degeneration was obvious include the motor cerebral cortex, the dentate gyrus, the CA1 and CA3 subfields of the hippocampus, and the Purkinje cell layer of the cerebellum. Other areas of the brain, though, showed occasional dying (eosinophilic) neurons in some animals; the overall cytoarchitecture remained comparable to that of control (vehicle-treated) rats. Therefore, detailed investigation of neuropathological alterations using quantitative methods was performed only on the above brain regions.

Alterations in the Cytoarchitecture of the Motor Cerebral Cortex

In animals treated with either DEET or permethrin alone, both superficial and deeper regions of the motor cortex exhibited degenerating neurons in H&E-stained sections. In superficial regions (layers I–III, Fig. 2), degenerating neurons were conspicuous in both layers II and III. The majority of degenerating neurons in these layers were of the pyramidal type with prominent eosinophilic apical dendrites (Fig. 2 (A_2, A_3)). The overall degree of neuronal degeneration was comparable in animals treated with DEET and animals treated with permethrin. In deeper regions of the cortex (layers IV–VI), degenerating neurons were observed mostly in layer V. These are larger pyramidal neurons with prominent apical and basal dendrites emanating from a larger pyramid-shaped cell body (Fig. 3 (A_2, A_3)). The extent of degeneration appeared greater with exposure to DEET than with exposure to permethrin. Further, in addition to the presence of many degenerating neurons, both superficial and deeper regions of the cortex in animals treated with either DEET or permethrin exhibited clearly reduced packing density of surviving neurons, in comparison to the cortex of control animals (Figs. 2 (A_1–A_3) and 3 (A_1–A_3)). The adjacent sections stained for MAP-2 revealed significantly reduced MAP-2 positive dendrites in layers III and V of animals treated with either DEET or permethrin, in comparison to control animals (Figs. 2 (B_1–B_3) and 3 (B_1–B_3)). MAP-2 expression in dendrites also appeared somewhat disrupted and scarcer. Further, immunostaining with GFAP demonstrated hypertrophy of astrocytes with increased GFAP expression in animals treated with either DEET or permethrin compared with control animals (Figs. 2 (C_1–C_3) and 3 (C_1–C_3)). Thus, both MAP-2- and GFAP-immunostained sections clearly corroborated the DEET- and permethrin-induced neurodegeneration, as observed in H&E-stained samples.

In animals treated with both DEET and permethrin, degenerating (or eosinophilic) neurons were infrequent in both superficial and deeper regions of the cortex (Figs. 2 (A_4) and 3 (A_4)). The packing density of surviving neurons, however, appeared less than that of control animals (Figs. 2 (A_1, A_4) and 3 (A_1, A_4)). Areas devoid of neurons were conspicuous in layers III and V of the cortex (Figs. 2 (A_4) and 3 (A_4)). Thus, the lack of degenerating (eosinophilic) neurons in animals receiving both DEET and permethrin appeared to be due to early cell death of neurons following the combined exposure in comparison to animals receiving either DEET or permethrin alone. The adjacent sections stained for MAP-2 substantiated the above finding by exhibiting reduced MAP-2 staining of dendrites, particularly in layer V of the cortex (Figs. 2 (B_4, C_4) and 3 (B_4, C_4)). The GFAP-positive astrocytes were of reactive type and exhibited characteristic GFAP expression in their soma. In addition, the pattern of MAP-2 expression differed from those of both control animals and animals treated with either DEET or permethrin by showing a lack of expression in soma of neurons, and by their wavy and fragmented appearance in dendrites throughout the thickness of the cortex (Figs. 2 (B_4) and 3 (B_4)).

Extent of Neuron Loss, Reductions in MAP-2 Immunoreactivity, and Upregulation of GFAP Immunoreactivity in the Motor Cortex

Quantification of healthy (or surviving) neurons per square millimeter of layers III and V of the motor cortex revealed that animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibited a significant decrease in the number of surviving neurons in both layers III and V ($P < 0.01$; Fig. 4), in comparison to control animals. Further comparison between treated groups revealed that the extent of reductions in the density of healthy neurons within the motor cortex was similar in the three treatment groups. Analysis of dying neurons revealed that, in layer III of the motor cortex, animals treated with DEET exhibited a significant number of dying neurons, in comparison to control animals ($P < 0.01$) (Fig. 4). With respect to layer V of the motor cortex, animals treated with either DEET or permethrin exhibited a significant number of dying neurons compared with control animals ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 4). Thus, subchronic dermal application of DEET and permethrin, alone or in combination, leads to a significant reduction in the number of surviving neurons in the motor cortex, and the extent of overall reductions in neurons is similar in the three treatment groups. This suggests that concurrent application of DEET and permethrin does not induce enhanced neu-

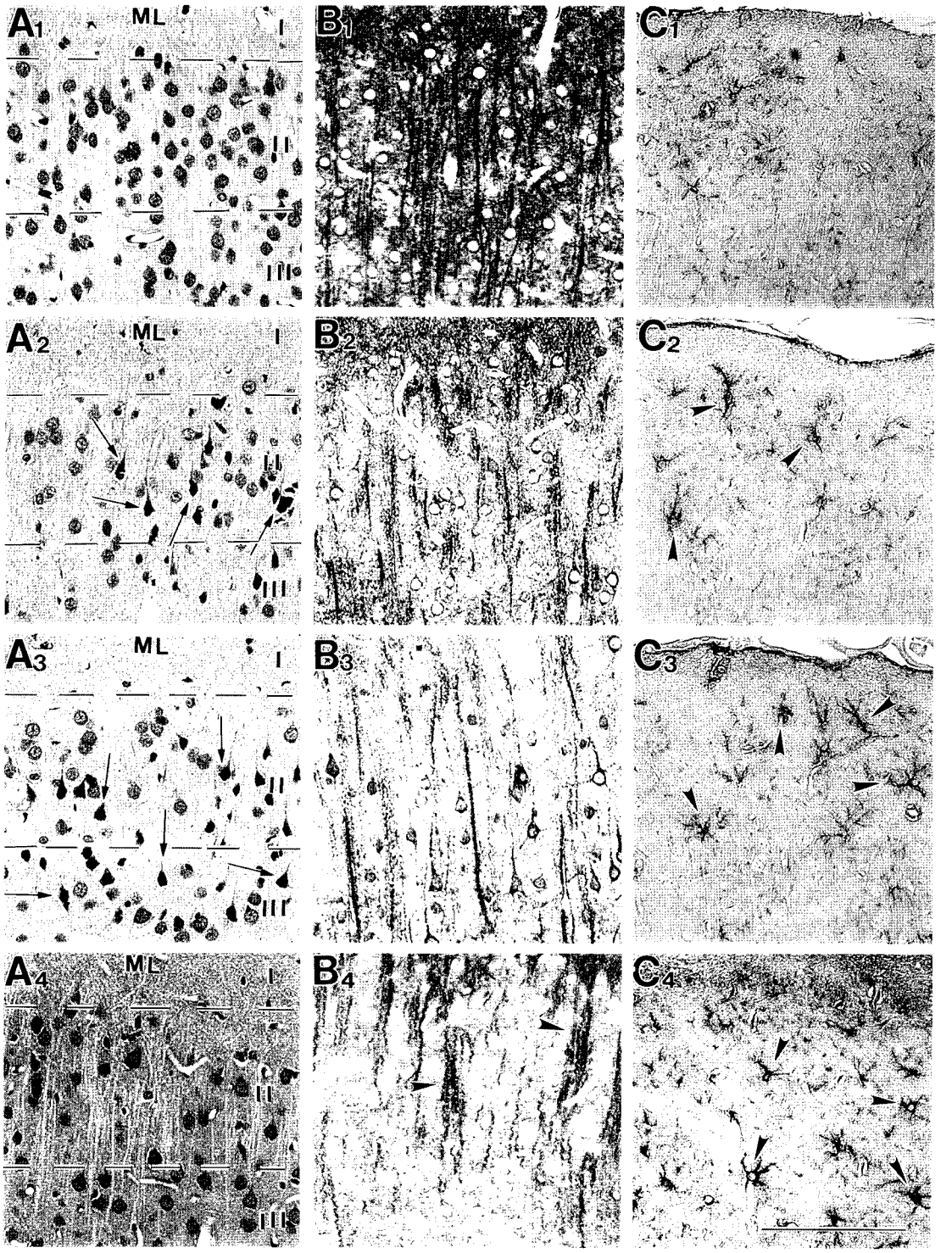

FIG. 2. Alterations in the superficial layers (layers I–III) of the motor cortex following daily dermal application of DEET and permethrin. (A_1–A_4) H&E staining, (B_1–B_4) MAP-2 immunostaining, (C_1–C_4) GFAP immunostaining. (A_1, B_1, C_1) Examples from a control rat. (A_2, B_2, C_2) Examples from a rat treated with DEET. (A_3, B_3, C_3) Examples from a rat treated with permethrin. (A_4, B_4, C_4) Examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in rats treated with either DEET or permethrin (arrows in A_2 and A_3). Whereas in the rat treated with both DEET and permethrin, vacant areas devoid of neurons (A_4) and wavy appearance of dendrites (arrows in B_4) are conspicuous. Note that, in all three treated groups, the overall MAP-2 immunoreactivity is significantly reduced (B_2–B_4) and GFAP immunoreactivity of astrocytes is significantly enhanced (arrowheads in C_2–C_4), in comparison to the control group (B_1, C_1). Bar = 100 μm.

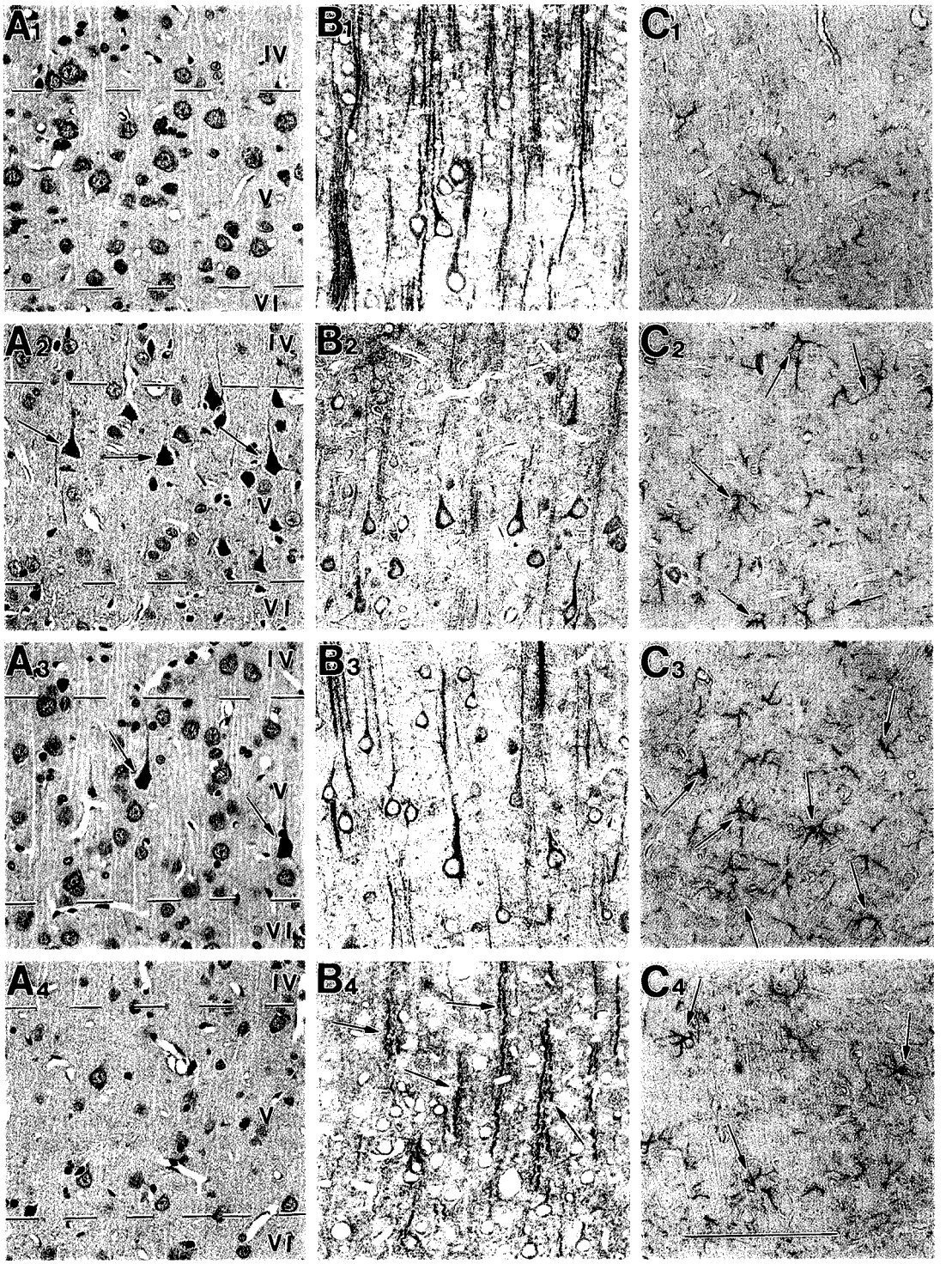

FIG. 3. Changes in the deeper layers (layers IV and V) of the motor cortex following daily application of DEET and permethrin. (A_1–A_4) H&E staining, (B_1–B_4) MAP-2 immunostaining, (C_1–C_4) GFAP immunostaining. (A_1, B_1, C_1) Examples from a control rat. (A_2, B_2, C_2) Examples from a rat treated with DEET. (A_3, B_3, C_3) Examples from a rat treated with permethrin. (A_4, B_4, C_4) Examples from a rat treated with both DEET and permethrin. A large number of degenerating pyramidal neurons are clearly visible in layer V of the cortex in rats treated with either DEET or permethrin (arrows in A_2 and A_3). In the rat treated with both DEET and permethrin, vacant areas devoid of neurons (A_4) and wavy appearance of dendrites (arrows in B_4) are conspicuous. Note that, in all three treated groups, the overall MAP-2 immunoreactivity is significantly reduced (B_2–B_4) and GFAP immunoreactivity of astrocytes is significantly enhanced (arrows in C_2–C_4), in comparison to the control group (B_1, C_1). Bar = 100 μm.

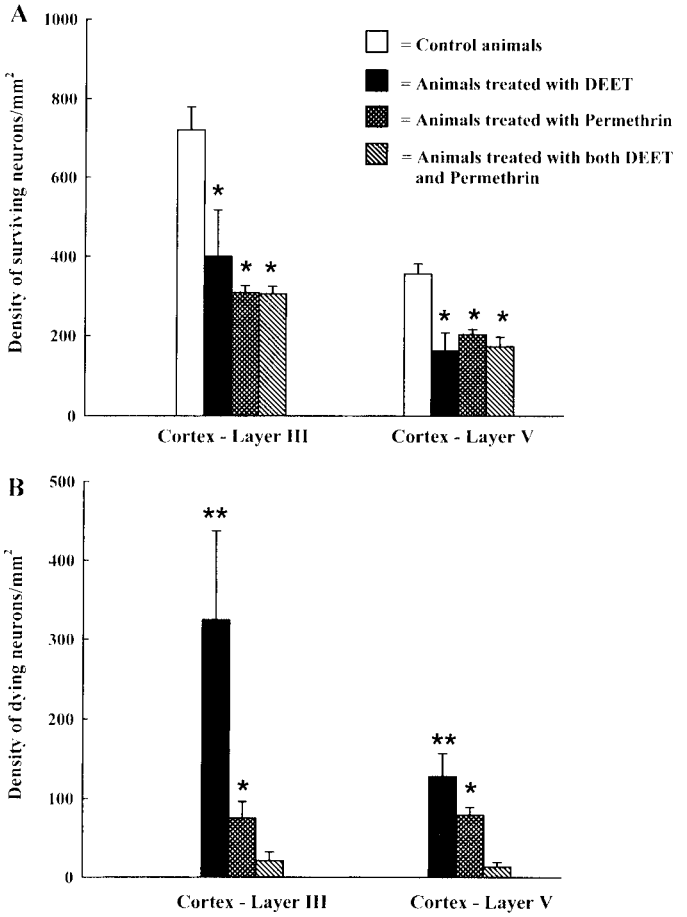

FIG. 4. Histograms show the density of surviving (A) and dying (B) neurons per square millimeter of layers III and V of the motor cortex. Values represent means and standard errors ($n = 5$ per group). Analyses with one-way ANOVA show significant differences for surviving neurons between groups ($P < 0.01$ in layer III, $P < 0.001$ in layer V). The post hoc analysis with the Student Newman–Keuls multiple comparison test further revealed that animals treated with DEET or permethrin alone exhibit a significant decrease in the number of surviving neurons, in comparison to control animals (layer III, $P < 0.05$; layer V, $P < 0.01$). Analysis of dying neurons revealed that, in layer III of the motor cortex, animals treated with DEET exhibit a significant increase in the number of dying neurons, in comparison to control animals, animals treated with permethrin, and animals treated with both DEET and permethrin ($P < 0.01$). In layer V of the motor cortex, animals treated with either DEET or permethrin exhibit a significant increase in dying neurons compared with control animals ($P < 0.001$ and $P < 0.01$, respectively). Further, exposure to DEET alone results in a significantly increased number of dying neurons than exposure to permethrin alone ($P < 0.05$) and exposure to both DEET and permethrin ($P < 0.001$).

ron loss in the motor cortex, compared with exposure to either DEET or permethrin.

Quantification of the area of MAP-2-immunoreactive elements per unit area of layers III and V of the motor cortex showed that there were fewer MAP-2-positive structures in the motor cortex of all treated groups (Fig. 5A). In layer III of the cortex, the MAP-2-immunoreactive structures exhibited 27–28% reduction with exposure to DEET or permethrin alone ($P < 0.05$) and 15% reduction with exposure to both DEET and permethrin ($P > 0.05$), whereas in layer V of the motor cortex, the MAP-2-immunopositive structures showed 52% reduction with exposure to DEET alone ($P < 0.01$), 35% reduction with exposure to permethrin alone ($P < 0.05$), and 49% reduction with exposure to both DEET and permethrin ($P < 0.01$). The measurement of GFAP-immunoreactive structures per unit area of layer V of the motor cortex revealed a signification upregulation in GFAP-positive elements ($P < 0.05$) with exposure to either DEET (74% increase) or permethrin (63% increase) (Fig. 5B). However, with

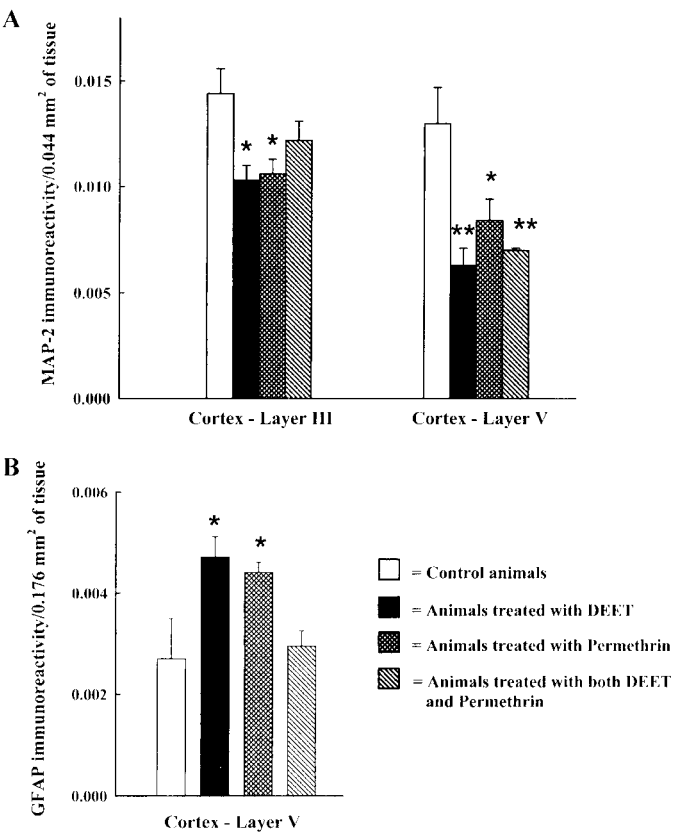

FIG. 5. Histograms in (A) show the area of MAP-2-immunoreactive elements in square millimeter per unit area (0.044 mm²) of layers III and V of the motor cortex. Values represent means and standard errors ($n = 5$ per group). In layer III of the cortex, the MAP-2-immunoreactive structures exhibited 27–28% reduction with exposure to DEET or permethrin alone ($P < 0.05$), and 15% reduction with exposure to both DEET and permethrin ($P > 0.05$). Whereas in layer V of the motor cortex, the MAP-2-immunopositive structures showed 52% reduction with exposure to DEET alone ($P < 0.01$), 35% reduction with exposure to permethrin alone ($P < 0.05$), and 49% reduction with exposure to both DEET and permethrin ($P < 0.01$). Histograms in (B) show the area of GFAP-immunoreactive elements (in mm²) per unit area (0.176 mm²) of layer V of the motor cortex. Values represent means and standard errors ($n = 5$ per group). Note a signification upregulation in GFAP-positive elements ($P < 0.05$) with exposure to either DEET (74% increase) or permethrin (63% increase). However, with combined exposure to DEET and permethrin, there was only a 10% increase in GFAP immunoreactivity ($P > 0.05$).

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Alterations in the Hippocampal Formation

Neuronal degeneration was obvious in the dentate gyrus and CA1 and CA3 subfields of the hippocampal formation following exposure to DEET and permethrin, alone or in combination. In all treated groups, the thickness and cell packing density of the granule cell layer appeared reduced compared with that of control animals. Further, in dentate gyrus of animals treated with DEET and permethrin, degenerating neurons were observed in both the granule cell layer and the dentate hilus (Fig. 6 (A_2, A_3)). GFAP immunoreactivity was enhanced in the molecular layer and the hilus of all three treated groups, in comparison to control animals (Fig. 6 (B_1–B_4)). MAP-2 staining of the granule cell layer and the molecular layer did not show any significant differences between animals belonging to different treated groups but appeared slightly reduced compared with that of the control animals (data not shown).

In the CA1 subfield of the hippocampus, the thickness and cell packing density of stratum pyramidale appeared reduced in treated groups compared with control animals (Fig. 7). Further, degenerating neurons were clearly observed in the stratum pyramidale of animals treated with either DEET or permethrin alone (Fig. 7 (A_1–A_3)). MAP-2 staining of adjacent sections demonstrated a conspicuously reduced density of MAP-2-positive dendrites in animals belonging to all three treated groups compared with control animals (Fig. 7 (B_1–B_4)). In animals treated with DEET, MAP-2-positive apical dendrites in stratum radiatum were thinner and appeared disrupted, whereas in animals treated with permethrin, MAP-2-positive dendrites appeared to be either beaded or arranged in aggregates, with highly conspicuous vacant spaces between them. In animals treated with both DEET and permethrin, MAP-2-positive dendrites were wavy and thinner. The appearance of MAP-2 staining of apical dendrites in all treated groups highly contrasted with the homogenous MAP-2 staining observed in control animals. Immunostaining of neighboring sections for GFAP demonstrated enhanced GFAP immunoreactivity in animals belonging to all three treated groups compared with control animals (Fig. 7 (C_1–C_4)).

In the CA3 subfield of the hippocampus, the thickness and neuronal density of stratum pyramidale appeared reduced in all three treated groups compared with control animals (Fig. 8). The degenerating neurons were conspicuous in the stratum pyramidale of animals treated with DEET alone (Fig. 8 (A_1, A_2)). MAP-2 staining of adjacent sections demonstrated only a slightly reduced density of MAP-2-positive dendrites in animals belonging to all three treated groups compared with control animals (data not shown). GFAP immunostaining of neighboring sections showed enhanced GFAP immunoreactivity in animals belonging to all three treated groups compared with control animals (Fig. 8 (C_1–C_4)).

Extent of Neuron Loss, Reductions in MAP-2 Immunoreactivity, and Upregulation of GFAP Immunoreactivity in the Hippocampal Formation

Quantification of surviving and dying neurons per square millimeter of granule cell layer of the dentate gyrus and pyramidal cell layer of subfields CA1 and CA3 (Fig. 9) demonstrated the following. In dentate granule cell layer, animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibited a significant decrease in surviving neurons, in comparison to control animals ($P < 0.01$) (Fig. 9). Further, animals treated with either DEET or permethrin exhibited a significant decrease in surviving neurons, in comparison to animals treated with combined DEET and permethrin ($P < 0.01$) (Fig. 9). Analysis of dying neurons showed that animals treated with either DEET or permethrin exhibited a significant number of dying neurons ($P < 0.05$) (Fig. 9). In the CA1 subfield, only animals treated with either DEET or permethrin exhibited a significant decrease in the number of surviving neurons compared with control animals ($P < 0.05$) (Fig. 9). Analysis of dying neurons also showed the same trend. In the CA3 subfield, animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibited a significant decrease in surviving neurons, in comparison to control animals ($P < 0.01$) (Fig. 9). Analysis of dying neurons revealed that animals treated with either DEET or permethrin exhibited a significant number of dying neurons ($P < 0.01$). Thus, a significant reduction in surviving neurons occurs in the dentate gyrus and CA3 subfield of the hippocampal formation following subchronic dermal application of DEET and permethrin, alone or in combination; however, the overall neuron loss in the dentate gyrus is significantly greater when DEET and permethrin are applied separately. In the CA1 subfield of the hippocampus, a significant decrease in the number of surviving neurons occurs with exposure to DEET or permethrin alone but not with exposure to both DEET and permethrin.

Quantification of the area of MAP-2-immunoreactive elements per unit area of the CA1 stratum radiatum showed that there were fewer MAP-2-positive structures in the CA1 subfield of all treated groups (Fig. 10A). The MAP-2-immunoreactive structures exhibited 28% reduction with exposure to DEET alone ($P < 0.05$), and 16% reduction with exposure to permethrin alone or exposure to both DEET and permethrin ($P < 0.05$). The measurement of GFAP-immunoreactive

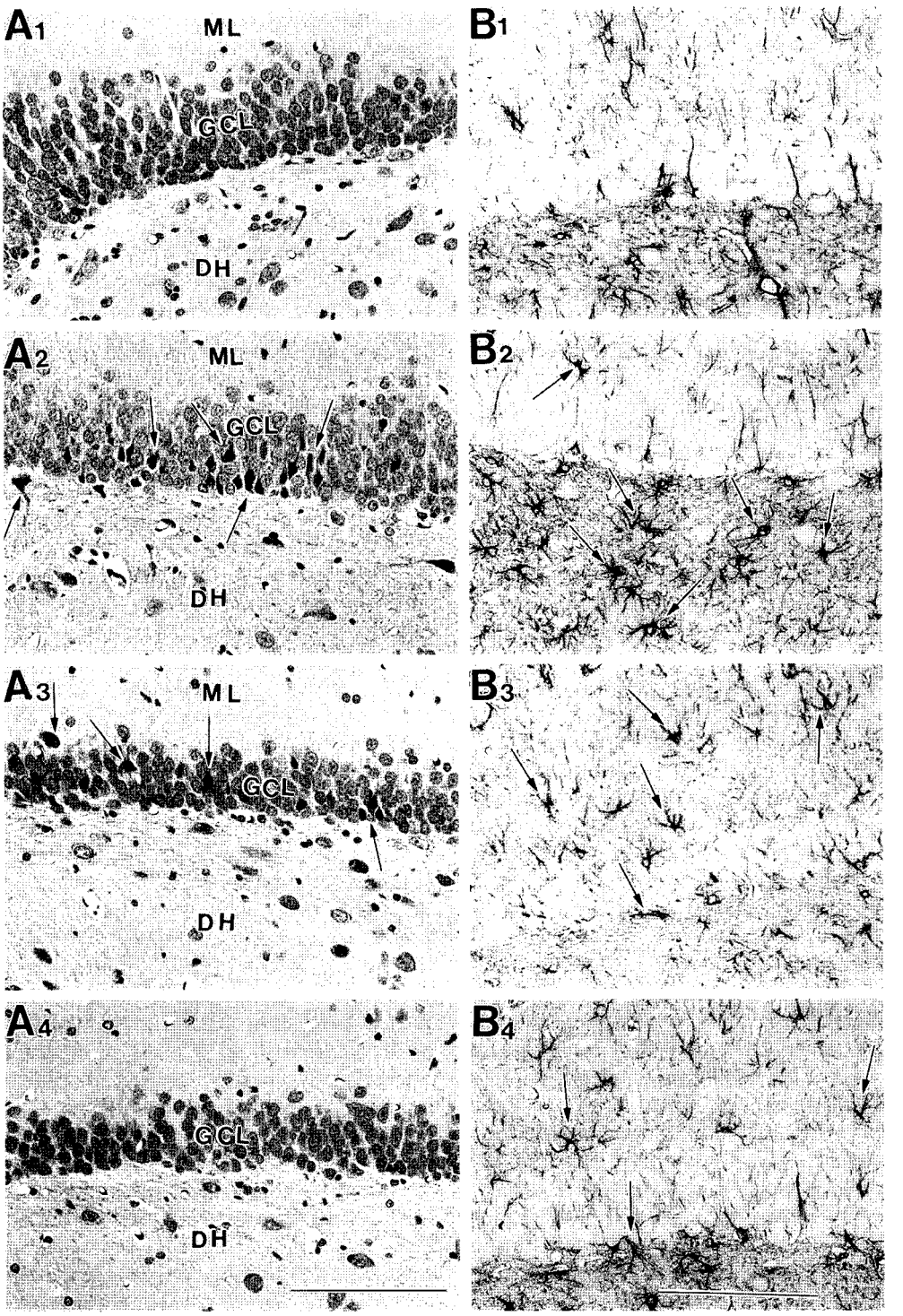

FIG. 6. Alterations in the dentate gyrus following daily application of DEET and permethrin. (A_1–A_4) H&E staining, (B_1–B_4) GFAP immunostaining. (A_1, B_1) Examples from a control rat. (A_2, B_2) Examples from a rat treated with DEET. (A_3, B_3) Examples from a rat treated with permethrin. (A_4, B_4) Examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in the dentate granule cell layer (GCL) and the dentate hilus (DH) of rats treated with either DEET or permethrin (arrows in A_2 and A_3). In the rat treated with both DEET and permethrin (A_4), both thickness and cell packing density of granule cell layer are reduced compared with the control rat. Note that GFAP immunoreactivity is upregulated in all three treated groups (B_2, B_3, B_4). ML, molecular layer. Bar = 100 μm.

combined exposure to DEET and permethrin, there was only a 10% increase in GFAP immunoreactivity ($P > 0.05$).

Alterations in the Hippocampal Formation

Neuronal degeneration was obvious in the dentate gyrus and CA1 and CA3 subfields of the hippocampal formation following exposure to DEET and permethrin, alone or in combination. In all treated groups, the thickness and cell packing density of the granule cell layer appeared reduced compared with that of control animals. Further, in dentate gyrus of animals treated with DEET and permethrin, degenerating neurons were observed in both the granule cell layer and the dentate hilus (Fig. 6 (A_2, A_3)). GFAP immunoreactivity was enhanced in the molecular layer and the hilus of all three treated groups, in comparison to control animals (Fig. 6 (B_1–B_4)). MAP-2 staining of the granule cell layer and the molecular layer did not show any significant differences between animals belonging to different treated groups but appeared slightly reduced compared with that of the control animals (data not shown).

In the CA1 subfield of the hippocampus, the thickness and cell packing density of stratum pyramidale appeared reduced in treated groups compared with control animals (Fig. 7). Further, degenerating neurons were clearly observed in the stratum pyramidale of animals treated with either DEET or permethrin alone (Fig. 7 (A_1–A_3)). MAP-2 staining of adjacent sections demonstrated a conspicuously reduced density of MAP-2-positive dendrites in animals belonging to all three treated groups compared with control animals (Fig. 7 (B_1–B_4)). In animals treated with DEET, MAP-2-positive apical dendrites in stratum radiatum were thinner and appeared disrupted, whereas in animals treated with permethrin, MAP-2-positive dendrites appeared to be either beaded or arranged in aggregates, with highly conspicuous vacant spaces between them. In animals treated with both DEET and permethrin, MAP-2-positive dendrites were wavy and thinner. The appearance of MAP-2 staining of apical dendrites in all treated groups highly contrasted with the homogenous MAP-2 staining observed in control animals. Immunostaining of neighboring sections for GFAP demonstrated enhanced GFAP immunoreactivity in animals belonging to all three treated groups compared with control animals (Fig. 7 (C_1–C_4)).

In the CA3 subfield of the hippocampus, the thickness and neuronal density of stratum pyramidale appeared reduced in all three treated groups compared with control animals (Fig. 8). The degenerating neurons were conspicuous in the stratum pyramidale of animals treated with DEET alone (Fig. 8 (A_1, A_2)). MAP-2 staining of adjacent sections demonstrated only a slightly reduced density of MAP-2-positive dendrites in animals belonging to all three treated groups compared with control animals (data not shown). GFAP immunostaining of neighboring sections showed enhanced GFAP immunoreactivity in animals belonging to all three treated groups compared with control animals (Fig. 8 (C_1–C_4)).

Extent of Neuron Loss, Reductions in MAP-2 Immunoreactivity, and Upregulation of GFAP Immunoreactivity in the Hippocampal Formation

Quantification of surviving and dying neurons per square millimeter of granule cell layer of the dentate gyrus and pyramidal cell layer of subfields CA1 and CA3 (Fig. 9) demonstrated the following. In dentate granule cell layer, animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibited a significant decrease in surviving neurons, in comparison to control animals ($P < 0.01$) (Fig. 9). Further, animals treated with either DEET or permethrin exhibited a significant decrease in surviving neurons, in comparison to animals treated with combined DEET and permethrin ($P < 0.01$) (Fig. 9). Analysis of dying neurons showed that animals treated with either DEET or permethrin exhibited a significant number of dying neurons ($P < 0.05$) (Fig. 9). In the CA1 subfield, only animals treated with either DEET or permethrin exhibited a significant decrease in the number of surviving neurons compared with control animals ($P < 0.05$) (Fig. 9). Analysis of dying neurons also showed the same trend. In the CA3 subfield, animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibited a significant decrease in surviving neurons, in comparison to control animals ($P < 0.01$) (Fig. 9). Analysis of dying neurons revealed that animals treated with either DEET or permethrin exhibited a significant number of dying neurons ($P < 0.01$). Thus, a significant reduction in surviving neurons occurs in the dentate gyrus and CA3 subfield of the hippocampal formation following subchronic dermal application of DEET and permethrin, alone or in combination; however, the overall neuron loss in the dentate gyrus is significantly greater when DEET and permethrin are applied separately. In the CA1 subfield of the hippocampus, a significant decrease in the number of surviving neurons occurs with exposure to DEET or permethrin alone but not with exposure to both DEET and permethrin.

Quantification of the area of MAP-2-immunoreactive elements per unit area of the CA1 stratum radiatum showed that there were fewer MAP-2-positive structures in the CA1 subfield of all treated groups (Fig. 10A). The MAP-2-immunoreactive structures exhibited 28% reduction with exposure to DEET alone ($P < 0.05$), and 16% reduction with exposure to permethrin alone or exposure to both DEET and permethrin ($P < 0.05$). The measurement of GFAP-immunoreactive

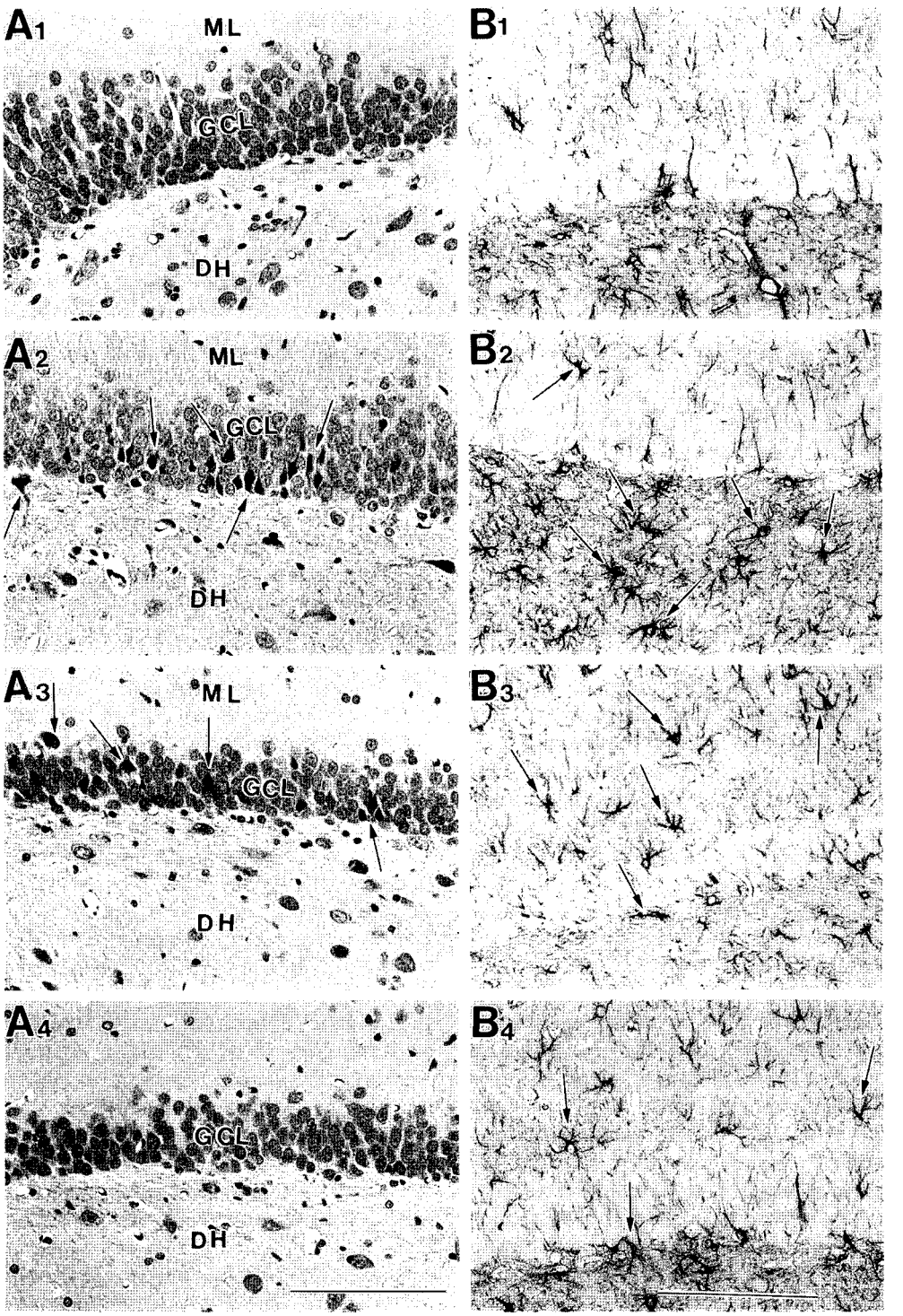

FIG. 6. Alterations in the dentate gyrus following daily application of DEET and permethrin. (A_1–A_4) H&E staining, (B_1–B_4) GFAP immunostaining. (A_1, B_1) Examples from a control rat. (A_2, B_2) Examples from a rat treated with DEET. (A_3, B_3) Examples from a rat treated with permethrin. (A_4, B_4) Examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in the dentate granule cell layer (GCL) and the dentate hilus (DH) of rats treated with either DEET or permethrin (arrows in A_2 and A_3). In the rat treated with both DEET and permethrin (A_4), both thickness and cell packing density of granule cell layer are reduced compared with the control rat. Note that GFAP immunoreactivity is upregulated in all three treated groups (B_2, B_3, B_4). ML, molecular layer. Bar = 100 μm.

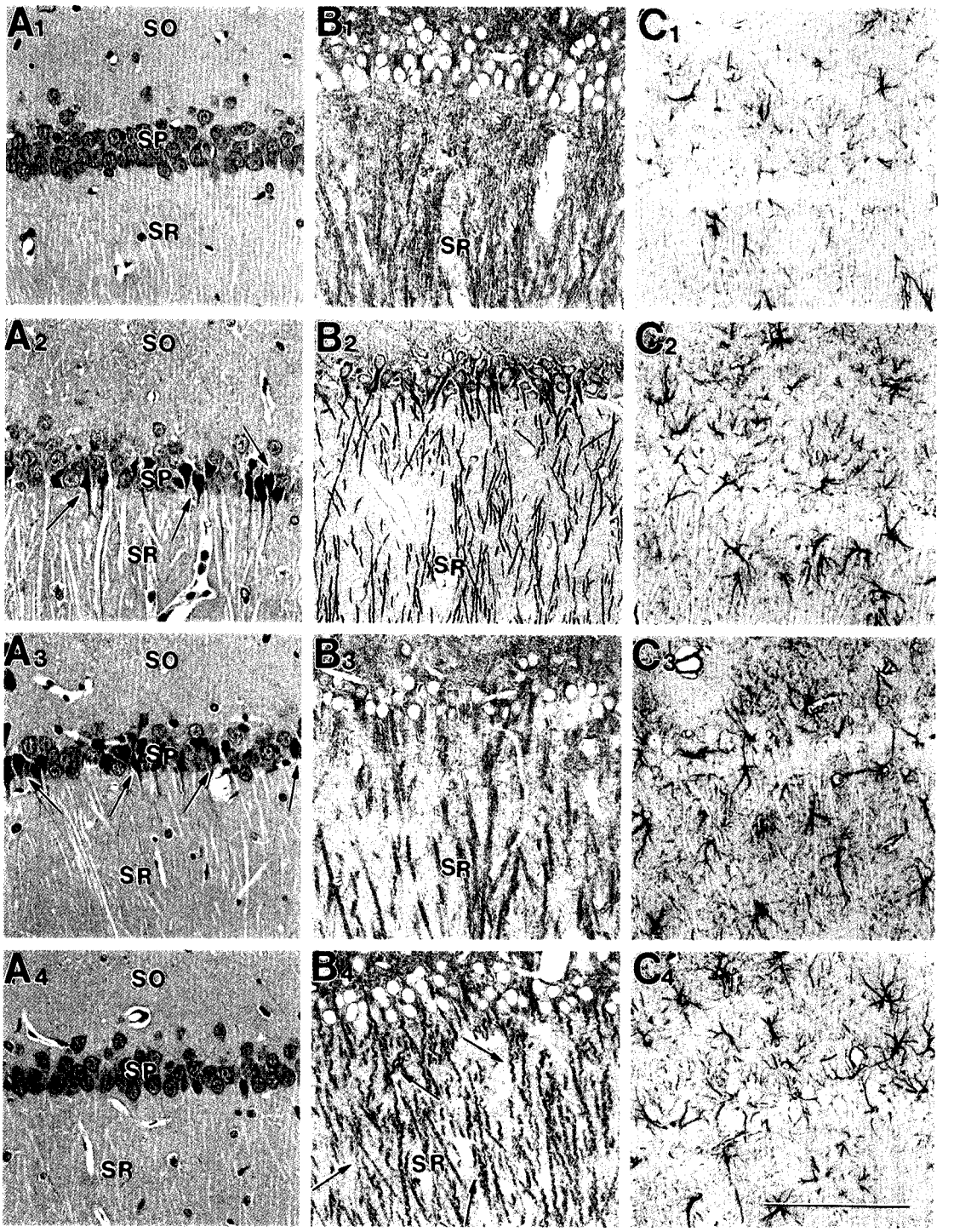

FIG. 7. Alterations in the CA1 subfield of the hippocampus following daily dermal application of DEET and permethrin. (A_1–A_4) H&E staining, (B_1–B_4) MAP-2 immunostaining, (C_1–C_4) GFAP immunostaining. (A_1, B_1, C_1) Examples from a control rat. (A_2, B_2, C_2) Examples from a rat treated with DEET. (A_3, B_3, C_3) Examples from a rat treated with permethrin. (A_4, B_4, C_4) Examples from a rat treated with both DEET and permethrin. A large number of degenerating pyramidal neurons are clearly visible in the stratum pyramidale (SP) of rats treated with either DEET or permethrin (arrows in A_2 and A_3). In the rat treated with both DEET and permethrin, both thickness and cell packing density of CA1 cell layers are reduced compared with the control rat (A_1), and dendrites in stratum radiatum have a wavy appearance (B_4). Note that, in all three treated groups, the overall density of MAP-2-immunoreactive elements is significantly reduced (B_2–B_4) and the pattern of MAP-2 expression in dendrites is altered, in comparison to the control group (B_1). Further, in all treatment groups (C_2–C_4), GFAP-immunoreactive astrocytes are significantly increased in both stratum oriens (SO) and stratum radiatum (SR). Bar = 100 μm.

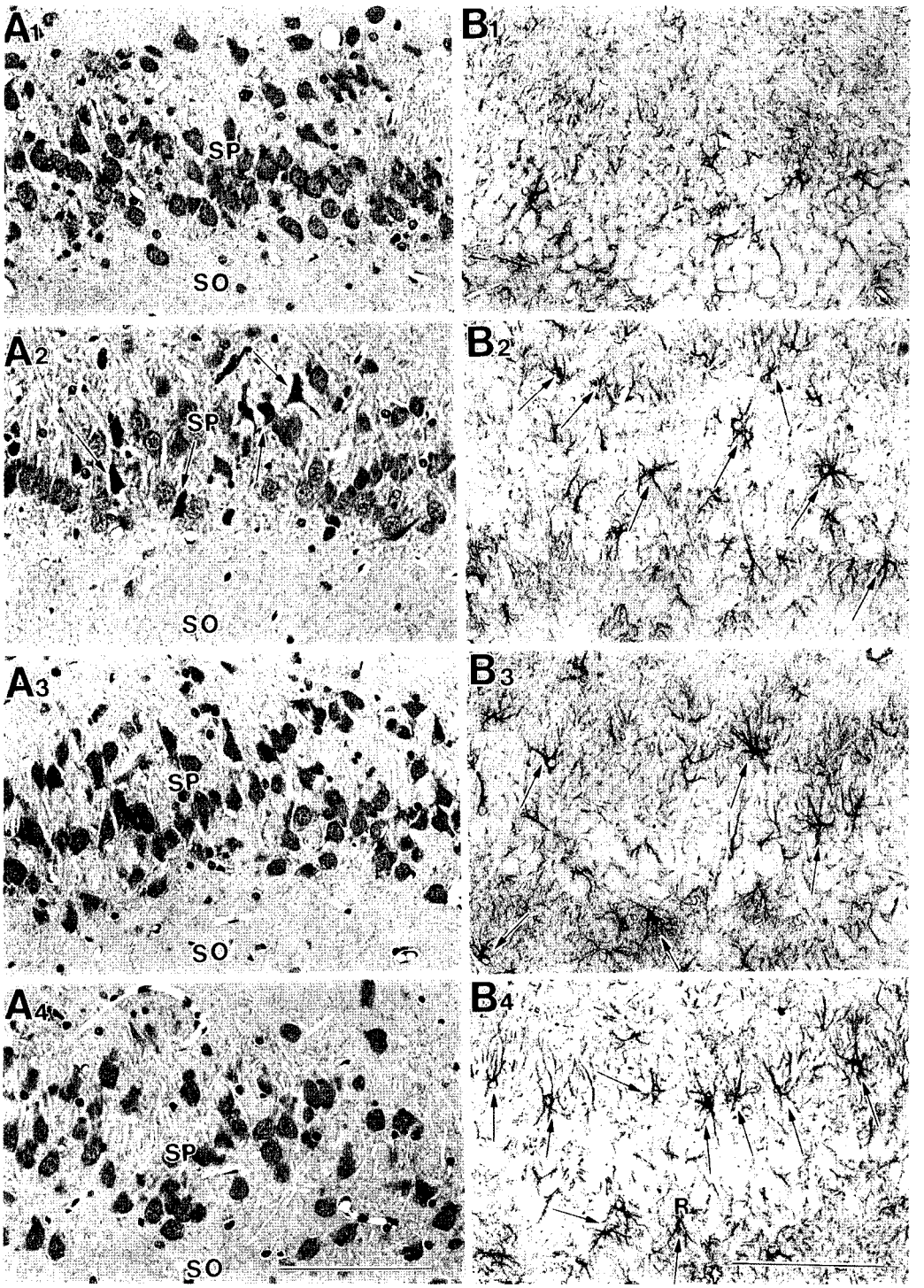

FIG. 8. Changes in the CA3 subfield of the hippocampus following daily application of DEET and permethrin. (A_1–A_4) H&E staining, (B_1–B_4) GFAP immunostaining. (A_1, B_1) Examples from a control rat. (A_2, B_2) Examples from a rat treated with DEET. (A_3, B_3) Examples from a rat treated with permethrin. (A_4, B_4) Examples from a rat treated with both DEET and permethrin. A large number of degenerating neurons are clearly visible in the stratum pyramidale (SP) of the CA3 subfield of the rat treated with DEET alone (arrows in A_2). In rats treated with permethrin alone (A_3) and both DEET and permethrin (A_4), the thickness and cell packing density of the CA3 cell layer are reduced compared with the control rat. Note that GFAP immunoreactivity is upregulated in all three treated groups (B_2, B_3, B_4). SO, stratum oriens. Bar 100 μm.

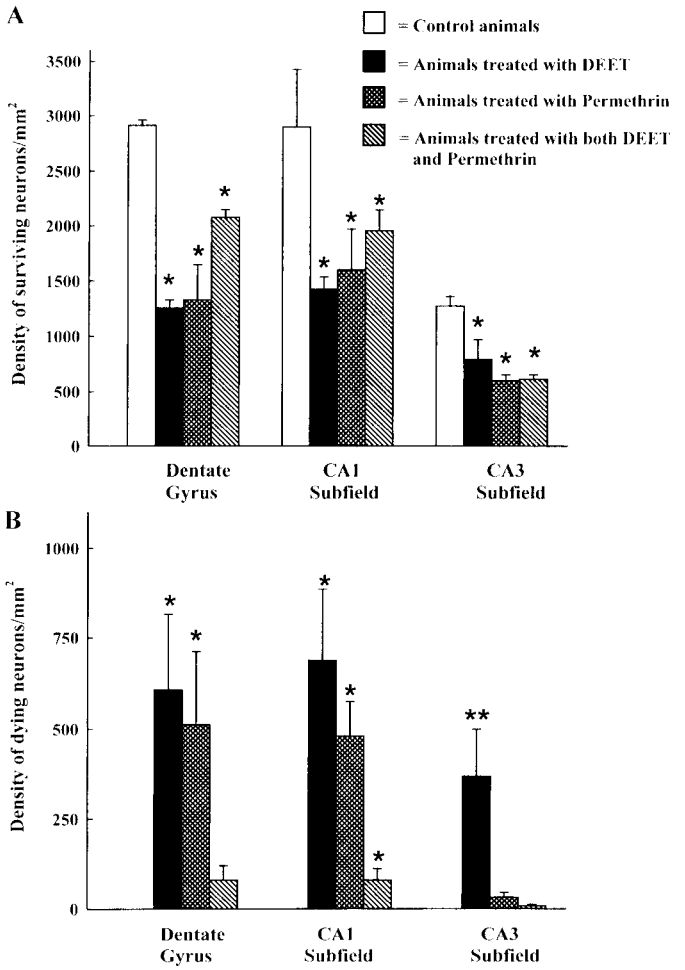

FIG. 9. Histograms show the density of surviving (A) and dying (B) neurons per square millimeter of area of different cell layers of the hippocampal formation. Values represent means and standard errors ($n = 5$ per group). Analyses with one-way ANOVA revealed significant differences between groups for both surviving neurons (dentate gyrus, $P < 0.001$; CA1 subfield, $P < 0.05$; CA3 subfield, $P < 0.001$) and dying neurons (dentate gyrus, CA1 and CA3 subfields, $P < 0.01$). The post hoc analysis with Student Newman–Keuls multiple comparisons test further revealed the following. In dentate granule cell layer, animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibit a significant decrease in surviving neurons, in comparison to control animals ($P < 0.01$). Further, animals treated with either DEET or permethrin exhibit a significant decrease in surviving neurons, in comparison to animals treated with both DEET and permethrin ($P < 0.01$). Analysis of dying neurons (B) shows that animals treated with either DEET or permethrin exhibit a significant number of dying neurons compared with control animals ($P < 0.05$); the number of dying neurons with exposure to DEET alone is greater than with exposure to both DEET and permethrin ($P < 0.05$). In CA1 subfield, only animals treated with either DEET or permethrin exhibit a significant decrease in the number of surviving neurons compared with controls ($P < 0.05$). Analysis of dying neurons shows that animals treated with either DEET or permethrin exhibit a greater number of dying neurons than both controls ($P < 0.01$) and animals treated with both DEET and permethrin ($P < 0.05$). In CA3 subfield, animals treated with DEET, permethrin, or a combination of DEET and permethrin exhibit a significant decrease in surviving neurons, in comparison to controls ($P < 0.01$). Analysis of dying neurons reveals that animals treated with either DEET or permethrin exhibit a greater number of dying neurons compared with both controls ($P < 0.01$) and animals treated with both DEET and permethrin ($P < 0.05$).

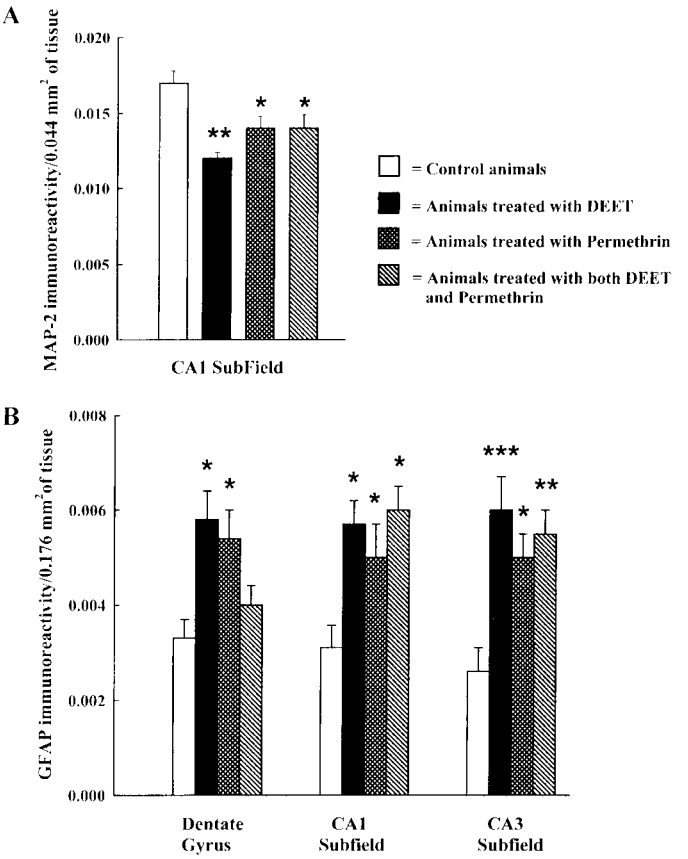

FIG. 10. Histograms in (A) show the area of MAP-2-immunoreactive elements (in mm^2) per unit area (0.044 mm^2) of CA1 stratum radiatum of the hippocampus. Values represent means and standard errors ($n = 5$ per group). Note that there are fewer MAP-2-positive structures in the CA1 subfield of all treated groups. The MAP-2-immunoreactive structures exhibited 28% reduction with exposure to DEET alone ($P < 0.05$), and 16% reduction with exposure to permethrin alone or exposure to both DEET and permethrin ($P < 0.05$). Histograms in (B) show the area of GFAP-immunoreactive elements (in mm^2) per unit area (0.176 mm^2) of different regions of the hippocampus. Values represent means and standard errors ($n = 5$ per group). Note the upregulation in GFAP-positive elements within all regions of the hippocampus in all treatment groups. In dentate gyrus, the increase was 77% with DEET exposure ($P < 0.05$), 65% with permethrin exposure ($P < 0.05$), and 24% with exposure to both DEET and permethrin ($P < 0.05$). In the CA1 subfield, the increase in different treatment groups varied from 81 to 91% ($P < 0.05$). In the CA3 subfield, the increase in GFAP immunoreactivity varied from 60 to 93% ($P < 0.05$).

structures per unit area of different regions of the hippocampal formation demonstrated upregulation in GFAP-positive elements in all treatment groups (Fig. 10B). In dentate gyrus, the increase was 77% with DEET exposure ($P < 0.05$), 65% with permethrin exposure ($P < 0.05$), and 24% with exposure to both DEET and permethrin ($P < 0.05$). In the CA1 subfield, the increase in different treatment groups varied from 81 to 91% ($P < 0.05$). In the CA3 subfield, the increase in GFAP immunoreactivity varied from 60 to 93% ($P < 0.05$).

Alterations in the Cytoarchitecture of the Cerebellum

In the cerebellum, the most conspicuous damage following exposure to DEET and permethrin, alone or in combination, was in the Purkinje cell layer. A large number of degenerating neurons were observed in animals treated with either DEET or permethrin compared with control animals (Fig. 11 (A_1–A_3)). In animals treated with combined DEET and permethrin, dying neurons were infrequent. However, the Purkinje cell density per length of Purkinje cell layer appeared reduced in comparison to control animals (Fig. 11 (A_4)). Wide areas of the Purkinje cell layer lacking Purkinje neurons were frequently encountered in animals treated with both DEET and permethrin (Fig. 11 (A_4)). Both thickness and cell packing density in the granule cell layer appeared comparable in control animals and animals belonging to three treated groups. GFAP immunostaining of neighboring sections showed significantly enhanced GFAP immunoreactivity in the cerebellar white matter of animals belonging to the three treatment groups compared with control animals (Fig. 11 (B_1–B_4)). However, the maximal enhancement of GFAP immunoreactivity was observed in animals treated with both DEET and permethrin (Fig. 11 (B_4)).

Extent of Purkinje Neuron Loss and Upregulation of GFAP Immunoreactivity in the Cerebellum

Quantitative analysis of Purkinje cells showed that animals treated with DEET and permethrin, alone or in combination, exhibited a significant decrease in surviving neurons, in comparison to control animals (76–83% in lobule 2 of the cerebellar vermis, and 36–58% in crus 2 ansiform lobule of the cerebellar hemisphere, $P < 0.001$) (Fig. 12). However, the degree of neuron loss in the cerebellar hemisphere with combined exposure to DEET and permethrin was greater than that with exposure to DEET or permethrin alone ($P < 0.05$) (Fig. 12). Analysis of dying neurons in the cerebellar vermis revealed that animals treated with either DEET or permethrin exhibited a significant number of dying neurons ($P < 0.001$) (Fig. 12). However, in the cerebellar hemisphere, all treated groups exhibited a significant number of dying neurons ($P < 0.05$). Thus, as in the cerebral cortex and the hippocampal formation, significant neuronal cell death occurs in the Purkinje cell layer of the cerebellum following subchronic dermal application of DEET and permethrin, alone or in combination; however, the overall neuron loss in the cerebellar hemisphere is significantly greater with combined application of DEET and permethrin, compared with exposure to DEET or permethrin alone. The measurement of GFAP-immunoreactive structures per unit area of the central white matter of the cerebellum demonstrated significant upregulation in GFAP-positive elements in all treatment groups: 53–60% increase with exposure to DEET or permethrin ($P < 0.05$) (Fig. 12C), and 106% increase with exposure to both DEET and permethrin ($P < 0.01$) (Fig. 12C).

DISCUSSION

The present study was designed to investigate the effects of daily dermal application of DEET and permethrin, alone or in combination, for 60 days on histopathological changes in the brain of male rats. The route of exposure and dose levels of test compounds were chosen to closely reflect those present during the Persian Gulf War (1). The test compounds were applied dermally at a dose that was approximately equivalent to the exposure that may have occurred to army personnel during the Gulf War (18; W. McCain, Department of Defense, personal communication]. Our data suggest that exposure to DEET and permethrin, alone or in combination, for 60 days causes the following: (1) a diffuse neuronal cell death in the motor cortex, the different subfields of the hippocampal formation, and the Purkinje cell layer of the cerebellum; (2) a significant reduction in MAP-2-positive immunoreactive structures associated with atypical expression of MAP-2 in dendrites of surviving neurons within the cerebral cortex and the hippocampus (the expression of MAP-2 within apical dendrites of pyramidal neurons of the cortex and the CA1 subfield was characterized by a beaded, disrupted, or wavy appearance); (3) a significant upregulation of GFAP-positive structures (this was exemplified by GFAP expression in soma of astrocytes and hypertrophy of astrocytic processes emanating from the soma).

To determine the overall extent of neuron loss, we quantified the density of surviving (healthy) and dying neurons in layers III and V of the motor cerebral cortex, granule cells of the dentate gyrus, pyramidal neurons of hippocampal CA1 and CA3 subfields, and Purkinje cells of the cerebellum. Neuronal cell death was evident in all three treated groups by a significant decrease in the density of surviving neurons. In animals treated with DEET or permethrin alone, the occurrence of neuronal cell death was also confirmed by the presence of a significant number of dying (eosinophilic) neurons after the 60-day exposure regimen. However, in animals treated with both DEET and permethrin, the number of dying neurons after the same exposure regimen was significantly less than in animals treated with DEET or permethrin alone. A lack of significant number of dying neurons but a clear reduction in the number of surviving neurons (in comparison to control animals) in animals exposed to both DEET and permethrin suggests that, in animals receiving combined DEET and permethrin, neuronal cell death occurs earlier than in animals receiving either DEET or permethrin alone. However, analysis of dying neurons at multiple time points during the exposure period employed in this study is necessary to clearly address the above issue. Further, the extent of reductions in

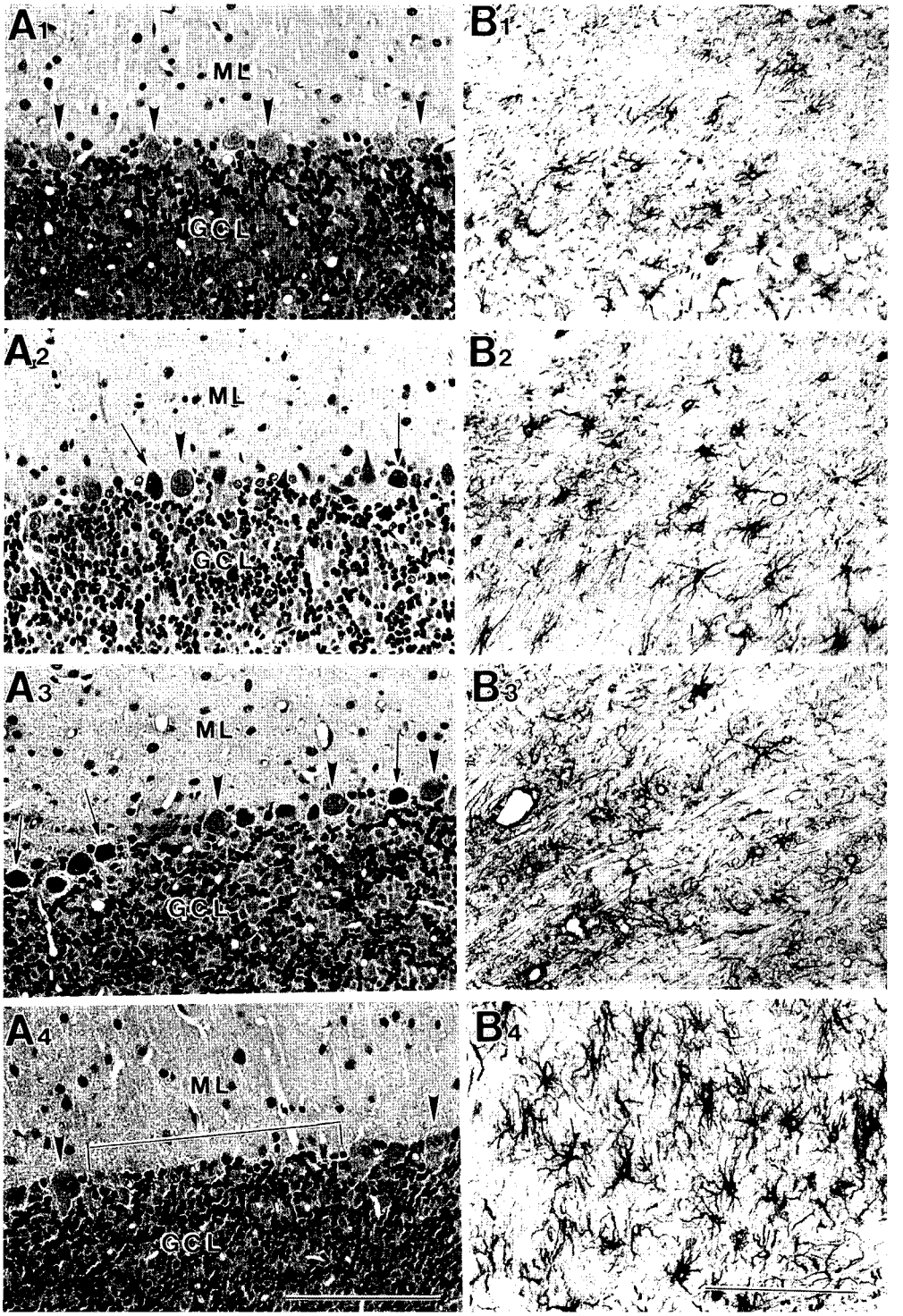

FIG. 11. Alterations in the cerebellum following daily application of DEET and permethrin. (A_1–A_4) H&E staining of the cerebellar cortex, (B_1–B_4) GFAP immunostaining of the cerebellar white matter. (A_1, B_1) Examples from a control rat. (A_2, B_2) Examples from a rat treated with DEET. (A_3, B_3) Examples from a rat treated with permethrin. (A_4, B_4) Examples from a rat treated with both DEET and permethrin. A large number of degenerating Purkinje neurons are clearly visible in the Purkinje cell layer of rats treated with either DEET or permethrin (arrows in A_2, A_3). Arrows in (A_1)–(A_4) point to surviving neurons. In rats treated with both DEET and permethrin (A_4), a large area of Purkinje cell layer is devoid of Purkinje neurons (bracketed area, A_4). Note that GFAP immunoreactivity in the white matter of the cerebellum is significantly upregulated in all three treated groups (B_2, B_3, B_4), with maximal upregulation in the group treated with both DEET and permethrin. GCL, granule cell layer; ML, molecular layer. Bar = 100 μm.

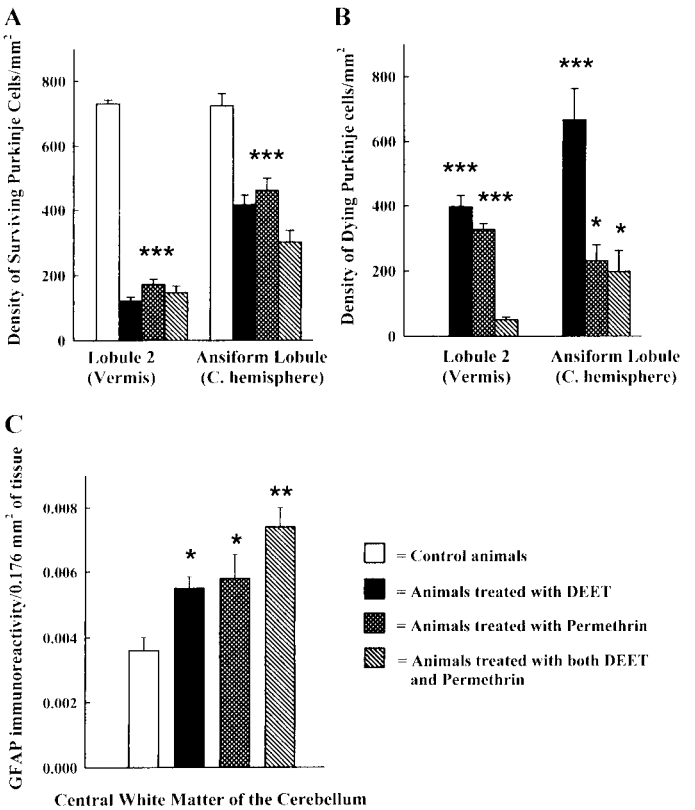

FIG. 12. Histograms show the density of surviving (A) and dying (B) neurons per square millimeter of area of the Purkinje cell layer in lobule 2 of the cerebellar vermis and crus 2 ansiform lobule of the cerebellar hemisphere. Values represent means and standard errors ($n = 5$ per group). Note that animals treated with DEET and permethrin, alone or in combination, exhibit a significant decrease in surviving neurons, in comparison to control animals (76–83% in lobule 2 of the cerebellar vermis, and 36–58% in crus 2 ansiform lobule of the cerebellar hemisphere, $P < 0.001$). And, the degree of neuron loss in the cerebellar hemisphere with combined exposure to DEET and permethrin is greater than that with exposure to DEET or permethrin alone ($P < 0.05$). Also, animals treated with either DEET or permethrin exhibit a significant number of dying neurons in the cerebellar vermis ($P < 0.001$; B). In the cerebellar hemisphere, all treated groups exhibit a significant number of dying neurons ($P < 0.05$). Histograms in (C) show GFAP-immunoreactive elements (in mm^2) per unit area (0.176 mm^2) of the central white matter of the cerebellum. Note that a significant upregulation in GFAP-positive elements occurs in all treatment groups (53–60% increase with exposure to DEET or permethrin, $P < 0.05$; 106% increase with exposure to both DEET and permethrin, $P < 0.01$).

surviving neurons within some regions of the brain varied between the three treatment groups. Layers III and V of the motor region of the cerebral cortex exhibited similar levels of reductions (43–57%) following exposure to DEET and permethrin, alone or in combination. The dentate gyrus of the hippocampal formation demonstrated a significantly greater level of reduction with exposure to DEET or permethrin alone, in comparison to combined DEET and permethrin exposure (54% with DEET alone, 54% with permethrin alone, and 29% with combined DEET and permethrin); however, the CA1 and CA3 subfields of the hippocampus exhibited statistically similar level of reductions (CA1, 33–51% reduction; CA3, 38–53% reduction) following exposure to DEET and permethrin, alone or in combination. The Purkinje cells of the cerebellar vermis exhibited similar levels of reductions with all three exposures (76–83% reduction). However, the Purkinje cells of the cerebellar hemisphere demonstrated a significantly greater reduction with combined exposure to DEET and permethrin (58% decrease) than with exposure to DEET or permethrin alone (36–43% decrease). Thus, in the motor cerebral cortex, the hippocampal subfields CA1 and CA3, and the Purkinje cell layer of the cerebellar vermis, exposure to DEET and permethrin, alone or in combination, causes similar levels of neuronal cell death. In contrast, in the dentate gyrus, exposure to DEET alone causes greater neuronal cell death than exposure to both DEET and permethrin. And, in the Purkinje cell layer of the cerebellar hemisphere, exposure to combined DEET and permethrin causes more damage than exposure to DEET or permethrin alone.

A greater level of neuron loss in the dentate gyrus following exposure to DEET alone compared with exposure to a combination of DEET and permethrin suggests that the extent of DEET-induced neuronal loss in some regions of the brain wanes significantly when both DEET and permethrin are applied together. The reduced neuronal loss with exposure to both DEET and permethrin likely reflects a decrease in effective concentration of chemicals at the neurotoxicity target, as concurrent exposure to chemicals can decrease their absorption (18, 24). This may also suggest that there is some protective effect with concurrent exposure to DEET and permethrin. Nevertheless, animals treated with both DEET and permethrin exhibited maximal cytoarchitectural alterations in the expression of MAP-2 within the motor cerebral cortex and the CA1 subfield of the hippocampus though the overall reductions in MAP-2 immunoreactivity were mostly comparable in all treated groups. The upregulation of GFAP immunoreactivity was comparable in the three treatment groups in the CA3 subfield of the hippocampus. In the motor cortex and the dentate gyrus, the upregulation was greater with exposure to DEET or permethrin alone compared with combined exposure, whereas, in the cerebellum, the upregulation was greater with combined exposure than with exposure to DEET or permethrin alone. The above pattern of cytoskeletal alterations suggests that degenerative changes induced by co-exposure to DEET and permethrin are significant and detrimental to the normal functioning of the central nervous system, despite a slight reduction in the extent of overall neuronal cell loss.

In our previous studies in hens using the subcutaneous route of exposure at relatively higher doses, we demonstrated that co-exposure to DEET and permethrin results in an enhanced level of toxicity com-

pared with exposure to each chemical alone (2). Our recent behavioral studies in rats showed that exposure to DEET or permethrin or both DEET and permethrin for 60 days, using the same dose levels used in the current study, leads to significant deficits in sensorimotor functions (1). In addition, our previous study demonstrated subtle changes in the blood–brain barrier following exposure to either DEET or both DEET and permethrin, and suggested that additional approaches such as histopathological evaluations may provide definitive proof of the changes in the CNS occurring as a consequence of DEET treatment alone or a combination with permethrin. Our present data provide clear histopathological evidence that subchronic exposure to DEET and permethrin, alone or in combination, leads to significant neuronal cell death and cytoskeletal abnormalities in surviving neurons that could compromise functions of the brain.

The neurotoxic effects of DEET may be augmented both by its increased localization into the CNS because of its lipophilicity and because of decrease in the transport of otherwise critical molecules. Severe signs of CNS toxicity due to DEET and permethrin are apparent only at high doses; e.g., DEET-induced signs of CNS depression, death, and protracted seizure activity were observed at several dose levels in rats (39). Similar complications have been observed in DEET poisoning in humans (21, 26). Symptoms such as daytime sleepiness and impaired cognitive functions have been shown to result from heavy DEET exposure, whereas gait balance and dexterity were moderately affected (21). Additionally, lethargy has been noted as a prominent feature in severe acute DEET intoxication (33, 35). A relatively recent study found a decrease in motor activity in male and female rats after a single dose of DEET treatment (39). Permethrin-induced behavioral changes have also been documented in animals (16). Permethrin-induced neurotoxic changes are characterized by aggressive sparring, increased sensitivity to external stimuli, and fine tremors that progresses to whole-body tremors and prostration (4, 37, 38). McDaniel and Moser (22) reported a decrease in grip strength and induced head and forelimb shaking. Additionally, decreased operant response rate and a decrease in turning-wheel activity have been observed (6, 14). Studies by Crofton and Reiter (9) have shown a decrease in locomotor activity in rats exposed to permethrin.

The cytoarchitecture of the CNS is maintained by a complex cellular milieu that involves neurons and a variety of cells of glial origin. For the CNS to function properly and to respond to external stimuli, it is absolutely required that proper communication is maintained within these cells. A major determinant of neuronal morphology is the cytoskeleton. Different components of cytoskeleton within the neurons and astrocytes provide forces to maintain the appropriate cellular structure; e.g., neuronal dendrites and axons are maintained in stable conditions by the force provided by the elements of cytoskeleton (13). Such interactions are essential for proper synapse formation. The components of cytoskeleton are microfilaments, intermediate filaments, and microtubules. An important neuronal component, MAP-2, is enriched in dendrites and cell bodies (36), in which it stabilizes the polymerized tubulin. Abnormal regulation of expression of MAP-2 causes suppression of neurite outgrowth and reduction in the number of neurites in cultured neurons (7). Similarly, aberrant intermediate filament proteins have been linked to diseases of neurodegeneration (11). Our data clearly show both decrease and abnormalities in MAP-2 expression following treatment with DEET or permethrin or both DEET and permethrin in the brain, particularly the motor cerebral cortex and the CA1 subfield of the hippocampal formation. A decreased expression and beaded appearance of MAP-2 in dendrites would lead to destabilization of dendrites and can result in abnormal functioning of neurons, particularly loss of synapses due to resorption of postsynaptic specializations such as dendritic spines. Such aberrant dendritic organization and the consequently altered connectivity in the cerebral cortex and the hippocampal formation could respectively have profound adverse influence on motor function and learning and memory.

A major component of astrocytic intermediate filament, GFAP is upregulated in response to reactive gliosis as a consequence of a variety of insults, such as exposure to neurotoxic chemicals, trauma, and neurodegenerative diseases that affect the CNS (12). The precise function of GFAP is not well understood, but it is believed to play an important role in the long-term maintenance of brain cytoarchitecture (19), proper functioning of the blood–brain barrier (25), and modulation of neuronal functions (31). Increased expression of GFAP in the soma and processes of astrocytes in various brain regions exhibiting neuronal cell death indicates that neurodegenerative changes induced by exposure to DEET and permethrin, either alone or in combination, are quite robust and lead to a significant hypertrophy of astrocytes. This is because hypertrophied astrocytes (or reactive astrocytes) represent transformed resting astrocytes with increased GFAP accumulation, and this transformation occurs as a consequence of injury to the brain. The accumulation of reactive astrocytes can lead to increased generation of toxic mediators that may cause further pathological damages in the brain (27).

CONCLUSIONS

Most of the earlier studies on the neurotoxic effects of DEET or permethrin used routes of exposure that are not directly germane to the contact exposure, as is believed to have occurred during the Gulf War. The results of this study, however, clearly suggest that

subchronic dermal application of these chemicals leads to diffuse neuronal cell death and significant neuronal cytoskeletal abnormalities in the motor cerebral cortex, the hippocampal formation, and the cerebellum. Taken together, these alterations can lead to many physiological and behavioral abnormalities, particularly motor deficits and learning and memory dysfunction. The above alterations are likely the contributory factors for neurobehavioral abnormalities observed earlier in adult rats following exposure to DEET and permethrin, alone or in combination. Thus, it is likely that subchronic exposure to DEET and permethrin experienced by service personnel during the Persian Gulf War has played an important role in the development of illnesses in some veterans after the Gulf War.

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