Pergamon

PII: S0306-4522(97)00606-4

Neuroscience Vol. 85, No. 1, pp. 123–133, 1998

0306–4522/98 $19.00+0.00

IMMUNOHISTOCHEMICAL LOCALIZATION AND QUANTIFICATION OF GLIAL FIBRILLARY ACIDIC PROTEIN AND SYNAPTOSOMAL-ASSOCIATED PROTEIN (MOL. WT 25000) IN THE AGEING HIPPOCAMPUS FOLLOWING ADMINISTRATION OF 5,7-DIHYDROXYTRYPTAMINE

A. DUGAR,* C. PATANOW,* J. P. O'CALLAGHAN† and J. M. LAKOSKI*‡

*Departments of Pharmacology and Anesthesia, The Pennsylvania State University College of Medicine, 500 University Drive, Hershey, PA 17033-0850, U.S.A.

†Centers for Disease Control and Prevention, NIOSH, Health Effects Laboratory Division TMBB (MS 3014), 1095 Willowdale Road, Morgantown, WV 26505-2888, U.S.A.

Abstract—Responses to injury in the ageing hippocampus were assessed utilizing the synaptic markers glial fibrillary acidic protein and synaptosomal-associated protein (mol. wt 25,000) following administration of the neurotoxin, 5,7-dihydroxytryptamine, into the fimbria–fornix and cingulum bundle to denervate serotonergic afferent input to the dorsal hippocampus. Age-dependent alterations in hippocampal immunohistochemical localization of glial fibrillary acidic protein and synaptosomal-associated protein were evaluated in female Fischer 344 rats following serotonergic deafferentation with 5,7-dihydroxytryptamine. Across the lifespan, as indicated by measurements taken at three, 18, 21 and 29 months, marked increases in glial fibrillary acidic protein, but not synaptosomal-associated protein immunoreactivity, occurred throughout the hippocampus at 21 and 29 months compared to three and 18 months. Following three weeks pretreatment with 5,7-dihydroxytryptamine (20 μg total dose) or vehicle (0.1% ascorbic saline; 2 μl total volume) infused in the fimbria–fornix/cingulum bundle, immunohistochemical analysis demonstrated marked increases of glial fibrillary acidic protein, but not synaptosomal-associated protein, in the 18-month 5,7-dihydroxytryptamine group compared to the 18-month vehicle and 3-month 5,7-dihydroxytryptamine groups. Additionally, a significant increase in glial fibrillary acidic protein concentration was found by enzyme-linked immunosorbent asssay in the 18-month 5,7-dihydroxytryptamine group compared to the 18-month vehicle and three-month 5,7-dihydroxytryptamine groups. These results demonstrate that selective neurotoxicant damage of the hippocampal serotonergic system differentially alters the expression of glial fibrillary acidic protein. This approach may provide a valuable tool to determine the ability of the hippocampus to respond to age-related neurodegenerative injury. © 1998 IBRO. Published by Elsevier Science Ltd.

Key words: serotonin (5-hydroxytryptamine), 5,7-dihydroxytryptamine, fimbria–fornix/cingulum bundle, hippocampus, glial fibrillary acidic protein, synaptosomal-associated protein (mol. wt 25,000).

The hippocampus is a target for injury produced by ischemia and hypoxia,[5] as well as several neurochemical toxins.[2,6,16,36,50] Cellular responses following damage of hippocampal neurons include the astrocytic response, known as reactive gliosis, which is characterized by increased expression of glial fibrillary acidic protein (GFAP).[22,25,33,36,48,53] Another synaptic marker often elevated after neuronal injury is synaptosomal-associated protein of mol. wt 25,000 (SNAP-25); this is a t-snare protein required for synaptic vesicle exocytosis and synaptogenesis.[40,41] Both mechanical lesions[11,12,24,43] and neurotoxicant administration placed in afferent pathways to the hippocampus, including kainate, trimethyltin (TMT), colchicine or 5,7-dihydroxytryptamine (5,7-DHT), produce neuronal degeneration in the hippocampus characterized by alterations in GFAP and SNAP-25 localization and mRNA levels.[1,4,8,29,41,47] These experimental models have utilized the mature adult brain and have not systematically addressed the cellular adaptive responses to neuronal injury in the ageing nervous system.

Ageing affects the expression of GFAP and SNAP-25 as indicators of neuronal damage. The use of enzyme-linked immunosorbent assays (ELISAs) has demonstrated an enhanced concentration of

‡To whom correspondence should be addressed.

Abbreviations: CB, cingulum bundle; DAB, diaminobenzidine; 5,7-DHT, 5,7-dihydroxytryptamine; ELISA, enzyme-linked immunosorbent assay; FF, fimbria–fornix; GFAP, glial fibrillary acidic protein; 5-HT, serotonin (5-hydroxytryptamine); mAb, monoclonal antibody; pAb, polyclonal antibody; PBS, phosphate-buffered saline; SNAP-25, synaptosomal-associated protein of mol. wt 25000; TBS, Tris-buffered saline; TMT, trimethyltin.

GFAP in the hippocampus and cortex in the ageing rat.[26,38] Enhanced GFAP immunoreactivity and mRNA levels in the mouse cerebral cortex and hippocampus have also been reported.[14] Upon analysis of GFAP expression, the number of astrocytes in the hippocampus has been demonstrated to be elevated with ageing,[15,49,52] although others have reported no alteration in number but, instead, an increase in size and fibrous character of these astrocytes.[3,13,18,21] In contrast, SNAP-25 immunoreactivity in hippocampal mossy fibers remains constant across the lifespan in male Fischer 344 rats.[23] To date, studies examining concomitant age-related changes in both GFAP and SNAP-25 localization in the hippocampus following neurotoxic damage are limited. The use of synaptic markers to assess neuronal damage may be a valuable tool to determine the ability of the hippocampus to respond to age-related neurodegenerative injury.

In this study, the consequences of injury in the ageing hippocampus following administration of the neurotoxicant 5,7-DHT were assessed with GFAP and SNAP-25. The neurotoxicant was placed into the fimbria–fornix/cingulum bundle (FF/CB), the major serotonergic afferent pathways to the dorsal hippocampus,[44,51] to deplete 80–90% of the 5-hydroxytryptamine (5-HT) in the hippocampus.[44] GFAP and SNAP-25 levels were evaluated across the lifespan in the female Fischer 344 rat to elucidate cellular changes in the ageing hippocampus which occur in response to discrete neuronal injury of the sertonergic system. Careful consideration of pyramidal subfield-specific immunohistochemical localization was given due to known differences in CA1 and CA3 responses to injury.[6,17,19]

EXPERIMENTAL PROCEDURES

Animals

Virgin female Fischer 344 rats were obtained from the National Institute on Aging colony (Indianapolis, IN). Four age groups were utilized, two to three, 17–18, 21 and 29 months, representing young (sexually mature), middle-aged (reproductively senescent) and old (senescent) groups, respectively.[9,54] Animals were housed under standard conditions and maintained on a 12-h light/dark cycle (lights on at 07.00) in a temperature-controlled environment. Food and water were available *ad libitum*.

Experimental design

Two experimental designs for immunohistochemical analysis were utilized. In the first, intact animals at age three, 18, 21 and 29 months were used to characterize the effect of advancing age on the immunohistochemical distribution of GFAP and SNAP-25. In the second design, two- and 17-month-old animals were randomly assigned to 5,7-DHT or vehicle treatment groups and maintained for three weeks following neurotoxicant treatment; this paradigm was used to assay GFAP content in the hippocampus and cortex by an ELISA technique. For immunohistochemical studies, animals were transcardially perfused with freshly prepared phosphate-buffered saline (PBS; pH 7.4) and 4% paraformaldehyde (500 ml), decapitated and the brain stored in 4% paraformaldehyde overnight at 4°C. The tissue was transferred to Tris-buffered saline (TBS; pH 7.4) and stored at 4°C (one week). For the second design, animal ages at the time of analysis were three and 18 months, and the experimental groups were three-month vehicle, three-month 5,7-DHT, 18-month vehicle and 18-month 5,7-DHT.

5,7-Dihydroxytryptamine lesion

The neurotoxicant 5,7-DHT (RBI, Natick, MA) was administered into the FF/CB according to Lakoski *et al.*[20] Briefly, animals were anesthetized with Nembutal (40 mg/kg, i.p.; Abbott Laboratories, North Chicago, IL), immobilized using a small animal stereotaxic instrument (Kopf Instruments, Tujunga, CA) and body temperature was maintained at 37°C with a heating pad (Braintree Scientific, Inc., Braintree, MA). The skin over the skull was retracted and a 2-µl syringe (Model 88500, Hamilton Co., Reno, NV) was angled at 15° toward the midline and the tip aligned with bregma; coordinates of 1.0 mm posterior and 1.3 mm lateral from the bregma suture[42] were used for bilateral injection of the neurotoxicant 5,7-DHT or vehicle.

Animals were pretreated with the catecholaminergic uptake blocker nomifensine (15 mg/kg, i.p.; RBI, Natick, MA) 30 min prior to injection of 5,7-DHT (10 µg/µl dissolved in 0.1% ascorbic saline) or vehicle [0.1% ascorbic acid prepared in saline (0.9% NaCl)]. This compound was used to protect the catecholaminergic reuptake sites to ensure selectivity of the neurotoxin for the serotonergic system. Following removal of the bone over the dura, the syringe was filled with 5,7-DHT (prepared fresh, kept on ice and shielded from light to prevent oxidation-induced inactivation) and lowered 4.5 mm ventral from the surface of the brain into the FF and then 2.5 mm ventral into the CB. To reduce cytoarchitectual damage, bilateral microinjections were made at a rate of 0.5 µl/min (total volume=2 µl). The needle remained in position for 3 min following each injection to limit non-specific diffusion of 5,7-DHT. The incision was sutured and body temperature maintained at 37°C with a heating pad during recovery from anesthesia. In an identical manner, this procedure was used for groups injected with vehicle solution (0.5 µl/site; total=2 µl). All treatment groups were maintained for a period of three weeks to ensure depletion of hippocampal 5-HT levels. Body weight and general health were assessed on a daily basis following surgery.

Immunohistochemical analysis of glial fibrillary acidic protein and synaptosomal-associated protein (mol. wt 25,000)

Coronal hippocampal slices (30 µm) were obtained using a vibratome from both vehicle- and 5,7-DHT-treated animals, and immunohistochemical localization was performed as reported previously.[41] Experiments included samples from each age and treatment group in order to control for inter-assay variation in immunostaining. According to the coordinates obtained from the Paxinos and Watson rat atlas (4.0 mm posterior, 3.8 mm lateral from bregma),[42] five coronal sections from the dorsal hippocampus of each animal were used for immunohistochemical localization studies. Briefly, slices were stored in TBS at 4°C until ready for analysis. Sections were placed in cold TBS (4°C) containing 30% H_2O_2 (Fisher Scientific, Fair Lawn, NJ) and 1 mg/ml sodium azide for 10 min (shaken on a rocker) to quench endogenous peroxidase activity. Slices were rinsed thoroughly (five times for 15 min) in TBS at room temperature to remove all traces of sodium azide. Sections used for immunohistochemical analysis with the GFAP mouse monoclonal antibody (mAb; Boehringer Mannheim, Indianapolis, IN) were washed in TBS containing 0.5% Triton X-100 overnight at 4°C; all rinses and incubations occurred with sections placed on a rocker. Adjacent slices analysed with the SNAP-25 rabbit polyclonal antibody (pAb; courtesy of Dr M. L. Billingsley, Pennsylvania State University College of Medicine) were placed in TBS with

0.5% Triton X-100, 1% bovine serum albumin and 0.1 mg/ml avidin (STC Laboratories, Canada) overnight at 4°C. All sections were rinsed the following day in TBS (four times for 15 min) at room temperature. Free avidin sites were blocked in the sections to be analysed with the SNAP-25 pAb with a 60-min incubation in TBS containing 1 mg/ml D-biotin (Sigma Chemical Co., St Louis, MO). Slices were then rinsed in TBS (four times for 15 min) at room temperature and then incubated for 60 min in diluted normal serum (1:50) from the animal in which the appropriate secondary antibody was produced (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Sections were rinsed (once for 15 min) at room temperature, then incubated in primary antibody for 48 h at 4°C. Slices were then rinsed in TBS (four times for 15 min) at room temperature. The sections incubated with the mouse mAb were incubated in diluted secondary antibody (1:500) for 2 h at room temperature followed by rinses in TBS (four times for 15 min) at room temperature.

Immunoreactive sites were visualized using diaminobenzidine (DAB; 0.5 mg/ml; Sigma Chemical Co.) activated with 0.02% H_2O_2. After a final rinse in TBS, the slices were mounted on gelatin-subbed slides and allowed to air dry overnight. Slides were dehydrated through a graded series of alcohol washes and a final xylene wash. The sections were coverslipped with Cytoseal (Stephens Scientific, Riverdale, NJ) and examined under a light microscope using Nemarski differential interference contrast optics (Olympus Model BH-2, Olympus Corporation, Lake Success, NY).

Slices incubated with SNAP-25 rabbit pAbs were incubated in secondary antibody (1:1000) and washed as described previously. Preformed avidin–biotinylated peroxidase complexes (Vector Laboratories, Burlingame, CA) were added for 1 h, followed by washing in TBS (4 × 15 min) at 37°C. Immune complexes in these sections were detected using DAB as mentioned above. Sections were dehydrated and coverslipped as described above. Brain slices not incubated with primary antibodies were carried as negative controls in all experiments.

Product density analysis

A semi-quantitative determination of immunoreactive product densities at the level of the dorsal hippocampus (coordinates from bregma: 4.0 mm posterior, 3.8 mm lateral[42]) was performed using NIH Image Version 1.6. Slide-mounted sections were placed on a light microscope (Olympus BH-2), captured using a CCD camera (NEC TI-324A) and visualized (Macintosh IIci personal computer). Sections from each immunohistochemical experiment, consisting of samples from each age, treatment and control group, were captured under identical lighting conditions. Typically, five sections from each brain were used and the CA1 and CA3 pyramidal subfields were outlined for quantitation of the product densities. The product densities were averaged across the five sections and expressed as mean percentage change; the percentage change across the age and treatment groups was obtained and expressed as mean ± S.E.M. for the various age and treatment groups. Statistical analysis was conducted only on sample sizes of three or greater. Although the intensity of staining varied from one experiment to another, within a single experiment the application of primary and secondary antibodies and DAB exposure times were uniform. This approach provides a measurement of the relative percentage change among different age and treatment groups based on the density of staining in a given brain region.

Enzyme-linked immunosorbent assay for glial fibrillary acidic protein determination

Three weeks following 5,7-DHT or vehicle treatment, animals were rapidly decapitated using a small animal guillotine, brains removed and the frontal cortex and hippocampus rapidly dissected on ice. Tissues were weighed, placed in 1.5-ml tubes (Eppendorf) and frozen on dry ice until assay using the sandwich ELISA technique as described by O'Callaghan.[35] The samples were sonified in 1% sodium dodecyl sulfate and diluted in sample buffer [pH 7.4; PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM $KH_2PO_4 \cdot 7H_2O$) containing 0.5% Triton X-100] to a concentration of 10 µg/ml. Standard curve samples were prepared in sample buffer at dilutions between 0.05 and 10 µg/µl.

Following sample preparation, Immulon-2 flat-bottomed microtiter plates were coated with an immunoglobulin G fraction of a polyclonal anti-GFAP antibody (1.0 µg total immunoglobulin protein/100 µl/well, diluted in PBS) and then incubated for 1 h at 37°C. All other incubations are at room temperature with moderate shaking on a reciprocating shaker. Next, the plates were emptied, tapped on absorbent paper to remove excess reagent and washed four times with PBS (200 µl/well, tapped and blotted between each wash). To decrease non-specific binding, the plates were incubated with Blotto (5% non-fat powdered milk in PBS) for 1 h (100 µl/well). Again, the plates were emptied, tapped on absorbent paper to remove excess Blotto, and the standards and samples were loaded in duplicate for 1 h (100 µl/well) and incubated for 1 h at room temperature. The plates were rinsed four times with PBS (containing 0.5% Triton X-100). The plates were then incubated for 30 min in alkaline phosphatase-conjugated anti-mouse immunoglobulin G (1:3000 dilution; Jackson ImmunoResearch Laboratories) made in Blotto (with 0.5% Triton X-100, 100 µl/well). These plates were rinsed four times with PBS containing 0.5% Triton X-100 (200 µl/well). Finally, 100 µl of *p*-nitrophenylphosphate substrate (Bio Rad) was added to the reaction and terminated 10 min later with addition of 0.4 M NaOH (100 µl/well). The optical densities of the standards and samples were read at 405 nm using a Molecular Devices UV Max Microplate Reader (Menlo Park, CA) coupled to a MacIntosh computer running a Soft Max (Molecular Devices) program. Quantity of GFAP (µg/mg total protein) in each sample was determined from a standard curve which had a known total protein concentration (11.5 mg/ml) and a known concentration of GFAP (2.4 µg GFAP/mg total protein). The detection limit of the sandwich ELISA was 0.25 ng GFAP/mg total protein.

Statistical analysis

A two-way ANOVA was performed to determine age- and treatment-related differences obtained from ELISA and product density analysis results. The Student–Newman–Keuls test ($P < 0.05$ level of significance) was used for *post hoc* comparisons between treatments and across ages for each brain region using SigmaStat (Version 1.0, Jandel Corporation, San Rafael, CA).

RESULTS

Immunohistochemical localization of glial fibrillary acidic protein in the ageing rat hippocampal CA1 subfield

Immunohistochemcial localization of GFAP demonstrated increased immunostaining in the ageing hippocampus of female Fischer 344 rats (aged three, 18, 21 and 29 months). Three- and 18-month animals showed similar patterns of immunostaining in the stratum radiatum and stratum lacunosum moleculare. In contrast, a marked increase in the 21-month group and a striking increase in the 29-month group occurred in these layers of the hippocampal CA1

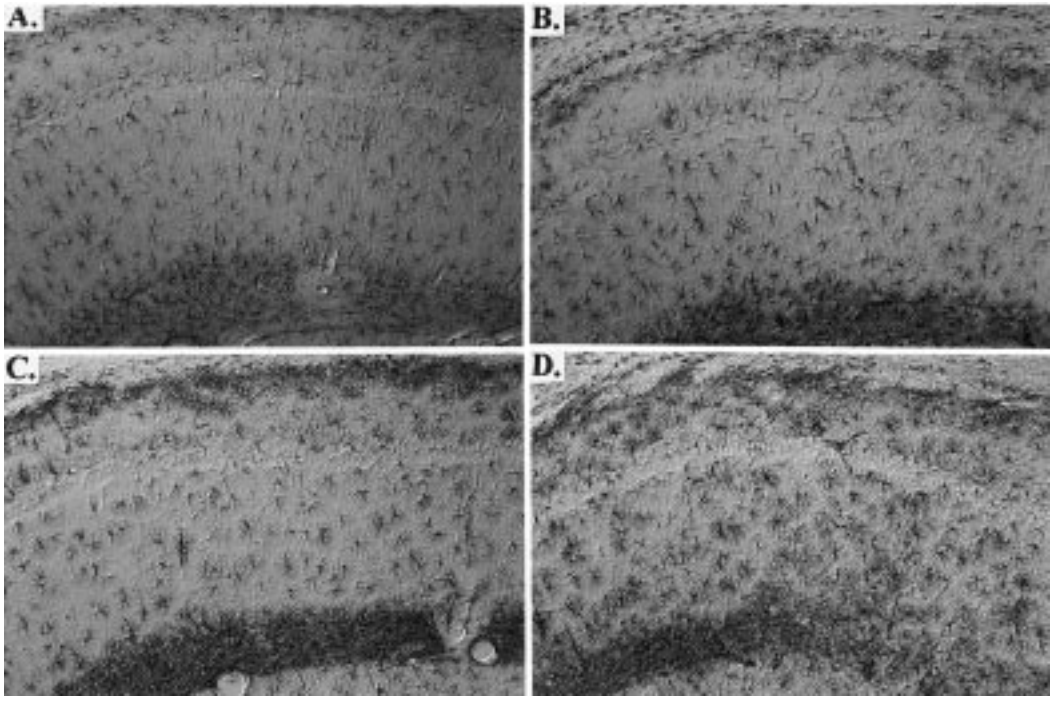

Fig. 1. GFAP immunoreactivity in the ageing rat hippocampal CA1 region. Hippocampal coronal sections (30 μm) from female Fischer 344 rats were incubated with a GFAP monoclonal antibody. (A) Three-month animal. (B) Eighteen-month animal. (C) Twenty-one-month animal. (D) Twenty-nine-month animal (N=4, 4, 2 and 2, respectively). An increase in CA1 subfield GFAP immunoreactivity was seen in 21- and 29-month animals compared to three- and 18-month animals. Magnification: ×100.

subfields (Fig. 1). These results agree with previous findings showing increased GFAP expression with age.[26] It must be noted that the sample size for the 21- and 29-month Fischer 344 rats were limited due to availability of aged animals.

Immunohistochemical localization of glial fibrillary acidic protein in the ageing rat hippocampal CA1 subfield following 5,7-dihydroxytryptamine or vehicle pretreatment

We performed immunohistochemical analysis of GFAP distribution in the hippocampus using the following four treatment groups: three-month vehicle, three-month 5,7-DHT, 18-month vehicle and 18-month 5,7-DHT. Cellular localization of GFAP showed little difference in the stratum radiatum and stratum lacunosum moleculare layers of the CA1 subfield among the three-month vehicle, three-month 5,7-DHT and 18-month vehicle groups (Fig. 2). However, there was a significant increase in the density of GFAP staining in these layers of the hippocampal CA1 region of the 18-month 5,7-DHT group compared to the other treatment groups. The CA1 region of the 18-month 5,7-DHT group revealed significant increases of $31 \pm 3.6\%$, $29 \pm 1.9\%$ and $26 \pm 2.0\%$ in the density of GFAP staining compared to the three-month vehicle, three-month 5,7-DHT and 18-month vehicle groups, respectively.

Immunohistochemical localization of glial fibrillary acidic protein in the ageing rat hippocampal CA3 subfield

In the hippocampus, immunohistochemical staining and localization of GFAP increased in the ageing female Fischer 344 rat (aged three, 18, 21 and 29 months). Three- and 18-month animals showed similar patterns of immunostaining in the stratum moleculare and stratum granulosum, whereas a marked increase in the 21-month group and a striking increase in the 29-month group occurred in these layers of the hippocampal CA3 subfields (Fig. 3). These results agree with previous findings showing increased GFAP expression with age.[26] It must be noted that the sample size for the 21- and 29-month Fischer 344 rats were limited due to availability of aged animals.

Immunohistochemical localization of glial fibrillary acidic protein in the ageing rat hippocampal CA3 subfield following 5,7-dihydroxytryptamine or vehicle pretreatment

We performed immunohistochemical analysis of GFAP distribution in the hippocampus using the following four treatment groups: three-month vehicle, three-month 5,7-DHT, 18-month vehicle and 18-month 5,7-DHT. Cellular localization of GFAP showed no difference in the stratum moleculare and

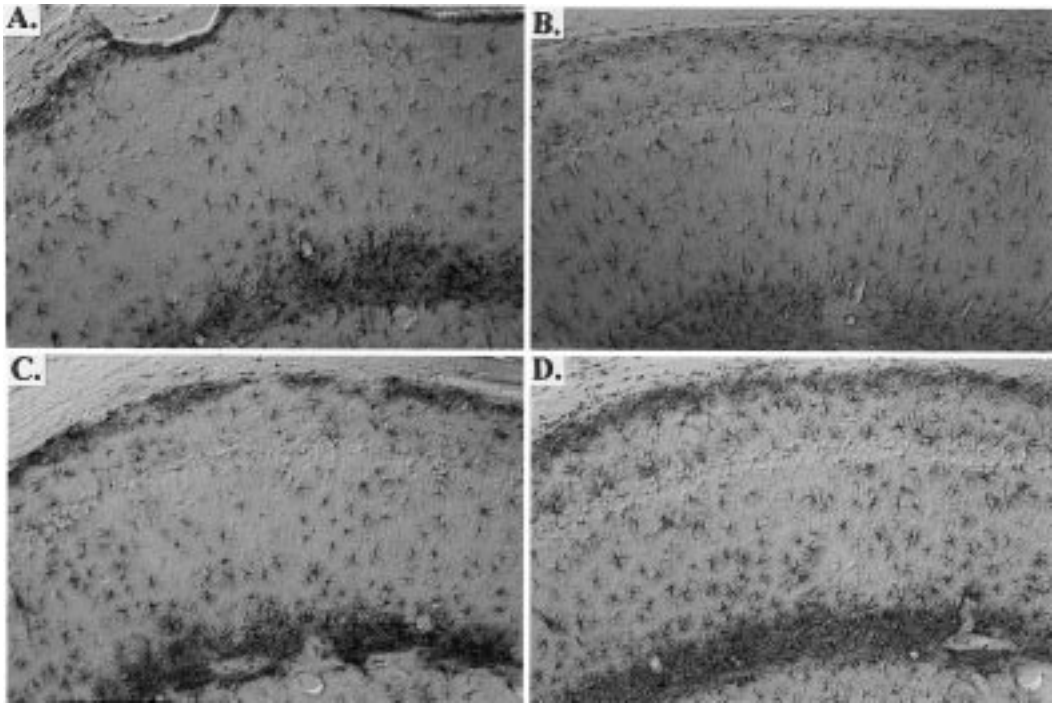

Fig. 2. GFAP immunoreactivity in the ageing rat hippocampal CA1 region following administration of vehicle or 5,7-DHT. Three weeks following lesion treatment, hippocampal coronal sections (30 μm) from female Fischer 344 rats were incubated with a GFAP mAb. (A) Three-month vehicle. (B) Three-month 5,7-DHT. (C) Eighteen-month vehicle. (D) Eighteen-month 5,7-DHT (N=4, 4, 4 and 4, respectively). An increase in CA1 subfield GFAP immunoreactivity was seen in 18-month 5,7-DHT animals compared to age- and treatment-matched animals. Magnification: ×100.

stratum granulosum layers in the CA3 subfield among the three-month vehicle, three-month 5,7-DHT and 18-month vehicle groups (Fig. 4). However, there was a significant increase in the density of GFAP staining in these layers of the hippocampal CA3 region of the 18-month 5,7-DHT group compared to the other treatment groups. The CA3 region of the 18-month 5,7-DHT group demonstrated significant increases of 35±3.0%, 33±3.7% and 26±1.8% in density of GFAP staining compared to the three-month vehicle, three-month 5,7-DHT and 18-month vehicle groups, respectively. Additionally, the GFAP localization in the 18-month 5,7-DHT group was strikingly similar to the distribution in the 21- and 29-month intact groups.

Enzyme-linked immunosorbent assay determination of glial fibrillary acidic protein content in the ageing rat hippocampus and cortex following 5,7-dihydroxytryptamine or vehicle treatment

GFAP concentration (μg/mg total protein) was determined by sandwich ELISA for three-month vehicle, three-month 5,7-DHT, 18-month vehicle and 18-month 5,7-DHT groups (Table 1). The concentrations of GFAP in the hippocampal regions of the three-month vehicle, three-month 5,7-DHT and 18-month vehicle groups were similar. However, GFAP concentration in this brain region in the 18-month 5,7-DHT group increased significantly (29% and 32%) compared to age- and treatment-matched groups (18-month vehicle and three-month 5,7-DHT groups, respectively). Additionally, no changes occurred in cortical GFAP concentration in all four experimental groups (Table 1).

Immunohistochemical localization of synaptosomal-associated protein (mol. wt 25,000) in the ageing rat hippocampus

In the ageing hippocampus, immunohistochemical localization of SNAP-25 in the CA3 subfield demonstrated no alterations in immunostaining as a function of age (three, 18, 21 and 29 months; Fig. 5). Terminals of the mossy fibers in the CA3 region stained consistently for SNAP-25 throughout the different age groups. Additionally, image analyses of this brain region indicated no changes in product density for SNAP-25 antibody immunostaining.

Immunohistochemical localization of synaptosomal-associated protein (mol. wt 25000) in the ageing rat hippocampus following 5,7-dihydroxytryptamine or vehicle treatment

Immunohistochemical analysis for SNAP-25 localization was performed in three-month vehicle, 18-month vehicle, three-month 5,7-DHT and

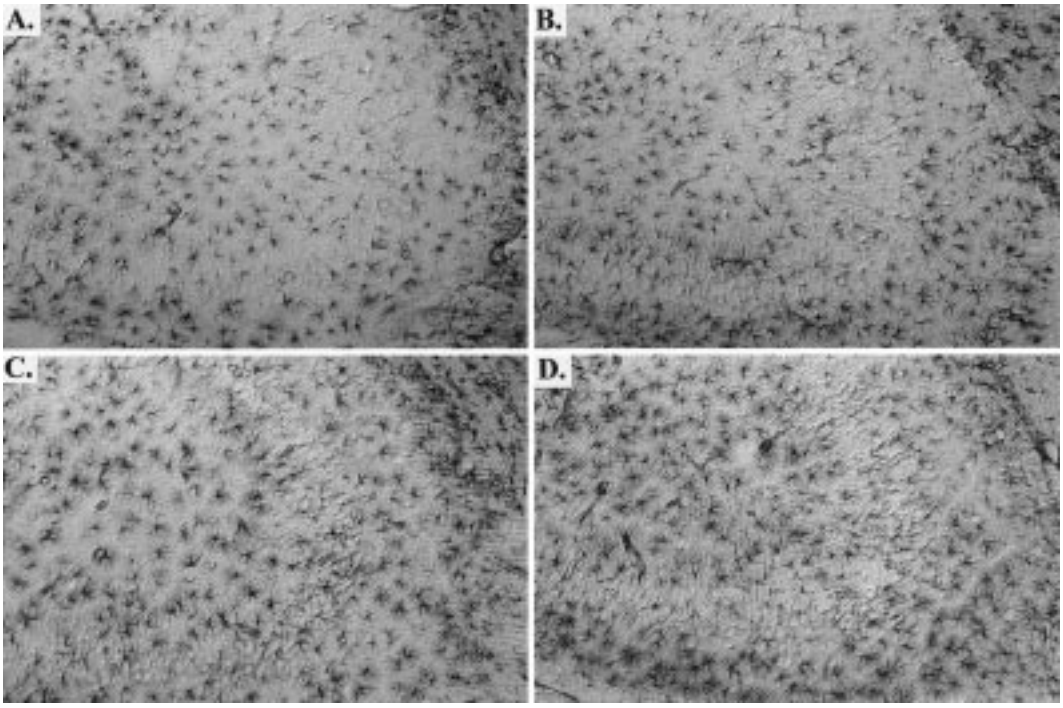

Fig. 3. GFAP immunoreactivity in the ageing rat hippocampal CA3 region. Hippocampal coronal sections (30 μm) from female Fischer 344 rats were incubated with a GFAP monoclonal antibody. (A) Three-month animal. (B) Eighteen-month animal. (C) Twenty-one-month animal. (D) Twenty-nine-month animal (N=4, 4, 2 and 2, respectively). An increase in CA3 subfield GFAP immunoreactivity was seen in 21- and 29-month animals compared to three- and 18-month animals. Magnification: × 100.

18-month 5,7-DHT groups. No differences in the distribution and density of SNAP-25 immunoreactivity in the CA3 region were detected among the four experimental groups (Fig. 6). Product density following immunostaining with antisera to SNAP-25 was not altered in any of the treatment groups.

DISCUSSION

Neurochemical lesion of the serotonergic system at the level of the FF/CB produced age-dependent increases in GFAP expression in the hippocampus. The hippocampal CA1 and CA3 subfields of young treatment groups showed unaltered GFAP expression after depletion of 5-HT in this brain region. In contrast, a marked enhancement of GFAP localization and a significant increase in the levels of this synaptic marker occurred in the hippocampus of old groups receiving the neurotoxicant compared to control groups of the same age. These data demonstrate an age-dependent increase in hippocampal damage following selective serotonergic injury.

Older age groups revealed greater reactive astrocytic responses following administration of 5,7-DHT into the FF/CB. This enhanced astrocytic response may indicate a decreased regenerative response in the ageing hippocampus. Concomitantly, the attenuated response of astrocytic proliferation to the neurochemical lesion in younger groups may facilitate regeneration of damaged neuronal and/or synaptic connections. Other studies utilizing similar lesion paradigms have shown that reactive astrocytes, together with microglia and macrophages, phagocytose and remove degenerating terminals and, thus, make room for collateral reactive sprouting from the remaining healthy neurons.[17] Microglia can also be used as markers of neuronal damage;[29] however, they appear as much smaller cells than astrocytes and are not localized using antibodies to GFAP. Reactive astrocytes may also produce neurotrophic factors needed for the induction of axon growth and sprouting,[11,34] or release substances that inhibit neurite growth.[10,28,30] Combined with other studies showing age-related declines in synaptic and neuronal regeneration following neuronal damage, the present studies suggest that the excessive astrocytic reaction observed in older groups may serve to inhibit regeneration or sprouting of new synaptic connections in the hippocampus.

Little change in SNAP-25 immunoreactivity was observed in the hippocampal CA3 subfield among the various intact age groups, as well as the lesioned animals. These results indicate that vesicle release and neuronal maturation processes that require this protein remain relatively stable during ageing and are not affected by this selective neuronal damage. In agreement with previous reports, an increase in GFAP expression was noted with increasing age[26] and no change was evident for SNAP-25 expression in intact animals.[23]

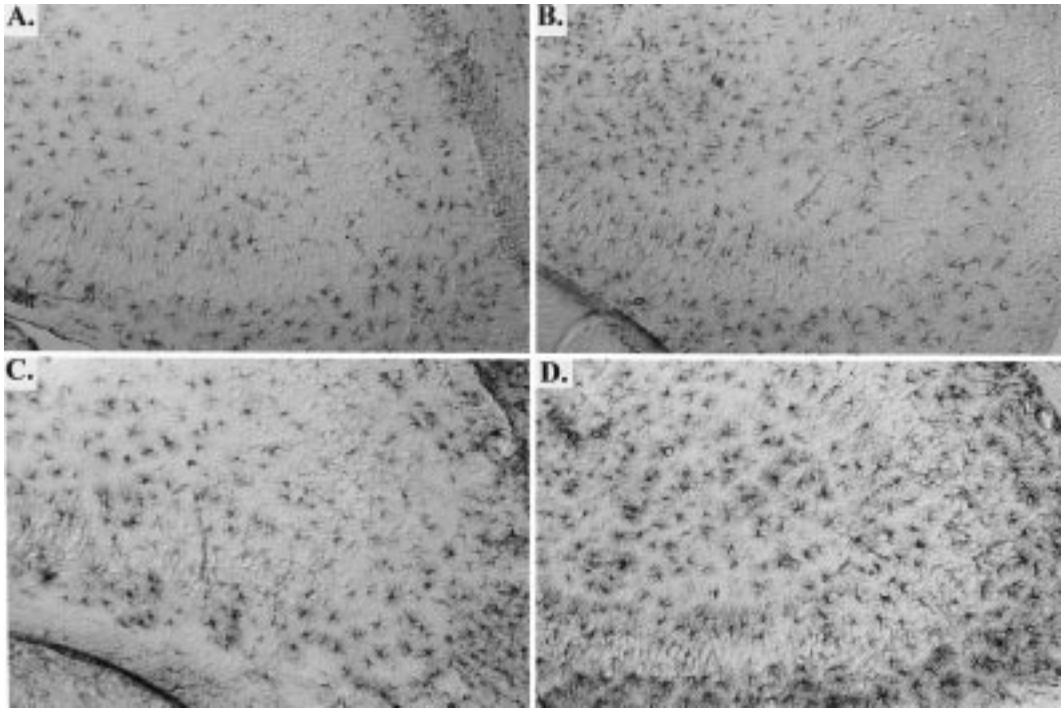

Fig. 4. GFAP immunoreactivity in the ageing rat hippocampal CA3 region following administration of vehicle or 5,7-DHT. Three weeks following lesion treatment, hippocampal coronal sections (30 μm) from female Fischer 344 rats were incubated with a GFAP mAb. (A) Three-month vehicle. (B) Three-month 5,7-DHT. (C) Eighteen-month vehicle. (D) Eighteen-month 5,7-DHT (N=4, 4, 4 and 4, respectively). An increase in CA3 subfield GFAP immunoreactivity was seen in 18-month 5,7-DHT animals compared to age- and treatment-matched animals. Magnification: ×100.

Table 1. Glial fibrillary acidic protein levels in specific brain regions of ageing female Fischer 344 rats following 5,7-dihydroxytryptamine or vehicle administration in the fimbria–fornix/cingulum bundle determined by sandwich enzyme-linked immunosorbent assay

	GFAP (μg/mg total protein) by ELISA			
Brain region	3-month VEH	3-month 5,7-DHT	18-month VEH	18-month 5,7-DHT
Hippocampus	2.79 ± 0.20	2.87 ± 0.22	2.95 ± 0.29	3.79 ± 0.08*
Frontal cortex	1.11 ± 0.34	1.29 ± 0.15	1.23 ± 0.29	1.53 ± 0.14

Each value represents the mean ± S.E.M. (N=3 for each group). Vehicle (VEH)-treated animals received 0.1% ascorbic saline.
*Significantly different from 18-month vehicle and three-month 5,7-DHT (P<0.05).

Neurochemical (e.g., kainate, colchicine) and mechanical (e.g., entorhinal cortex, fornix) lesion studies in aged animals have shown astrocytic responses similar to the present observations in the hippocampus.[17,34,45,46] Our results with SNAP-25 suggest that this protein linked to synaptogenesis is unaltered in the ageing hippocampus. However, these results do not preclude age-dependent alterations for other proteins required for synaptogenesis. Alternatively, recovery of reductions in SNAP-25 expression may have been completed within the three-week time-course of this study for both young and old groups.

The question of the ability of the aged hippocampus to compensate on a cellular level after injury is raised by these findings. For example, GFAP localization in denervated middle-aged animals was remarkably similar to the intact 29-month animals. In contrast to the young lesioned group, the older lesioned group did not compensate for this neurotoxic lesion which damaged the hippocampus. Although the 18-month saline-treated rats appeared similar to the young adult animals (both saline and 5,7-DHT treated), the older drug-treated animals may no longer have the ability to correct the selective serotonergic deafferentation produced by 5,7-DHT administration. Furthermore, the anatomical changes may occur in parallel with functional changes identified previously at the cellular physiological level in CA3 pyramidal neurons following denervation of hippocampal serotonergic input. Dugar and Lakoski[6] showed that 18-month female

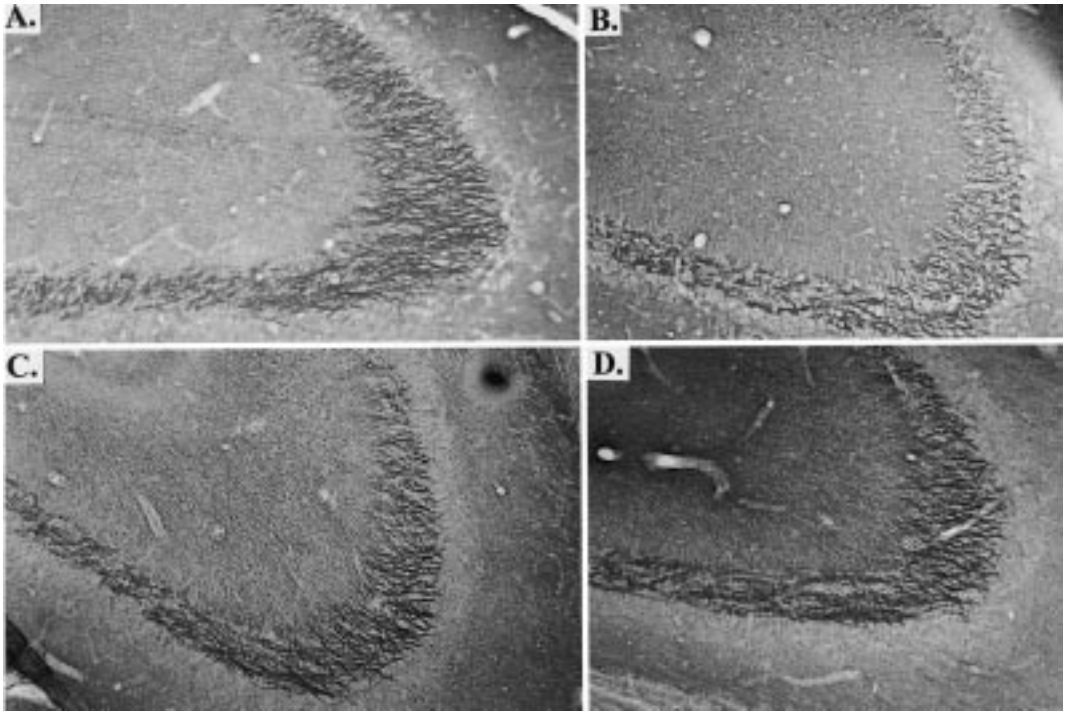

Fig. 5. SNAP-25 immunoreactivity in the ageing rat hippocampus. Hippocampal coronal sections (30 μm) from female Fischer 344 rats were incubated with a SNAP-25 polyclonal antisera. (A) Three-month animal. (B) Eighteen-month animal. (C) Twenty-one-month animal. (D) Twenty-nine-month animal (N=4, 4, 2 and 2, respectively). No consistent changes were demonstrated in CA3 subfield SNAP-25 immunoreactivity with age. Magnification: × 100.

Fischer 344 rats which received 5,7-DHT in the FF/CB demonstrated a compromised recovery response following 5-HT application, indicative of alterations in presynaptic uptake processes in old treatment groups. These results suggest that the middle-aged animal cannot compensate on a functional and anatomical basis for the selective neurotoxic insult.

Numerous experimental lesion models produce synaptic degeneration with subsequent glial and/or SNAP-25 reactions distal to the site of degeneration. For example, major deafferentation of the hippocampus by entorhinal cortex and/or FF lesions consistently increased levels of GFAP and SNAP-25 message in the hippocampus.[14,41] However, our model provides a selective denervation at the level of the FF/CB, which does not change SNAP-25 localization in the mossy fibers of the hippocampus with ageing. Damage to the hippocampus following intraventricular administration of 5,7-DHT has been shown to include reactive gliosis, with an increase in GFAP expression[39] and mRNA, but not proliferation of astrocytes.[2,8,16] Studies with kainate lesions have also suggested that increased GFAP expression in the hippocampus is a common response to hippocampal injury.[19] Lesion studies further suggest that mossy fibers are involved in the susceptibility of CA3 pyramidal cells to epileptic processes and in degeneration of these neurons after kainate administration.[55] Developmental studies in Long–Evans rats in which TMT, a neurotoxicant which degenerates CA3 and CA4 pyramidal cells, was administered during early postnatal periods showed persistent increases in GFAP concentration in the hippocampus and cortex up to two months post-drug treatment.[37] Likewise, Sprague–Dawley rats showed enhanced SNAP-25 immunoreactivity and increased message in the hippocampus following TMT application.[41] These models provide important information to help understand how neuronal insults may affect brain regions like the hippocampus.

GFAP immunoreactivity and message are increased in age-related disorders including Alzheimer's disease, as well as other neurodegenerative diseases, including epilepsy, multiple sclerosis and Pick's disease.[7,32] Compared to young patients, old patients with Down's syndrome have increased numbers of GFAP-immunoreactive cells, suggesting the presence of gliosis related to senile plaques and neurofibrillary tangles.[31] Furthermore, injections of kainate or colchicine into the cerebral ventricles resulted in neurodegeneration which, in turn, induced expression of amyloid precursor protein in hippocampal astrocytes.[47] Experimental lesions frequently serve as paradigms for age-related neurodegenerative diseases. For example, lesions of the entorhinal cortex and FF are used to model Alzheimer's disease and lesions of the substantia nigra using

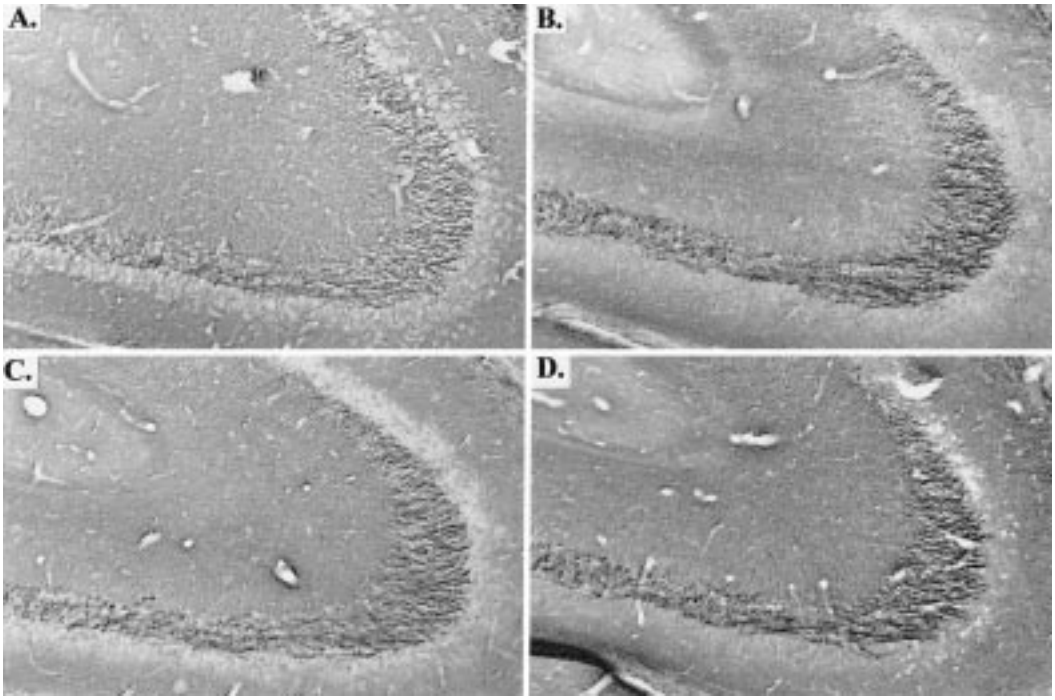

Fig. 6. SNAP-25 immunoreactivity in the ageing rat hippocampus following administration of vehicle or 5,7-DHT. Three weeks following lesion treatment, hippocampal coronal sections from female Fischer 344 rats were incubated with a SNAP-25 polyclonal antisera. (A) Three-month vehicle. (B) Three-month 5,7-DHT. (C) Eighteen-month vehicle. (D) Eighteen-month 5,7-DHT (N=4, 4, 4 and 4, respectively). No consistent changes were demonstrated in CA3 subfield SNAP-25 immunoreactivity across ages or treatment groups. Magnification: $\times$100.

6-hydroxydopamine to mimic Parkinson's. However, most of these models have examined only young adults and may consequently overlook effects of advanced age on cellular response to neuronal injury.

CONCLUSION

These data support the hypothesis that, with ageing, the hippocampus demonstrates altered reactive gliosis responses as a result of a neurotoxic insult. Younger animals compensated both functionally and anatomically to denervation of the serotonergic input to the hippocampus, while the older animals lost recovery responses and revealed greater astrocytic proliferation from this insult. Whether the mechanism underlying the increased astrocytic response to neurotoxic insult is harmful or beneficial still remains unclear. Some studies suggest that this response may involve inhibition of neurite regrowth,[27] whereas other reports indicate that astrocytic reactivity may produce neurotrophic factors required for induction of axon growth and sprouting.[11,34] These issues of neuronal compensation and regeneration are important to consider when investigating age-related neurodegenerative diseases, and in particular, when utilizing neurochemical lesion models in the ageing animal.

Acknowledgements—We would like to thank Dr M. Billingsley for providing SNAP-25 antibodies for immunohistochemical studies and Elizabeth Boykin for conducting the ELISAs for quantification of GFAP levels. Publication no. 94 supported by U.S.P.H.S. grant PO1 AG10514 (J.M.L.), The Pennsylvania State University Gerontology Faculty Project (J.M.L.) and the Merck Company Foundation (A.D.).

REFERENCES

1. Balaban C. D., O'Callaghan J. P. and Billingsley M. L. (1988) Trimethyl-tin-induced neuronal damage in the rat: comparative studies using silver degeneration stains, immunocytochemistry and immunoassay for neuronotypic and gliotypic proteins. *Neuroscience* **26,** 337–361.
2. Bendotti C., Baldessari S., Pende M., Tarizzo G., Miari A., Presti M. L., Mennini T. and Samanin R. (1994) Does GFAP mRNA and mitochondrial benzodiazepine receptor binding detect serotonergic neuronal degeneration in rat? *Brain Res. Bull.* **34,** 389–394.
3. Bjorklund H., Eriksdotter-Nilsson M., Dahl D., Rose G., Hoffer B. and Olson L. (1985) Image analysis of GFA-positive astrocytes from adolescence to senescence. *Expl Brain Res.* **58,** 163–170.

4. Brock T. O. and O'Callaghan J. P. (1987) Quantitative changes in the synaptic vesicle proteins, synapsin I and p38, and the astrocyte specific protein, GFAP, are associated with chemical-induced injury to the rat central nervous system. *J. Neurosci.* **7,** 931–942.
5. Burtrum D. and Silverstein F. S. (1994) Hypoxic–ischemic brain injury stimulates glial fibrillary acidic protein mRNA and protein expression in neonatal rats. *Expl Neurol.* **126,** 112–118.
6. Dugar A. and Lakoski J. M. (1997) Serotonergic function of aging hippocampal CA3 pyramidal neurons: electrophysiological assessment following administration of 5,7-dihydroxytryptamine in the fimbria–fornix and cingulum bundle. *J. Neurosci. Res.* **47,** 58–67.
7. Duguid J. R., Boumont C. W., Liu N. and Tourtellotte W. W. (1989) Changes in brain gene expression shared by scrapie and Alzheimer's disease. *Proc. natn. Acad. Sci. U.S.A.* **86,** 7260–7264.
8. Fages C., Le Prince G., Didier-Bazes M., Rolland B., Hardin H. and Tardy M. (1994) Long-term astroglial reaction to serotonergic fiber degeneration. *Brain Res.* **639,** 161–166.
9. Finch C. E., Felicio L. S., Mobbs C. V. and Nelson J. F. (1984) Ovarian and steroidal influences on neuroendocrine aging processes in female rodents. *Endocr. Rev.* **5,** 467–497.
10. Frederickson R. C. A. and Silver J. (1991) Glial cells, the unsung heroes of the brain. *Biotechnology* **9,** 1–4.
11. Gage F. H., Olejniczak P. and Armstrong D. M. (1988) Astrocytes are important for sprouting in the septohippocampal circuit. *Expl Neurol.* **102,** 2–13.
12. Gall C., Rose G. and Lynch G. (1979) Proliferative and migratory activity of glial cells in the partially deafferented hippocampus. *J. comp. Neurol.* **183,** 539–550.
13. Gordon M. N. and Morgan D. G. (1991) Increased GFAP expression in the aged rat brain does not result from increased astrocyte density. *Soc. Neurosci. Abstr.* **17,** 53.
14. Goss J. R. and Morgan D. G. (1995) Enhanced glial fibrillary acidic protein RNA response to fornix transection in aged mice. *J. Neurochem.* **64,** 1351–1360.
15. Hansen L. A., Armstrong D. M. and Terry R. D. (1987) An immunohistochemical quantification of fibrous astrocytes in the ageing human cerebral cortex. *Neurobiol. Aging* **8,** 1–6.
16. Hardin H., Bernard A., Rajas F., Fevre-Montage M., Derrington E., Belin M. F. and Didier-Bazes M. (1994) Modifications of glial metabolism of glutamate after serotonergic neuron degeneration in the hippocampus of the rat. *Brain Res.* **26,** 1–8.
17. Hoff S. F., Scheff S. W., Bernardo L. S. and Cotman C. W. (1982) Lesion-induced synaptogenesis in the dentate gyrus of aged rats. I. Loss and reacquistion of normal synaptic density. *J. comp. Neurol.* **205,** 246–252.
18. Hughes C. C. W. and Lantos P. L. (1987) A morphometric study of blood vessel, neuron and glial cell distribution in young and old rat brain. *J. neurol. Sci.* **79,** 101–110.
19. Kesslak J. P., Yuan D., Neeper S. and Cotman C. W. (1995) Vulnerability of the hippocampus to kainate excitotoxicity in the aged, mature and young adult rat. *Neurosci. Lett.* **188,** 117–120.
20. Lakoski J. M., Keck B. J. and Dugar A. (1996) Neurochemical lesions: tools for functional assessment of serotonin neuronal systems. In *Paradigms of Neural Injury* (ed. Perez-Polo J. R.), pp. 115–123. Academic, San Diego.
21. Landfield P. W., Braum L. D., Pitler T. A., Lindsey J. D. and Lynch G. (1981) Hippocampal aging in rats: a morphometric study of multiple variables in semithin sections. *Neurobiol. Aging* **2,** 265–275.
22. Landfield P. W. and Lynch G. (1977) Impaired monosynaptic potentiation in *in vitro* hippocampal slices from aged, memory-deficient rats. *J. Geront.* **32,** 523–533.
23. Lapchak P. A., Araujo D. M., Beck K. D., Finch C. E., Johnson S. A. and Hefti F. (1993) BDNF and trkB mRNA expression in the hippocampal formation of aging rats. *Neurobiol. Aging* **14,** 121–126.
24. Laping N. J., Nichols N. R., Day J. R. and Finch C. E. (1991) Corticosterone differentially regulates the bilateral response of astrocyte mRNAs in the hippocampus to entorhinal cortex lesions in male rats. *Molec. Brain Res.* **10,** 291–297.
25. Lindsay, R. M. (1986) Reactive gliosis. In *Astrocytes* (eds S. Federoff and A. Vernadakis), pp. 231–262. Academic, San Diego.
26. Linnemann D. and Skarsfelt T. (1994) Regional changes in expression of NCAM, GFAP, and S100 in aging rat brain. *Neurobiol. Aging* **15,** 651–655.
27. Luizzi F. J. (1990) Proteolysis is a critical step in the physiological stop pathway: mechanisms involved in the blockade of axonal regeneration by mammalian astrocytes. *Brain Res.* **512,** 277–283.
28. Luizzi F. J. and Lasek R. J. (1987) Astrocytes block axonal regeneration in mammals by activating the physiological stop pathway. *Science* **237,** 642–645.
29. McCann M. J., O'Callaghan J. P., Martin P. M., Bertram T. and Streit W. J. (1996) Differential activation of microglia and astrocytes following trimethyl tin-induced neurodegeneration. *Neuroscience* **72,** 273–281.
30. McKeon R. J., Schreiber R. C., Rudge J. S. and Silver J. (1991) Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *J. Neurosci.* **11,** 3398–3411.
31. Mito T. and Becker L. E. (1993) Developmental changes of S-100 protein and glial fibrillary acidic protein in the brain in Down syndrome. *Expl Neurol.* **120,** 170–176.
32. Morgan D. G., Finch C. E. and Johnson S. A. (1991) RNA metabolism in Alzheimer's disease: selective increase in GFAP RNA. In *Alzheimer's Disease: Basic Mechanism, Diagnosis and Therapeutic Strategies* (ed. K. Iqbal), pp. 407–416. John Wiley, New York.
33. Mucke L. and Eddleston M. (1993) Astrocytes in infectious and immune-mediated disease of the central nervous system. *Fedn Proc. Fedn Am. Socs exp. Biol.* **7,** 1226–1232.
34. Nieto-Sampedro M. and Cotman C. W. (1985) Growth factor induction and temporal order in CNS repair. In *Synaptic Plasticity* (ed. C. W. Cotman), pp. 407–455. Guilford, New York.
35. O'Callaghan J. P. (1991) Quantification of glial fibrillary acidic protein: comparison of slot-immunobinding assay with a novel sandwich ELISA. *Neurotoxicol. Teratol.* **13,** 275–281.
36. O'Callaghan J. P. (1993) Quantitative features of reactive gliosis following toxicant-induced damage of the CNS. *Ann. N. Y. Acad. Sci.* **679,** 195–210.
37. O'Callaghan J. P. and Miller D. B. (1989) Assessment of chemically-induced alteration in brain development using assays of neuron- and glia-localized proteins. *Neurotoxicology* **10,** 393–406.

38. O'Callaghan J. P. and Miller D. B. (1991) The concentration of glial fibrillary acidic protein increases with age in the mouse and rat brain. *Neurobiol. Aging* **12,** 171–174.
39. O'Callaghan J. P. and Miller D. B. (1993) Quantification of reactive gliosis as an approach to neurotoxicity assessment. In *Assessing Neurotoxicity of Drugs of Abuse* (ed. Erinoff L.), pp. 188–212. U.S. Government Printing Office, Washington, DC.
40. Oyler G. A., Polli J. W., Wilson M. C. and Billingsley M. L. (1991) Developmental expression of the 25-kDa synaptosomal-associated protein (SNAP-25) in rat brain. *Proc. natn. Acad. Sci. U.S.A.* **88,** 5247–5251.
41. Patanow C. M., Day J. and Billingsley M. L. (1997) Alterations in hippocampal expression of SNAP-25, GAP-43, stannin, and glial fibrillary acidic protein following mechanical and trimethyltin-induced injury in the rat. *Neuroscience* **76,** 187–202.
42. Paxinos G. and Watson C. (1986) *The Rat Brain in Stereotaxic Coordinates*. Academic, New York.
43. Poirier J., May P. C., Osterburg H. H., Geddes J., Cotman C. and Finch C. E. (1990) Selective alterations of RNA in rat hippocampus after entorhinal cortex lesion. *Proc. natn. Acad. Sci. U.S.A.* **87,** 303–307.
44. Quik M. and Azmitia E. (1983) Selective destruction of the serotonergic fibers of the fornix–fimbria and cingulum bundle increases 5-HT_1 but not 5-HT_2 receptors in rat midbrain. *Eur. J. Pharmac.* **90,** 377–384.
45. Scheff S. W., Bernardo L. S. and Cotman C. W. (1978) Decrease in adrenergic axon sprouting in the senescent rat. *Science* **202,** 775–778.
46. Scheff S. W., Bernardo L. S. and Cotman C. W. (1980) Decline in reactive fiber growth in the dentate gyrus of aged rats compared to young adult rats following entorhinal cortex removal. *Brain Res.* **199,** 21–38.
47. Siman R., Card J. P., Nelson R. B. and Davis L. G. (1989) Expression of beta-amyloid precursor protein in reactive astrocytes following neuronal damage. *Neuron* **3,** 275–285.
48. Sivron T. and Schwartz M. (1995) Glial cell types, lineages, and response to injury in rat and fish: implications for regeneration. *Glia* **13,** 157–165.
49. Sturrock R. R. (1980) A comparative quantitative and morphological study of ageing in the mouse neostriatum, injusium griseum and anterior commissure. *Neuropath. appl. Neurobiol.* **6,** 51–68.
50. Toggas S. M., Krady J. K., Thompson T. A. and Billingsley M. L. (1993) Molecular mechanisms of selective neurotoxicants: studies on organotin compounds. *Ann. N. Y. Acad. Sci.* **679,** 157–177.
51. Van de Kar L. D. and Lorens S. A. (1979) Differential serotonergic innervation of individual hypothalamic nuclei and other forebrain regions by the dorsal and median midbrain raphe nuclei. *Brain Res.* **162,** 45–54.
52. Vaughan D. W. and Peters A. (1974) Neuroglial cells in the cerebral cortex of rats from young adulthood to old age: an electron microscope study. *J. Neurocytol.* **3,** 405–429.
53. Wagner A. P., Reck G. and Platt D. (1993) Evidence that V+ fibronectin, GFAP and S100 beta mRNAs are increased in the hippocampus of aged rats. *Expl Geront.* **28,** 135–143.
54. Wise P. M. (1983) Aging of the female reproductive system. *Rev. biol. Res. Aging* **1,** 195–222.
55. Yang Q., Wang S., Hamberger A. and Haglid K. G. (1996) Plasticity of granule cell–mossy fiber system following kainic acid induced seizures: an immunocytochemical study on neurofilament proteins. *Neurosci. Res.* **26,** 57–64.

(*Accepted* 31 *October* 1997)