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Expression of microtubule-associated protein 2 by reactive astrocytes

(regeneration/reactive gliosis)

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ABSTRACT **After an injury to the central nervous system, a dramatic change in the astrocytes bordering the wound occurs. The most characteristic feature of this process, termed reactive gliosis, is the upregulation of the intermediate filament protein, glial fibrillary acidic protein. In the present study, we show that reactive astrocytes express high levels of microtubule-associated protein 2 (MAP-2), a protein normally found in the somatodendritic compartment of neurons. When sections of injured brain are double-stained with antibodies directed against MAP-2 and glial fibrillary protein, all of the reactive astrocytes are found to contain MAP-2. The high levels of this protein appear to represent a permanent change in reactive astrocytes. In parallel quantitative studies, an elevated level of MAP-2 in the injured brain is confirmed by an immunoblot analysis of injured and normal white matter. This report demonstrates the direct involvement of a microtubule protein in the process of reactive gliosis.**

When the central nervous system (CNS) is injured, a sequence of pathological changes occurs, termed reactive gliosis. During the initial phases of this process, astrocytes become hypertrophied, increase their mitotic activity, and increase their content of the intermediate filament protein, glial fibrillary acidic protein (GFAP) (1, 2). These reactive astrocytes are an integral component of the CNS scar, and several lines of evidence indicate that these cells may contribute to the lack of regeneration seen in the mammalian brain after injury (3–5). Because these cells play a critical role in the response of the CNS to trauma, defining the changes that occur when an astrocyte becomes reactive is fundamental to understanding the mechanisms of CNS scar formation. Despite the importance of understanding reactive gliosis, surprisingly little is known about the molecular events that characterize this process.

The most prominent alteration that occurs during reactive gliosis is a dramatic increase in the number of intermediate filaments within the astrocytes (2). Although the intermediate filaments are a major component of the astrocytic cytoskeleton, microtubules are also present (6). Both of these cytoskeletal elements are important in the organization and maintenance of cell shape as well as in other cellular functions. The microtubules interact with a group of proteins termed microtubule-associated proteins that are known to modulate their stability (7). The most abundant of these proteins is a large, heat-stable protein (8, 9), microtubule-associated protein 2 (MAP-2). In the brain, with rare exceptions (see *Discussion*), MAP-2 is found in the somatodendritic compartment of neurons. While studying the process of CNS wound healing with a panel of monoclonal antibodies (mAbs) directed against cytoskeletal proteins, we observed that reactive astrocytes express high levels of MAP-2. The dramatic upregulation of MAP-2 in reactive astrocytes appears to be a fundamental change in these cells, reflecting their altered role in CNS wound healing.

METHODS

Antibodies. Three mouse mAbs directed against MAP-2 were used in this study. Two mouse mAbs, AP-14 and AP-18, were raised by using bovine MAP-2 as the immunogen. mAb AP-14 (10) and AP-18 (11) were characterized and used in previous studies. The third mAb, AP-15, was derived from a fusion by using rat microtubules as an immunogen. All of the antibodies are of the IgG1 subclass. The mAb TuJ1, specific for class III β-tubulin, was a gift from A. Frankfurter (Department of Biology, University of Virginia) (12, 13).

Electrophoretic Procedures. Brain-tissue samples were homogenized in phosphate-buffered saline with a portion of each sample being dissolved in NaOH for protein determination (14). The balance was prepared for electrophoresis (15) and stored at −20°C. Known amounts of each sample were loaded onto 4–16% polyacrylamide linear gradient gels (8.3 × 5.3 × 1.5 cm). Electrophoresis and immunoblotting with a second antibody–peroxidase conjugate were performed as described (10). For quantitative analysis, immunoblots were processed with the unlabeled antibody procedure of Sternberger (16) by using peroxidase–antiperoxidase (PAP) obtained from Sternberger-Meyer Immunochemicals (Whitehall, MD). The antibody binding to these blots was visualized with diaminobenzidine and hydrogen peroxide. Measurements of the relative amounts of the reaction product on the immunoblots were performed with a computerized reflected light densitometer (Bio-Rad, model 620).

Epitope Mapping. Purified rat MAP-2 was a gift from G. V. W. Johnson, Department of Neurology, University of Alabama at Birmingham. An aliquot of this preparation was incubated at 37°C for 10 min at a concentration of 0.5 mg/ml in a solution containing 50 mM Pipes, 1 mM EGTA, 0.5 mM $MgSO_4$, 0.1 mM GTP, and α-chymotrypsin at 1 μg/ml (pH 6.9 with NaOH). The proteolytic digestion was terminated by mixing the sample 1:1 with electrophoresis sample buffer and immediately placing it in a boiling water bath for 5 min. This sample was then loaded onto a slotless SDS/polyacrylamide gel, and after electrophoresis, the proteins were transferred to nitrocellulose. Adjacent strips of the immunoblot were probed with the three different MAP-2 mAbs.

Surgery. Lesions of the CNS were made in the internal capsule and the fimbria of 18 rats and the corpus callosum of five rats. The general surgical procedures have been described (17). Sprague–Dawley rats (250 g) were anesthetized with sodium pentobarbital, and sterile surgery was performed. Lesions of the internal capsule were made unilaterally with a knife cut in the coronal plane. After a survival

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Abbreviations: MAP-2, microtubule-associated protein 2; GFAP, glial fibrillary acidic protein; mAb, monoclonal antibody.

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period from 0 to 50 days, the animals were deeply anesthetized with sodium pentobarbital and perfused through the heart with either 4% paraformaldehyde/phosphate buffer, pH 7.4, or with 4% paraformaldehyde/0.5% glutaraldehyde/phosphate buffer, pH 7.4.

The relative increase in MAP-2 was determined by a quantitative analysis of immunoblots. The corpus callosa of four rats were lesioned bilaterally in the sagittal plane 1 mm from the midline. After a 10-day survival period, the animals were perfused with saline followed by 2% paraformaldehyde/phosphate buffer, pH 7.4. The corpus callosum was dissected free of the overlying cerebral cortex and the underlying hippocampus. The tissue was homogenized in phosphate-buffered saline, an aliquot was set aside for protein determination, and the balance was prepared for electrophoresis. The corpus callosa of four normal rats were prepared in a similar manner and used as control samples.

Immunohistochemistry. The brains were sectioned on a freezing microtome at 40 μm. The sections were stained by rabbit antiserum directed against GFAP (Accurate Chemicals, Westbury, NY) and mouse mAbs specific for MAP-2 (AP-14, AP-15, and AP-18). The mAb TuJ1 was used to stain neuronal tubulin. Sections to be stained by the peroxidase–antiperoxidase method were rinsed in borate-buffered saline (pH 8.4) and placed in a blocking solution. The sections were then placed in the primary antibody at 4°C overnight. The next day the sections were rinsed in borate-buffered saline and placed in peroxidase-labeled secondary antisera (HyClone) at a dilution of 1:500 for 2 hr at room temperature. After being rinsed, the sections were placed in peroxidase–anti-peroxidase (Sigma) for 2 hr, rinsed with phosphate-buffered saline (pH 7.4) and reacted with diaminobenzidine and hydrogen peroxide for 15 min.

Selected sections were processed for double immunofluorescence by using a rabbit anti-GFAP antiserum and a mouse mAb (AP-14) directed against MAP-2. These primary antibodies were followed by a rhodamine-labeled goat anti-rabbit secondary antibody that had been absorbed against rat IgG (HyClone) and a fluorescein-labeled goat anti-mouse secondary antibody that had been absorbed with rat IgG (HyClone).

RESULTS

The process of reactive gliosis was studied by examining frozen sections of the brain from animals that received

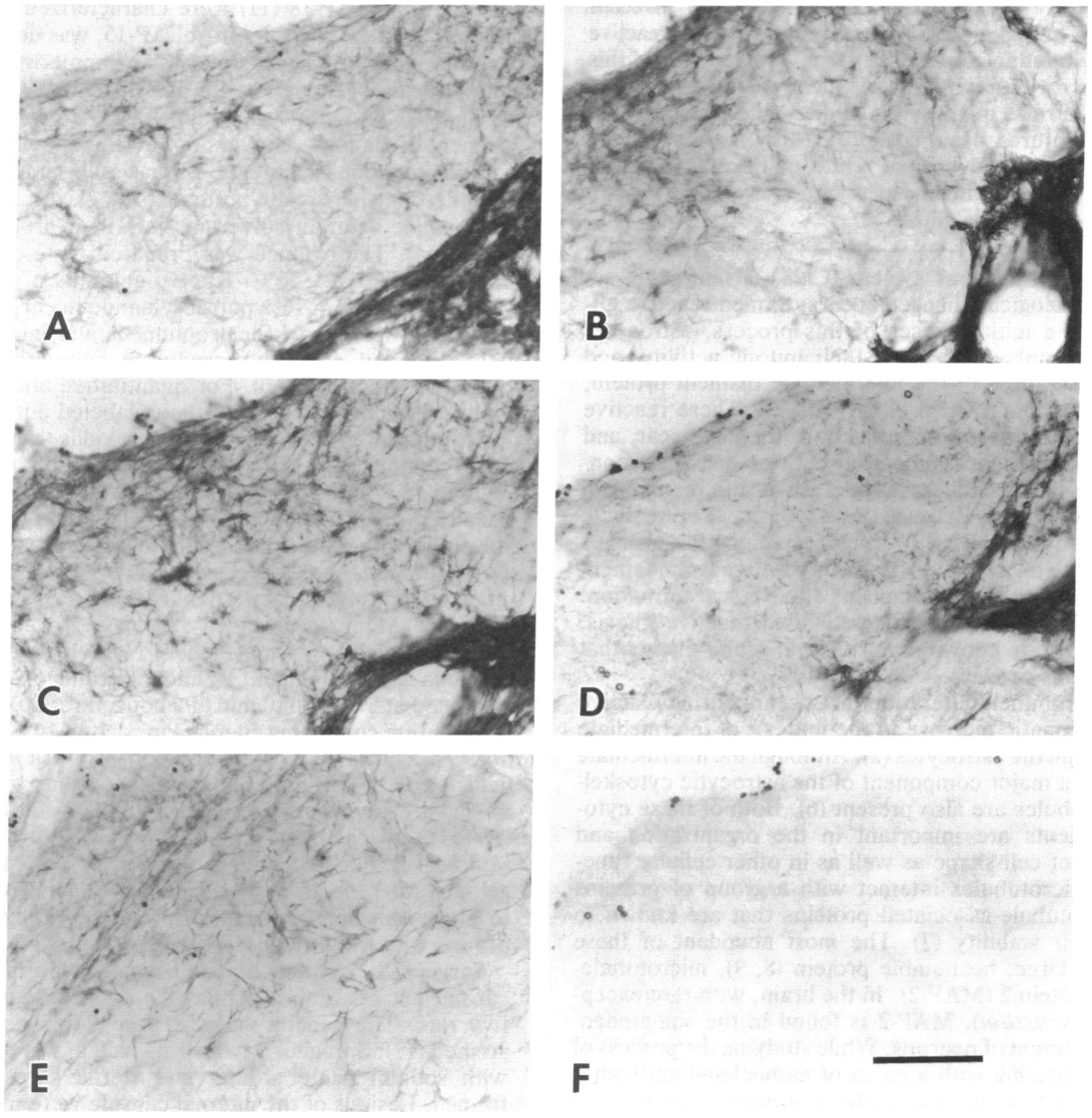

FIG. 1. Frozen sections from a rat brain, 10 days after injury, were stained with the MAP-2 mAbs AP-14 (*A*), AP-15 (*B*), and AP-18 (*C*) and compared to the immunostaining pattern seen with TuJ1 (a mAb directed against class III β-tubulin) (*D*), and the staining pattern for GFAP (a polyclonal antiserum) (*E*). The edge of the site of injury is in the upper left corner, and the caudate–putamen is in the lower right corner. (*F*) Photomicrograph of a control section stained with the secondary antibody only, showing background labeling. (Bar = 100 μm.) All photomicrographs are at the same magnification.

lesions of the internal capsule. When these sections were stained with mAbs directed against MAP-2 (AP-14, AP-15, and AP-18), neuronal cell bodies and dendrites were heavily labeled (Fig. 1). In addition, many cells within the white matter near the site of injury were also labeled (Fig. 1). These cells in the white matter were not stained by an antibody directed against class III β-tubulin, a tubulin isotype found in neurons and their processes, but not in glia (Fig. 1*D*). In adjacent sections stained for GFAP (Fig. 1*E*), the size and distribution of the reactive astrocytes were similar to the MAP-2-positive cells seen in the white matter. When the reactive GFAP-positive astrocytes were restricted to the area immediately adjacent to the lesion, the MAP-2-positive cells within the white matter demonstrated a similar restricted distribution. In the cases where the reactive gliosis extended further away from the initial site of injury, the MAP-2-positive cells mimicked a more extensive distribution. Both MAP-2 and GFAP immunoreactivity were seen as early as 1 day after the lesion. By 5 days postlesion, the relative intensity of staining increased and remained at this high level up to 50 days postlesion, the longest survival period examined.

To show that the MAP-2-positive cells in the white matter were reactive astrocytes, selected sections from lesioned animals were double-stained for GFAP and MAP-2 (Fig. 2). All cells near the edge of the lesion that stained for GFAP also stained intensely for MAP-2, whereas on the nonlesioned side of the brain no detectable amount of MAP-2 staining was seen in GFAP-positive cells of the internal capsule. Double-labeled cells were observed in the white matter of the internal

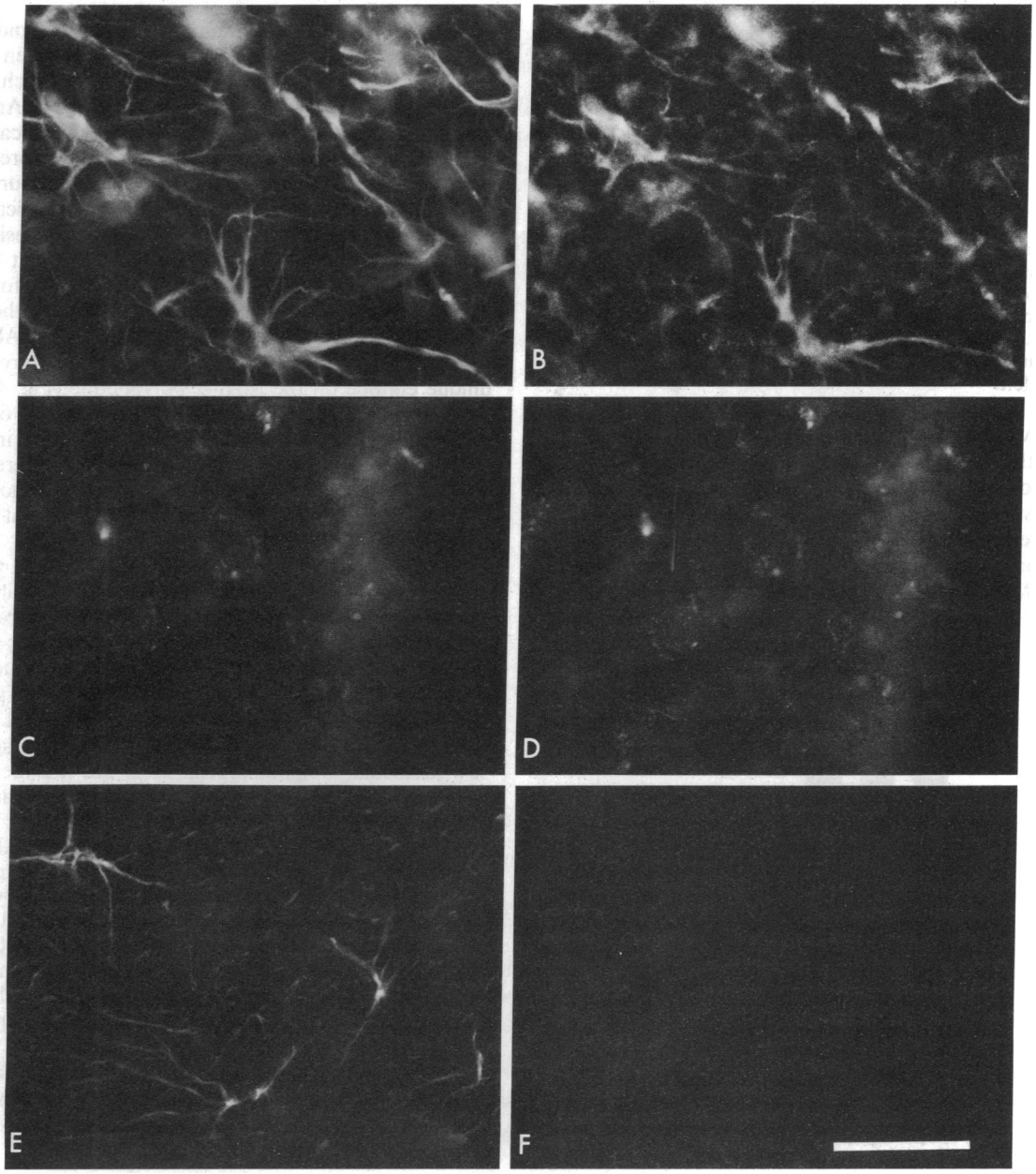

FIG. 2. Sections from a rat 10 days after injury were double-labeled with a rabbit anti-GFAP antiserum and a mouse mAb (AP-14) directed against MAP-2. On the lesioned side of the brain reactive astrocytes within the internal capsule are labeled by antibodies directed against both GFAP (*A*) and MAP-2 (*B*). The region of injury from a control section that was not incubated with primary antibodies shows the labeling observed with the rhodamine-tagged secondary antibody (*C*) and the fluorescein-tagged secondary antibody (*D*). The edge of the lesion is to the right of each of these figures. On the unlesioned side of the brain from the same section illustrated in *A* and *B*, astrocytes in the internal capsule contained GFAP (*E*) but no detectable amount of MAP-2 (*F*). (Bar = 50 μm.) All photomicrographs are at the same magnification.

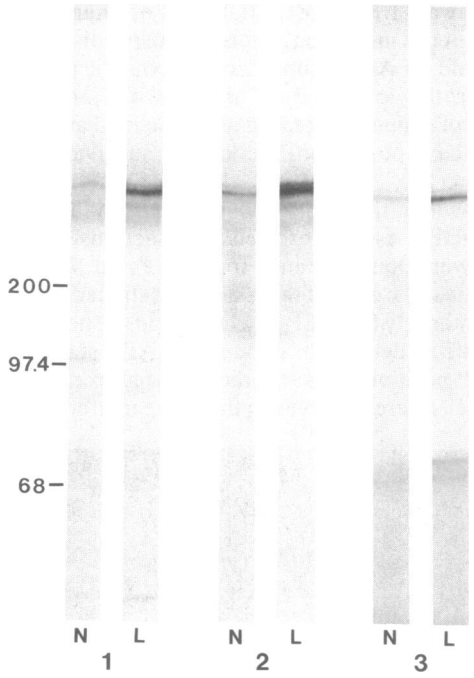

FIG. 3. Representative immunoblots of one control and one lesioned rat corpus callosum (10 days after injury) were stained with the three different MAP-2 mAbs, with 14 μg of normal (N) and lesioned (L) corpus callosum loaded onto gels and transferred to nitrocellulose. The immunoblots were probed with mAb AP-14 (lane 1), AP-15 (lane 2), and AP-18 (lane 3). Molecular weight ($\times 10^{-3}$) values are at left.

capsule, as well as in the gray matter of the cerebral cortex adjacent to the injury. Within astrocytes near the edge of the injured region the staining patterns for MAP-2 and GFAP were very similar, demonstrating that the MAP-2-positive cells were reactive astrocytes.

In addition to astrocytes within the internal capsule and cortex, the MAP-2 content of astrocytes in the corpus callosum was examined with immunohistochemical and biochemical methods. This region of the brain was chosen for immunochemical analysis due to the ease with which it can be dissected away from the adjacent gray matter. An immunohistochemical analysis of the lesioned corpus callosum revealed a dramatic increase in MAP-2 immunoreactivity in reactive astrocytes, similar to that seen in the injured internal capsule (data not shown). In a parallel biochemical analysis, protein samples were prepared for electrophoresis from the dissected corpus callosum of four animals that received a bilateral lesion of the corpus callosum 10 days before sacrifice and from four normal animals. Immunoblots of these protein samples were probed with each of the three mAbs directed against MAP-2 (Fig. 3). Each of these mAbs recognizes a unique epitope on the MAP-2 polypeptide (Fig. 4). For this analysis care was taken to load the same amount of protein in each lane of the gel, and all reactions were done in one batch so that the concentrations of all reagents were identical. Immunoblots probed with these three mAbs demonstrated an increase in the high- and the low-molecular-weight isoforms of MAP-2 in lesioned corpus callosum (Fig. 3).

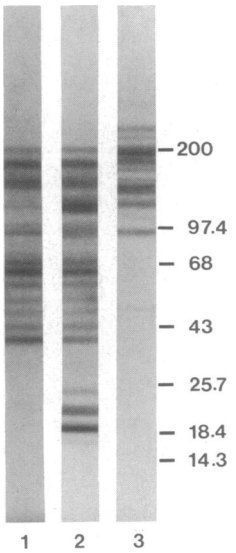

FIG. 4. Purified rat MAP-2 partially digested with chymotrypsin before electrophoresis was run on a slotless gel and transferred to nitrocellulose. Adjacent strips were challenged with mAb AP-14 (lane 1), AP-15 (lane 2), and AP-18 (lane 3). Although the total fragment complement was identical on each strip, unique patterns were recognized by each mAb, proving that the mAbs were binding to different MAP-2 sites. Molecular weight values ($\times 10^{-3}$) are at right.

Table 1. Quantitative immunoblot analysis of MAP-2 in rat corpus callosum

	Normal, *A* units ($\bar{x} \pm$ SEM)	Lesioned, *A* units ($\bar{x} \pm$ SEM)
	1.42 ± 0.03	2.85 ± 0.00
	1.48 ± 0.00	3.30 ± 0.07
	2.15 ± 0.01	3.47 ± 0.11
	1.40*	3.17 ± 0.11
Combined	1.65 ± 0.34	3.20 ± 0.25

Reflected-light densitometric readings for samples from normal and lesioned corpus callosa are shown. Each sample, adjusted to the same protein concentration, was run in triplicate, blotted, and scanned; data are expressed in relative absorbance units. A statistically significant difference ($P < 0.001$) exists between normal and lesioned callosa by the Mann–Whitney *U* test.
*Data based on a single sample.

Perfusions with paraformaldehyde were used to facilitate the dissection of the injured and normal corpus callosum from the overlying gray matter, resulting in an alteration in the electrophoretic mobility of the high-molecular-weight isoforms of MAP-2. In unfixed samples of white matter, MAP-2a and MAP-2b could be identified as two distinct species (data not shown); however, in perfused samples the high-molecular-weight isoforms migrated as a single species. The low-molecular-weight isoform, MAP-2c, was identified with mAb AP-18 only as reported (11) and was unaffected by paraformaldehyde perfusion (Fig. 3).

In an attempt to quantify the increase in MAP-2 seen on immunoblots of the injured corpus callosum, triplicate samples obtained from each injured and normal rat were immunoblotted and probed with mAb AP-14. The blots were then scanned to determine the relative amounts of the reaction product; the data from all eight animals are presented in Table 1. The corpus callosum from the normal animals contained consistently lower levels of MAP-2 than that from any of the lesioned rats. An average 2-fold increase in the relative amount of MAP-2 was seen in the lesioned animals when compared to the normal animals assayed. The difference between these groups was significant ($P < 0.001$) by the Mann–Whitney *U* test.

DISCUSSION

One of the most profound changes that occurs during reactive gliosis is the increase in cytoskeletal proteins within the astrocytes. The best defined of these effects is the elevation of the intermediate filament protein GFAP (1, 2, 18). When sections of normal adult CNS are stained by antibodies

directed against GFAP, fibrous astrocytes within the white matter are very immunoreactive, whereas protoplasmic astrocytes in the gray matter are weakly labeled. After a stab wound, there is a significant increase in GFAP within the fibrous astrocytes as well as the protoplasmic astrocytes (2). In addition to the alterations in the intermediate filaments, microtubule content within the reactive astrocytes appears to increase (6, 19). In the present study, we demonstrate an increase of the microtubule-associated protein MAP-2. This increase was seen by using both immunohistochemical methods and a quantitative immunoblot analysis.

The quantitative immunoblot analysis of the MAP-2 content in the injured versus the normal corpus callosum revealed an apparent 2-fold increase in the high-molecular-weight isoforms of MAP-2. This increase in MAP-2 is readily demonstrable in pure white matter samples because of the normally low constitutive levels of MAP-2 within myelinated pathways (10). This 2-fold increase in MAP-2 is undoubtedly an underestimate of the MAP-2 expression occurring within the reactive astrocytes. The immunohistochemical analysis of the injured corpus callosum shows that the reactive astrocytes do not extend throughout the entire corpus callosum but that they are concentrated near the lesion edge. Thus, because the whole lesioned corpus callosum is dissected for the immunoblot analysis, the large number of normal astrocytes within the sample must dilute the relative elevation of astrocytic MAP-2 measured, thus underestimating the increase within the reactive astrocytes.

Within the nervous system, MAP-2 is primarily, though not exclusively, localized to the somatodendritic compartment of neurons. Detectable levels of the high-molecular-weight isoforms of MAP-2 are found in SDS extracts of white matter (10), indicating MAP-2 presence in axons, glia, or the few neurons found in white matter. The high-molecular-weight isoforms of MAP-2 are found in the peripheral axons of motor neurons (20) and in a subpopulation of normal astrocytes found in the optic nerve, the fornix, or the infundibular stalk (21). Furthermore, avian Bergman glia appear to contain the low-molecular-weight isoform MAP-2c (11). These data indicate that MAP-2 is not associated with a physiological process occurring exclusively within the somatodendritic compartment of neurons and that specific populations of resting glial cells do contain MAP-2.

Two proposed functional roles of MAP-2 are (*i*) the direct stabilization of microtubules (7, 8) and (*ii*) the cross-linking of microtubules with themselves or with intermediate filaments (20, 22–25). Although MAP-2 does not appear to interact with GFAP, it does bind to vimentin (26, 27), and vimentin is a component of the intermediate filaments of reactive astrocytes (28, 29). By crosslinking cytoskeletal elements and increasing the stability of microtubules, the MAP-2 within the long, elaborate processes of the reactive astrocytes may stabilize the astrocytic cytoskeleton, providing structural integrity to the glial scar at the site of injury within the brain.

In summary, astrocytes are intimately involved in the response of the CNS to injury. The increase in size, increased mitotic rate, and upregulation of intermediate filament proteins suggest that reactive astrocytes have acquired a functional role not present in normal resting astrocytes. In the present study, we demonstrate that reactive astrocytes in the internal capsule, the corpus callosum, and the cerebral cortex express MAP-2 that is not detectable in these cells under normal, resting conditions. At 10 days after injury, sections double-stained for both GFAP and MAP-2 show MAP-2 within all of the astrocytes that have increased levels of GFAP. This population includes the astrocytes at the edge of the damaged area associated with the CNS scar as well as the reactive astrocytes in the tissue surrounding the lesion. Furthermore, the presence of MAP-2 within the reactive astrocytes appears to parallel the time course of GFAP upregulation. These reactive astrocytes maintain high levels of MAP-2 and GFAP immunoreactivity up to 50 days after injury, indicating that upregulation of MAP-2, like the upregulation of GFAP, is a relatively permanent alteration in the astrocytic cytoskeleton.

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