Hypoxic–Ischemic Brain Injury Stimulates Glial Fibrillary Acidic Protein mRNA and Protein Expression in Neonatal Rats

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Accumulation of glial fibrillary acidic protein xk(G-FAP) in reactive astrocytes is a characteristic neuropathologic feature of ischemic brain injury. We examined injury-induced changes in GFAP mRNA and protein in a well-characterized model of focal hypoxic-ischemic injury in perinatal rodent brain. Postnatal Day (PND) 7 rats underwent right carotid artery ligation followed by 2.5 h exposure to 8% oxygen, which results in injury to ipsilateral cortex, hippocampus, and striatum in the majority of animals. Using Northern analysis, we assayed GFAP mRNA in samples from the lesioned and contralateral hemispheres of animals killed 1 h to 14 days later, and from animals treated with the neuroprotective glutamate antagonist MK-801. GFAP immunoreactivity in tissue homogenates from the lesioned and contralateral hemispheres was also compared with an immunoblot assay. One and 4 h posthypoxia GFAP mRNA expression was barely detectable. In the lesioned cortex, increased GFAP mRNA was detected at 24 h postinjury; over the next 2 weeks GFAP mRNA was consistently higher (at least 2-fold) in lesioned than in contralateral cortex. In contrast, in lesioned hippocampus and striatum, consistent increases in GFAP mRNA were first detected on PND 12. Immunoassays of GFAP demonstrated early (PND 8) and sustained (to PND 21) up to 10-fold increases in lesioned cortex, hippocampus, and striatum. In this perinatal stroke model regionally specific increases in GFAP mRNA expression and GFAP immunoreactivity are detected in the first 2 weeks after hypoxic-ischemic injury; intrinsic properties of glia and/or neurons in different brain regions may influence the timing and mag- xe03nitude of stimulation of this response. © 1994 Academic Press, Inc.

INTRODUCTION

Accumulation of glial fibrillary acidic protein (GFAP), the major intermediate filament protein of reactive astrocytes (11), is a characteristic neuropathologic feature of ischemic brain injury (10, 22). The cellular and molecular signals that initiate this pathophysiologic response to neuronal injury *in vivo* are largely unknown. To understand the factors that induce GFAP accumulation after ischemic brain injury, an important primary step is to identify the level(s) of molecular regulation of GFAP expression.

In this study we began to address this question in a well-characterized model of focal hypoxic–ischemic brain injury in perinatal rodents (3, 23) elicited by unilateral carotid artery ligation followed by 2.5 h exposure to 8% oxygen (O$_2$) in Postnatal Day (PND) 7 rats. This lesioning method results in ipsilateral forebrain injury in the majority of animals, and preliminary immunohistochemical studies had demonstrated increased GFAP immunoreactivity in the lesioned hemisphere 5–10 days later (27).

In the developing rat brain, there is a programmed developmental switch from vimentin to GFAP as the major astrocyte intermediate filament protein in the second postnatal week (8, 9), and GFAP content progressively increases in the first postnatal month (20). Yet, even in fetal rodent brain, focal cortical injury induced GFAP immunoreactivity prenatally in brain regions where normally GFAP immunoreactivity was absent; the intensity of immunoreactivity increased in animals lesioned in similar fashion in the early postnatal period (19). Recently, we found that in PND 7 rats, focal excitotoxic lesioning, elicited by direct intracerebral injection of NMDA on PND 7, stimulated GFAP mRNA expression within 24 h in the lesioned hemisphere, and that these increases were sustained over the next 2 weeks (4).

Results based on a variety of *in vitro* and *in vivo* experimental approaches indicate the complexity of regulation of GFAP expression both during normal maturation and after injury. Developmental studies in mouse brain suggested that although GFAP mRNA increased in the early postnatal period, these rises were insufficient to account for the progressive rise in GFAP accumulation in mature astrocytes, and that post-transcriptional regulatory mechanisms contributed to this developmental process (25). Yet, in an experimental allergic encephalomyelitis model, increases in GFAP mRNA of considerably greater magnitude than increases in GFAP content were detected in spinal cord after immunization.(1). In addition, a study comparing immunocytochemical assays of GFAP with measure-

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ments of protein synthesis in the same tissue suggested that increases in GFAP immunoreactivity did not necessarily indicate increased synthesis of the protein (13).

In rat brain, GFAP mRNA expression increases in the first postnatal month, and the timing of increases in GFAP mRNA expression differs among brain regions (12, 26); GFAP mRNA rises and plateaus earlier in cortex than in hippocampus or striatum (Silverstein and Burtrum, unpublished observation). In this perinatal stroke model, prominent injury evolves unilaterally in cortex, hippocampus, and striatum. Our previous studies of perinatal excitotoxic injury suggested that there were differences in the timing of injury-induced stimulation of GFAP mRNA expression in these three regions (4). In this study, we determined injury-induced changes in GFAP mRNA accumulation in these three brain regions in the first 2 weeks after injury; we also estimated corresponding changes in GFAP immunoreactivity in tissue homogenates and evaluated the impact of treatment with the neuroprotective NMDA antagonist MK-801 (17) on GFAP expression.

METHODS

Animal methods. In ether-anesthetized PND 7 Sprague–Dawley rats the right carotid artery was ligated; after a 1-h recovery period animals were exposed to 8% oxygen (balance nitrogen) for 2.5 h in plastic chambers, partially submerged in a water bath (temperature maintained at 36.5°C). In experiments in which MK-801 was tested, all animals were lesioned, half received ip injections (1 mg/kg) 15 min prehypoxia, while the remainder were injected with equal volumes of saline.

RNA samples were prepared from lesioned animals sacrificed on PND 7 (1 or 4 h postinjury) (8, 10, 12, 17, 21); at least two independent experiments were done at each time interval (with the exception of a single experiment at 1 and 4 h postinjury). MK-801-treated animals were studied at PNDs 8 and 12. Protein samples were prepared from additional animals sacrificed on PNDs 8, 9, 12, 14, 17, and 21.

In a single experiment, in ether-anesthetized PND 7 rats, 12.5 nmol NMDA was injected in the right posterior striatum [as previously described (4, 18)], and RNA samples were obtained 5 days later. This lesioning method results in neuronal necrosis and atrophy ipsilaterally in striatum, overlying dorsal hippocampus and cortex.

RNA isolation. Total RNA was prepared from pooled tissue samples of cortex, hippocampus, and striatum immediately postmortem by an acid guanidinium thiocyanate-phenol-chloroform extraction method (6). Since the optimal sample size for processing was >100 mg, typically, brain samples derived from the hemispheres ipsilateral and contralateral to the injection were pooled independently (from 3 to 5 animals less than PND 14,

and from 2 to 3 animals in older animals). RNA concentrations were estimated from optical density measurements at 260 nm. Typical yields were 0.6– 0.8 mg/g tissue.

Northern analysis. RNA samples (10 μg) were fractionated by electrophoresis through 1% agarose–6% formaldehyde gels. Gels were stained with ethidium bromide to visualize ribosomal RNA and ensure equal loading of RNA/lane. After destaining, RNA was transferred to nylon filters (Nytran, Schleicher and Schuell, Keene, NH) and baked (80°C for 2 h). A 2.5-kb cDNA probe that encoded >97% of the translated sequence and a 1.4-kb portion of the 3' untranslated region [generously provided by Dr. N Cowan, New York University (16)] was labeled with [^{32}P]CTP (S.A.: 3000 Ci/mMol, Dupont, NEN, Boston, MA) by the random prime method (Prime-It kit, Stratagene, La Jolla, CA). Filters were prehybridized (42°C for 2 h in 50% formamide with 5× SSC, 1× PE, 150 μg/ml salmon sperm DNA) and hybridized (overnight in the same buffer with 3×10^6 cpm of probe/10 ml buffer), washed in 2× SSC/0.1% SDS (2×20 min at room temperature, 20 min at 55°C), and exposed to x-ray film to generate autoradiograms. A single band (2.8 kb) was consistently detected. In selected experiments the Nytran membranes were stripped and reprobed with a ^{32}P-labeled 28S ribosomal probe (from Dr. V. Dixit, University of Michigan) to estimate the amounts of total RNA/sample. In most experiments, side-to-side and interregion differences in GFAP mRNA expression were readily apparent. To estimate the magnitudes of differences in hybridization intensities selected autoradiograms (of blots hybridized with cDNAs for GFAP mRNA and 28S ribosomal RNA) and corresponding photographs of ethidium bromide- stained gels were scanned using a Sharp flatbed scanner (Adobe Photoshop, Mountain View, CA); values for GFAP mRNA were normalized based on estimates of ribosomal RNA/sample.

Assay of GFAP immunoreactivity. Pooled tissue samples were prepared from animals killed 1–14 days after lesioning; GFAP was solubilized (sonication in 20-fold excess volume of 1% SDS at 90°C) and protein content was assayed (14). Using a dot-blot manifold, 5, 1, and 0.5 μg total protein/sample were loaded, in duplicate, on nitrocellulose membranes. A Western assay was used to compare GFAP content of tissue samples. In preliminary experiments, samples were resolved by electrophoresis in a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and incubated sequentially with a polyclonal rabbit, anti-bovine GFAP primary antibody (Dakopatts) (1:5000 or 1:10,000), HRP-conjugated secondary antibody (1:50,000 or 1:100,000), and a chemiluminescent substrate (ECL kit, Amersham); a single 58- kDa band corresponding with GFAP was detected. The same conditions were used for the dot-blot membranes: sequential incubation with the GFAP primary

antibody (1:10,000), HRF-conjugated secondary antibody (1:100,000), and the chemiluminescent substrate, followed by exposure to x-ray film (15–30 s); the intensity of light emission/sample was directly proportional with immunoreactive GFAP.

RESULTS

One and 4 h posthypoxia GFAP mRNA were barely detectable in samples from lesioned tissue. Twenty-four h posthypoxia GFAP mRNA content was increased in the lesioned right cortex in comparison with the level of expression in the contralateral cortex (Fig. 1). Figure 1 compares GFAP mRNA expression in samples from the lesioned and contralateral hemispheres 1–14 days after injury; increases in GFAP mRNA expression in lesioned cortex were sustained during this time interval and a progressive increase in GFAP mRNA expression was apparent in samples from the left cortex (which was similar to that seen in samples from unlesioned animals). At PND 8, there were no increases in GFAP mRNA levels in lesioned hippocampus or striatum; similarly, in samples prepared from lesioned animals on PND 10, GFAP mRNA expression was consistently increased only in lesioned cortex.

Figure 2 compares GFAP mRNA expression in tissue samples obtained on PND 12 from two groups of lesioned animals, those that received MK-801 and saline- injected controls. In samples prepared from the saline- treated group, GFAP mRNA was highest in the right cortex and was also elevated in lesioned hippocampus; scans of blots indicated that GFAP mRNA was twofold higher in lesioned than in contralateral cortex. In samples from MK-801-treated animals the magnitude of stimulation of GFAP mRNA in right cortex and hippocampus was considerably attenuated in comparison with saline-treated groups. Of note, GFAP mRNA expression was also reduced in the contralateral cortex
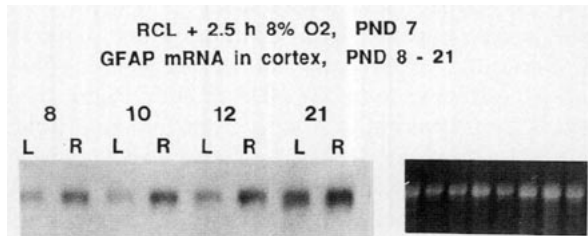

FIG. 1. Comparison, by Northern analysis, of GFAP mRNA in samples (10 µg RNA) from left (L) and right (R) cortex of animals that underwent right carotid ligation (RCL) and 2.5 h 8% O_2 exposure on PND 7 and were killed 1–14 days later (see Methods for details of assay). At these four postinjury time intervals, GFAP mRNA was two- to threefold higher in samples from the lesioned (R) cortex than in samples from the contralateral (L) hemisphere; the progressive rise in GFAP mRNA expression in samples from the left cortex was similar to that observed in unlesioned animals of the same ages. The panel on the right demonstrates ethidium bromide staining of 28S ribosomal RNA bands and confirms the equivalency of RNA content in these samples.
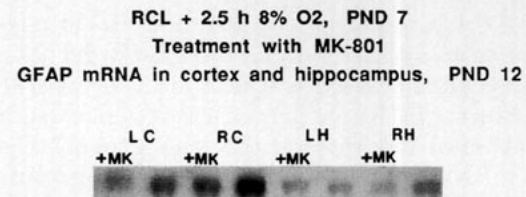
FIG. 2. Comparison, by Northern analysis (see Methods), of GFAP mRNA content in RNA samples (10 µg) from the left and right cortex (LC, RC) and hippocampus (LH, RH) of two groups of littermates that underwent right carotid ligation (RCL) and 2.5 h 8% O_2 exposure on PND 7 and were killed 5 days later. Half the animals were treated with MK-801 (1 mg/kg) immediately before hypoxic exposure ("""+MK"), while the others were injected with equal volumes of saline. In samples from the saline-treated group, GFAP mRNA expression was increased in lesioned cortex (RC), and, to a lesser degree, hippocampus (RH). MK-801 treatment markedly attenuated stimulation of GFAP mRNA expression in RC and RH; in addition, in the unlesioned contralateral cortex (LC), GFAP mRNA expression was reduced in comparison with the corresponding sample from the saline-treated groups.

[a pattern we also found in MK-801-treated lesioned animals examined on PND 8 (not shown)]. In this experiment, striatal GFAP mRNA was barely detectable in any of the samples and no side-to-side or treatment-related differences were apparent (not shown). However, in samples prepared from another group of animals that underwent hypoxic–ischemic lesioning, slight increases in right striatal GFAP mRNA were detectable at PND 12 (see Fig. 3).

Figure 3 compares the patterns of GFAP mRNA expression in cortex, hippocampus, and striatum on PND 12 after hypoxic–ischemic lesioning and excitotoxic lesioning (elicited by direct intracerebral injection of NMDA into right posterior striatum). Typically, in
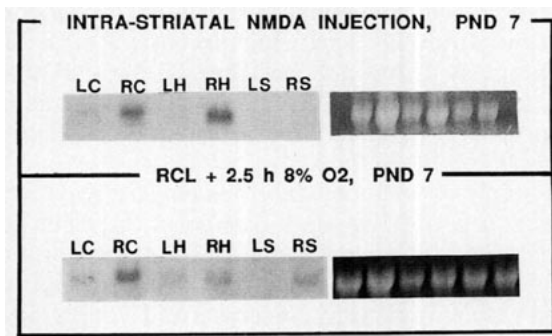
FIG. 3. Composite photograph compares regional distributions of GFAP mRNA in two groups of PND 12 animals, lesioned either by direct intrastriatal injection of NMDA (12.5 nmol) or by right carotid ligation (RCL) and 2.5 h 8% O_2 exposure on PND 7. GFAP mRNA content was assayed in RNA samples (10 µg) from left and right cortex (LC, RC), hippocampus (LH, RH), and striatum (LS, RS) in both groups (see Methods). In both groups, in samples from the lesioned hemisphere, stimulation of GFAP mRNA is greatest in the cortex and least evident in the striatum. The panels on the right demonstrate ethidium bromide staining of 28S ribosomal RNA bands to confirm the equivalency of RNA content in the samples.

both injury models the extent of irreversible injury is similar in the three regions studied; yet, cortical GFAP mRNA induction was more pronounced than in hippocampus or in striatum in the first 5 days after injury.

In contrast, 10 days after hypoxic–ischemic injury (PND 17), three- to sixfold increases in GFAP mRNA were detected ipsilaterally in samples from these three regions (Fig. 4).

In the same brain regions, injury-induced increases in GFAP immunoreactivity were more consistent, of greater magnitude, and demonstrated considerably less regional heterogeneity than injury-induced changes in GFAP mRNA. Figure 5 compares equivalent protein samples (5, 1, or 0.5 µg) from the lesioned and contralateral cortex, hippocampus, and striatum of animals sacrificed 1–14 days postinjury; the sequential dilutions facilitate estimates of relative amounts/sample (but do not reflect differences in total protein content among samples, regions, or different developmental stages). In samples from the left (unlesioned) hemisphere, developmental trends are readily apparent; GFAP immunoreactivity increased with age in all three regions. At PND 8, GFAP was detected in all samples, and as noted with respect to mRNA in lesioned tissue, was increased only in right cortex. Subsequently, from PNDs 9–21, GFAP immunoreactivity was higher in all samples from the lesioned than from the contralateral hemisphere (with the exception of PND 17 right hippocampus, which was likely artifactual). Based on comparison of signal intensities of the sample dilutions, in lesioned tissue peak signal intensities were at least 10-fold higher than in samples from the contralateral hemisphere. Of particular note, in hippocampal and striatal samples, early and sustained increases in GFAP immunoreactivity were of similar magnitude to those in cortex. Included in this assay were samples from a group of lesioned animals

that had been treated with MK-801 ('''12 + MK''); surprisingly, GFAP immunoreactivity was markedly increased in the lesioned hemisphere, and only in striatum was the magnitude of stimulation somewhat less than in untreated animals.

DISCUSSION

These results suggest that in this perinatal stroke model (1) increased GFAP mRNA accumulation contributes to induction of GFAP immunoreactivity in the first 2 weeks after injury, (2) intrinsic properties of glia and/or neurons in different brain regions may influence the timing and extent of stimulation of GFAP mRNA levels, and (3) initial injury-induced increases in GFAP immunoreactivity are of greater magnitude than increases in GFAP mRNA.

In lesioned cortex, GFAP mRNA increased markedly within 24 h and increases were sustained over the next 2 weeks. In lesioned hippocampus and striatum, the initial stimulation of GFAP mRNA levels was delayed, relative to cortex; yet, in the second week after injury, increases in GFAP mRNA were detected in all three regions. The relatively delayed increases in GFAP mRNA in lesioned hippocampus and striatum could, in part, reflect regional ontogenetic differences in glial maturation (12, 26). The similar temporal and anatomic features of GFAP mRNA stimulation by excitotoxic and hypoxic–ischemic injury in perinatal rodent brain also suggested that intrinsic features of cell populations, in the three brain regions examined, influenced the differential responses observed. Yet, since GFAP mRNA was readily detectable in striatum and hippocampus by PND 12, it is unlikely that ontogenetic differences alone accounted for the region-specific differences observed at this time.
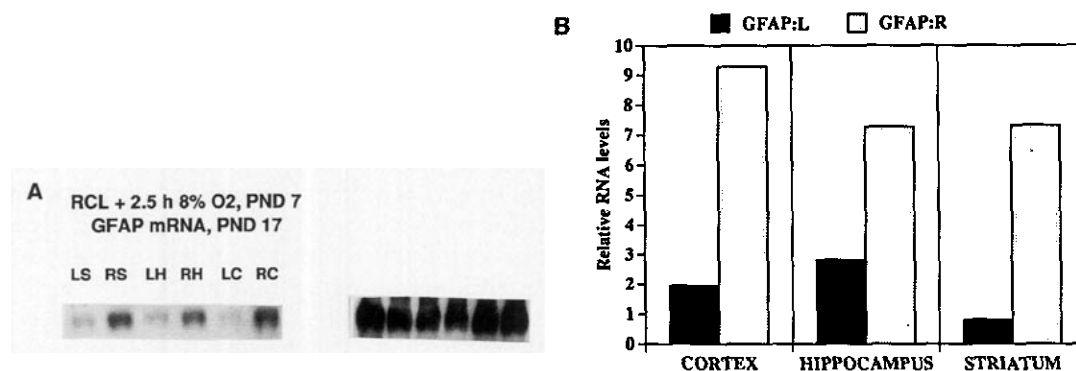
FIG. 4. (A) Comparison of GFAP mRNA in samples (10 µg RNA) prepared from animals that underwent right carotid ligation (RCL) and 2.5 h 8% O₂ exposure on PND 7 and were killed 10 days later (PND 17) (see Methods for details of assay). The panel on the right is an autoradiogram prepared from the same blot, stripped and rehybridized with a cDNA probe for 28S RNA to confirm the equality of total RNA in the samples. (B) Semiquantitative estimates of GFAP mRNA content (expressed as arbitrary units), derived from computerized scanning of Northern blots, and normalizing values based on total ribosomal RNA/sample; normalized values obtained by scanning a photograph of the ethidium bromide-stained gel yielded virtually identical results (not shown). Three- to sixfold increases in GFAP mRNA expression were detected in lesioned tissue.
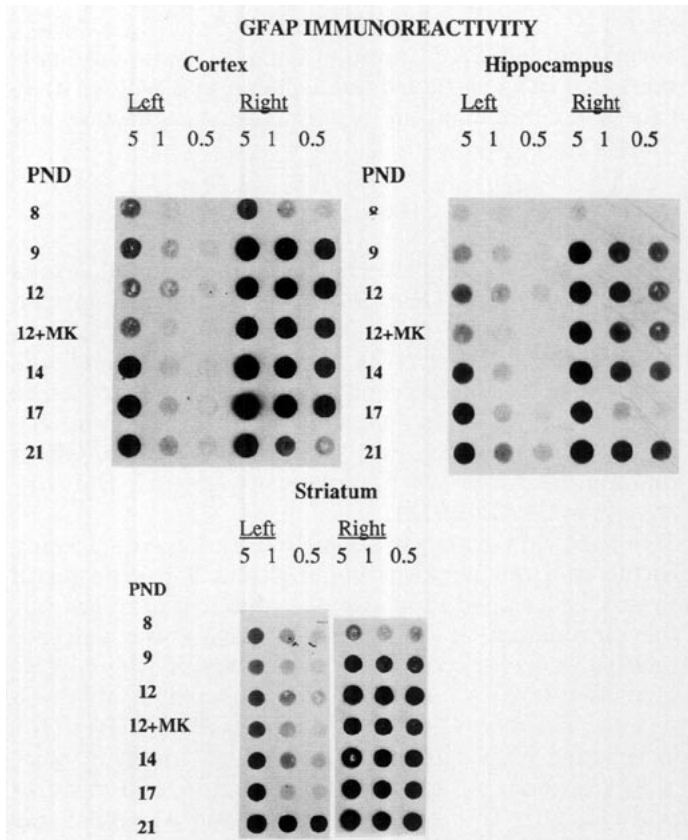
FIG. 5. Composite photograph of immunoblots prepared from samples of lesioned and contralateral cortex, hippocampus, and striatum of animals that underwent right carotid ligation (RCL) and 2.5 h 8% O_2 exposure on PND 7 and were killed on PNDs 8–21. One group of samples (PND 12 + MK) were derived from animals that received 1 mg/kg MK-801 15 min prehypoxia. Serial dilutions yielding 5, 1, or 0.5 µg total protein/well/sample were loaded on nitrocellulose membranes; membranes were incubated sequentially with a polyclonal rabbit, anti-bovine GFAP primary antibody (1:10,000), HRP-conjugated secondary antibody (1:100,000), and a chemiluminescent substrate, and then exposed to x-ray film (see Methods). In all three regions, comparison of GFAP immunoreactivity in samples from the left hemisphere demonstrates the expected developmental rise in GFAP over PNDs 8–21. In samples from the lesioned right hemisphere, increased GFAP immunoreactivity is first apparent in the cortex at PND 8. At all subsequent time intervals (PNDs 9–21) GFAP immunoreactivity is increased up to 10-fold in all three regions; the only exception, PND 17 hippocampus, is likely artifactual. GFAP immunoreactivity is also markedly increased in samples from the right hemisphere of MK-801-treated animals; only in striatum is the magnitude of stimulation somewhat less than in untreated animals.

The regional heterogeneity in the onset and magnitude of stimulation of GFAP mRNA did not correspond predictably with the known distribution of injury in this model. In animals that undergo this lesioning procedure, although the extent of injury is somewhat variable, in each animal, the severity of neuronal injury and substance loss is generally similar in cortex, striatum, and hippocampus (23). A limitation of the tissue homogenate-derived RNA assays was that the distribution and severity of neuronal injury could not be assessed concur-

rently. On-going experiments, using an *in situ* hybridization histochemistry assay (4), should provide the anatomic resolution required to determine if stimulation of GFAP mRNA expression is directly related to the severity of local tissue injury, and if injury in specific regions is more likely to induce GFAP mRNA.

The observed increases in GFAP mRNA in lesioned tissue could reflect increased mRNA synthesis and/or stability of the transcripts, and our data could not distinguish these possibilities. A potential confounding factor in interpreting these data analysis is that as a result of neuronal damage and coincident gliosis, the relative proportions of neurons and glia in tissue samples are likely to change; thus, in RNA samples prepared from tissue homogenates, glia-derived mRNA may represent an increasing fraction of total RNA (and protein) over time, and this may, in part, account for the more pronounced increases in GFAP mRNA levels observed on PND 17 than on PND 12. Yet, overall the results suggest that increased GFAP mRNA accumulation likely represents an important level of regulation of GFAP expression in the developing brain after injury. Previous studies in adult brain have also demonstrated acute injury-induced increases in GFAP mRNA, peaking 1–5 days after cortical stab wounds (7, 15) and 11–14 days after immunization to induce experimental autoimmune encephalomyelitis (2).

In this perinatal stroke model treatment with the noncompetitive NMDA antagonist MK-801 attenuates but does not eliminate brain injury (17); in MK-801-treated animals, GFAP mRNA accumulation in lesioned cortex and hippocampus at PND 12 was considerably reduced. The inhibitory effects of MK-801 treatment suggest that the signals leading to stimulation of GFAP mRNA synthesis in lesioned brain are attributable to neuronal injury and/or to overexcitation of NMDA receptors. Recent studies have provided evidence of a link between glial gene expression and abnormal neuronal activity (24). Transient electrographic seizures occur in the first 24 h after injury (5) and could conceivably contribute to the initial stimulation of GFAP mRNA in lesioned cortex. Yet, there is no behavioral evidence of seizures in the next 2 weeks, when sustained increases in GFAP mRNA were observed, and it is thus unlikely that seizures contributed to the sustained stimulation of GFAP mRNA expression. Alternatively, hypoxia–ischemia could also directly injure astrocytes, and the contribution of astrocyte dysfunction/ or activation in the progression of ischemic brain injury is currently uncertain.

Whether injury induced GFAP mRNA expression at sites remote from tissue injury was not adequately resolved by our data. Based on the comparisons of GFAP mRNA content in tissue derived from the unlesioned hemispheres of untreated and MK-801-treated animals, it appeared that MK-801 also prevented contralateral

increases in GFAP mRNA in cortex and hippocampus. Of note, we found a similar pattern after perinatal excitotoxic lesioning: MK-801 treatment resulted in suppression of GFAP mRNA in both ipsilateral and contralateral cortex (4). Since rates of total RNA synthesis were not assessed, the possibility that MK-801 treatment inhibited RNA synthesis could not be excluded; yet, it is more likely that MK-801 treatment suppressed hypoxia–ischemia-related effects in the contralateral hemisphere. Observations of transient increases in GFAP immunoreactivity in undamaged brain regions (22) in a global ischemia model in adult rat brain may reflect a similar glial response to injury.

Assays of GFAP in tissue homogenates yielded results that were, in some respects, discrepant with results of mRNA analysis. GFAP immunoreactivity increased markedly within 48 h in all three regions, whereas in hippocampus and striatum injury-induced increases in GFAP mRNA were initially detected at later times. Five days after injury, in lesioned cortex GFAP immunoreactivity was over 10-fold higher than in the contralateral cortex, whereas GFAP mRNA levels in lesioned tissue were increased by only 2-fold. In contrast with the marked suppression of injury-related increases in GFAP mRNA by treatment with MK-801 [a drug that significantly reduces but does not completely eliminate injury (17)] in a single experiment the same treatment only minimally attenuated increases in GFAP (in striatum). It is conceivable that the minimal residual injury in MK-801-treated animals was sufficient to elicit the stimuli that result in increased GFAP immunoreactivity (some of which may be independent of mRNA). In several recent neurotoxicology studies evaluating the sensitivity of GFAP as an indicator of neurotoxicity, doses of toxic chemicals that were below the threshold to elicit evidence of neuronal injury (by Nissl staining) nonetheless elicited robust increases in GFAP immunoreactivity (21). Considerable data indicate that increased local tissue GFAP immunoreactivity is an extremely sensitive indicator of neuronal injury (28). Yet, an important potentially confounding factor in interpreting these data is presented by studies indicating that increased GFAP immunoreactivity does not necessarily correspond directly with increased protein synthesis (13).

Understanding the molecular signals that regulate GFAP expression may improve our ability to modify the developing brain's response to injury. Our data indicate that increases in mRNA levels represent a major potential mechanism underlying induction of GFAP accumulation after hypoxic–ischemic injury, and that increases in immunoreactive GFAP cannot be attributed solely to changes in mRNA. Important unanswered questions include: which cellular and molecular signals initiate and sustain increased GFAP mRNA synthesis and/or stability after injury, which factors account for the accelerated and pronounced increases in GFAP mRNA in lesioned cortex (relative to hippocampus and striatum, where injury is equally or more severe), what other glial genes are concurrently activated, what are the other determinants of injury-induced increases in GFAP, and ultimately, how do these glial events influence neuronal healing and reorganization?

ACKNOWLEDGMENTS

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