

Excitotoxic Injury Stimulates Glial Fibrillary Acidic Protein mRNA Expression in Perinatal Rat Brain

DOUGLAS BURTRUM AND FAYE S. SILVERSTEIN¹*Departments of Pediatrics and Neurology, University of Michigan, Ann Arbor, Michigan 48109-0570*

To study the molecular mechanisms contributing to glial fibrillary acidic protein (GFAP) accumulation after neuronal injury in the developing brain, we used a reproducible and pharmacologically modifiable model of excitotoxic injury, intracerebral injection of *N*-methyl-D-aspartate (NMDA) in Postnatal Day 7 rats. Injection of NMDA into the posterior striatum elicits dose-dependent ipsilateral injury to striatum, hippocampus, and overlying cortex; treatment with the non-competitive NMDA antagonist MK-801 is neuroprotective. To examine regionally specific changes in GFAP mRNA expression after lesioning, GFAP mRNA content was assayed, by Northern analysis, in pooled tissue samples of striatum, hippocampus, and cortex, derived from the injected and contralateral hemispheres of animals killed 1-16 days after lesioning with NMDA (12.5 nmol), and in samples derived from lesioned animals and littermates treated with MK-801. In addition, *in situ* hybridization assays were done to visualize the anatomic distribution of GFAP mRNA expression in NMDA-lesioned ($n = 5$) and lesioned/MK-801-treated animals ($n = 3$) 5 days postinjection. There was a marked rise in GFAP mRNA in lesioned cortex within 24 h, and increases were sustained over the next 2 weeks. In contrast, in striatum and hippocampus, in which severe histologic damage evolves, at 24 h postlesioning there was little stimulation of GFAP mRNA expression. Subsequently, 5-16 days postinjury increases in GFAP mRNA were detected in both brain regions. In animals examined 5 days postlesioning, MK-801 treatment markedly attenuated stimulation of GFAP mRNA expression. *In situ* hybridization assays revealed a marked increase in GFAP mRNA unilaterally, in cortex, hippocampus, and striatum; in similarly lesioned animals treated with MK-801, there was no evidence of tissue injury, and no increase in GFAP mRNA was apparent. Thus, in perinatal rodent brain, focal excitotoxic injury predictably stimulates GFAP mRNA expression in a temporally and regionally specific fashion. © 1993 Academic Press, Inc.

INTRODUCTION

Reactive gliosis, accompanied by increased accumulation of glial fibrillary acidic protein (GFAP), is a char-

acteristic response of both the immature and adult brain to many diverse forms of neuronal injury. In addition to mechanical injury, excitotoxic lesions, ischemia, encephalitis, hippocampal deafferentation, and intense neuronal activity all induce expression of GFAP (mRNA and/or protein) (2, 4, 6, 9, 24, 25). These recent data suggest that different biological mechanisms may induce and maintain reactive gliosis, depending on the type of injury, age, and brain region affected. The molecular mechanisms that signal the initiation and sustained GFAP accumulation in injured brain are unknown. Marked increases in glial trophic factors have been identified in the extracellular fluid soon after brain injury (21); cytokines such as interleukin-1 may be important mediators of glial activation in the developing brain (9).

In the developing rodent brain, initially vimentin is the major glial filament protein (7). In rats, there is a programmed developmental switch from vimentin to GFAP in early postnatal life. GFAP, a 58-kDa protein, is the major intermediate filament protein of mature astrocytes. GFAP mRNA expression in brain increases abruptly in the second postnatal week (8, 27); peaks are detected earlier in cortex than in hippocampus or striatum (23). GFAP mRNA content then plateaus, whereas GFAP immunoreactivity progressively increases in the first postnatal month (22). Significant discrepancies between developmental trends in GFAP mRNA and protein content have been delineated (26); increased stability of the protein with maturation contributes to its progressive accumulation (22).

In immature rodent brain, unilateral intrastriatal injections of the excitatory amino acid receptor agonist *N*-methyl-D-aspartate (NMDA) (5-25 nmol) elicit predictable, dose-related focal excitotoxic brain injury in striatum, overlying hippocampus, and cortex (15). The severity of injury is predictably attenuated by systemic administration of competitive and noncompetitive NMDA receptor antagonists (14-18). Susceptibility to NMDA neurotoxicity peaks at PND 7 in rats (14); the cellular and molecular mechanisms that underlie this enhanced vulnerability are uncertain, but are probably related to the critical roles of NMDA receptor activation in normal developmental processes (17). Biochemical analysis of brain tissue after direct intracerebral NMDA injections has enabled us to characterize some

¹ To whom reprint requests should be addressed.

of the distinctive features of the perinatal brain's response to excitotoxic injury (18). Similarly, this model facilitates direct examination of the relationship between neuronal injury and regulation of GFAP expression in developing brain.

In this study, we determined the major anatomic and temporal features of stimulation of GFAP mRNA accumulation after intracerebral injections of NMDA in perinatal rodents and the impact of neuroprotective treatment with MK-801 on GFAP mRNA accumulation.

METHODS

Animal lesioning. Postnatal Day (PND) 7 Sprague-Dawley rats were anesthetized with ether. NMDA (12.5 nmol/0.5 μ l) was injected, under stereotaxic guidance, into the right posterior striatum, using a 26-gauge Hamilton syringe (15). Injection coordinates (referenced to bregma: AP, -2.0 mm; L, 2.5 mm; D, 4 mm) were such that the needle track passed through the overlying cortex, corpus callosum, and dorsal hippocampus into posterior striatum. After injection of saline using this method, mechanical defects (along the needle track) can sometimes be detected, but there is no adjacent cell loss. After injections of NMDA, the extent of damage is determined by the injection site and the dose administered; the toxicity threshold is close to 5 nmol. Damage to hippocampus and cortex, after intrastriatal injections, likely reflect leakage of drug along the needle track, rather than remote effects of NMDA.

Animals were killed, by decapitation, 1-16 days later. For each time interval evaluated, at least two independent experiments were performed. In experiments in which the effects of MK-801 were tested, all animals received intracerebral injections of NMDA, half received ip injections of MK-801 (1 mg/kg) 15 min post-NMDA, while the remainder received injections of equal volumes of saline. Animal use protocols were approved by the University of Michigan Committee on the Care and Use of Animals.

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 (5). Since the optimal sample size for processing was >50 mg, typically, brain samples derived from the hemispheres ipsilateral and contralateral to the injection were pooled independently (from three to five animals less than PND 14, and from two to three 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. Ten-microgram RNA samples were fractionated by electrophoresis through 1% agarose-6% formaldehyde gels. Gels were stained with ethidium bromide to visualize ribosomal RNA and en-

sure equal loading of RNA/lane. After destaining, RNA was transferred to nylon filters (Nytran, Schleicher & Schuell, Keene, NH) and baked (80°C 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 (from Dr. N. Cowan, New York University) was labeled with [³²P]CTP (sp act 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 \times SSC, 1 \times PE, 150 μ g/ml salmon sperm DNA) and hybridized (overnight in the same buffer with 3 \times 10⁶ cpm of probe/10 ml buffer), washed in 2 \times SSC/0.1% SDS (2 \times 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 situ hybridization. Brains from eight lesioned animals (three of which were also treated with MK-801) were removed intact on PND 12 for *in situ* hybridization studies.

A *SalI-HindIII* 1.2-kb fragment from the 5' coding region of the GFAP cDNA was subcloned into the plasmid Bluescript IISK⁺ (Stratagene). The transcription cassette was excised with *BssHIII*, antisense and sense riboprobes of similar lengths, using T7 and T3 RNA polymerases, respectively (Stratagene RNA transcription kit), were prepared, incorporating [³⁵S]UTP (sp act 1100-1400 Ci/mmol, Dupont, NEN, Boston, MA). To verify the specificity of hybridization, in each experiment, adjacent sections were incubated with antisense and sense strand probes.

Fourteen-micrometer frozen sections, mounted on polylysine-coated slides, were postfixed in formaldehyde, treated with proteinase K (5 μ g/ml), and acetylated. Sections were covered with 40 μ l hybridization buffer (50% formamide, 10% dextran sulfate, 1 mM EDTA, 10 mM Tris, 0.1 mM dithiothreitol) with 10⁶ cpm of labeled probe, coverslipped, hybridized for 21 h at 55°C, rinsed with 2 \times SSC at room temperature for 30 min, 50% formamide/2 \times SSC at 55°C for 30 min, treated with RNase A, and dried before exposure to X-ray film for 10 days (24).

RESULTS

Within 24 h of injection of 12.5 nmol NMDA into posterior striatum, ipsilateral edema and loss of Nissl substance are evident in striatum, hippocampus, and cortex. Yet, at this time interval, consistent prominent increases in GFAP mRNA content were evident only in cortex; in lesioned hippocampus and striatum, only subtle and inconsistent increases in GFAP mRNA were detected (Fig. 1). Over the next 2 weeks, increases in GFAP mRNA content in lesioned cortex were sustained and were accompanied by marked increases in GFAP mRNA levels in lesioned hippocampus and striatum

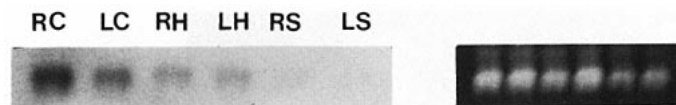


FIG. 1. Comparison, by Northern analysis, of cortical (C), hippocampal (H), and striatal (S) GFAP mRNA content in RNA samples (10 μ g) derived from the right (R) and left (L) hemispheres of animals that received right intracerebral injections of NMDA (12.5 nmol) on PND 7, and were sacrificed 24 h later (see Methods for details of mRNA assay). A marked increase in GFAP mRNA content is evident only in the right cortex. The right demonstrates ethidium bromide staining of 28S ribosomal RNA bands and confirms the equivalency of RNA content in samples obtained from the left and right hemispheres.

(Fig. 2). In the first week after injury results of all assays showed that GFAP mRNA content was markedly increased in lesioned cortex, whereas the increases in hippocampus and striatum were somewhat less consistent. It is, however, conceivable that difficulties in accurate dissection of lesioned striatum at this developmental stage may have limited the reliability of some of the tissue homogenate assays.

In comparison with saline-treated lesioned littermates, in MK-801-treated animals there was marked attenuation of GFAP mRNA accumulation in lesioned cortex and hippocampus (Fig. 3); in this experiment, GFAP mRNA was not detectable in striatal samples. Of note, in two independent experiments, it appeared that in MK-801-treated animals, GFAP mRNA expression in tissues derived from the contralateral hemisphere was also reduced relative to saline-treated controls (as in Fig. 3, comparing GFAP mRNA in left cortical and hippocampal samples of saline and MK-801-treated animals).

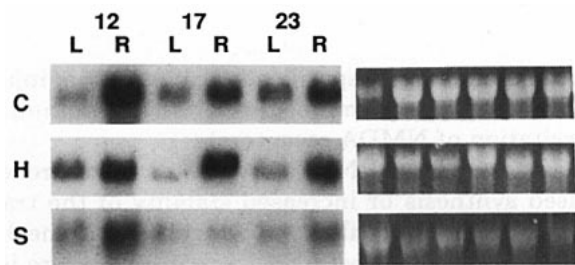


FIG. 2. Comparison, by Northern analysis, of cortical (C), hippocampal (H), and striatal (S) GFAP mRNA content in RNA samples (10 μ g) derived from left (L) and right (R) hemispheres of animals that received right intracerebral injections of NMDA (12.5 nmol) on PND 7, and were killed 5 to 16 days later (PND12-23) (see Methods for details of mRNA assay). At all three time intervals, in all three regions, GFAP mRNA content is increased in samples from the lesioned hemisphere. The right demonstrates ethidium bromide-stained 28S ribosomal RNA bands from the corresponding samples. In the sample prepared from the left cortex on PND 12 total RNA appears reduced relative to the right cortex; additional experiments (not shown) confirmed that this disparity did not account for the increase GFAP mRNA content detected in the right cortex.

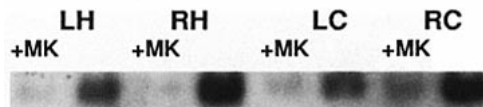


FIG. 3. Comparison, by Northern analysis, of GFAP mRNA content in RNA samples (10 μ g) from the left and right cortex (LC, RC) and hippocampus (LH, RH) of two groups of littermates (see Methods for details of mRNA assay). All animals received right intrastriatal injections of 12.5 nmol NMDA on PND 7; half were treated with MK-801 (“+MK”, 1 mg/kg ip 15 min post-NMDA) and the remainder received saline injections. All were killed 5 days later. In comparison with saline-injected animals, in the MK-801-treated group, GFAP mRNA is markedly reduced in both the lesioned and contralateral hemispheres in both brain regions. In these animals no increases in striatal GFAP mRNA content were detected in either group (not shown).

In situ hybridization assays enabled concurrent visualization of GFAP mRNA distribution and the extent of injury in each brain. Multiple sections, through the levels of striatum and hippocampus from five NMDA-lesioned animals were examined; in all, GFAP mRNA hybridization signal was increased in the lesioned hemisphere. In adjacent sections from the same brains, hybridized with the sense-strand probe, there was no hybridization signal (not shown). Figure 4 presents representative autoradiograms that demonstrate the distribution of injury and GFAP mRNA in two of these animals (A-D), and in an NMDA-lesioned animal treated with MK-801 (E and F). In the coronal brain sections presented in A-D, tissue injury is confined to the injected hemisphere; there is striatal atrophy, corresponding dilatation of the adjacent lateral ventricle, and hippocampal and cortical atrophy. In the lesioned hemisphere there are prominent increases in GFAP mRNA in cortex, striatum, and hippocampus, whereas in the contralateral hemisphere there is minimal hybridization signal except in white matter tracts. In the thalamus, increased GFAP mRNA was often noted ipsilateral to the injection (as in B and D); although tissue injury in the thalamus was not readily discernible in these autoradiograms, histologic evaluation of adjacent sections demonstrated neuronal loss in this region (not shown). Overall, there was some variability in the distribution of tissue injury, which was readily explained by variations in the site of the NMDA injection. There was also variability in the distribution of maximal GFAP mRNA signal, which did not clearly correspond with site of maximal injury in each brain. Of note, no increases in GFAP mRNA were apparent in the contralateral hemispheres of NMDA-lesioned animals (as had been suggested by the findings in tissue homogenate assays, illustrated in Fig. 3). In three NMDA-lesioned animals treated with MK-801, there was no histologic evidence of tissue injury, and there was no increase in GFAP mRNA hybridization signal in the injected hemisphere relative to the contralateral hemisphere (as illustrated in Figs. 4E and 4F).

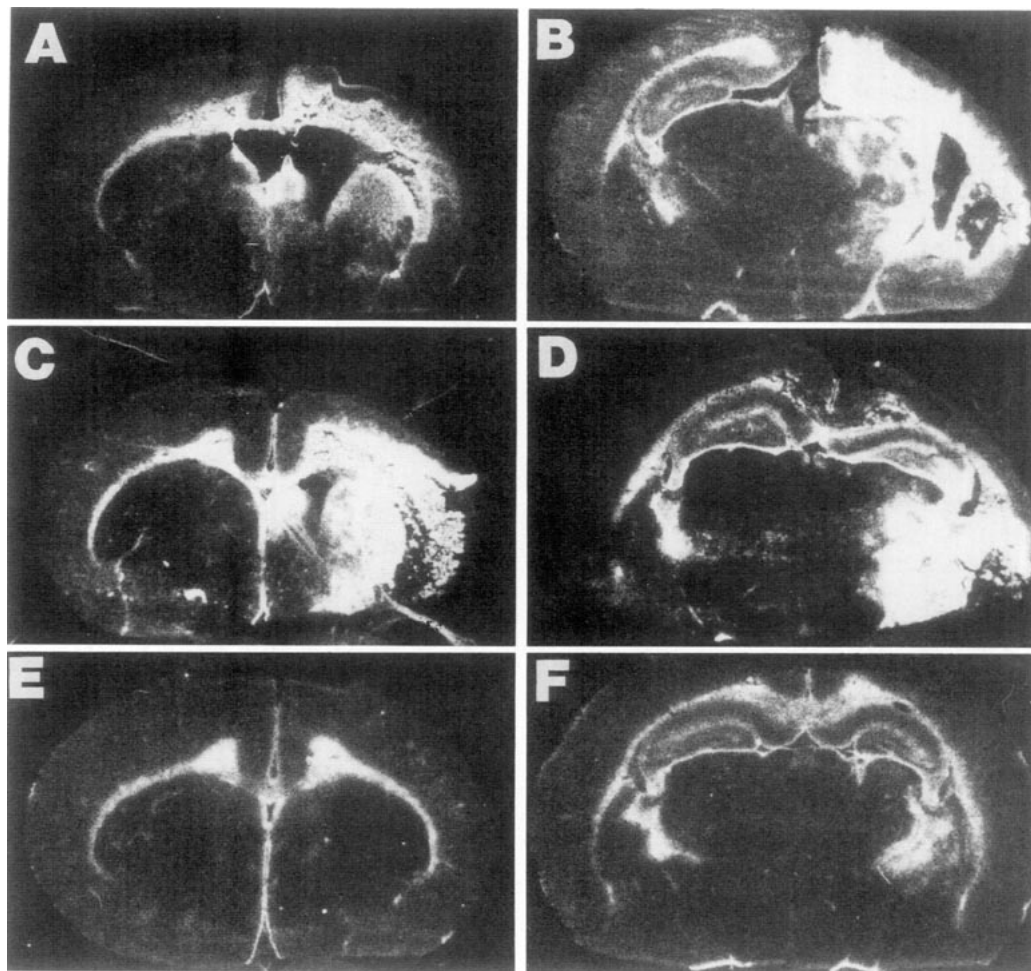


FIG. 4. Dark-field autoradiograms demonstrate the distribution of GFAP mRNA, visualized by *in situ* hybridization histochemistry in coronal brain sections, at the levels of striatum (A, C, E) and hippocampus (B, D, F), in brains prepared from three animals (see Methods for details of *in situ* hybridization assay). All three received right intrastriatal injections of 12.5 nmol of NMDA on PND 7 and were sacrificed on PND 12; the animal presented in E and F was also treated with MK-801 (1 mg/kg ip) 15 min post-NMDA injection. In A–D, ipsilateral tissue injury with substance loss in striatum, hippocampus, and cortex are evident, and there are coincident focal increases in GFAP mRNA expression. In contrast, in E and F, tissue integrity is preserved, and there are symmetrical low levels of GFAP mRNA expression.

DISCUSSION

Gliosis, accompanied by increased immunoreactive GFAP, has long been recognized neuropathologically as the hallmark of the brain's response to many diverse forms of injury. Our data indicate that in immature rodent brain focal excitotoxic injury induced accumulation of GFAP mRNA: in lesioned cortex, GFAP mRNA rose markedly within 24 h and increases were sustained over the next 2 weeks, whereas in lesioned hippocampus and striatum, the initial stimulation of GFAP mRNA levels was delayed relative to cortex. Treatment with the neuroprotective NMDA antagonist MK-801 effectively suppressed stimulation of GFAP mRNA accumulation in lesioned tissue. Thus, in this experimental model, increased mRNA synthesis and/or stability represents a major level of regulation of GFAP expression; based on the effects of MK-801 treatment, the initiating

signal appears to relate specifically to blockade of injury (and conceivably also more directly to prevention of overexcitation of NMDA receptors).

Increased GFAP mRNA tissue content could reflect increased synthesis or increased stability of the transcripts, and our data could not distinguish these mechanisms. In the second postnatal week in rats, there is a progressive, physiologic increase in GFAP mRNA content in cortex, hippocampus, and striatum; peak levels of GFAP mRNA are attained several days earlier in the cortex than in the other two regions (8, 23, 27; Silverstein and Burtrum, unpublished observations). The delayed increase in GFAP mRNA in lesioned hippocampus and striatum may, to some degree, reflect regional ontogenetic differences in glial maturation. Yet, in the second week after injury, stimulation of GFAP mRNA expression was also detected in these regions. Of interest, studies of the temporal and anatomic features of

GFAP mRNA stimulation in a perinatal rodent stroke model revealed strikingly similar patterns (23). In tissue derived from the lesioned hemisphere, GFAP mRNA increased within 24 h in cortex, whereas in the hippocampus and striatum, increases were delayed (inconsistently detected at PND 12, and considerably more robust in the third postnatal week). In both injury models, the extent of irreversible injury is similar in the three regions studied; yet, cortical GFAP mRNA induction occurred more rapidly and was often more pronounced and more sustained than in hippocampus or striatum. Of note, in fetal rodent brain, focal cortical injury induced GFAP immunoreactivity prenatally in brain regions where normally GFAP immunoreactivity was absent; the intensity of immunoreactivity did increase in animals lesioned in similar fashion in the early postnatal period (19).

In situ hybridization assays provided important complementary information. Prominent increases in GFAP mRNA in the lesioned forebrain were confirmed. Concurrent assessment of tissue injury (substance loss) and GFAP mRNA expression in the autoradiograms revealed considerable regional heterogeneity in the magnitude of stimulation of GFAP mRNA, which did not necessarily correspond directly with the severity of injury in each brain region. This pattern suggested that there were intrinsic differences in the response to excitotoxic injury within forebrain. It has been suggested that GFAP immunoreactivity can serve as a sensitive indicator of selective neuronal vulnerability (29). In perinatal rodents, with a modified lesioning method such that NMDA injections were directed more selectively to discrete brain regions, it should be possible to determine if stimulation of GFAP mRNA expression is directly related to the severity of local tissue injury, if injury in specific regions is more likely to induce GFAP, and if different lesioning methods induce distinctive region-specific changes.

Two recent studies (24, 25) that used *in situ* hybridization to delineate GFAP mRNA expression provided important new insights about potential neuronal influences on GFAP expression. After electrically induced seizures, GFAP mRNA was induced both locally and transynaptically; induction occurred without neuronal necrosis. In a denervation injury model, at the time of lesion-induced synaptic turnover, there was marked induction of GFAP mRNA both in denervated regions, and also in areas not obviously connected with the lesion. These studies provided evidence of a link between glial gene expression and abnormal neuronal activity. However, it is unlikely that NMDA-induced seizures stimulated the focal increases in GFAP mRNA we observed, since, based on analysis of electrocorticograms previously in this model, we found that unilateral intracerebral NMDA injection induced generalized synchronous epileptiform activity over both cerebral hemispheres (16). It is also unlikely that the increases in

GFAP mRNA reflected a primary astrocytic response to NMDA, since NMDA does not directly activate astrocytic excitatory amino acid receptors (3).

Whether focal excitotoxic 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 neuroprotection also prevented contralateral increases in GFAP mRNA in cortex and hippocampus. However, *in situ* hybridization assays did not demonstrate increased GFAP mRNA expression in the contralateral hemisphere; it is conceivable that subtle but significant increases remote from the area of injury were not detectable at this level of anatomic resolution.

Previous studies have demonstrated acute increases in GFAP mRNA, peaking 3–5 days after cortical stab wound injury in adult brain (6, 10). Moreover, several recent studies have identified discrepancies between tissue content of GFAP mRNA and immunoreactive protein, both during normal development (26) and after injury (2). In some settings post-translational changes in protein stability (or immunogenicity) may represent a major mechanism accounting for detected increases in GFAP. Our data indicate that in the immature brain increases in mRNA synthesis (and/or stability) occur acutely after excitotoxic injury and represent a major potential mechanism underlying induction of GFAP accumulation for at least 2 weeks after injury, intrinsic properties of specific cell populations (glia and/or neurons) in different brain regions influence the timing and magnitude of increases in GFAP mRNA, and, effective neuroprotective interventions attenuate increases in GFAP mRNA.

The sequelae of reactive gliosis may encompass both detrimental effects [e.g., limiting axonal sprouting (13)] and beneficial effects [e.g., production of neuronal trophic factors (20)]. Important unanswered questions include what cellular and molecular signals initiate GFAP mRNA synthesis after injury, what accounts for the accelerated and pronounced effects in lesioned cortex (relative to hippocampus and striatum where injury is equally or more severe), and ultimately, how do these glial events influence neuronal healing and reorganization? Understanding the molecular signals that regulate GFAP expression may improve our ability to modify the developing brain's response to injury. This reproducible excitotoxic injury model provides an optimal system for analysis of regulation of reactive gliosis in the developing brain.

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