Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6819–6823, August 1991
Neurobiology

Neuronal activity up-regulates astroglial gene expression

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Communicated by Richard F. Thompson, May 6, 1991 (received for review November 27, 1990)

ABSTRACT **Neuronal gene expression is known to be modulated by functional activity. This modulation is thought to play a key role in determining the differentiation of developing neurons and regulating the operation of mature neurons. Here we describe a regulation of astroglial gene expression by neuronal activity. We report that intense neuronal activity (electrically induced seizures) in rat hippocampus leads to rapid and dramatic increases in mRNA for glial fibrillary acidic protein (GFAP), an astroglia-specific intermediate filament protein. GFAP mRNA levels increased at sites of stimulation as well as in areas that were synaptically activated by the resultant seizures. When seizures were induced repetitively for many days, levels of GFAP mRNA remained chronically elevated. However, GFAP mRNA returned to control levels within a few days after the cessation of stimulation. The coupling between astroglial gene expression and neuronal activity may be a mechanism through which neuronal activity modulates the function of supporting cells that are responsible for regulating the extracellular microenvironment of the brain.**

Astrocytes are one class of glial cells that play an important supportive role in the central nervous system. In the mature brain, astrocytes are thought to buffer the extracellular environment by taking up ions and other small molecules from the extracellular milieu (1, 2). Astrocytes are also well known for their reaction to injury, during which they undergo substantial hypertrophy and phagocytose degeneration debris from dying neurons (3). Because astrocytes play such a key role in regulating the microenvironment of the brain, it is of considerable interest to understand the signals that induce their growth and regulate their gene expression.

In a previous study of changes in astroglial gene expression following injury (4), we described a biphasic response of astroglia following electrolytic lesions, which suggested that a number of different signals might regulate astrocyte gene expression. There was a rapid transient increase in glial fibrillary acidic protein (GFAP) mRNA in areas that were distant from the site of injury but received multisynaptic projections from the damaged region. These increases in GFAP mRNA in areas remote from the injury were surprising because it had been thought that astrocyte hypertrophy occurred specifically in response to degeneration debris. Our results thus suggested that astroglial gene expression might be regulated by neuronal activity, so that GFAP mRNA levels were up-regulated following electrolytic lesions because of the burst of activity generated during lesion production. We test this hypothesis in the present study by evaluating whether direct electrical activation of central nervous system pathways leads to an up-regulation of GFAP mRNA in astrocytes.

Our experiments focused on the hippocampus, which offers special advantages because its circuitry is extremely well characterized, because the patterns of activity induced by different types of stimulation are known, and because it is possible to repetitively elicit intense neuronal activity (seizures). We were interested in evaluating the effects of intense (seizure) activity on glial gene expression in order to optimize our chances to detect an activity-mediated change if one occurred. In the hippocampus, seizure activity can be elicited using modest stimulation by taking advantage of the kindling phenomenon, in which repeated electrical stimulation produces a state of chronically enhanced responsiveness (5). In "kindled" animals, stimuli that initially produce only a brief electrical discharge come to elicit robust electrographic and behavioral seizures (5, 6).

MATERIALS AND METHODS

Stimulating electrodes were implanted unilaterally into the CA3 region of the hippocampus of adult male rats (7). Because neuronal damage induces GFAP mRNA, it was important to carry out our experiments in animals with chronically implanted electrodes, in which any response to injury produced by electrode implantation would be resolved prior to the time that stimulation was delivered. Accordingly, animals were allowed to recover for a minimum of 7 days after electrode implantation. Kindling was then induced via a "rapid kindling paradigm" (7). Kindling stimulation (50-Hz stimulus trains 10 sec in duration) was given 12 times per day until kindled seizures were reliably elicited. Thereafter, seizures were elicited 12 times per day every other day until the day before the animals were euthanized for evaluation of GFAP mRNA levels. Once the kindled state has been achieved in this way, stable kindled seizures can be elicited many times per day for many days (7).

GFAP mRNA levels were assessed in animals that were killed 1 day following the first kindled seizure and in animals in which kindled seizures had been elicited every other day for 12–30 days. Surgical controls were implanted with electrodes but left unstimulated for 7 days. Levels of GFAP mRNA were assessed by *in situ* and dot blot hybridization using ^{35}S-labeled cRNA probes (riboprobes) produced from a cDNA for GFAP.

The preparation of the cRNA probe for GFAP mRNA has been described (4). The probe was derived from a 2.5-kilobase cDNA clone for mouse GFAP (8). A 1.26-kilobase fragment from the 5′ portion of this clone was recloned into the *Hin*dIII site of a Bluescript M13 vector by D. Chikaraishi (Tufts University) and was provided to us as a gift. ^{35}S-labeled antisense cRNA probes of about 1000 bases in length were synthesized from linearized plasmid by using the T3 promoter. The specific activity of the probes ranged from about 4×10^8 to 6×10^8 cpm/μg of RNA.

***In Situ* Hybridization.** The detailed protocol for *in situ* hybridization with the GFAP probe and the controls for nonspecific hybridization have been described (4). Animals were perfused under anesthesia (sodium pentobarbital) with 0.4% or 4% paraformaldehyde. We found that the lower concentrations of fixative improved the histological quality without compromising specific hybridization. This protocol

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Abbreviation: GFAP, glial fibrillary acidic protein.

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was used for routine *in situ* hybridization. Some animals were fixed with 4% paraformaldehyde so that sections could be used both for *in situ* hybridization and for silver staining (by the Fink–Heimer procedure; ref. 32) to reveal the extent of neuronal degeneration induced by the implanted electrodes and the seizures. Brains that were to be prepared only for *in situ* hybridization were immersed in 15% sucrose/4% paraformaldehyde for 1–4 hr. Brains that were to be prepared for both *in situ* hybridization and silver staining were immersed overnight in 4% paraformaldehyde and then were placed in 30% sucrose until they sank. The brains were then mounted on the chuck of a cryostat and frozen by immersion in methylbutane chilled with liquid nitrogen.

Cryostat sections were cut at 20 μm, and a 1-in-10 series of sections was collected on microscope slides that had been coated with polylysine. In the case of animals that had been perfused with 4% paraformaldehyde, a separate series of sections was collected in 4% phosphate buffer (pH 7.4) and was later stained by a modification of the Fink–Heimer technique in order to reveal degeneration debris. Microscope slides with affixed sections were stored frozen at −80°C until use. The slides were then thawed, postfixed in 4% paraformaldehyde, treated with proteinase K [1 μg/ml in RNase buffer (0.5 M NaCl/10 mM Tris, pH 8.0) for 30 min], washed twice in 0.5× standard saline/citrate (SSC), and dried with a Kimwipe. Sections were placed in humidified Petri dishes, covered with 120 μl of hybridization buffer containing 1 μl of probe (0.5–1 × 10^6 cpm) and 2 μg of tRNA. Sections were hybridized overnight at 55°C, treated with RNase A (Sigma R5503; 2 mg/ml in RNase buffer) for 30 min at room temperature, and washed for 2 hr at 55°C in a high-stringency buffer (0.1× SSC/10 mM 2-mercaptoethanol/1 mM EDTA). Slides were dipped in photographic emulsion, exposed for 1 week, developed in D19 (Kodak) and stained with cresyl violet.

mRNA Isolation and Dot Blot Analyses. The detailed protocols for mRNA isolation, dot blot hybridization with the GFAP probe, and the controls for nonspecific hybridization have been described (4). Animals were killed by sodium pentobarbital overdose and hippocampi were rapidly removed. Total cellular RNA was isolated from hippocampi ipsi- and contralateral to the stimulation by guanidinium isothiocyanate extraction followed by CsCl gradient centrifugation (9). The RNA was dissolved in 10× SSC/7.5% formaldehyde, pH 7.4, and samples containing 4 μg of RNA were spotted onto Nytran membranes and fixed to the membrane by crosslinking with ultraviolet light. Previous studies have defined the relationship between the amount of RNA added to the membrane and the extent of probe binding (4) and have revealed that samples containing 4 μg of RNA are nonsaturating. Membranes were hybridized at 65°C for 24 hr with ^{35}S-labeled probe [2.5 ng/ml, 10^6 cpm/ml in a hybridization buffer consisting of 50% formamide, 5× standard saline/phosphate/EDTA (SSPE), 5× Denhardt's solution, 1% SDS, 100 μg of tRNA per ml, and 100 μg of poly(A) per ml]. The membrane was then washed three times in 1× SSPE/1% SDS at 65°C, treated with RNase (10 μg/ml in 5× SSC) for 30 min at 35°C, and washed for 30 min at 65°C in a high-stringency buffer (0.1× SSPE/1% SDS). The membranes were then dried and exposed to Kodak X-Omat film for autoradiography. Sections of the membrane containing individual spots were cut out for measurement of radioactivity with a scintillation counter.

RESULTS

Fig. 1 illustrates the increases in GFAP mRNA in the hippocampus ipsilateral (*A*, *C*, and *E*) and contralateral (*B*, *D*, and *F*) to the implanted electrodes. In rats that had been implanted with stimulating electrodes but left unstimulated

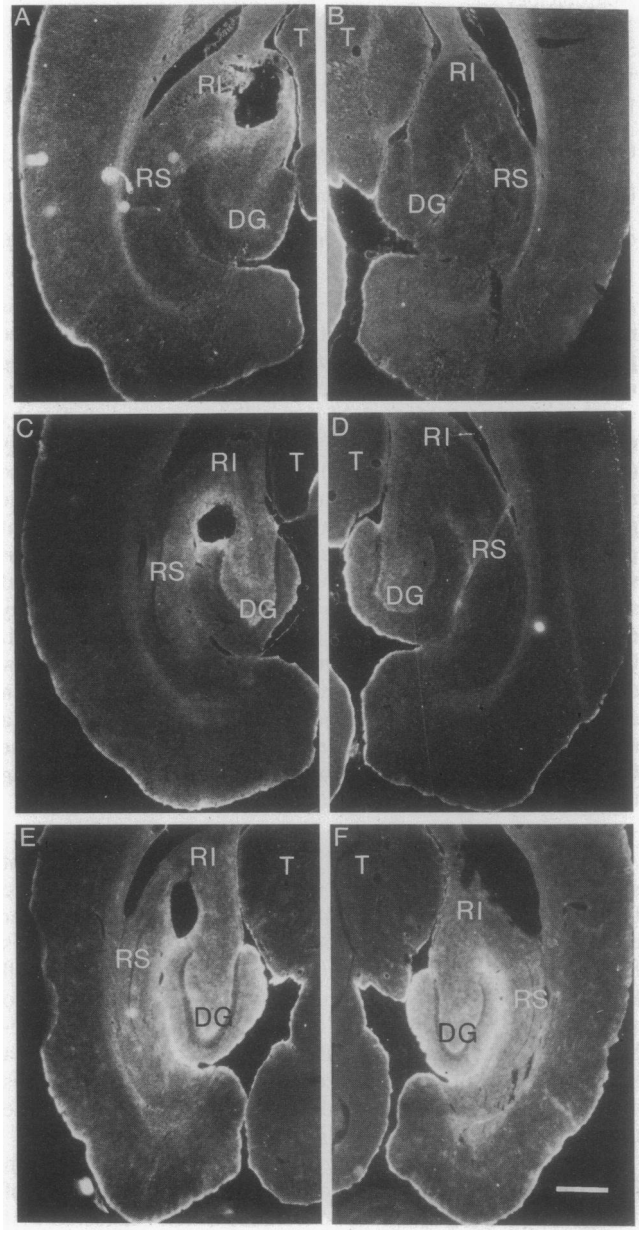

FIG. 1. Increases in GFAP mRNA in response to seizures as revealed by *in situ* hybridization. (*A* and *B*) GFAP mRNA ipsilateral (*A*) and contralateral (*B*) to a chronically implanted stimulating electrode (7 days after implantation, when no stimulation had been delivered). (*C* and *D*) GFAP mRNA ipsilateral (*C*) and contralateral (*D*) to the stimulation, 1 day after a single seizure. (*E* and *F*) GFAP mRNA ipsilateral (*E*) and contralateral (*F*) to the stimulation, 1 day after the last of a total of 300 kindled seizures delivered at a rate of 12 per day every other day. RS, regio superior of the hippocampus; RI, regio inferior of the hippocampus; DG, dentate gyrus; T, thalamus. The position of the stimulating electrode is made obvious by the hole near RI. (Bar = 300 μm.)

(Fig. 1 *A* and *B*), the levels of GFAP mRNA were slightly elevated near the electrode; however, throughout most of the hippocampus, GFAP mRNA levels were indistinguishable from control levels. In contrast, GFAP mRNA levels were substantially elevated ipsilateral to the stimulation in the animal that was killed 1 day after the first seizure (Fig. 1 *C* and *D*). The increases were particularly prominent in areas that would be synaptically activated by the stimulation (for example, the stratum radiatum of regio superior of the hippocampus, which receives input from neurons in CA3).

In animals that had experienced 12 seizures per day every other day for many days, GFAP mRNA was markedly

increased both ipsilateral and contralateral to the stimulation (Fig. 1 *E* and *F*). In addition, GFAP mRNA was increased throughout the hippocampus and dentate gyrus. This is consistent with the known pattern of seizure activity that occurs in kindled animals, where kindled seizures occur bilaterally and involve both the hippocampus proper and the dentate gyrus (10). This pattern of increased GFAP mRNA was observed in all three animals that were evaluated after having received multiple seizures (12 per day every other day) for 25–30 days. It is interesting that the levels of GFAP mRNA also appeared to be somewhat higher over periventricular regions, as is also true following lesions (4). However, this potential change was not evaluated further.

Quantitative assessments of grain density in the autoradiographs from the animals illustrated in Fig. 1 confirmed the qualitative impressions (Fig. 2). Grain counts were carried out in the stratum radiatum of the CA1 region about 0.5 mm from the stimulating electrode (counts of three to four slides per case). To normalize across cases and hybridization runs, grain density in the hippocampus was expressed as a percentage of a control region (the thalamus). In the chronically kindled animal illustrated in Fig. 1 *E* and *F*, grain density was an average of 13-fold higher than control levels on the side ipsilateral to the stimulation, and 11-fold higher on the contralateral side. In the three chronically kindled animals that were analyzed by *in situ* hybridization, grain density was increased an average of 11-fold ipsilateral to the stimulation and 7-fold on the contralateral side.

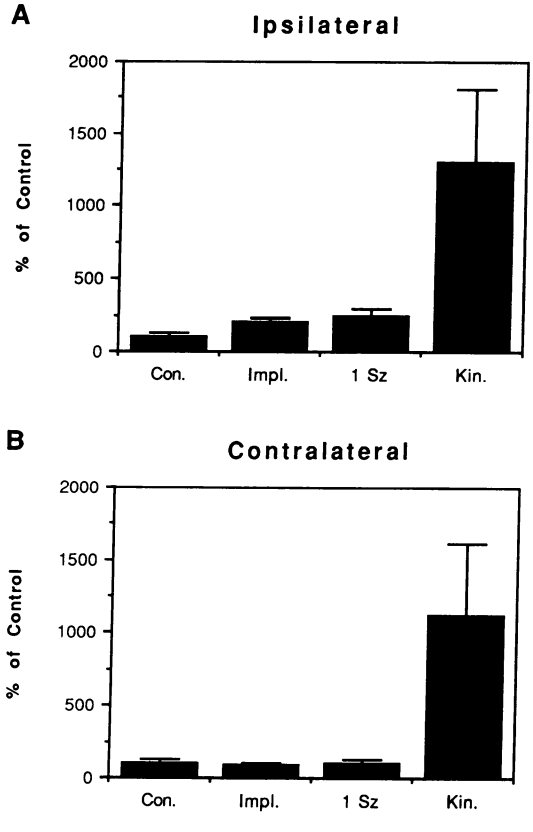

FIG. 2. Quantitative analyses of the increases in GFAP mRNA as revealed by *in situ* hybridization. Average grain density in the hippocampus ipsilateral (*A*) and contralateral (*B*) to the stimulating electrode is shown for the three rats illustrated in Fig. 1. Con., grain density in an area distant from the electrode (thalamus). The values are expressed as a percentage, with the error bars indicating the average SD obtained in the three to four measurements per case. Impl., grain density in the implanted control animal 7 days after electrode implantation (from the case illustrated in Fig. 1 *A* and *B*). 1 Sz, grain density 24 hr after a single seizure (from the case illustrated in Fig. 1 *C* and *D*). Kin., grain density 24 hr after the last of a total of 300 kindled seizures delivered at a rate of 12 per day every other day (from the case illustrated in Fig. 1 *E* and *F*). The values represent the mean and SD of the measurements from a total of five measuring sites in a total of three separate slides per case. Grain counts were carried out using a Gould IP8500 image-analysis system and software developed by the Biomedical Image Processing Center at the University of Virginia.

Dot blot analyses of a separate set of animals confirmed the results of the *in situ* hybridization experiments and provided additional information. Fig. 3 illustrates the results of several dot blot analyses in which samples from stimulated animals were compared with samples from intact controls. The levels of GFAP mRNA in all implanted animals were then expressed as a percentage of the control value, and treatment groups were compared. A one-factor analysis of variance revealed a significant effect of treatment ($F = 20.15$, $P < 0.0001$; for details on statistical comparisons between treatment groups, see legend to Fig. 3). GFAP mRNA was slightly increased as a result of electrode implantation in nonstimulated animals but was not significantly increased when animals received "spaced" stimulation (which involved the delivery of a number of stimuli equal to the kindling stimuli, but at low frequency). GFAP mRNA was increased 24 hr following a single seizure. In these cases, however, the increases were observed only ipsilateral to the stimulation. However, GFAP mRNA was increased about 5-fold in animals that were killed 1 day after the induction of 12 seizures over the course of 1 day. In these cases, GFAP mRNA was increased both ipsilateral and contralateral to the stimulation.

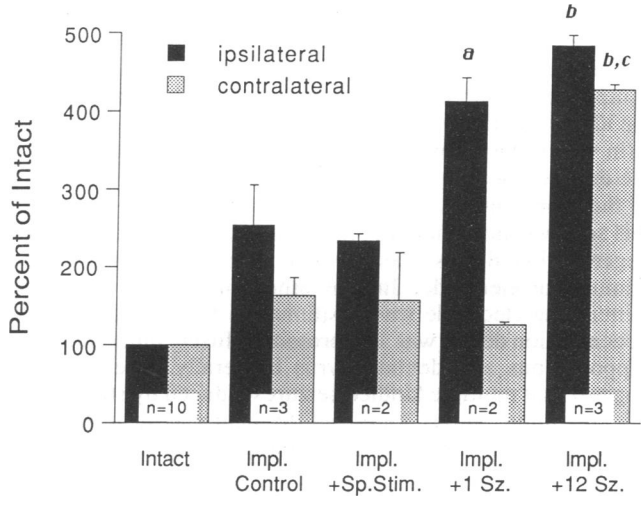

FIG. 3. Quantitative analyses of the increases in GFAP mRNA as revealed by dot blot hybridization. Shown are the combined results of several dot blot analyses in which samples from stimulated animals were compared with samples from intact controls. The levels of GFAP mRNA in all implanted animals were then expressed as a percentage of intact control. Bars represent the mean ± SEM from two to three quadruplicate experiments. A one-factor analysis of variance revealed a significant effect of treatment ($F = 20.15$, $P < 0.0001$). GFAP mRNA was slightly increased as a result of electrode implantation (Impl. Control). GFAP mRNA was not significantly increased when animals received "spaced" stimulation, which involved the delivery of a number of stimuli equal to the kindling stimuli, but at low frequency (Impl. + Sp. Stim). GFAP mRNA was significantly increased 24 hr after the induction of a single seizure (Impl. + 1 Sz.; for bar *a*, $P < 0.05$ in comparison to Impl. Control). The increases were observed only ipsilateral to the stimulation. GFAP mRNA was significantly increased bilaterally in animals that were killed 1 day after the induction of 12 seizures (Impl. + 12 Sz.) over the course of 1 day (bars *b*, $P < 0.01$ in comparison to Impl. Control and Sp. Stim; bar *c*, $P < 0.001$ in comparison to the contralateral side of Impl. + 1 Sz.). Numbers within the bars indicate the number of animals analyzed for each treatment condition.

The combined results clearly indicate that seizure activity leads to dramatic increases in GFAP mRNA not only near the site of stimulation but also in distant structures in which seizures occur as a result of synaptic activation.

We did not systematically evaluate the time course of the increases in GFAP mRNA after stimulation. However, the GFAP mRNA level remained elevated in one chronically kindled animal killed 3 days after the final day on which seizures were induced, although the increase was not as pronounced as at 24 hr postseizure. In an animal that was killed 5 days after the final day of seizures, the GFAP mRNA level was near control levels (data not shown). Thus, the increases in GFAP mRNA that occur as a result of seizures are transient, returning to near control levels within a few days.

An important issue is whether the increases in GFAP mRNA occur in response to neuronal degeneration induced by the seizures. Our results clearly indicated that the increases in GFAP mRNA were not related to the presence of degeneration debris. Fig. 4 illustrates the pattern of degeneration debris in the same animal as illustrated in Fig. 1 *E* and *F*. As is evident, degeneration debris was not detectable in many areas in which there were dramatic increases in GFAP mRNA (i.e., in the dentate gyrus bilaterally and in the hippocampus contralateral to the stimulation). An important internal control is that terminal degeneration was readily detected in the stratum radiatum of the hippocampus ipsilateral to the stimulation (near the implanted stimulating electrode).

Although the increases in GFAP mRNA did not appear to be related to the presence of degeneration debris, the studies did indicate an increase in degeneration debris in the animals that had experienced 12 seizures per day for many days, in comparison to kindled animals that had not been stimulated for 30 days. In the animal illustrated in Fig. 1 *E* and *F* and Fig. 4 (in which the stimulating electrode was localized in CA3), the degeneration debris was limited to the stratum radiatum of the hippocampus ipsilateral to the stimulation. Debris of this sort was not evident in nonstimulated animals, although degeneration debris was present immediately around the stimulating electrode. In one other animal (in which the stimulating electrode was positioned in the fimbria/fornix), degeneration debris was also present diffusely throughout the hippocampus and dentate gyrus bilaterally. These results suggest that multiple kindled seizures induced by stimulation of the hippocampus can lead to neuronal degeneration, although the pattern and extent of degeneration are variable.

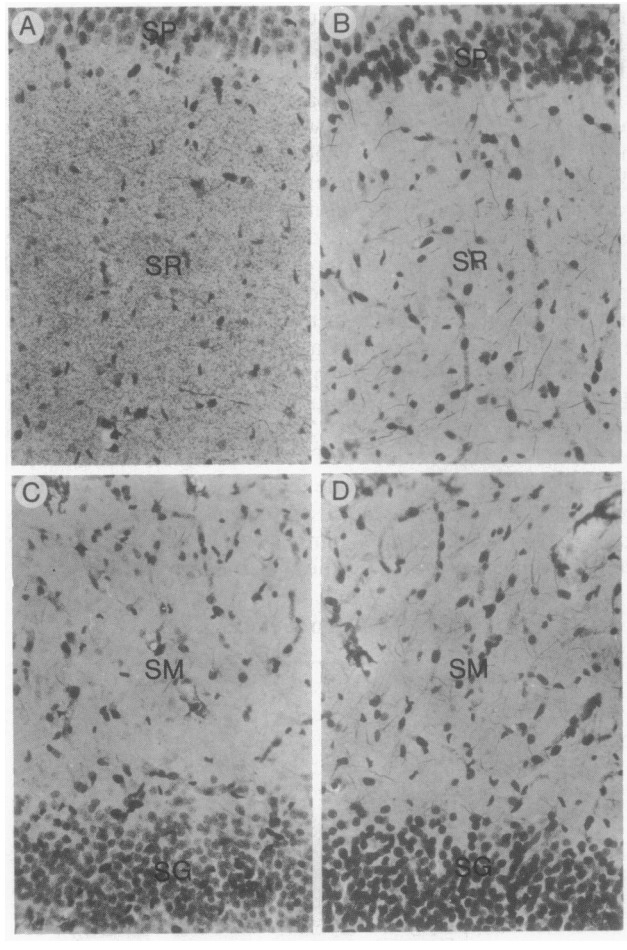

FIG. 4. Terminal degeneration in an animal that experienced 12 seizures per day for many days, as revealed by Fink–Heimer silver staining. These sections are from the same animal illustrated in Fig. 1 *E* and *F*, in which levels of GFAP mRNA were elevated throughout the hippocampus and dentate gyrus bilaterally. (*A* and *B*) Regio superior of the hippocampus ipsilateral (*A*) and contralateral (*B*) to the stimulating electrode. (*C* and *D*) Dentate gyrus ipsilateral (*C*) and contralateral (*D*) to the stimulating electrode. Terminal degeneration is evident in the stratum radiatum of the hippocampus near the implanted stimulating electrode. However, there is no detectable terminal degeneration in the hippocampus on the contralateral side or in either dentate gyrus. SP, stratum pyramidale; SR, stratum radiatum; SG, stratum granulosum; SM, stratum moleculare. These photomicrographs were taken from a section through the same dorsoventral level of the hippocampus as the one illustrated in Fig. 1 *E* and *F*. (×125.)

DISCUSSION

The results demonstrate that the expression of a major astrocyte-specific intermediate filament protein is up-regulated by intense neuronal activity. Thus, intense activity in neurons regulates gene expression in a cell type that plays a key role in regulating the extracellular microenvironment of the brain. Since increased GFAP expression is a hallmark of glial hypertrophy (11), our results suggest that intense activity of neurons can induce glial growth. This interpretation is reminiscent of studies showing that astrocytes in culture extend filopodia in response to neurotransmitters (12). This response to neurotransmitters occurs within seconds and thus cannot involve gene induction. However, because neurotransmitters would normally be released as a consequence of neuronal activity, and because our results indicate that neuronal activity regulates gene expression, the combined data indicate that functional activity of neurons can induce rapid changes in the structure of glial cells that are followed by long-term changes due to modulation of glial gene expression.

Our experiments do not identify the signaling mechanisms responsible for this transcellular regulation but do suggest that neuronal degeneration is not likely to be the principal signal. Intense seizure activity can certainly lead to neuronal loss and axonal degeneration (13, 14). However, previous studies have indicated that the stimulation paradigm used in the present study does not lead to detectable loss of neurons (15). Our results suggest that repeated seizures can lead to an increase in the amount of axonal degeneration in the hippocampus, although the extent and pattern of the degeneration is variable. Additional studies will be required to define the relationship between the degeneration debris and the position of the stimulating electrode, the duration and intensity of seizures, and other variables. However, the large increases in GFAP mRNA in areas that do not contain degeneration debris clearly indicate that other intercellular signals related to neuronal activity play the primary role in elevating GFAP mRNA levels.

Candidates for the intercellular signals include neurotransmitters, for which glial cells express receptors (16), or ions released by active neurons, especially K^+, which is released by active neurons and taken up by astrocytes (1, 2). The observation that rapid filopodial extension occurs in response to glutamate (12) suggests a role for excitatory amino acids, which are the principal transmitters of hippocampal pathways (17). In addition, *in vitro* studies have indicated that when astrocytes are grown in medium containing a high concentration of K^+, there is a down-regulation of GFAP rather than an up-regulation (18). However, there may be important differences in the consequences of a transient increase in K^+ (as would occur during seizures) and a sustained increase as would result from a particular concentration of K^+ in culture medium.

Previous studies have revealed that seizure activity dramatically up-regulates the expression of several "immediate early genes," including c-*fos* and c-*jun* (19, 20). These and other immediate early gene products are thought to act as transcription factors that presumably activate certain target genes. *In situ* hybridization studies strongly suggest that the increases in expression of the immediate early genes that have been studied to date occur primarily if not exclusively in neurons (20). The neuronal target genes that may be regulated by these transcription factors have not been identified. Considered within this context, the present results reveal a "late" gene that is regulated by activity in a cell type in which there is as yet no evidence for activity-induced up-regulation of immediate early gene expression. It may be that immediate early genes other than those studied to date are up-regulated by neuronal activity in glial cells.

GFAP mRNA levels were up-regulated following a single occurrence of intense neuronal activity and remained high when seizures occurred repeatedly. This persistent gene induction is in contrast to the transient induction of immediate early genes by seizure activity, where the increases in expression are not maintained when seizures occur repetitively (21).

The significance of the activity-induced increases in GFAP expression is not clear. One speculation is that reactive changes in glia are related to the synaptic reorganization that occurs in response to activity. It is well established that certain patterns of neuronal activity lead to a substantial remodeling of synapses (22–25). Indeed, dramatic synapse remodeling occurs in response to certain types of kindled seizures (26). Activity-induced synapse remodeling may involve a rapid synapse turnover similar to that which occurs following injury (where existing synapses are eliminated and replaced by new ones). In this case, the induction of astrocyte gene expression may be similar to that which occurs during lesion-induced synapse turnover, where reactive astrocytes play a central role in stripping degenerating synapses from neurons (27).

One important implication of our results is that it may be possible to use stimulation to modify the time course or extent of the reactive response of astrocytes following injury. This is important because a regulatory role for reactive astrocytes in the response of neurons to injury has long been suspected. On the one hand, it has been proposed that reactive astroglia form an inhospitable environment for growing axons and thus actually impede axon growth (28, 29). On the other hand, there is evidence that astrocytes in injured brains produce neuronotrophic factors that promote the survival and growth of injured neurons (30, 31). The present study suggests an approach to testing these hypotheses, since it should be possible, by using manipulations that affect neuronal activity, to modify astrocyte gene expression so as to increase or decrease glial hypertrophy or perhaps increase the production of particular glial factors.

We thank L. Whitmore and P. M. Falk for excellent technical help, N. Cowan for the GFAP cDNA clone, D. Chikaraishi for recloning the cDNA clone into a riboprobe vector, and T. Mifflin and the staff of the Molecular Probe Laboratory of the University of Virginia for producing the labeled cRNA probes. This work was supported by National Science Foundation Grant BNS8818766 (to O.S.) and National Institutes of Health Grants NS21671 and NS25605 (to E.L.). R.T. was the recipient of a postdoctoral fellowship from National Institutes of Health Training Grant T32 NS07199.

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