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Journal
Proceedings of the National Academy of Sciences of the United States of America, 87(19)

ISSN
0027-8424

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Publication Date
1990-10-01

Peer reviewed

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Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7643–7647, October 1990
Neurobiology

Levels of mRNA for a putative kainate receptor are affected by seizures

(glutamate receptor/hippocampus/dentate gyrus/cerebellum/neurotransmitter)

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Communicated by James L. McGaugh, July 2, 1990 (received for review May 11, 1990)

ABSTRACT **In situ hybridization and RNA blot-hybridization techniques were used (i) to examine the regional distribution of mRNA for a putative kainate receptor in adult rat brain and (ii) to test the possibility that seizures affect expression of the receptor gene. The highest densities of hybridization were distributed within hippocampal pyramidal and granule cells, medial habenula, Purkinje cells and the molecular layer of cerebellum, and olfactory bulb. Recurrent limbic seizures caused a massive, delayed, and reversible reduction in levels of the kainate receptor mRNA in dentate gyrus; lesser decreases were found in pyramidal cell fields of hippocampus and superficial cortex. These findings provide evidence that unusual patterns of physiological activity can alter genomic expression for a subclass of glutamate receptors in brain.**

A growing body of evidence indicates that synaptically induced muscle activity can affect genomic expression of the cholinergic receptor (see ref. 1 for a review). These observations lead naturally to the idea that physiological activity might also affect transcription of mRNA for neurotransmitter receptors in brain, either as part of pathogenesis or in some manner related to the encoding of experience. Unfortunately, a continued lack of success in the purification of glutamate receptors (the predominant form of excitatory receptor in much of mammalian brain) has precluded efforts to test this possibility. Recently, however, Hollmann *et al.* (2) defined the sequence for a cDNA clone that transcribes a functional kainate receptor, one of the major subclasses of glutamate receptors. This led us to prepare complimentary RNA (cRNA) probes using the sequence data provided by Hollmann *et al.* and then to test if aberrant physiological activity alters the expression of a glutamate receptor gene.

Previous studies have established that recurrent limbic seizures elicited by strategically placed focal lesions cause dramatic activity-dependent changes in the expression of mRNAs that encode (*i*) several neuroactive peptides (3, 4), (*ii*) a variety of immediate-early gene (IEG) products (5, 6), and, most recently, (*iii*) nerve growth factor (NGF) (7). Regional distributions and time courses have been mapped carefully for some of these effects (see ref. 8 for a review). The limbic seizure paradigm is thus an appropriate vehicle for attempting to manipulate expression of the putative kainate receptor gene. Here we report that seizures cause a pronounced and delayed down-regulation of kainate receptor mRNA expression in specific telencephalic cell groups.

MATERIALS AND METHODS

Materials. Adult male Sprague–Dawley rats (300–350 g) were used. The studies used three RNA probes complementary to aspects of the kainate receptor cDNA sequence reported by Hollman *et al.* (2). Kainate receptor cDNA of about 2.8-kilobase (kb) length was synthesized by using the polymerase chain reaction (PCR; ref. 9) with two oligonucleotide primers and was subcloned into the *Eco*RI site of transcription vector pGEM-4Z. Partial sequence analysis of the cDNA determined that it was identical to the sequence reported by Hollman *et al.* (2). Electrophysiological studies of *Xenopus* oocytes injected with capped mRNA (10), prepared by using phage SP6 RNA polymerase, showed that the cloned cDNA coded for a functional kainate receptor.

For *in situ* hybridization analysis, three cRNA probes were prepared. The first (about 720 nucleotides) was transcribed from the 3′ end of the cDNA linearized with *Bgl* II by using phage T7 RNA polymerase in the presence of ^{35}S-labeled UTP. For the preparation of antisense RNA probes for the 5′ end of the receptor mRNA, *Eco*RI–*Bam*HI (about 1442 nucleotides) and *Eco*RI–*Kpn* I (about 720 nucleotides) fragments were excised from the cloned kainate receptor cDNA and subcloned into *Eco*RI/*Bam*HI- and *Eco*RI/*Kpn* I-cleaved pGEM-4Z vectors, respectively. Two antisense RNA probes were then prepared from these clones by linearizing the cDNA with *Eco*RI and transcribing with T7 RNA polymerase in the presence of ^{35}S-labeled UTP.

Experimental Treatments. Unilateral electrolytic lesions were placed in the hilus of the dentate gyrus of anesthetized rats by using insulated stainless steel electrodes. Such lesions cause recurrent seizures of the limbic kindling type for 2–10 hr after lesion formation with the greatest number clustered in the first 3 hr of seizure onset. All experimental animals (n = 16) included here exhibited at least two behavioral seizures (11). Control rats (n = 10) were anesthetized, but no surgery was performed. At 6 (n = 3), 12 (n = 1), 21 (n = 1), 24 (n = 4), 30 (n = 3), 48 (n = 1), 72 (n = 1), and 96 (n = 2) hr after lesion placement, experimental and paired control rats were sacrificed by overdose with sodium pentobarbital and intracardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). An extra rat was given an electrolytic lesion with a platinum iridium wire and sacrificed 30 hr later; this type of lesion does not cause seizure activity (12).

***In Situ* Hybridization and Northern Blot Analysis.** For localization of kainate receptor mRNA by *in situ* hybridization, brains were sectioned at a thickness of 25 μm on a freezing microtome, and free-floating tissue sections were processed as described (7). Briefly, sections were treated with proteinase K at 1 μg/ml, rinsed in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7), placed in hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.7% Ficoll, 0.7% polyvinylpyrrolidone, bovine serum albumin at 350 mg/ml, yeast tRNA at 0.15 mg/ml, denatured herring sperm DNA at 0.33 mg/ml, and 20 μM dithiothreitol. After 1 hr of incubation at 60°C, sections were transferred to fresh hybridization buffer containing an addi-

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Abbreviations: NGF, nerve growth factor; IEG, immediate early gene; cRNA, complementary RNA.
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tional 20 μM dithiothreitol and ^{35}S-labeled antisense RNA probe (1 × 10^6 cpm/100 μl). After 36–40 hr of hybridization at 60°C, sections were rinsed in 4× SSC, treated with ribonuclease A at 20 μg/ml for 30 min at 45°C, and then rinsed through descending concentrations of SSC to a final wash in 0.1× SSC at 60°C. Hybridization was evaluated by using both Amersham B-Max film and Kodak NTB2 emulsion autoradiography with exposure times of 5–12 hr and 5–9 days, respectively. Hybridization density was quantified by calibrated densitometric analysis of film autoradiograms relative to radiolabeled brain paste standards. As controls for specificity, alternate sections were processed with ^{35}S-labeled sense RNA sequences (transcribed from the 5′ cDNA templates by using SP6 RNA polymerase in the presence of ^{35}S-labeled UTP) or were treated with ribonuclease A at 20 μg/ml for 30 min at 37°C prior to normal hybridization. No cellular labeling was observed in control tissue.

Two experimental rats and two paired controls were sacrificed 24 hr after lesion placement for Northern blot analysis. The hippocampi were longitudinally bisected into field CA3 vs. combined field CA1/dentate gyrus samples, and RNA was isolated by the method of Chomcyznski and Sacchi (13). Fifteen micrograms of RNA from each region was heated to 65°C for 15 min and cooled on ice. Ethidium bromide solution was then added for RNA visualization after gel separation and transfer to a GeneScreen*Plus* membrane (14). Hybridization was carried out at 42°C for about 24 hr in a solution containing denatured salmon sperm DNA (180 μg/ml) and kainate receptor probe (2 × 10^8 cpm/μg; 1 × 10^5 cpm/ml) prepared from the 1442-bp *Eco*RI/*Bam*HI fragment by the random primer method (15).

RESULTS

Equivalent results were obtained with the three kainate receptor cRNA probes in all aspects of the present study.

Distribution of mRNA for Kainate Receptor. In a preliminary study (16), we found that the density of hybridization to the putative kainate receptor mRNA varies markedly across brain regions and systems. Present results with emulsion autoradiography add important information to the earlier maps.

Neocortex. Hybridization of the ^{35}S-labeled cRNA probe labeled cells in all layers of neocortex with notably lesser intensity in layer IV (Fig. 1). There were no obvious regional variations in hybridization, although this point was not explored in detail.

Olfactory system. The olfactory system alone among the sensory pathways contained high levels of message. Within the olfactory bulb, cells of the glomerular, mitral, and granule layers were well-labeled, although the periglomerular and mitral cells, both innervated by the olfactory nerve, were more densely labeled than the inhibitory granule neurons. The anterior olfactory nucleus and layer II of olfactory cortex exhibited dense hybridization; a gradient was present in the latter structure such that rostral subdivisions were more densely labeled than caudal zones.

Striatal complex. The dorsal and ventral (e.g., nucleus accumbens) striatum were moderately labeled but with some evidence of heterogeneity—i.e., within the caudate/putamen, "gaps" were present in which hybridization was much lower than in the surrounding areas (Fig. 1*A*). Based on the present material alone, it was not possible to determine if this reflected the striosome organization (17). In contrast to striatum, the globus pallidus was unlabeled with the exception of a few large scattered cells. Between ventral striatum and superficial olfactory tubercle, patches of hybridization were seen overlying loose clusters of medium-size perikarya and, on occasion, appearing as "striatal bridges" (18).

Limbic system. The cells in each subdivision of hippocampal formation (dentate gyrus, field CA3, field CA1, and subiculum) were very densely labeled (Fig. 1 *B* and *C*). Cells in two target areas of hippocampal efferents, the dorsolateral septum and the lateral mammillary bodies, were also sites of unusually dense hybridization as was the septofimbrial nucleus. In contrast, only very sparse labeling was found in the medial septal nucleus–diagonal bands complex, which projects to hippocampus. Each of the major subdivisions of the amygdala (medial, centrome-

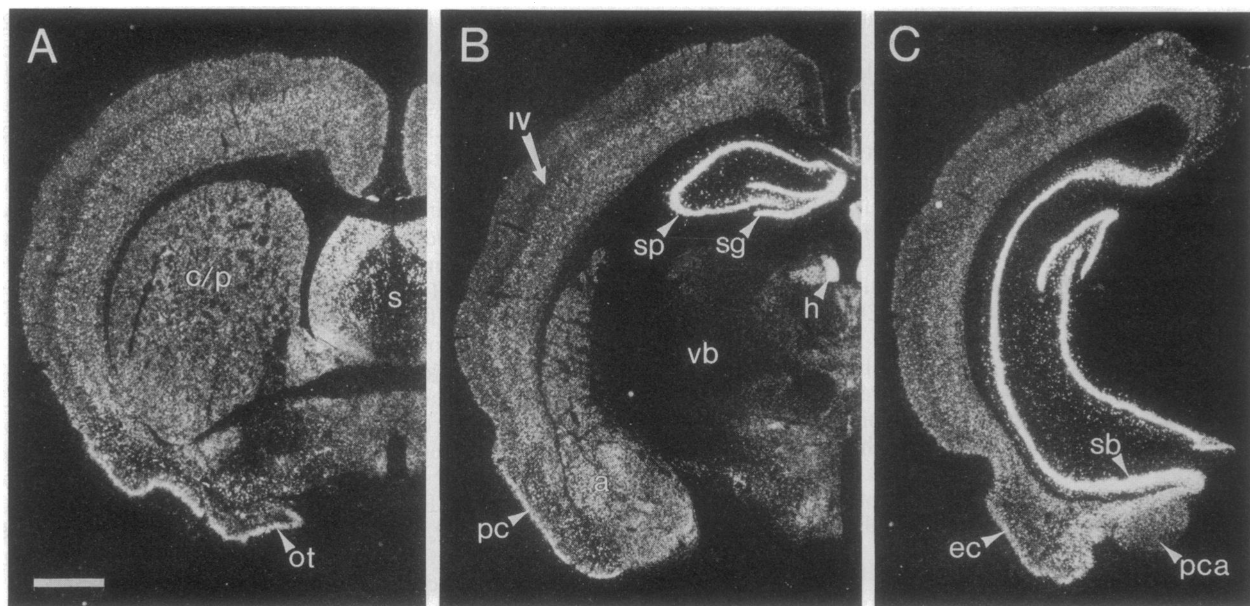

FIG. 1. Dark-field photomicrographs showing the autoradiographic localization of hybridization of the putative kainate receptor cRNA (≈720 bases, 5′ sequence) in coronal sections through the septum (s) (*A*), rostral hippocampus (*B*), and caudal hippocampus (*C*) of a normal rat. Highest densities of hybridization (evident as white) can be seen in hippocampal stratum granulosum (sg) and stratum pyramidale (sp), subiculum (sb), and lateral septum. The medial habenula (h) and superficial layers of piriform cortex (pc), entorhinal cortex (ec), and olfactory tubercle (ot) are also densely labeled. Within neocortex, layer IV is less densely labeled than surrounding fields. Relatively low levels of hybridization are evident in *B* within thalamic intralaminar nuclei, but the ventrobasal complex (vb) is strikingly devoid of hybridization. a, Amygdaloid complex; c/p, caudate/putamen; pca, posterocorticomedial amygdala. (Bar = 1200 μm.)

dial, and basolateral) exhibited moderate-to-high levels of labeling (Fig. 1B). The bed nucleus of stria terminalis, which is interconnected with several amygdaloid nuclei, was the site of substantial hybridization and in this regard was unique among basal forebrain structures.

Thalamus and hypothalamus. The primary sensory relay nuclei of the thalamus were strikingly devoid of labeled cells; the anterior nuclear group and the dorsomedial nucleus also had very low levels of hybridization (Fig. 1B). However, cells in the intralaminar complex and the parafascicular nucleus were moderately well labeled as were cells in the ventral lateral geniculate nucleus. Labeling in hypothalamus was moderate and scattered except for higher densities in the ventromedial nucleus and, as noted earlier, the lateral mammillary bodies. Finally, the habenulo–interpeduncular system (medial habenula, lateral habenula, and interpeduncular nucleus) at the meso–diencephalic border exhibited high levels of hybridization (Fig. 1B).

Cerebellum. Intense hybridization, equivalent to that found in olfactory bulb and hippocampus, was present in the cerebellar Purkinje cells, while the immediately subjacent granule cells were virtually unlabeled (Fig. 2). Examination of the emulsion material strongly suggested that mRNA for the kainate receptor was present not only in the somata of the Purkinje cells but in their dendritic trees as well; dense autoradiographic labeling extended well into the molecular layer although decreasing somewhat proximodistally to the layer of cell bodies. The inferior olivary complex, which provides the climbing fiber input to the Purkinje cells, was also densely labeled and clearly stood out from the surrounding brain stem. It is noteworthy that the apparent localization of kainate receptor mRNA within the dendrites of the Purkinje cells contrasts sharply with the strictly perikaryal hybridization in other areas such as hippocampus.

Experimental Seizure Results. A reliable time-dependent decrease in hybridization to kainate receptor mRNA was observed in hippocampus of experimental-seizure rats processed with each of the antisense probes. In all instances, the reduction was most dramatic in stratum granulosum. This is illustrated in Fig. 3 *A* and *B*, which show the dentate gyrus of an experimental animal sacrificed 30 hr after a lesion of the contralateral hilus was formed and of a paired control rat. As can be seen, hybridization is reduced to very low levels within the granule cells at this time point, while neurons of the hilus and CA3 stratum pyramidale are still well labeled. Quantitative analysis of film autoradiographs (Fig. 4) revealed that the decrease in hybridization within stratum granulosum develops between 12 and 21 hr after lesion placement and thus occurs after the major phase of seizure activity within hippocampus (2–10 hr after lesion placement). Hybridization recovers to near normal levels between 30 and 48 hr after lesion placement.

Reductions in kainate receptor mRNA were not restricted to the dentate gyrus. Smaller and less reliable decreases were observed within the pyramidal cell layer of hippocampal regions CA3 and CA1 (Fig. 4), whereas hybridization within superficial layers of neocortex and piriform cortex was consistently lower than normal 24 and 30 hr after lesion placement (Fig. 3 *C–E*). The lesion made with a platinum iridium electrode did not cause seizures and produced no detectable changes in hybridization (data not shown).

Northern blot analysis corroborated the *in situ* hybridization results. Relative to paired controls, there was a large reduction in hybridization to kainate receptor mRNA within the dentate gyrus/CA1 sample and a more modest reduction in the CA3 sample from rats sacrificed 24 hr after lesion placement (Fig. 5). However, when the same RNA blot was used for measurement of the mRNA encoding the γ_2 subunit of the γ-aminobutyric acid receptor type A, no clear differences were found between experimental and control samples (data not shown).

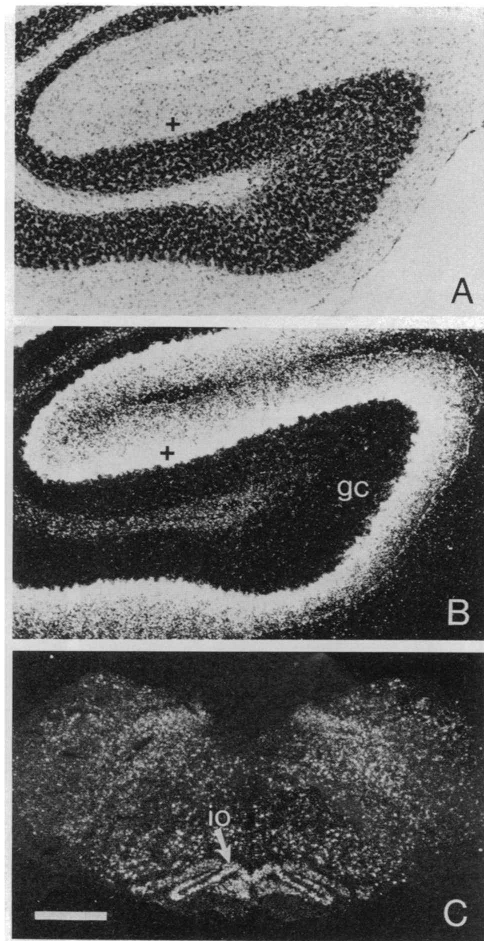

FIG. 2. Photomicrographs showing the distribution of hybridization of the ^{35}S-labeled cRNA (3′ sequence) within cerebellar cortex (*A* and *B*) and brain stem (*C*). (*A* and *B*) A single folia of cerebellar cortex is shown in light-field (*A*) and dark-field (*B*) illumination to illustrate the distribution of autoradiographic labeling relative to the Nissl-stained granule cell layer (*A*); hybridization is clearly dense over the Purkinje and molecular layers, whereas the granule cell layer (gc) is virtually unlabeled ("+" marks identical tissue positions in *A* and *B*). (*C*) Section through the medulla showing moderately dense labeling of the inferior olive nucleus (io) and neurons scattered across the reticular formation. (Bar = 250 μm for *A* and *B* and 870 μm for *C*.)

DISCUSSION

Regional Distribution of Cells Labeled by the Kainate Receptor Probe. The regional distribution of hybridization indicates high levels of kainate receptor mRNA in serial links of particular brain systems. For example, the two distinctly different types of neurons (mitral and periglomular cells) innervated by the olfactory nerve exhibited intense hybridization as did the cortical neurons contacted by one of these populations. This theme was repeated at all levels of the neuroaxis, with a particularly striking case being found in the cerebellum. The inferior olivary complex was unusual for pontine brain stem in being densely labeled. Cells in this region project massively to the cerebellar Purkinje cells, and those target neurons also had extremely dense hybridization. In contrast, neither the cerebellar granule cells nor the various brain stem nuclei which project to them (e.g., lateral vestibular nucleus) had significant labeling.

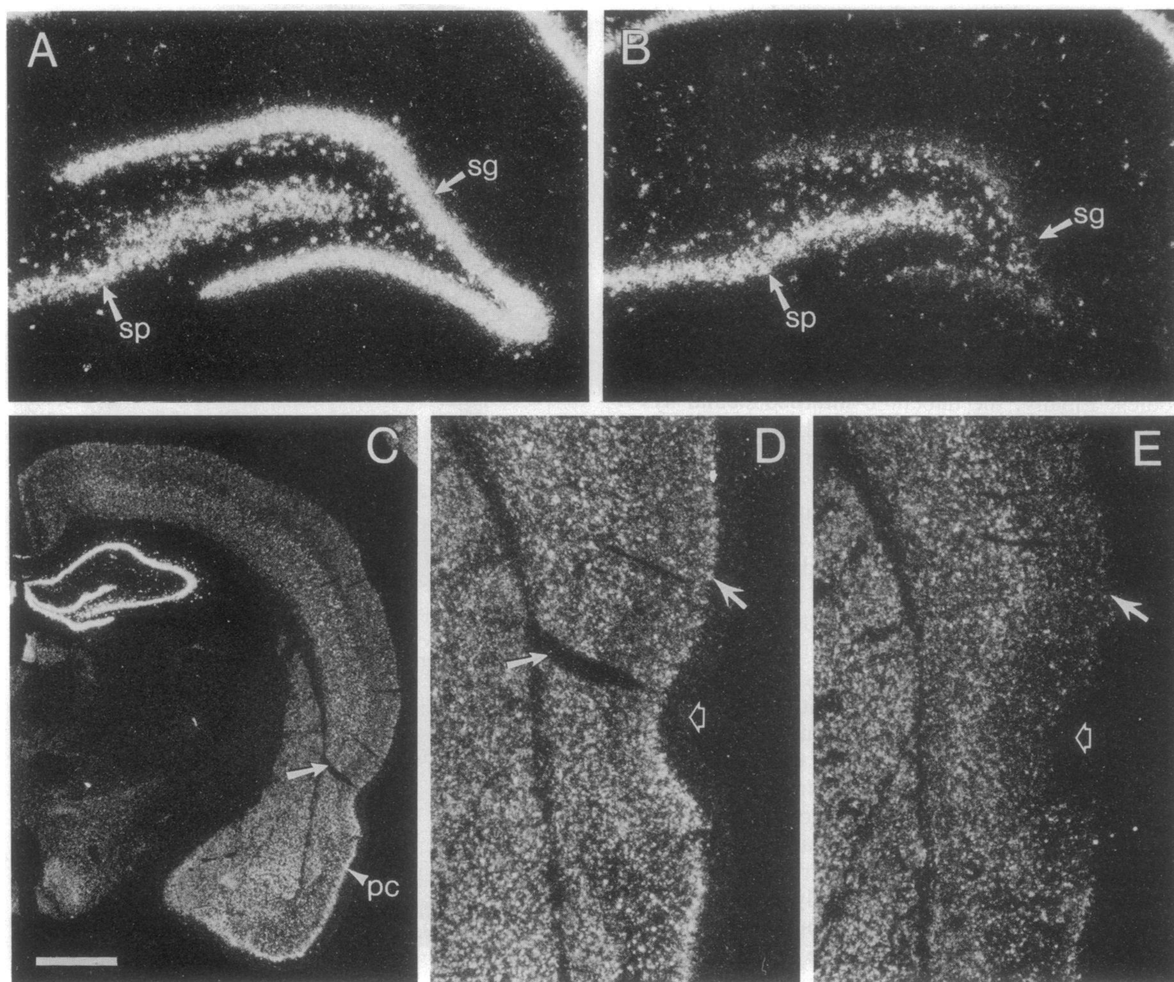

FIG. 3. Dark-field photomicrographs showing the autoradiographic localization of RNA hybridization (≈720 bases; 5′ cRNA) in tissue sections from a control rat (*A*, *C*, and *D*) and a paired experimental-seizure rat sacrificed 30 hr after a contralateral hilus lesion (*B* and *E*). (*A* and *B*) In rostral dentate gyrus, hybridization is dense within stratum granulosum (sg) and field CA3 stratum pyramidale (sp) of the normal rat (*A*) but dramatically reduced within stratum granulosum of the experimental animal (*B*). (*C* and *D*) Photomicrographs show labeling within a coronal section through rostral hippocampus (*C*) and, at higher magnification, the cortical region surrounding the rhinal fissure (the small white arrow indicates same blood vessel in each). In a comparison of *D* and *E* (showing a comparable cortical field from the experimental seizure rat), one can see a clear reduction in hybridization density within superficial layers of piriform, pararhinal, and neocortex in the experimental rat. In *D* and *E*, the large white arrow indicates neocortical layer II, and the open arrow indicates the tissue surface at the rhinal fissure. (Bar = 300 μm for *A* and *B*, 1200 μm for *C*, and 450 μm for *D* and *E*.)

The functional significance and cellular origins of the arrangements suggested by these observations need investigation. The cells in the successive links of the olivo–cerebellar circuit or in the bulbar–cortical–hippocampal network have distinctly different morphologies and in some cases are known to differ by a number of neurochemical criteria (e.g., relative levels of neuroactive peptides). However, physiological activity in the olfactory–hippocampal system from bulb to field CA1 is synchronized and time-locked to the sniffing rhythm (19), and it is conceivable that this pattern provides a type of trophic signal for the kainate receptor gene. The olivo–cerebellar network is also notable for its physiological coupling. It will be of considerable interest to test the effects of disrupting normal activity in the olfactory–hippocampal circuit or in the olivo–cerebellar complex on levels of kainate receptor mRNA.

In many respects the regional distribution of hybridization to the putative kainate receptor mRNA reported here is in agreement with the distribution of [^{3}H]kainic acid binding. By both techniques, labeling is moderate to dense in cortex, caudate/putamen, hippocampus, and olfactory bulb and very low in dorsal thalamus, globus pallidus, and brain stem (20, 21). The clearest discrepancies are evident in hippocampus septum and cerebellum. In the latter region, [^{3}H]kainic acid binding is dense in the granule cell layer and less dense in the molecular layer (20), while kainic acid-displaceable [^{3}H]-glutamate binding is relatively low in all layers (22). In contrast, hybridization of the kainate receptor cRNA is very dense in the Purkinje cell and molecular layers. These differences suggest that, at least in the cerebellum, the putative kainate receptor mRNA studied here does not encode the ligand binding site(s). However, such conclusions are premature until further characterization of these proteins and their ultrastructural localization is available.

Causes and Significance of Down-Regulation. Changes in concentrations of kainate receptor mRNA were restricted to certain populations of cells found in different parts of the forebrain. The most pronounced and reliable reductions were obtained in the dentate gyrus, where other changes in genomic expression following limbic seizures are greatest (8), but lesser effects were also evident in superficial neocortex and olfactory cortex and in the pyramidal cells of hippocampus. These are clearly activity-dependent modifications, since anatomical studies (23) have not detected degeneration in these fields contralateral to hilus lesions. Moreover, as reported here, lesions that do not induce seizures do not alter

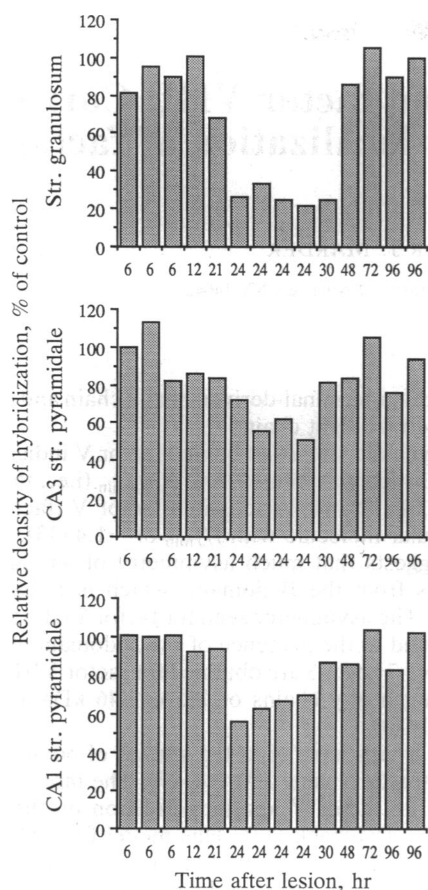

FIG. 4. Bar graph showing the relative density of RNA hybridization, determined from densitometric analysis of film autoradiograms, within stratum (Str.) granulosum (*Top*), field CA3 stratum pyramidale (*Middle*), and field CA1 stratum pyramidale (*Bottom*) in experimental seizure rats as compared with paired controls. Each bar indicates the relative density of hybridization for an individual experimental animal sacrificed at the time indicated after lesion placement.

gene expression (7, 8). The question thus arises as to whether the distribution of reactive neurons reflects the intensity of seizure activity experienced by different regions or instead is due to phenotypic differences. Resolution of this question, and many of the other issues regarding regulation, will probably require studies using subseizure stimulation of specific synaptic pathways. Such experiments could also provide clues about which of the many physiological effects that accompany seizures are responsible for the down-regulation of the kainate receptor gene. It is of interest in this regard that induction of IEG and NGF mRNA expression requires relatively modest periods of afferent stimulation (C.G., unpublished data). Whether this holds true for the delayed down-regulation of kainate receptor mRNA is a subject of some considerable importance.

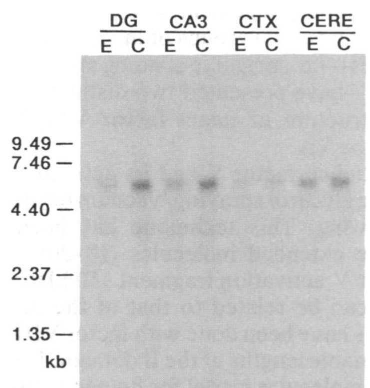

FIG. 5. Northern blot analysis of total RNA from field CA1/dentate gyrus (DG), field CA3, neocortex (CTX), and cerebellum (CERE) of experimental (lanes E) and control (lanes C) animals using a kainate receptor cDNA probe. The RNA ladder (BRL) was used as a size marker and is indicated on the left. The patterns and intensities of RNAs on the GeneScreen*Plus* membrane visualized by ethidium bromide were very similar among samples.

It is noteworthy that the time course for seizure-induced changes in kainate receptor gene expression does not correspond to any of those established for IEGs (5, 6), NGF (7), or the neuropeptides (8). Changes in IEG and NGF mRNA content are maximal approximately 30 min and 2 hr after seizure onset, respectively. Suppression of kainate receptor mRNA expression thus represents a relatively delayed event, possibly a late step in a cascade of changes in gene expression that begins within minutes of the first seizure episode.

The functional consequences of the genomic regulation described here will be determined by both the location of the kainate receptor (i.e., synaptic vs. extrasynaptic) and the signal needed to effect altered expression. If dependent upon seizures, then it may serve as a safety device, reducing excitability and hence the probability of future seizure activity. Conversely, if the effects can be produced by subseizure stimulation it is conceivable that down-regulation represents a means through which activity levels affect the functional properties of neuronal circuits.

This work was supported by National Institutes of Health Grants NS26448 to C.G. and NS23716 to K.S., and Office of Naval Research Grant N00014-89-J-1255 to G.L.

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