The Expression of Kainate Receptor Subunits in Hippocampal Astrocytes After Experimentally Induced Status Epilepticus

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Abstract

Astrocytes have emerged as active participants of synaptic transmission and are increasingly implicated in neurologic disorders including epilepsy. Adult glial fibrillary acidic protein (GFAP)-positive hippocampal astrocytes are not known for ionotropic glutamate receptor expression under basal conditions. Using a chemoconvulsive status epilepticus (SE) model of temporal lobe epilepsy, we show by immunohistochemistry and colocalization analysis that reactive hippocampal astrocytes express kainate receptor (KAR) subunits after SE. In the CA1 region, GluK1, GluK2/3, GluK4, and GluK5 subunit expression was observed in GFAP-positive astrocytes during the seizure-free or “latent” period 1 week after SE. At 8 weeks after SE, a time after SE when spontaneous behavioral seizures occur, the GluK1 and GluK5 subunits remained expressed at significant levels. Kainate receptor subunit expression was found in astrocytes in the hippocampus and surrounding cortex but not in GFAP-positive astrocytes of striatum, olfactory bulb, or brainstem. To examine hippocampal KAR expression more broadly, astroglial-enriched tissue fractions were prepared from dissected hippocampi and were found to have greater GluK4 expression after SE than controls. These results demonstrate that astrocytes begin to express KARs after seizure activity and suggest that their expression may contribute to the pathophysiology of epilepsy.

Key Words: Astrogliosis, Epilepsy, Glutamate receptor, Kainate acid.

INTRODUCTION

Temporal lobe epilepsy (TLE) is one of the most common acquired seizure disorders. It is often unresponsive to antiseizure drugs, increases in severity over time, and can result in cognitive decline (1–3). Temporal lobe epilepsy is associated with neuronal cell death and pronounced astrogliosis that is characterized by hypertrophy of astrocyte processes, with concomitant disruption in domain organization, increases in the expression of intermediate filament proteins such as glial fibrillary acidic protein (GFAP), and changes in the expression of a variety of proteins essential for proper astrocyte function (4–6). Although numerous studies have focused on the role of neurons in contributing to seizure generation in models of TLE, the contribution of astrocytes to neuronal excitability and seizure generation has recently garnered more interest, which is not surprising because seizures can often initiate in or near gliotic tissue (7). As a consequence of the growing recognition that astrocytes play a dynamic role in normal CNS function via the release of gliotransmitters and other signaling molecules (e.g. tumor necrosis factor [8, 9]), many of the functional and structural changes observed in astrocytes in TLE are hypothesized to alter excitability in the same limbic structures that are involved in seizure generation.

Ionotropic glutamate receptors are essential mediators of excitatory drive in the central nervous system, and their study, primarily in neurons, has shed light on synaptic transmission mechanisms and alterations that occur in disease states such as epilepsy (10, 11). Interestingly, astrocytes have been shown to express ionotropic glutamate receptors in various preparations from different brain regions. Several groups have demonstrated α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor and kainate receptor (KAR) responses in astrocytes from hippocampal preparations (12–14). These responses are often identified in “complex” cells that display low gap junction coupling, voltage-dependent current responses, and absent or reduced GFAP staining; they are now understood to be a different glial cell type, that is, the NG2 glia (14, 15). Furthermore, ionotropic glutamate receptor expression is most often found within astrocytic cultures or in slice preparations from young animals (average age, ~postnatal day 10)—both preparations that can have substantially different expression profiles versus adult tissue (16, 17). In the adult hippocampus, electrically passive, GFAP-expressing, protoplasmic astrocytes do not seem to express ionotropic glutamate receptors to any significant degree under basal conditions. In this study, we were interested in determining whether KAR expression is present in astrocytes of the hippocampus after seizure activity in a model of human TLE. Interestingly, KARs and N-methyl-D-aspartate receptors have been reported to be expressed in reactive astrocytes after ischemic insult in rodents (18, 19).

Kainate receptors are ionotropic glutamate receptors composed of the “high-affinity” GluK4 and GluK5 subunits and the “low-affinity” GluK1, GluK2, and GluK3 subunits. Whereas the GluK1, GluK2, and GluK3 subunits can form functional homomeric KARs in expression systems, the...
GluK4 and GluK5 subunits cannot. However, GluK4 and GluK5 can assemble with GluK1, GluK2, or GluK3 to form functional heteromeric receptors (20). Furthermore, KARs can also couple to G-proteins, thus bestowing a metabotropic signaling mechanism onto these receptors (21, 22). Using immunohistochemistry on fixed whole-brain specimens and Western blot analysis on enriched astroglial tissue fractions, we report that GFAP-positive astrocytes in the hippocampus of adult animals dramatically and differentially increase the expression of KAR subunits after SE. These results, coupled with an increased understanding of altered properties of astrocytes after SE (23, 24), suggest that ionotropic glutamate receptor expression in reactive astrocytes may contribute to the pathophysiology of epilepsy and warrants future mechanistic studies to unravel the consequence of this expression in diseased astrocytes.

MATERIALS AND METHODS

Animals and Seizure Induction

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Utah. Chemical reagents used in the study were purchased from Sigma Aldrich, St. Louis, MO, unless indicated otherwise. Male Sprague-Dawley rats (42–45 days old) were injected with kainic acid (KA) Nanocs, Boston, MA) to induce SE using the previously described repeated low-dose KA model of acquired epilepsy (24–27). Briefly, injections of KA (5 mg/kg, intraperitoneally) occurred once every hour until animals exhibited their first-stage 4/5 seizure, as defined by the Racine scale (28). After the onset of the first-stage 4/5 seizure, injections were ceased and the time and stage of subsequent seizures were observed. To be included in the study, rats had to exhibit a minimum of 1-stage 4/5 seizure per hour during a 3.5-hour observation period. At the conclusion of behavioral monitoring, rats were given an injection of 0.9% saline (1 mL, subcutaneously) to prevent dehydration. Rats were then housed individually in a temperature- and light-controlled (12-hour light/dark cycle) environment and allowed access to food and water ad libitum until experimentation. Animals were killed 1 and 8 weeks after SE. Animals at the 8-week time point were video monitored for 48 hours before killing to confirm the presence of unprovoked spontaneous behavioral seizure activity in that cohort of animals. Animals at the 8-week time point included in the study displayed at least 1-stage 4/5 behavior seizure in a 48-hour period. A subset of animals was subjected to pilocarpine-induced SE, as previously described, to compare our results with those of a mechanistically different model of SE (n = 3) (29). Pilocarpine hydrochloride (50 mg/kg; intraperitoneally) was administered 24 hours after a lithium chloride injection (20 mg/kg; intraperitoneally), which reduces the dose of pilocarpine needed to induce SE. Animals were observed and scored for seizure severity for 1.5 hours before being returned to their home cages. All animals were given 1 mL of 0.9% saline to compensate for the fluid loss induced by excessive cholinergic activation. Numbers of animals used for each experiment are summarized in the Table.

### Immunohistochemistry

For immunofluorescence, animals at 1 and 8 weeks after KA-induced SE and age-matched controls were anesthetized with pentobarbital and killed by transcardial perfusion. Animals were quickly perfused with 1× PBS to remove blood from the brain. The brains were then immediately removed and flash frozen in 2-methylbutane. Horizontal cryostat sections 30 μm thick containing the hippocampus were prepared. Tissue samples from KA-treated and control animals were batch processed by placing cryosections from each condition on the same slide. The staining and imaging were performed in triplicate for each individual KAR subunit and GFAP (Table). Frozen slides were brought to room temperature and immersed in 4% paraformaldehyde for 1 hour to fix. Sections were then rinsed with TRIS buffer (0.1 mol/L TRIS) then placed into heated TRIS buffer (~85°C) for 1 minute to retrieve antigens. Sections were blocked in a TRIS B solution (0.1% Triton, 0.25% bovine serum albumin, in 0.1 mol/L TRIS buffer) for 1 hour and then incubated overnight at room temperature with goat polyclonal primary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) directed against GluK1 (sc-7617, 1:100), GluK2/3 (sc-7620, 1:100), GluK4 (sc-8916, 1:100), and GluK5 (sc-8914, 1:100) made up in TRIS B. Sections were rinsed with TRIS B and then incubated for 2 hours in anti-goat secondary antibody conjugated to Alexa 488, Chemicon, Billerica, MA) for 2 additional hours. Finally, slides were rinsed with TRIS and coverslipped using Prolong Gold with 4',6-diamidino-2-phenylindole counterstain (Molecular Probes).

A subset of animals 1 week post-KA-induced SE and age-matched controls and animals 1 to 4 weeks post-pilocarpine treatment and age-matched controls were processed for bright field microscopy in a similar manner as above but instead using avidin-biotin complexes and 3,3'-diaminobenzidine (DAB) immunoreactivity. Animals were anesthetized and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were removed and allowed to postfix overnight in 4% paraformaldehyde. Brains were cut on a vibratome into 30-μm sections and stored in TRIS buffer until processing. Sections were

### TABLE. Summary of Numbers of Animals Used in Each Component of the Study

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Methods</th>
<th>No. Animals</th>
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<tbody>
<tr>
<td>1 week control</td>
<td>IHC, IF</td>
<td>5</td>
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<tr>
<td>1 week control</td>
<td>IHC, DAB</td>
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<tr>
<td>1 week after KA</td>
<td>IHC, IF</td>
<td>6</td>
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<tr>
<td>1 week after KA</td>
<td>IHC, DAB</td>
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<tr>
<td>8 weeks control</td>
<td>IHC, IF</td>
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<tr>
<td>8 weeks after KA</td>
<td>IHC, IF</td>
<td>5</td>
</tr>
<tr>
<td>1–4 weeks after pilocarpine</td>
<td>IHC, DAB</td>
<td>3</td>
</tr>
<tr>
<td>1 week control</td>
<td>WB</td>
<td>4</td>
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<tr>
<td>1 week after KA</td>
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DAB, 3,3'-diaminobenzidine; IF, immunofluorescence; IHC, immunohistochemistry; KA, kainic acid; WB, Western blotting.
mounted onto slides and air-dried for 15 minutes. For staining, slides were placed into heated TRIS buffer (~85°C) for 1 minute to retrieve antigens, blocked for 1 hour in TRIS B, then incubated overnight in primary antibody directed against NeuN (Chemicon, 1:10,000) or polyclonal antibodies from Santa Cruz Biotechnology directed against GluK1 (sc-7617, 1:100), GluK2/3 (sc-7620, 1:100), GluK4 (sc-8916, 1:100), and GluK5 (sc-8914, 1:100) using TRIS B. Slides were washed, then incubated in species-specific biotinylated secondary antibody for 2 hours, followed by incubation for 1 hour with a Standard ABC Elite Kit (1:1000, Vector Laboratories, Burlingame, CA). After an additional wash step, slides were incubated in a DAB Elite kit (1:1000; Vector Laboratories). Finally, sections were dehydrated in graded ethanols and then mounted in DPX mounting medium.

The KAR subunit antibodies used for immunohistochemistry were tested for specificity by preabsorption of the primary antibody, with blocking peptides provided by the manufacturer as well as primary omission of the antibody as a negative control. Because certain non-N-methyl-D-aspartate glutamate receptors can be sensitive to fixation (30), it is sometimes necessary to use either a non-formaldehyde-based fixative or an antigen retrieval step (30, 31). We used an antigen retrieval step that resulted in maximal detection using these antibodies. It was not possible to distinguish between GluK2 and GluK3 expression.

FIGURE 1. Neuron loss and reactive astrocytes in the hippocampus after KA-induced SE. (A, B) Bright field images of immunostaining for the neuronal marker NeuN in an age-matched control animal (A) and 1 week after KA-induced SE (B). Prominent cell loss and disruption of cell layers are indicated by black arrows. (C, D) Optical sections (1 μm thick) of immunofluorescence staining for GFAP in the CA1 region of age-matched control (C) and increased GFAP expression in astrocytes of the CA1 region after KA-induced SE (D). Black boxes in (A) and (B) indicate regional location of images in (C) and (D). Inset, increased magnification of selected regions in (C) and (D). SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 50 μm.

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Imaging and Colocalization

Images were captured with an Olympus FV1000 confocal microscope (IX81 inverted microscope; Olympus America) using a 20×/NA 0.75 air and 60×/NA 1.20 water immersion objectives and using FluoView Software. Sections stained with DAB were imaged in bright field mode. For immunofluorescence, the stratum radiatum of CA1 and other regions of interest were initially identified using a 100W halogen lamp and

**FIGURE 2.** Antibody specificity using bright field microscopy of immunostaining with goat polyclonal KAR subunit antibodies. (A-K) Labeling with antibodies to GluK1, GluK2/3, GluK4, and GluK5 in the hippocampal CA1 region from age-matched control animals (A, D, G, J) and animals 1 week after KA-induced SE (B, E, H, K). Note the neuronal cell loss and increased immunoreactivity in cells resembling astrocytes in KA-treated animals (black arrows). Staining was abolished in KA-treated tissue sections when the primary antibody was omitted (not shown) and when the primary antibody was preadsorbed with appropriate blocking peptide (C, F, I, L). SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 50 μm.
a filter set for 4',6-diamidino-2-phenylindole to avoid selection bias. Once the region was selected, laser scanning mode was used to collect images in the z-axis from the GFAP and KAR subunit channels. Laser output, photomultiplier, gain, and offset settings were corrected to minimize saturated pixels and maximize signal with respect to noise. Once optimized, the laser settings were held constant between images acquired from control and KA-treated tissue sections of the same slide. Each fluorescent channel was excited and captured sequentially to minimize bleed-through. A minimum of 12 z-stack optical images (1 μm thick) were imaged for each subunit stain from triplicate sections from animals at 1 and 8 weeks after SE and from age-matched controls. We focused optical imaging and colocalization analysis on the CA1 region of the hippocampus, but additional regions within and outside the hippocampus were also imaged. Finally, only z-stack images collected from the center of the section were further analyzed to avoid nonspecific antibody interactions at the surfaces of the tissue sections.

Raw gray scale 16-bit images from the KAR subunit and GFAP channels were used to perform colocalization analysis. To remove bias in the analysis, an automated macro was created using ImageJ software (National Institutes of Health) that performed the following functions to each raw image: each image was first converted to 8-bit, then a rolling ball background subtraction was performed. Images were then automatically thresholded using the triangle algorithm (32), and corresponding images from each channel were processed through the “colocalization” plug-in (http://rsb.info.nih.gov/ij/plugins/colocalization.html). The area of the resulting colocalized points was measured for each optical section through the stained tissue section. These values were averaged for each tissue section and then averaged across the triplicate sections stained from each brain for each KAR subunit. Glial fibrillary acidic protein–positive hippocampal astrocytes showed little to no colocalization with any of the KAR subunits in naive control animals. The control values were found not to be statistically different from each other across all control animals measured and thus were pooled. An analysis of variance with Tukey posttest was used to find statistical significance between the colocalization areas of each KAR subunit in KA-treated animals compared with control. In colocalization images, the primary antibody omission slides were used to determine the background autofluorescence (mean +1 SD of control sections) and therefore, the level of detection for the colocalization analysis is indicated as the dotted horizontal line on bar graphs.

**Western Blotting**

Protein analysis was performed using the Bradford assay (Bio-Rad, Hercules, CA). Samples were diluted into loading buffer (final concentration, 2.25% SDS, 18% glycerol, 180 mmol/L Tris base [pH 6.8], and bromophenol blue).

### Enriched Astroglial Tissue Fraction Preparation

Enriched astroglial tissue fractions were prepared from the hippocampus using modifications of previously established methods (33–36). Briefly, the tissue was homogenized in 0.32 mol/L sucrose, buffered at pH 7.4 with Tris-HCl, using glass-Teflon tissue grinders. The homogenate was then centrifuged for 5 minutes (1,000 × g at 4°C) to remove nuclei and debris. The resulting supernatant was then gently stratified on to a discontinuous Percoll gradient (2%, 6%, 10%, and 20% vol/vol in Tris-buffered sucrose) and centrifuged for an additional 5 minutes (33,500 × g at 4°C). The layers between 2% and 6% Percoll (glial fraction) and 10% and 20% Percoll (neuronal fraction) were collected and washed by centrifugation. The pellets were resuspended in suspension buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA), supplemented with a mammalian protease arrest protease inhibitor cocktail (G-Biosciences, St. Louis, MO), and frozen (−80°C) until use.

**FIGURE 3.** Kainic acid receptor (KAR) subunit expression in pilocarpine-treated rats. (A) Neuronal GluK4 KAR subunit expression in the CA1 region of control animals shows a similar pattern to that in the KA-treated control group. (B) At 1 to 4 weeks after pilocarpine treatment (in addition to neuronal cell loss), there is GluK4 immunoreactivity in cells resembling astrocytes in the stratum radiatum of the hippocampus (black arrows). SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 50 μm.
Western blot analysis was performed as previously described (24, 37). Briefly, equal amounts of protein were loaded onto SDS-polyacrylamide gels. After electrophoresis, samples were transferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences, Waltham, MA) and blocked in StartingBlock blocking buffer (Pierce, Rockford, IL). Membranes were then probed with primary antibodies against GFAP (610565, 1:1000; BD Scientific, San Jose, CA), PSD-95 (1:1000; NeuroMab, Davis, CA), and GluK4 (ab10101, 1:1000; Abcam, Cambridge, MA) overnight and

FIGURE 4. Kainic acid receptor subunits colocalize with GFAP-positive astrocytes after KA-induced SE. Immunofluorescence staining for the GluK4 receptor subunit and GFAP in the CA1 region from a control (A) and an animal 1 week after KA-induced SE (B). Immunofluorescence staining for the GluK1 receptor subunit and GFAP in the CA1 region from a control (C) and animal 1 week after KA-induced SE (D). Images are 1-μm-thick confocal optical sections. Notice neuronal cell layer disruption and the colocalization (in white) of GFAP-positive astrocytes with KAR subunits after KA-induced SE. SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 50 μm.
then washed in Tris buffered saline with Tween (0.05% Tween-20). Membranes were then incubated for 1 hour with species-specific secondary antibodies conjugated with horse-radish peroxidase. Antigen-antibody complexes were visualized by chemiluminescence (PerkinElmer Life Sciences). Bands were quantified with an Alpha-Innotech FluorChem SP digital imager with AlphaEase software. Data are expressed as mean ± SEM, unless otherwise indicated. As a lane loading control, blots were stripped and reprobed with an anti-actin antibody (sc-1616, 1:100; Santa Cruz Biotechnology). Statistical significance was determined with Student t test for independent and unpaired samples unless otherwise indicated.

FIGURE 5. Example of colocalization analysis and automated quantification. (A) Gray scale 8-bit optical section (1 μm thick) of GFAP staining in the CA1 region from an animal 1 week after KA-induced SE that has been autothresholded using the triangle algorithm. (B) Optical section of the corresponding KAR subunit stain channel that has also been thresholded. (C) Output of the colocalization plug-in. (D) The plug-in also generates an 8-bit image of the areas of colocalization.
RESULTS

Astrogliosis and Neuronal Cell Loss in the Hippocampus After KA Treatment

The KA-treated rat is a well-established model of TLE that mimics many of the pathologic findings seen in human TLE (25, 27, 38). Specific neuronal cell loss, tissue shrinkage, and reactive astrogliosis are all recapitulated in the KA-treated rat. Neuron loss and reactive astrogliosis are seen in the hippocampus 1 week after SE induced by repeated low doses of KA (Fig. 1A–D). Consistent with previous reports from our laboratory as well as others, neuron cell loss was observed in the CA1 and CA3 cell layers, the hilar region of the dentate gyrus, and the entorhinal cortex in KA-treated animals (Fig. 1B) versus control (Fig. 1A) (26, 39). In addition to cell loss, prominent reactive astrogliosis is observed in this model (Fig. 1D). During 1 week post-SE, astrocytes display isomorphic gliosis characterized by pronounced hypertrophy and increased GFAP expression but without overt scar formation (i.e. no special orientation, palisading of processes, or gross overlap) in or around the damaged cell fields of CA1 (40, 41). Because the 1-week time point represents a period when spontaneous seizures are rarely observed (27), the cell loss and accompanying glial response are attributed to the excitotoxic cell damage resulting from the initial SE insult.

Increased Expression of KAR Subunits in Astrocytes After SE

We first determined the specificity of the KAR subunit antibodies. Tissue sections from animals at 1 week post-KA and age-matched controls were stained for each KAR subunit. Figure 2 demonstrates the antibody specificity imaged in the CA1 region of the rat hippocampus using standard avidin-biotin complex immunohistochemistry with DAB. Neuronal staining of the stratum pyramidale layer was present in naive age-matched control tissue for the GluK1, GluK2/3, GluK4, and GluK5 antibodies (Fig. 2A, D, G, J). Neuron labeling is reduced in animals at 1 week post-KA treatment most likely caused by a loss of primary cells and cell layer dispersion (Fig. 2B, E, H, K). Interestingly, we noticed GluK1, GluK2/3, GluK4, and GluK5 KAR subunit expression in cells resembling astrocytes after SE in the hippocampal CA1 region, with GluK1 being most prominent (Fig. 2B, E, H, K). Primary antibody omission (not shown) and preabsorption of the primary antibody with antibody-specific blocking peptides abolished staining (Fig. 2C, F, I, L). We next tested whether a mechanistically different but equally valid model of TLE, the pilocarpine-treated model, displayed similar results. Tissue sections from rats 1 to 4 weeks after pilocarpine treatment, and controls, were processed with DAB staining and GluK4 antibody. Figure 3 shows GluK4 staining with a similar pattern as that in the KA-treated tissue, with prominent neuronal cell layer disruption and cell loss and concomitant expression in cells that resemble astrocytes.

To confirm KAR subunit expression in astrocytes in KA-treated tissue, we performed immunofluorescence colocalization studies. Horizontal brain sections containing the hippocampus were processed for immunofluorescence using antibodies directed against GluK1, GluK2/3, GluK4, GluK5, and GFAP. Figure 4 shows example images stained for the GluK1 and GluK4 receptor subunits from control animals and animals at 1 week post-KA treatment. Optical sectioning of the signal reveals that KAR subunits colocalized with GFAP-positive astrocytes after insult (Fig. 4B, D), which does not occur in control tissue (Fig. 4A, C).

Quantification of KAR Subunit Expression in Astrocytes After KA Treatment

Panels A and B of Figure 5 show the resulting images from both GFAP and KAR channels that were thresholded using the triangle algorithm in hippocampal tissue from animals that experienced SE. A representative output image from the colocalization plug-in used is shown in Figure 5C. The plug-in superimposes 1-μm-thick 8-bit gray scale confocal images and displays the resulting areas of colocalization in white. The plug-in also generates an additional 8-bit image in which the colocalization area was measured using the Analyze Particles function within ImageJ (Fig. 5D).

Results from the colocalization analysis from control animals (n = 5) and animals at 1 week post-SE (n = 6) are shown in Figure 6. In agreement with the current literature, there seemed to be no KAR subunit expression in astrocytes in naive control adult rats, but at 1 week after SE, there was significant colocalization of KAR subunits with GFAP-positive astrocytes. GluK1 was the KAR subunit colocalized with astrocytes to the greatest extent followed by GluK4, GluK2/3, and GluK5.
KAR Expression Persists in Astrocytes 8 Weeks After KA-Induced SE

At 8 weeks post-SE (after the repeated low-dose KA model of acquired epilepsy), animals begin to express behavioral motor seizure activity. To confirm this, animals were placed into observation boxes, given access to food and water ad libitum, and video monitored for 48 hours to confirm the presence of spontaneous behavioral seizures. All animals examined displayed at least 1 spontaneous stage 4/5 behavioral seizure based on the Racine scale during the observation period. GluK1 and GluK5 continued to demonstrate significant astrocyte expression, as evidenced by colocalization with GFAP.

**FIGURE 7.** Kainic acid receptor subunit colocalization with GFAP-positive astrocytes persists 8 weeks after KA-induced SE. (A, B) Immunofluorescence staining for the GluK1 receptor subunit and GFAP in the CA1 region from a control (A) and an animal 8 weeks after KA-induced SE (B). (C, D) Staining for the GluK5 receptor subunit and GFAP in the CA1 region from a control (C) and 8 weeks after KA-induced SE (D). Images are 1-μm-thick confocal optical sections. SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 50 μm.
GFAP at this time point (Fig. 7B, D), although the overall extent of colocalization of KAR subunits at this time point was less than that observed in animals 1 week after SE. Similar to the 1-week time point, GluK1 continued to be colocalized to the greatest extent at the 8-week time point. The GluK2/3 and GluK4 subunits did not display statistically different colocalization with GFAP compared with controls at the 8-week time point. Results of analyzed sections from control animals (n = 5) and animals 8 weeks after SE (n = 5) are summarized in Figure 8. We hypothesized that, at the 8-week time point after SE (when animals begin to express spontaneous behavioral seizures), there might be a more intense glial reaction including scar formation caused by the seizure activity. However, this was not the case, and at the 8-week post-SE time point, astrocytes appear to remain isomorphic in their cytoarchitecture (Fig. 7B, D) (GFAP channel). Furthermore, analysis of the GFAP channel immunofluorescence from animals 1 and 8 weeks after SE revealed no statistically significant difference in GFAP expression area (data not shown), suggesting that the intensity of astrogliosis did not significantly change at the 8-week post-SE time point.

KAR Expression in Astrocytes Does Not Occur in All Brain Regions After SE

Kainate receptor subunits consistently colocalized with GFAP-positive astrocytes in the CA1 region of the hippocampus after SE. A qualitative assessment of GFAP-positive astrocytes in other regions of the hippocampus, including CA3, the hilus, and entorhinal cortex, also demonstrated KAR subunit expression (Fig. 9). We wanted to determine whether other GFAP-positive astrocytes in areas not thought to be involved in seizure activity also express KARs after SE. To test this, we imaged astrocytes in such alternate regions from the 1-week time point data set. Figure 10 demonstrates the results of images taken from the striatum of rats 1 week after SE (n = 6) and control animals (n = 5). Figure 10A is a representative image from a KA-treated animal demonstrating a lack of colocalization with GFAP-positive astrocytes in striatum. Interestingly, GFAP expression is increased in the striatum, suggesting reactive astrogliosis in this brain region (Fig. 10B). Colocalization analysis of the GluK1 and GluK4 subunits (the subunits shown to be expressed in the hippocampus to the highest degree in astrocytes) demonstrated a clear lack of colocalization in the striatum, however (Fig. 10C). We also

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**FIGURE 8.** Colocalization analysis for KAR subunits at 8 weeks after KA-induced SE versus control. Very little to no colocalization was seen in GFAP-positive astrocytes in naive control tissue (n = 5) and was below the experimentally derived limit of detection (dotted line, mean ±1 SD of colocalization area from combined primary antibody omission slides). For the KA-treated tissue (n = 5 for each subunit), only the GluK1 and GluK5 subunits showed statistically significant colocalization. Data are expressed as mean ± SEM. Ns, not significant. * p < 0.05, *** p < 0.001.

**FIGURE 9.** Kainic acid receptor subunits colocalize with GFAP-positive astrocytes throughout the hippocampus and surrounding cortex after KA-induced SE. (A–C) Kainic acid receptor subunit colocalization was routinely observed (but not quantified) throughout the hippocampus and surrounding cortex after KA-induced SE. Each panel are resulting colocalization analysis images demonstrating GluK4 colocalization in CA3 (A), GluK1 colocalization in the dentate gyrus (B), and GluK1 colocalization in the entorhinal cortex (C) after KA-induced SE. Kainic acid receptor subunit channel (green), GFAP channel (red), colocalized pixels (white). GCL, granule cell layer; SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 50 μm.
imaged regions in the brainstem and olfactory bulb and determined that there was no colocalization of KAR subunits with GFAP-positive astrocytes in those areas after SE (data not shown). These results suggest that KAR subunit expression in astrocytes after SE is not a universal finding throughout the brain but is restricted to certain brain regions.

**GluK4 Expression Is Increased in Glial-Enriched Fractions of Hippocampus After SE**

An alternative technique was used to confirm the KAR subunit protein expression seen in post-SE astrocytes via immunohistochemistry. We used immunoblotting on glial-enriched tissue fractions (33–36). This preparation was appealing because it allows for the rapid enrichment of adult astroglial and neuronal tissue from rat brain for use in biochemical analysis without the use of cell culturing or transgenic labeling and fluorescent activated cell sorting approaches. Figure 11A demonstrates the enrichment accomplished using the Percoll density gradient technique. Although it is not a full separation, glial tissue fractions are enriched in GFAP and relatively devoid of the neuronal marker PSD-95 compared with synaptosomal fractions of the same preparation, indicating enrichment. In our hands, the glial enrichment technique had extremely low yield, making an exhaustive Western blot analysis for all the KAR subunits not possible. When glial-enriched fractions were analyzed using a Western blot–specific anti-GluK4 antibody, there was a significant increase in receptor expression in KA-treated tissue 1 week post-SE (n = 4) versus control (n = 4) (Fig. 11B). Thus, using an alternate technique, this result also suggests that KAR subunits are increased in astrocytes after SE.

**DISCUSSION**

In this study, we used dual-labeling immunohistochemistry and glial-enriched Western blot techniques to demonstrate that KAR subunit expression increases in astrocytes after KA-induced SE, a model of TLE. Indeed, at 1 week after SE, we observed significant increases in the GluK1, GluK2/3, GluK4, and GluK5 KAR subunits in GFAP-positive astrocytes in the hippocampal CA1 region. Furthermore, GluK1 and GluK5 KAR subunit expression was also evident 8 weeks after SE—when animals exhibited spontaneous behavioral seizures. We further identified the increased expression of the GluK4 receptor subunit in glial-enriched hippocampal tissue fractions using Western blotting in tissue 1 week after SE. Finally, increased KAR expression in astrocytes was also observed in

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**FIGURE 10.** Kainic acid receptor subunit colocalization with GFAP-positive astrocytes after KA-induced SE is not observed in all brain regions. The KAR subunits that showed the greatest colocalization in the hippocampal CA1 region 1 week after SE (GluK1 and GluK4) were analyzed for colocalization in alternate brain regions. (A) Example image of staining for the GluK4 receptor subunit in the striatum of an animal 1 week after KA-induced SE. (B) In this region, GFAP expression is significantly increased in animals 1 week after KA-induced SE (n = 6) compared with that in the control (n = 5). (C) Although GFAP expression is increased, the resulting colocalization analysis of the GluK1 and GluK4 subunits showed lack of colocalization 1 week after KA-induced SE (n = 6) versus control (n = 5) in the striatum. Lack of colocalization was also noted in the brain stem and olfactory bulb of KA-treated animals (data not shown). Dotted line, mean ±1 SD of colocalization area from combined primary antibody omission slide. NS, not significant. * p < 0.05. Scale bar = 50 μm.
of Na+-permeable ionotropic KARs in astrocytes might serve substrates to surrounding neurons (44). Therefore, activation of astrocyte has been shown to provide important metabolic support (43). Through the astrocyte-to-neuron lactate shuttle, inward rectifying potassium channel Kir 4.1, and gap junction protein connexin 43, along with increased levels of the KAR subunits GluK4 and GluK5 (57). However, this study could not distinguish which cell types might be contributing to the increased KAR subunit expression levels observed. Our present findings suggest that astrocytic expression could contribute to the observed increase in KARs. Indeed, in the present experiments, GluK5 expression was shown to remain significantly elevated in astrocytes at the 8-week time point after KA treatment when animals became epileptic.

The neuronal expression pattern of KAR subunits in this study conforms well to previously reported observations. The mRNA message for the different KAR subunits is strongly detectable for GluK2 and GluK5 in the CA1 region of the adult rat hippocampus, whereas GluK1, GluK3, and GluK4 expression is lower but still detectable (58, 59). In our hands, using immunohistochemistry with DAB (a more sensitive staining method than immunofluorescence), we found consistent labeling of the CA1 pyramidal cell layer with the different KAR subunit antibodies used. However, in immunofluorescence staining, pyramidal cell labeling for GluK1 was often very low, but detectable, similar to mRNA expression reports (58, 59). We attempted to stain for GluK2 using similar polyclonal goat antibodies but did not find expression in the CA1 region (data not shown). Conversely, we detected positive labeling using the GluK3 antibody; however, the expression of GluK3 mRNA is primarily restricted to dentate gyrus granule cells and is only weakly expressed in CA1 (58). Significant cross-reactivity of GluK2 and GluK3 antibodies has been documented previously in the literature, and by using KAR subunit–specific knockout animals, it was shown that GluK2 expression most likely accounts for labeling in the CA1 cell fields using GluK2/3 antibodies (60). Therefore, in the present study, it was not possible to distinguish between GluK2 and GluK3 expression. In both DAB and immunofluorescence experiments, we saw consistent pyramidal cell layer expression of GluK4. Although the mRNA message for GluK4 is low in CA1, others reports have demonstrated protein expression of GluK4 in the CA1 of mouse and rat (60, 61).

In conclusion, we have demonstrated, in an experimental model of TLE, that adult GFAP-positive hippocampal astrocytes begin to express KAR subunits after SE. In the primary cultures of astrocytes taken from the sclerotic hippocampus, as well as cortical astrocytes isolated from the epileptogenic foci of child patients with an intractable form of epilepsy, have been shown to have increased calcium signaling (55, 56). Therefore, we hypothesize that increased calcium signaling mediated in part by the pathologic expression of ionotropic KARs in reactive astrocytes could cause the synchronization of remaining neurons via the release of glutamate, thus contributing to hyperexcitability. Future experiments will determine if activation of KARs in astrocytes after KA-induced SE contributes to enhanced calcium signaling and hyperexcitability in brain regions that are known to participate in seizure generation and propagation.

A recent study conducted on human hippocampal tissue resected from patients with TLE found significant alterations in astrocyte-related proteins including GFAP, the inward rectifying potassium channel Kir 4.1, and gap junction protein connexin 43, along with increased levels of the KAR subunits GluK4 and GluK5 (57). However, this study could not distinguish which cell types might be contributing to the increased KAR subunit expression levels observed. Our present findings suggest that astrocytic expression could contribute to the observed increase in KARs. Indeed, in the present experiments, GluK5 expression was shown to remain significantly elevated in astrocytes at the 8-week time point after KA treatment when animals became epileptic.

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In conclusion, we have demonstrated, in an experimental model of TLE, that adult GFAP-positive hippocampal astrocytes begin to express KAR subunits after SE. In the

FIGURE 11. Western blot analysis of glial-enriched tissue fractions reveals KAR expression in animals 1 week after KA-induced SE. (A) Demonstration of enrichment technique. Equal amounts of protein from enriched neuronal and glial fractions from the same preparation were probed for their immunoreactivity to the neuronal marker PSD-95 and glial marker (GFAP). The glial fraction has less PSD-95 reactivity and greater GFAP reactivity versus the neuronal fraction. (B) Enriched glial fractions demonstrate increased GluK4 receptor subunit immunoreactivity in animals 1 week after KA-induced SE (n = 4) versus control animals (n = 4). Data are expressed as mean ± SEM. For sample bands, CTR, control; KAT, KA treated. * p < 0.05.
CA1 region and surrounding structures, the expression of KAR subunits in reactive astrocytes accompanies neuronal cell loss early in the 1-week time point after SE and persists 8 weeks after SE when spontaneous behavioral seizures occur. At the latter time point, KAR subunit expression is dominated by the GluK1 and GluK5 receptor subunits. Kainate receptor expression in astrocytes after SE occurs in brain regions involved in the generation of seizure, such as the hippocampus and surrounding cortex, but is absent in other brain regions, for example, striatum, even under conditions when GFAP levels are increased. The present results suggest an intriguing contribution of ionotropic glutamate receptor signaling in astrocytes but only after pathologic insult in the adult CNS. Indeed, changes in astrocyte function may contribute considerably to the process of epileptogenesis, and understanding these alterations may be key to the development of innovative therapies.

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