Loss of Perivascular Kir4.1 Potassium Channels in the Sclerotic Hippocampus of Patients With Mesial Temporal Lobe Epilepsy

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Abstract
Recent experimental data in mice have shown that the inwardly rectifying K+ channel Kir4.1 mediates K+ spatial buffering in the hippocampus. Here we used immunohistochemistry to examine the distribution of Kir4.1 in hippocampi from patients with medication-refractory temporal lobe epilepsy. The selectivity of the antibody was confirmed in mice with a glial conditional deletion of the gene encoding Kir4.1. These mice showed a complete loss of labeled cells, indicating that Kir4.1 is restricted to glia. In human cases, Kir4.1 immunoreactivity observed in cells morphologically consistent with astrocytes was significantly reduced in 12 patients with hippocampal sclerosis versus 11 patients without sclerosis and 4 normal autopsy controls. Loss of astrocytic Kir4.1 immunoreactivity was most pronounced around vessels and was restricted to gliotic areas. Loss of Kir4.1 expression was associated with loss of dystrophin and α-syntrophin, but not with loss of β-dystroglycan, suggesting partial disruption of the dystrophin-associated protein complex. The changes identified in patients with hippocampal sclerosis likely interfere with K+ homeostasis and may contribute to the epileptogenicity of the sclerotic hippocampus.

Key Words: Astrocytes, Aquaporin, Dystroglycan, Dystrophin, Hippocampal sclerosis, KCNJ10, Syntrophin, Glia.

INTRODUCTION
Temporal lobe epilepsy (TLE) is a serious chronic neurologic condition characterized by recurrent temporal lobe seizures. It is estimated to affect one third of all patients with epilepsy and thus represents a major health care issue (1). Temporal lobe epilepsy presents with a typical set of clinical characteristics, and a subgroup of these patients (~45%) is known to be particularly resistant to pharmacologic treatment (2). For many of these pharmacoresistant patients, surgical removal of the hippocampus is the only option presently available that can achieve seizure control. Hence, there is an urgent need to develop novel treatment strategies for this disabling condition.

The hippocampal pathology in TLE is distinguished into several subgroups, of which mesial TLE with hippocampal sclerosis (MTLE) is likely the most frequent type. The sclerotic hippocampus is epileptogenic, implying that it is critically involved in the initiation and maintenance of seizures in MTLE. Most patients with sclerotic hippocampi have excellent seizure control after surgical resection of the sclerotic hippocampus. One theory regarding the epileptogenicity of the sclerotic hippocampus holds that impaired K+ spatial buffering contributes to hyperexcitability and epileptogenesis. This theory has gained experimental support from electrophysiological studies that have demonstrated reduced inwardly rectifying K+ currents in astrocytes of patients with MTLE (3, 4).

We recently showed in the mouse hippocampus that deletion of the inwardly rectifying K+ channel Kir4.1 delays K+ clearance after high-frequency synaptic stimulation (5). This finding led us to hypothesize that perturbed Kir4.1 expression could underlie the electrophysiologic abnormalities previously identified in MTLE astrocytes. Here we show that patients with MTLE have a loss of perivascular Kir4.1 in the hippocampus and provide evidence suggesting that this loss is secondary to a disruption of the dystrophin-associated protein complex (DAPC) in astrocytic end-feet.

MATERIALS AND METHODS
Human Subjects and Tissue Preparation
Patients with medication-refractory TLE underwent phased presurgical evaluation at Yale-New Haven Hospital. Patients selected for surgery underwent anteromedial temporal lobectomy with en bloc resection of the hippocampus and...
adjacent structures according to the procedure developed by Spencer et al (6). The hippocampus was removed by the same neurosurgeon (Dennis D. Spencer) using the same surgical approach in all cases. Tissue samples used in the study were obtained after informed consent and with approval of the institutional human investigations committee at Yale University.

Hippocampal surgical specimens from 12 randomly selected MTLE patients and 11 TLE patients without sclerosis (non-MTLE) and tissue from 4 autopsy controls were used for this study (Tables 1, 2). The tissue has been characterized and classified previously (7). Careful neuropathologic evaluation revealed that none of the non-MTLE patients had a neoplasm involving the hippocampus. Each hippocampal tissue block (~5 mm thick) was dissected from the specimen immediately after removal from the brain. All tissue blocks were from the approximately same anteroposterior level of the hippocampus (i.e. the mid-anterior portion). Immediately after dissection, the tissue blocks were immersed in a fixative containing 4% formaldehyde and 15% (vol/vol) saturated picric acid in 0.1 mol/L phosphate buffer (PB), pH 7.4, for 1 hour. They were then transferred to 5% acrolein (Sigma Chemical Co, St Louis, MO) in PB for 3 hours. Fifty-micrometer-thick coronal sections were cut on a Vibratome and stored in a cryoprotection solution (FD Neuro Technologies, Catonsville, MD) at ~20°C until immunohistochemical labeling was performed. Random sampling of sections was performed to avoid bias and allow direct comparison. One section was selected for Nissl staining and neuropathologic evaluation, and 1 section was selected for each of the immunohistochemical labeling procedures.

**Tissue From Glial-Conditional Kir4.1**

Glial-specific conditional Kir4.1 knockout (cKir4.1−/−) mice were generated by crossing a recombinant Kir4.1 floxed line and a mouse line expressing Cre recombinase under the human glial fibrillary acidic protein promoter, as previously described (8). Young (P17–P23) cKir4.1−/− and wild-type controls were used. The experiments comply with Norwegian laws and were approved by the Animal Care and Use Committee of the Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway.

The animals were deeply anesthetized with a mixture of chloral hydrate, magnesium sulfate, and pentobarbital (142, 70, and 32 mg/kg body weight intraperitoneally, respectively) before transcardiac perfusion with 2% dextran in PB for 15 to 20 seconds (to avoid clotting) and 4% formaldehyde in PB for 10 to 15 minutes (flow rate, ~5 mL/minute). The perfused animals were kept at 4°C overnight, after which the brain was removed and stored in 0.4% formaldehyde in PB. Sections were cut by a Vibratome, as previously described.

**Immunohistochemistry**

Vibratome sections were incubated free floating in the respective antibody solutions and processed according to the avidin-biotin peroxidase (9) using the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA), with diaminobenzidine as chromogen. The immunostained sections were mounted on gelatin-coated glass slides for examination by light microscopy. The same method was used to confirm specificity of the Kir4.1 antibody in fixed mouse tissue from cKir4.1−/− and wild-type mice.

We used rabbit affinity-purified polyclonal antibodies against Kir4.1 (No. APC-035; Alomone Labs, Jerusalem, Israel; 0.8 µg/mL incubated 72 hours at 4°C), dystrophin (No. ab15277; Abcam, Cambridge, UK; 1 µg/mL incubated 24 hours at 20°C), α-syntrophin (Syn259, kindly provided by S.C. Froehner, University of Washington, Seattle [10]; 6 µg/mL incubated 24 hours at 20°C), and mouse monoclonal antibodies against β-dystroglycan (No. B-DG-CE, Novocastra, Newcastle, UK; 0.1 µg/mL incubated 72 hours at 4°C). Sections from patients with MTLE and non-MTLE and autopsy controls were immunostained simultaneously under the same incubation conditions. The sections were viewed and photographed with an Olympus BH-2 microscope equipped with a QImaging Retiga 2000R camera.

**Semiquantitative Analysis**

A semiquantitative analysis using bright field microscopy was performed to determine statistical differences in astrocytic Kir4.1 immunoreactivity between 12 MTLE and 11 non-MTLE hippocampal specimens as well as 4 autopsy controls. Nissl-stained sections were compared with anti-Kir4.1-stained sections to identify the subregions in the hippocampal formation, that is, hilus, CA3, CA2, CA1, and subiculum. Subregions were then marked at ×4 magnification with an area marking tool software (Neurolucida from Microbrightfield Inc, Burlington, VT). Counting of immunopositive astrocytes was performed at ×20 magnification in multiple randomly chosen squares within the respective subregions. The density of Kir4.1-immunopositive astrocytes was calculated to counts per square millimeter. Astrocyte counting was performed by an investigator blinded to the patient data. Mann-Whitney U test was used for statistical comparisons, and a value of p < 0.05 was considered statistically significant.

**RESULTS**

Clinical and neuropathologic data of the seizure patients are shown in Table 1. Patients categorized as MTLE displayed hippocampal sclerosis, as evidenced by a shrunken hippocampus with astrogial proliferation and neuron loss, predominantly in CA1, CA3, and the dentate hilus (7, 11, 12). Non-MTLE patients and nonepileptic autopsy controls did not have significant neuron loss or astrogliosis. The mean age at surgery was higher for MTLE versus non-MTLE cases (4.2 ± 3.6 years vs 2.8 ± 0.7 years, respectively; p = 0.048, unpaired t-test). The non-MTLE controls had on average a much shorter history of epilepsy than the MTLE cases (4.5 ± 4.5 years vs 10.7 ± 2.8 years, respectively; p = 0.007) (Table 1).

In non-MTLE hippocampi and in autopsy controls, immunocytochemical staining revealed perivascular Kir4.1 labeling and in cells with morphologic features typical of astrocytes in all hippocampal subfields (Fig. 1). Immunolabeled parenchymal and perivascular profiles were connected with the cytoplasm of nearby recognizable astrocytes, suggesting that the perivascular Kir4.1 labeling resides in astrocytic endfeet (Fig. 1).
<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Classification</th>
<th>Sex</th>
<th>Age at Surgery, Years</th>
<th>Years Since First Habitual Seizure</th>
<th>Seizure Type</th>
<th>MRI Findings</th>
<th>AEM Presurgery</th>
<th>Surgical Procedure</th>
<th>Histologic Findings</th>
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<td>M</td>
<td>57</td>
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<td>2</td>
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<td>F</td>
<td>51</td>
<td>48</td>
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<td>MTS, cerebellar atrophy</td>
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<td>45</td>
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<td>46</td>
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<td>CBZ, PRM</td>
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<td>3</td>
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<td>2</td>
<td>GTC</td>
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<td>13</td>
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<td>Frontotemporal lobe mass lesion</td>
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<tr>
<td>21</td>
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<td>F</td>
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<td>5</td>
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<td>R temporal tumor</td>
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There were regions with a substantial loss of Kir4.1 immunoreactivity in hippocampi from patients with MTLE (Fig. 1C, F, I, L). The regions with reduced Kir4.1 staining coincided with areas showing neuronal loss and gliosis. The loss was most conspicuous in area CA1 (Fig. 1I), where most vessels were almost devoid of Kir4.1 immunoreactivity. The hilus showed changes that were similar to those in CA1, albeit somewhat less extensive (Fig. 1F), whereas the subiculum, a region with no obvious neuronal loss, exhibited perivascular immunolabeling intensity to a similar extent as that observed in non-MTLE (Fig. 1K, L). Loss of perivascular labeling was associated with a reduction of the number of Kir4.1-immunolabeled cells (Table 3).

For assessment of antibody specificity, the antibody to Kir4.1 was applied to hippocampi of mice subjected to targeted disruption of the Kir4.1 gene in glial cells. Under incubation conditions identical to those used for the human material, the antibody provided distinct labeling of astrocytes and astrocytic processes in wild-type (Fig. 2A) animals but no labeling of such profiles in cKir4.1−/− animals (Fig. 2B). Weak residual staining in the tissue was considered to be nonspecific.

Changes in dystrophin labeling mimicked the changes in Kir4.1 labeling (Fig. 3A–H). Notably, perivascular labeling for dystrophin was lost once the vessels entered the gliotic areas, most pronounced in CA1 (Fig. 3F) and to a lesser degree in hilus (Fig. 3D). Vessels in the granule cell layer and subiculum showed strong dystrophin immunolabeling in both MTLE and non-MTLE hippocampi (Fig. 3G, H).

The antibody to α-syntrophin produced a labeling pattern that was strikingly similar to that found after application of the antibody to dystrophin (Fig. 4). This was true for sections obtained from MTLE patients (Fig. 4B, D, F, H) and for sections from non-MTLE patients (Fig. 4A, C, E, G).

Like α-syntrophin, β-dystroglycan is a member of the DAPC; however, β-dystroglycan immunostaining results differed from those of α-syntrophin and dystrophin. Perivascular β-dystroglycan labeling was as intense in MTLE (Fig. 5B, D, F, H) as in non-MTLE hippocampi (Fig. 5A, C, E, G).

Direct comparisons of adjacent sections from the same patient (Fig. 6A–D) further confirmed that β-dystroglycan differs from dystrophin and Kir4.1 by maintaining its expression even in the gliotic CA1 (Fig. 6).

**DISCUSSION**

Normal brain function is critically dependent on efficient mechanisms for clearance of excess K⁺ from the extracellular space. There were regions with a substantial loss of Kir4.1 immunoreactivity in hippocampi from patients with MTLE (Fig. 1C, F, I, L). The regions with reduced Kir4.1 staining coincided with areas showing neuronal loss and gliosis. The loss was most conspicuous in area CA1 (Fig. 1I), where most vessels were almost devoid of Kir4.1 immunoreactivity. The hilus showed changes that were similar to those in CA1, albeit somewhat less extensive (Fig. 1F), whereas the subiculum, a region with no obvious neuronal loss, exhibited perivascular immunolabeling intensity to a similar extent as that observed in non-MTLE (Fig. 1K, L). Loss of perivascular labeling was associated with a reduction of the number of Kir4.1-immunolabeled cells (Table 3).

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**DISCUSSION**

Normal brain function is critically dependent on efficient mechanisms for clearance of excess K⁺ from the extracellular space.
space (13). A buildup of extracellular K⁺ makes neurons hyperexcitable and may rapidly translate into epileptic seizures. Electrophysiological data indicate that MTLE may be associated with deficient K⁺ handling and have pointed to possible perturbations of K⁺ transport through the family of inwardly rectifying K⁺ channels (3, 4).

Here we provide evidence suggesting that one member of this family, Kir4.1, is lost from perivascular end-feet in hippocampi of MTLE patients. This loss occurs specifically in sclerotic hippocampi and is most pronounced in area CA1 and the hilus. The loss observed is not caused by loss of tissue or membrane integrity because β-dystroglycan, which is
normally localized in perivascular end-feet membranes (14–16), seemed to be unaffected by the sclerotic process. The loss of Kir4.1 showed a precise spatial coupling to regions of neuronal loss and gliosis. Thus, vessels seemed to lose their perivascular pool of Kir4.1 as soon as they enter the sclerotic region.

The non-MTLE patients had a shorter history of epilepsy than the MTLE patients. This was expected because, in non-MTLE patients, the underlying pathology (often an extrahippocampal tumor) commonly requires expedite surgical treatment. The intergroup difference in epilepsy duration helps

FIGURE 1. Distribution of Kir4.1 immunoreactivity in coronal sections of hippocampi from nonepileptic autopsy controls and patients with temporal lobe epilepsy (TLE). (A–L) Kir4.1 labeling in subfields of the hippocampal formation from autopsy controls (A, D, G, J), patients without hippocampal sclerosis (non-MTLE) (B, E, H, K), and patients with hippocampal sclerosis (MTLE) (C, F, I, L). In autopsy controls and non-MTLE cases, Kir4.1 immunoreactivity resides in cells with morphology typical of astrocytes, including their somata (arrowheads), processes (double arrowhead in [E]), and perivascular end-feet (arrows). Hippocampi from patients with MTLE show a substantial loss of astrocytic Kir4.1 immunoreactivity in areas with neuronal loss and gliosis (F, I). Thus, the sclerotic CA1 are almost devoid of Kir4.1 labeling (I). The hilus shows changes that are somewhat less extensive than those in CA1 (F). Loss of perivascular Kir4.1 immunoreactivity is associated with reduced labeling of astrocytes. The subiculum (sub) in MTLE (L) displays a labeling pattern similar to that observed in autopsy controls (J) and non-MTLE (K). The sclerotic area in CA1 in MTLE is marked with a dashed line (C). Vertical panels (D, G, J), (E, H, K), and (F, I, L) are high-magnification fields of the respective areas in (A, B, C). Scale bars = (A–C) 1 mm; (D–I) 50 μm; (J–L) 100 μm.
contrast the MTLE patients with the control patients. Thus, non-MTLE patients represented a more “normal” pattern of Kir4.1 distribution instead of representing a different form of epilepsy. The Kir4.1 immunostaining in non-MTLE patients was similar to that of the autopsy subjects who did not have a history of epilepsy.

Our data provide some insights into mechanisms that underlie the observed loss of Kir4.1. Notably, the immunocytochemical analysis reveals that the changes in Kir4.1 distribution are associated with parallel changes in the distribution of dystrophin and α-syntrophin. The most salient explanation of this finding is that the loss of Kir4.1 is secondary to a disruption of the DAPC, of which α-syntrophin is known to be a member (17). This would imply that Kir4.1 is anchored to the DAPC in humans, as previously reported in mice (18). The changes incurred by disruption of local anchoring mechanisms may have been accentuated by alterations at the transcriptional or translational level, as indicated by reduced Kir4.1 labeling intensity in cell bodies in gliotic areas.

A reduced rate of Kir4.1 synthesis could occur as an indirect effect of neuronal loss, through a reduced demand for K⁺ buffering. This demand is difficult to assess because it would depend not only on the number of surviving neurons but also on their physiologic activity. Thus, we cannot determine what comes first—neuronal death (leading to a reduced demand for K⁺ buffering and a downregulation of this) or Kir4.1 loss (leading to perturbed K⁺ clearance and secondarily to neuronal death). The occurrence of the latter sequence of events is evident in mice in which a primary loss of Kir4.1 causes a severe epilepsy phenotype (8) that is very similar to that seen in humans with mutations in the corresponding gene (19, 20). Moreover, single nucleotide polymorphisms in the human Kir4.1 gene (KCNJ10) are associated with temporal lobe epilepsy (21). Although the precise mechanisms remain to be established, the redistribution of Kir4.1 in hippocampi of MTLE patients is of considerable interest because this redistribution may be an important contributing factor to epileptogenesis.

Our finding complements previous electrophysiologic observations in MTLE hippocampi that have pointed to deficiencies in K⁺ homeostasis. Recent data from our laboratory clearly show that deletion of Kir4.1 in mice delays K⁺ clearance and interferes with K⁺ spatial buffering (5). Taken together, the available data suggest that the perivascular loss of Kir4.1 presently observed could be a crucial step in the cascade of events that culminate in the development of chronic epilepsy.

Our finding that dystrophin and α-syntrophin are lost while β-dystroglycan persists is consistent with the idea that MTLE is associated with activation of an intracellular protease that cleaves dystrophin. It is interesting in this regard that excitotoxicity has been shown to induce activation of calpain, an enzyme with known affinity for dystrophin (22, 23).

The present findings have a striking parallel in previous observations focusing on the water channel aquaporin-4 (AQP4). Electron microscopic studies in mice show that AQP4 colocalizes with Kir4.1 in perivascular end-feet membranes (16, 24). In MTLE patients, the perivascular AQP4 pool is lost in gliotic areas (25), thus mimicking the changes presently described for Kir4.1. The change observed in AQP4 distribution in MTLE can be reproduced in mice by targeted disruption of α-syntrophin (26). This adds support to the idea that disruption of the DAPC complex may underlie the present observation of a redistribution of Kir4.1 in the gliotic area of MTLE patients.

In summary, we demonstrate a redistribution of Kir4.1 in MTLE with a distinct loss of perivascular Kir4.1, as well as of perivascular α-syntrophin and dystrophin. These changes coincide with gliosis, that is, immunoreactivities for these proteins drop to very low levels around vessels in gliotic areas. The most likely explanation of these findings is that the loss of Kir4.1 is secondary to a disruption of the DAPC complex. This adds support to the idea that disruption of the DAPC complex may underlie the present observation of a redistribution of Kir4.1 in the gliotic area of MTLE patients.

Our finding that dystrophin and α-syntrophin are lost while β-dystroglycan persists is consistent with the idea that MTLE is associated with activation of an intracellular protease that cleaves dystrophin. It is interesting in this regard that excitotoxicity has been shown to induce activation of calpain, an enzyme with known affinity for dystrophin (22, 23).

The present findings have a striking parallel in previous observations focusing on the water channel aquaporin-4 (AQP4). Electron microscopic studies in mice show that AQP4 colocalizes with Kir4.1 in perivascular end-feet membranes (16, 24). In MTLE patients, the perivascular AQP4 pool is lost in gliotic areas (25), thus mimicking the changes presently described for Kir4.1. The change observed in AQP4 distribution in MTLE can be reproduced in mice by targeted disruption of α-syntrophin (26). This adds support to the idea that disruption of the DAPC complex may underlie the present observation of a redistribution of Kir4.1 in the gliotic area of MTLE patients.

In summary, we demonstrate a redistribution of Kir4.1 in MTLE with a distinct loss of perivascular Kir4.1, as well as of perivascular α-syntrophin and dystrophin. These changes coincide with gliosis, that is, immunoreactivities for these proteins drop to very low levels around vessels in gliotic areas. The most likely explanation of these findings is that the loss of Kir4.1 is secondary to a disruption of the DAPC complex. This adds support to the idea that disruption of the DAPC complex may underlie the present observation of a redistribution of Kir4.1 in the gliotic area of MTLE patients.
FIGURE 3. Distribution of dystrophin immunoreactivity in coronal sections of the hippocampus in patients with temporal lobe epilepsy (TLE) with hippocampal sclerosis (MTLE) and TLE patients without sclerosis (non-MTLE). (A–H) In non-MTLE cases (A, C, E, G), there is strong perivascular dystrophin immunoreactivity (arrows) in all subfields of the hippocampal formation. Astrocytic somata (arrowheads) and non-end-foot processes (double arrowheads) are less intensely labeled. In MTLE hippocampal formations (B, D, F, H), perivascular dystrophin immunoreactivity is markedly reduced in sclerotic regions, particularly in CA1 (F). In these subfields, dystrophin immunoreactivity is diffuse throughout the neuropil without demarcation of vessels and astrocytes. Subfields CA2 (B) and subiculum (sub) (H) exhibit a staining pattern similar to that of non-MTLE (A, G). The sclerotic area in CA1 in MTLE (B) is marked with a dashed line. Scale bars = (A, B) 1 mm; (C–F) 500 μm; (G, H, and inset) 50 μm.
FIGURE 4. Distribution of α-syntrophin immunoreactivity in coronal sections of the hippocampus in a patient with temporal lobe epilepsy (TLE) with hippocampal sclerosis (MTLE) and a TLE patient without sclerosis (non-MTLE). (A–H) In non-MTLE patients (A, C, E, G), there is distinct perivascular α-syntrophin immunoreactivity (arrows) and somewhat weaker labeling of astrocytic somata (arrowheads) in all subfields of the hippocampal formation. In MTLE patients (B, D, F, H), perivascular α-syntrophin labeling is markedly reduced in sclerotic areas, particularly in CA1 (F), but also in the dentate hilus (D) and in CA3. In these subfields, the staining is very weak, without demarcation of vessels or astrocytes. Subfields CA2 (B) and subiculum (sub) (H) display a similar labeling pattern to that in non-MTLE cases (A, G). The sclerotic area in CA1 in MTLE is marked with a dashed line. Scale bars = (A, B) 1 mm; (C, D) 500 μm; (E–H) 50 μm.
FIGURE 5. Distribution of β-dystroglycan immunoreactivity in coronal hippocampus sections from representative patients with temporal lobe epilepsy (TLE) with hippocampal sclerosis (MTLE) and TLE patients without sclerosis (non-MTLE). (A–H) In both non-MTLE (A, C, E, G) and MTLE patients (B, D, F, H), there is strong perivascular β-dystroglycan immunoreactivity (arrows) in all subfields of the hippocampal formation. Astrocytic somata show no β-dystroglycan labeling. The labeling pattern is indistinguishable between the 2 categories of patients. Sub, subiculum. Scale bars = (A, B) 1 mm; (C–H) 200 μm.
Kir4.1 occurs from glial end-feet membranes and that this loss is secondary to disruption of the DAPC. The changes observed are likely to interfere with K+ homeostasis and may contribute to the epileptogenic properties of the sclerotic hippocampus.

ACKNOWLEDGMENTS
The glial-conditional Kir4.1−/− mice were kindly provided by Ken D. McCarthy, University of North Carolina at Chapel Hill, Chapel Hill, NC. The authors thank Ilona Kovacs, Yale University School of Medicine, New Haven, CT, and Carina Knutsen and P. Johannes Helm of the Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway, for excellent technical assistance and Dr. Jung H. Kim, Yale University School of Medicine, New Haven, CT, for helpful comments on the article.

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