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Research article

Regulation of the cell surface expression of chloride transporters during epileptogenesis

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HIGHLIGHTS

- Differential alterations in the plasma membrane expression of cation-chloride transporters were observed during epileptogenesis.
- A shift in the cell surface expression of KCC2 and NKCC1 was detected during the early phase of epileptogenesis.
- No change in the cell surface expression of KCC2 or NKCC1 was detected at the chronic stage.
- Hyperexcitability during epileptogenesis may be a cumulative effect of multiple deficits impacting different aspects of GABAergic neurotransmission.

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ABSTRACT

The process is commonly known as epileptogenesis refers to the cascade of molecular and cellular changes that transform the brain to make it hyperexcitable and capable of generate recurrent spontaneous seizures. Unfortunately, our understanding of the molecular changes that affect the brain during epileptogenesis remains incomplete. Recent evidence suggests that dysfunction of cation-chloride transporters (CCCs) might be one of the factors that contribute to the deficits in inhibitory neurotransmission observed during epileptogenesis. This study analyzed the cell surface expression of CCCs during epileptogenesis and during chronic epilepsy to evaluate if a loss of CCCs from the plasma membrane might contribute to hyperexcitability. Alterations in the plasma membrane expression of CCCs were mostly detected during the early phase of the epileptogenic period, suggesting that dysfunction of CCCs might contribute to the alterations in the chloride gradient previously detected. Together, the findings presented here suggest that aberrant regulation of the plasma membrane levels of CCCs might contribute to the impartment of GABAergic neurotransmission and that CCCs dysfunction might be relevant for the initial appearance of spontaneous seizures.

1. Introduction

In humans, occurrence of a severe head injury, prolonged febrile seizures, stroke or status epilepticus (SE) may precede the appearance of overt spontaneous seizures by months or years [19]. In animal models, an experimental brain injury is followed by a latent period with no overt seizure activity yet a number of pathophysiological and structural alterations have been described [8,25,26]. The myriad of cellular and molecular events leading to formation of hyperexcitable networks and the manifestation of overt spontaneous seizures is, as a whole, known as epileptogenesis [8,11,25,26]. In rodents, experimental SE promotes a transient decrease in GABAergic drive that coincides with the appearance of spontaneous seizures [10]. The deficit in GABAergic neurotransmission and the increase in neuronal excitability detected during the early phase of epileptogenesis appear to result, at least in part, from a lack of functional $GABA_A$ receptors ($GABA_AR$) [9,11,18].

The generation of "classical" postsynaptic inhibition through $GABA_AR$ activation requires the maintenance of low intracellular chloride concentrations that is mostly set by cation-chloride cotransporters (CCCs) [2,17]. In mature neurons, the potassium-chloride cotransporter 2 (KCC2) is the main chloride extruder maintaining intracellular chloride concentrations below electrochemical equilibrium while the opposite effect, intracellular chloride accumulation, is facilitated by the sodium-potassium-

Abbreviations: CCCs, cation-chloride transporters; $GABA_AR$, $GABA_A$ receptors; SE, status epilepticus; CA1, cornus ammonis 1; DG, dentate gyrus; aCSF, artificial cerebrospinal fluid; sulfo-NH-SS-biotin, sulfosuccinimidyl-2-[biotinamido]ethyl-1,3-dithiopropionate; PBS, phosphate buffered saline.

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chloride cotransporter 1 (NKCC1) [3,17]. In central neurons from adult hippocampus and cortex KCC2 activity governs the chloride gradient and loss of KCC2 from the plasma membrane promotes a reversal in the chloride gradient and reversal of $GABA_AR$ potential (E_{GABA}) [17]. However, it is also possible that subtle changes in NKCC1 plasma membrane expression might exert a dominant effect on the chloride gradient and change E_{GABA} [20]. Some evidence that alterations in CCCs expression might heavily contribute to abnormal synchronization and hyperexcitability during epileptogenesis has been observed in adult rats following pilocarpine-induced SE [1,23]. Unfortunately, the full extent of the molecular remodeling affecting GABAergic neurotransmission and its contribution to epileptogenesis is not fully understood.

In previous independent studies, we analyzed the cell surface expression of $GABA_AR$ during the "latent" and "chronic" period of epilepsy and discovered deficits on the cell surface expression of $GABA_AR$ subunits [12,13]. Here, the cell surface expression of KCC2 and NKCC1 was analyzed in tissue obtained during the epileptogenic period and in tissue obtained from chronically epileptic animals enduring both frequent and infrequent seizures. The results obtained show a prominent shift in the cell surface expression of KCC2 and NKCC1 during the early phase of epileptogenesis, with little to no change observed during the chronic stage. The results presented here suggest that hyperexcitability during epileptogenesis may be a cumulative effect of multiple deficits impacting GABAergic neurotransmission.

2. Materials and methods

The methods described here are similar to those described elsewhere, for further details please refer to our previous reports [12,13]. Samples were isolated from animals described in the above-mentioned studies and repurposed for the analysis of KCC2 and NKCC1 immunoreactivity.

2.1. Induction of status epilepticus

Adult male Sprague Dawley rats (Charles River, Wilmington, MA) were housed in a temperature-controlled vivarium with food and water *ad libitum*. Rats were injected intraperitoneally with scopolamine methyl nitrate (1 mg/kg) 30 min before administration of pilocarpine hydrochloride (385 mg/kg), as previously described [4,12,13,27]. Diazepam (6 mg/kg; Hospira, Lake Forest, IL) was administered to stop seizure progression. Control rats were handled similarly but treated with a subconvulsive dose of pilocarpine (1/10 of the full dose, 38.5 mg/kg) and a reduced dose of diazepam (1/10 of the full dose, 0.6 mg/kg). Animal procedures were performed in accordance with Institutional Animal Care and Use Committee regulations and approved protocols by the University of Colorado Anschutz Medical Campus.

2.2. EEG acquisition and analysis

Rats were implanted with EEG electrodes as previously described [13]. Following recovery, rats were placed in a recording chamber equipped with flexible cables attached to a commutator (*i.e.*, electric swivel) and recorded 24 h/day using an automatic Pinnacle digital video-EEG system. Tissue samples were collected either $\leq$3 h from the last seizure or $\geq$24 h after the last seizure. Thus, tissue was collected only if seizures were observed during the previous 3 h (frequent/recent seizures group), or if no seizures were detected in the last 24 h (infrequent seizures group) [13].

2.3. Biotinylation procedure

The protocol used was a modification of methods previously reported [14,15]. Briefly, to label plasma membrane proteins, hippocampal slices were bathed in aCSF containing 1 mg/ml sulfo-NH-SS-biotin (Thermo Scientific, Rockford, IL). Slices were rinsed in aCSF containing 100 mM glycine (quenching buffer) to eliminate unreacted biotin. Hippocampal regions of interest were isolated by microdissection. Tissue was lysed in RIPA buffer containing protease and phosphatase inhibitors. An aliquot of cleared lysate was mixed with a half volume of Laemmli buffer and labeled as "lysate fraction". Biotinylated proteins were batch extracted using Ultralink avidin-conjugated beads (Thermo Scientific, Rockford, IL). Proteins in the biotinylated fraction were diluted to the same extent than proteins in the total lysate, so that immunoreactivity in the lysate and biotinylated fractions is proportional when equal volumes of lysate and biotinylated fraction are analyzed.

2.4. Western blot

Protein samples were separated in SDS-polyacrylamide gels, transferred to nitrocellulose membranes and incubated with primary and secondary antibodies. Polyclonal rabbit antibodies for KCC2 were obtained from Millipore (Billerica, MA). A monoclonal mouse antibody to detect NKCC (T4) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Department of Biology, Iowa City, IA). This monoclonal antibody (T4) was generated against a fusion protein that encompassed the 310 C-terminal residues of human NKCC [21,22]. Since the C-terminus of NKCC1 and NKCC2 share more than a 90% of identity, the T4 antibody recognizes both polypeptides. Because NKCC2 is not expressed in the vertebrate brain, the immunoreactivity detected by T4 antibody in brain samples corresponds to NKCC1 [22]. To estimate potential variability in protein content and loading, blots were also probed with an anti-actin antibody from Sigma (St. Louis, MO). Anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase were from GE Health Care (Piscataway, NJ) or Jackson Immunoresearch laboratories (West Grove, PA), respectively. Immunoreactive bands were visualized using Super Signal West Dura chemiluminiscent substrate (Pierce, Rockford, IL, USA) and film. After scanning the films, immunoreactive bands of the appropriate size were quantified using Image J (NIH, Bethesda, MD, USA). Immunoreactivity for the bands of interest was normalized to actin immunoreactivity and compared to control values. Data is presented as the mean $\pm$ SEM.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad InStat (GraphPad Software, Inc., San Diego, CA, USA). Differences between groups were determined by one-way analysis de variance (ANOVA) followed by Bonferroni *post hoc* test. For these analyses, p values < 0.05 were considered significant.

3. Results

To characterize the effects of SE on CCCs, the cell surface expression of KCC2 and NKCC1 was analyzed in hippocampal tissue collected at different time points after induction of SE. Western blot analysis of samples from the CA1 region of hippocampus obtained 1-day post-SE showed reduced KCC2 immunoreactivity in the biotinylated (cell surface) fraction (Fig. 1). KCC2 loss was also evident in the lysate and intracellular (non-biotinylated) fractions (Fig. 1), suggesting that after internalization there is a net loss of KCC2 expression. Blots were also probed with antibodies directed against NKCC transporters (T4 mAb) and a strong immunoreactive