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Impaired Astrocytic Gap Junction Coupling and Potassium Buffering in a Mouse Model of Tuberous Sclerosis Complex

Lin Xu, Ling-Hui Zeng, and Michael Wong

Department of Neurology and the Hope Center for Neurological Disorders, Washington University School of Medicine, St. Louis, MO 63110

Abstract

Abnormalities in astrocytes occur in the brains of patients with Tuberous Sclerosis Complex (TSC) and may contribute to the pathogenesis of neurological dysfunction in this disease. Here, we report that knock-out mice with *Tsc1* gene inactivation in glia (*Tsc1*^{GFAP}CKO mice) exhibit decreased expression of the astrocytic connexin protein, Cx43, and an associated impairment in gap junction coupling between astrocytes. Correspondingly, hippocampal slices from *Tsc1*^{GFAP}CKO mice have increased extracellular potassium concentration in response to stimulation. This impaired potassium buffering can be attributed to abnormal gap junction coupling, as a gap junction inhibitor elicits an additional increase in potassium concentration in control, but not *Tsc1*^{GFAP}CKO slices. Furthermore, treatment with a mammalian target of rapamycin inhibitor reverses the deficient Cx43 expression and impaired potassium buffering. These findings suggest that *Tsc1* inactivation in astrocytes causes defects in astrocytic gap junction coupling and potassium clearance, which may contribute to epilepsy in *Tsc1*^{GFAP}CKO mice.

Keywords

tuberous sclerosis; epilepsy; seizures; astrocyte; glia; connexin; potassium

Introduction

Tuberous sclerosis complex (TSC) is a multisystem genetic disease caused by mutation of either the *TSC1* or *TSC2* gene and typically featuring disabling neurological symptoms, such as mental retardation, autism, and seizures (Kwiatkowski, 2003; Crino et al., 2006; Holmes et al., 2007). Epilepsy in TSC is particularly severe and refractory to available treatments (Sparagana et al., 2003; Holmes et al., 2007). The pathological hallmark of brains from TSC patients are areas of disrupted cortical lamination, termed tubers, which may represent the epileptogenic foci for seizures. Neuropathological studies demonstrate abnormalities in astrocytes in cortical tubers, such as astrocytosis and abnormally differentiated giant cells with glial, as well as neuronal, features (Crino, 2004; Sosunov et al., 2008; Talos et al., 2008), suggesting that astrocyte dysfunction may be centrally involved in epileptogenesis and other neurological deficits in TSC.

Corresponding Author: Michael Wong, MD, PhD, Department of Neurology, Box 8111, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, Phone: 314-362-8713, Fax: 314-362-9462, Email: wong_m@wustl.edu.

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Recent studies have demonstrated novel physiological roles of glial cells in regulating neuronal excitability and preventing epilepsy (Binder and Steinhauser, 2006; Jabs et al., 2008; Wetherington et al., 2008). For example, astrocytes maintain normal homeostasis of excitatory substances, such as extracellular potassium. The immediate uptake of extracellular potassium is at least partially dependent on specific potassium channels and sodium-potassium pumps of astrocytes (Haglund and Schwartzkroin, 1990; Xiong and Stringer, 2000; D'Ambrosio et al., 2002). Furthermore, potassium homeostasis is also regulated by spatial buffering via astrocyte networks, in which potassium released at sites of high concentration is redistributed to distant sites of lower potassium concentration (Kofuji and Newman, 2004). This spatial buffering of potassium involves coupling of astrocytes through gap junctions. Studies of both animal models and human epilepsy demonstrate a variety of abnormalities in potassium buffering mechanisms of astrocytes that may predispose to neuronal hyperexcitability and seizures (Janigro et al., 1997; Bordey and Sontheimer, 1998; Gabriel et al., 1998a,b; Xiong and Stringer, 1999; Hinterkeuser et al., 2000). Changes in expression of astrocytic gap junction proteins have been reported in different epileptic animal models and human brain specimens (Naus et al., 1991; Elisevich et al., 1997; Sohl et al., 2000; Aronica et al., 2001; Fonseca et al., 2002). Furthermore, mice deficient in astrocytic gap junction proteins exhibit reduced thresholds for generating epileptiform activity (Wallraff et al., 2006), suggesting that spatial buffering mechanisms of astrocytes are important in preventing epilepsy.

Our previous work in a mouse model of TSC (*Tsc1*^{GFAP}CKO mice) has demonstrated that inactivation of the *Tsc1* gene in glia results in the development of seizures (Uhlmann et al., 2002). This epilepsy phenotype is associated with increased neuronal excitability in response to elevated extracellular potassium and decreased expression and function of astrocytic potassium channels (Jansen et al., 2005). In the present study, we tested the hypothesis that *Tsc1*-deficient astrocytes exhibit impaired gap junction coupling and spatial potassium buffering that may contribute to epileptogenesis in *Tsc1*^{GFAP}CKO mice.

Materials and Methods

Animals

All experiments were conducted according to an animal protocol approved by the Washington University Animal Studies Committee. *Tsc1*^{flox/flox}-GFAP-Cre knock-out (*Tsc1*^{GFAP}CKO) mice with conditional inactivation of the *Tsc1* gene in GFAP-positive cells starting around embryonic day 14.5 were generated as described previously (Uhlmann et al., 2002). *Tsc1*^{flox/+}-GFAP-Cre and *Tsc1*^{flox/flox} littermates, which have been shown to have normal phenotypes, were used as control animals in these experiments.

Western blot and immunohistochemical analysis of connexin expression

Western blotting was performed on 4–5 week old *Tsc1*^{GFAP}CKO mice and control mice to detect expression of astrocyte specific gap junction proteins, Connexin 43, 30, and 26, as well as the neuronal specific Connexin 36, using standard methods as described previously (Zeng et al., 2008). In additional experiments, 2–3 week old mice were used to assay Connexin 43 expression at a younger age that should precede seizure onset in these mice (Erbayat-Altay et al., 2007). In brief, neocortex and hippocampus were dissected, sonicated, and centrifuged. Equal amounts of total protein extract were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After incubating with primary antibodies specific to Cx43 (1:1000; ZYMED laboratories, San Francisco, CA), Cx26 (1:250; ZYMED laboratories), Cx30 (1:250; ZYMED laboratories), Cx36 (1:250; ZYMED laboratories), and actin (1:5,000; Sigma), the membranes were reacted with a peroxidase-conjugated secondary antibody. Signals were detected by using enzyme chemiluminescence reagent (Pierce, Rockford, IL) and quantitatively analyzed with ImageJ

software. In a separate set of experiments, rapamycin (3mg/kg/d, i.p), or vehicle, was given to 2 week old *Tsc1*^{GFAP}CKO mice for 3 weeks to inhibit mammalian target of rapamycin (mTOR) pathway activation (Zeng et al., 2008), and Western blotting was then performed to assess the expression of Cx43, as described above. In other experiments, immunohistochemistry for connexin 43 labeling was performed on hippocampal sections of 4–5 week old *Tsc1*^{GFAP}CKO and control mice, using connexin 43 antibody (1:100, Zymed); general methods for processing tissue are described below.

Hippocampal slice preparation and whole-cell recording

Horizontal entorhinal cortex–hippocampal slices were prepared from 2 to 3 week old control or *Tsc1*^{GFAP}CKO mice, using standard methods as described previously (Wong and Yamada, 2000). In brief, horizontal slices (400 μ m) were cut with a vibratome, while submerged in ice-cold, oxygenated (95% O₂/5% CO₂) solution containing (in mM): 87 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 7 MgCl₂, 0.5 CaCl₂, 25 NaHCO₃, 25 glucose, and 75 sucrose. Slices were maintained at room temperature for at least 1 hour before use in an oxygenated (95% O₂/5% CO₂) artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 2CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, pH 7.4. Slices were then transferred to a submerged recording chamber and perfused with oxygenated aCSF at ~32°C. Astrocytes in the stratum radiatum of the CA1 region were directly visualized with infrared differential interference contrast (IR-DIC) optics on a Nikon upright microscope. Astrocytes were identified by their characteristic morphology, location within stratum radiatum, negative resting membrane potentials (less than –70 mV), and lack of voltage-activated sodium currents. Furthermore, only cells with time- and voltage-independent currents responses were included for further study (GluT cells from Matthias et al., 2003; Wallraff et al., 2004; linear cells from D'Ambrosio et al., 1998), as these represent the subtype of astrocytes that exhibit gap junction coupling (Wallraff et al., 2004). Under whole-cell voltage clamp mode, electrical signals were acquired through a patch pipette, processed with an Axopatch-200B amplifier, digitized at 10 kHz, and stored and analyzed with pCLAMP software (Molecular Devices, Sunnyvale, CA).

Immunohistochemical assays of gap junction coupling and astrocyte density

To assay astrocyte coupling, single astrocytes were filled with biocytin through the patch pipette. Patch pipettes (4–7 M Ω) were filled with an internal solution containing the following (in mM): 140 K-gluconate, 4 NaCl, 1 CaCl₂, 10 HEPES, 10 EGTA, and 2 Mg-ATP. In addition, biocytin (*N* ϵ -biotinyl-L-lysine; Sigma, St. Louis, MO) was dissolved at 0.5–0.6% in the internal solution. After obtaining whole-cell mode, the biocytin was allowed to diffuse into the cell for exactly 20 minutes. Current-voltage plots were obtained and input resistance was monitored – any cells showing a greater than 20% change in resistance during the 20 minute recording period were discarded. Only one cell per slice was injected with biocytin. After the 20 minute labeling period, slices were removed from the recording chamber and immersion-fixed in a solution of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4, for 4–12 hr at 4°C. The slices were rinsed in 0.1 M PB and then infiltrated with 10% sucrose in 0.1 M PB for 1hr, followed by 30% sucrose for 8–12 hr. For further analysis, the 400 μ m slices were then sectioned into 40 μ m-thick sections using a microtome. Sections were rinsed in 0.1 M PB and then in 0.1 M Tris-HCl buffer (TB), pH 7.4. The sections were then incubated with 1% H₂O₂ in 0.1 M TB for 2hr to suppress endogenous peroxidase. Sections were then treated with 2% bovine serum albumin (BSA; Boehringer Mannheim, Indianapolis, IN), 0.25% dimethylsulfoxide (DMSO; Sigma, St. Louis, MO), and 0.05 M Tris-buffered saline (TBS), pH 7.4, for 1hr to reduce nonspecific background staining and to permeabilize membranes. Sections were rinsed in 0.1 M TBS for 30min and then incubated with an Elite ABC kit (Vector Laboratories, Burlingame, CA), diluted 1:500 in 0.5% BSA, 0.25% DMSO, and 0.05 M TBS for 36–48 hr at 4°C. Sections were then rinsed thoroughly in 0.1M TBS followed by 0.1 M TB, pH 7.6, and incubated in SIGMAFAST™ DAB with metal enhancer

tablet set (D-0426, Sigma, St. Louis, MO) for 10–15 min. The reaction was stopped by rinses in 0.1 M TB, and the sections were mounted on gelatin-subbed slides and imaged with a Zeiss Axioskop microscope and AxioCamHR digital camera (Carl Zeiss MicroImaging Inc., Thornwood, NY). All DAB-positive cells were counted in all sections.

To determine astrocyte density, brains from 2- to 3-week old control and *Tsc1*^{GFAP}CKO mice were perfusion-fixed with 4% paraformaldehyde and cut into 50 μ m sections with a vibratome. Sections were labeled with GFAP antibody (rabbit anti-mouse; 1:500; Sigma, St. Louis, MO) and then anti-rabbit Alexa Fluor 488 (1:800; Invitrogen, Carlsbad, CA, and mounted in Vectashield (Vector Laboratories). Stacks of optical sections (2 μ m intervals) were acquired through the entire depth of a slice with a Zeiss LSM PASCAL confocal microscope (Zeiss, Thornwood, NY). From merged images from the stack, GFAP-immunoreactive cells in a 200 $\times$ 200 $\times$ 50 μ m region from the stratum radiatum of hippocampus were counted from four sections per mouse from a total of four mice per group, to calculate astrocyte density.

Potassium concentration measurements with potassium-selective microelectrodes

Hippocampal slices (400 μ m) were prepared with a vibratome from control or *Tsc1*^{GFAP}CKO mice, placed in a submerged recording chamber, and perfused with oxygenated (95% O₂/5% CO₂) aCSF at a rate of ~2 ml/min at ~32°C, as described above. To block synaptic transmission, 50 μ M D,L-APV, 10 μ M CNQX, and 100 μ M bicuculline were also added to the aCSF, and slices were equilibrated for 1 h. Double-barreled glass microelectrodes were constructed to allow simultaneous recording of potassium concentration and extracellular field potentials, as previously described (Meeks and Mennerick, 2007). In brief, theta glass capillaries (1.5mm, Sutter Instrument, Novato, CA) were pulled to a tip diameter of 1–5 μ m with a horizontal puller. One barrel was silanized with Sigmacote (Sigma, St. Louis, MO) vapor. Immediately after silanization, the capillaries were baked at 120°C for 15–30 minutes. To create a potassium ion-sensitive barrel, the silanized barrel was tip filled with the K⁺-selective ionophore IE-190 (World Precision Instruments, Sarasota, FL) and then back-filled with 500 mM KCl. The non-silanized reference barrel was back-filled with 140 mM NaCl. The recording electrode was placed between the stratum pyramidale and stratum oriens in the CA1 region at a depth of approximately 100 μ m. Voltage signals from both barrels were recorded with an Axoclamp 2B amplifier in bridge mode and pClamp software. The signal from the reference barrel was subtracted from the signal from the potassium-selective barrel, and the resulting signal was correlated with the subsequent calibration curves to calculate potassium concentration (see below). The signal from the reference barrel was also used to measure extracellular field potentials in response to stimulation. A bipolar stimulation electrode was placed in the alveus of the CA1 region near the subiculum to trigger potassium release from antidromic action potentials by electrical stimulation, using trains of stimuli (0.1 ms pulses, 20 Hz for 10 s). After obtaining an input-output response to a range of stimulation intensities, the amplitude of stimulation was adjusted to evoke population spike amplitudes at ~50% and 100% of maximal response to produce comparable stimulation intensities between different experiments. After recording, the electrode was calibrated in the standard aCSF containing varying K⁺ concentrations (2.5, 4, 10, 25, and 50 mM). A log-linear fit to the [K⁺]₀ responses was used to calibrate the electrodes and calculate [K⁺]₀. Data from an experiment were not used for this study unless the electrodes displayed voltage changes of at least 40 mV per ten-fold increase in [K⁺]₀. The maximal increase in [K⁺]₀ following stimulation and the subsequent decay time ($t_{1/2}$ – time for [K⁺]₀ to decay to half the maximum value; τ – a weighted time constant from a two exponential fit) was calculated for each experiment, using pCLAMP software. To control for variability in the stimulation and neuronal activation of potassium release, the absolute rise in potassium concentration was normalized to the population spike amplitude (measured from base to peak) at each level of stimulation. In addition to comparing these parameters between control and *Tsc1*^{GFAP}CKO mice, to investigate if spatial buffering through gap junctions

contributed to extracellular potassium concentration, we also compared the $[K^+]_0$ changes in the hippocampal slices before and after perfusion with the gap junction inhibitor carbenoxolone (100 μ M).

Statistics

Data are expressed as mean values $\pm$ SEM. Student's two-tailed *t* test was used for quantitative comparisons between two groups and ANOVA for comparisons for more than two groups, with Tukey multiple comparisons post-tests. Statistical significance was defined as $p < 0.05$.

Results

***Tsc1*^{GFAP}CKO mice have an mTOR-dependent decrease in expression of the astrocytic gap junction protein, Connexin 43**

Connexin 43 (Cx43) is the predominant gap junction protein expressed in astrocytes and involved in astrocytic coupling, although other connexins (Cx26, Cx30) in astrocytes have also been reported (Nagy and Rash, 2000). By using western blotting, we found that *Tsc1*^{GFAP}CKO mice have decreased Cx43 expression in both neocortex and hippocampus compared with control mice (Figs. 1A and C). In contrast, expression of Cx36, a major neuronal-specific gap junction protein, appeared to be slightly increased in *Tsc1*^{GFAP}CKO mice, although this was not statistically significant (Figs. 1B and D). Furthermore, there were no significant differences in the astrocytic gap junction proteins, Cx30 and Cx26, between *Tsc1*^{GFAP}CKO and control mice (data not shown). Immunohistochemical studies also confirmed a decrease in Connexin 43 expression in *Tsc1*^{GFAP}CKO mice (Supplemental Fig. 1A).

Although video-EEG was not directly performed in these studies, the abnormal Cx43 expression was correlated to the onset and evolution of epilepsy in *Tsc1*^{GFAP}CKO mice as documented in detail in previous video-EEG studies (Erbayat-Altay et al., 2007). The reduction in Cx43 expression was observed in *Tsc1*^{GFAP}CKO mice not only at 4–5 weeks old (Fig. 1), which is around the age seizures start in these mice (Erbayat-Altay et al., 2007), but also at 2–3 weeks of age (Supplemental Fig. 1B), which should precede onset of seizures.

In an attempt to identify a mechanistic link between *Tsc1* gene inactivation and connexin expression in astrocytes, the effect of the mTOR inhibitor, rapamycin, on Cx43 expression in *Tsc1*^{GFAP}CKO mice was tested, as the mTOR pathway can regulate protein synthesis (Sandsmark et al., 2007). Rapamycin has previously been shown to reverse the abnormal hyperactivation of the mTOR pathway in *Tsc1*^{GFAP}CKO mice (Zeng et al., 2008). Pretreatment with rapamycin prevented the decreased Cx43 expression in *Tsc1*^{GFAP}CKO mice, but had no effect on control mice (Fig. 2), indicating that the mTOR pathway does mediate the effects of *Tsc1* gene inactivation on Cx43 expression.

Astrocyte gap junction coupling is impaired in *Tsc1*^{GFAP}CKO mice

Since *Tsc1*^{GFAP}CKO mice exhibited decreased levels of the astrocytic gap junction Cx43 expression, we next examined astrocyte gap junction coupling by assessing the extent of transcellular diffusion of biocytin from single, patch-clamped astrocytes in stratum radiatum of the CA1 of hippocampal slices. With whole-cell recording, only cells with time- and voltage-independent currents responses in response to voltage steps were included for further study (see Fig. 3E), as these represent the subtype of astrocytes that exhibit gap junction coupling (Wallraff et al., 2004). There was no significant difference between *Tsc1*^{GFAP}CKO and control mice with respect to resting membrane potential and input resistance of astrocytes ($V_m = -73.2 \pm 1.5$ mV, $R_m = 2.4 \pm 0.6$ M Ω , $n = 20$ KO astrocytes; $V_m = -69.4 \pm 1.6$ mV, $R_m = 2.2 \pm 0.2$ M Ω , $n = 16$ control astrocytes). However, the number of coupled astrocytes as detected by biocytin staining was significantly less in hippocampal slices from *Tsc1*^{GFAP}CKO mice

compared to control mice (Figs. 3A, B, and F). In *Tsc1^{GFAP}CKO* mice, biocytin spread from single injected astrocytes to 118 ± 57 encircling cells ($n=20$ injections) after 20 minutes of whole-cell recording, as opposed to 243 ± 87 cells in control mice ($n=16$ injections), indicating a significant impairment of astrocytic gap junction coupling in *Tsc1^{GFAP}CKO* mice (Fig. 3F, $p<0.001$ by t-test). This decrease in astrocyte coupling in *Tsc1^{GFAP}CKO* mice was observed, despite a very similar density of GFAP-positive astrocytes between *Tsc1^{GFAP}CKO* and control mice (Fig 3C and D; $14,550 \pm 1,220$ cells/mm³, $n=4$ *Tsc1^{GFAP}CKO* mice; $14,650 \pm 1,250$ cells/mm³, $n=4$ control mice), indicating that the changes in astrocyte coupling and Cx43 expression are not related to changes in astrocyte number that develop later in *Tsc1^{GFAP}CKO* mice (Uhlmann et al., 2002).

The decrease in astrocyte coupling in the hippocampal slices from *Tsc1^{GFAP}CKO* mice did not have a completely uniform spatial laminar distribution. When comparing parallel ~ 40 μ m sub-sections of the original 400 μ m hippocampal slice, the number of biocytin-positive cells decreased in sections more distant from the injected astrocyte in both *Tsc1^{GFAP}CKO* and control mice and was consistently lower in *Tsc1^{GFAP}CKO* in all sections (Fig. 3G). However, when comparing different strata within the hippocampus, control mice exhibited an expected decrease in coupled cells in the more distal stratum lacunosum-moleculare compared to the stratum radiatum, whereas *Tsc1^{GFAP}CKO* mice had equal numbers of coupled cells in these two strata (Fig. 3H). Thus, compared to control mice, the number of coupled cells in *Tsc1^{GFAP}CKO* mice was only decreased in the stratum radiatum, not the stratum lacunosum-moleculare. Although the cause or functional consequences of these laminar-specific differences were not specifically investigated in this study, laminar differences in spatial potassium buffering have previously been reported in connexin knock-out mice (Wallraff et al., 2006) and helped target our electrode placement in the subsequent potassium concentration studies.

***Tsc1^{GFAP}CKO* mice exhibit impaired potassium buffering related to deficient astrocyte coupling**

To determine whether the decreased astrocyte gap junction coupling in *Tsc1^{GFAP}CKO* mice might affect potassium buffering properties, we measured stimulus-evoked extracellular potassium concentrations ($[K^+]_0$) in hippocampal slices from *Tsc1^{GFAP}CKO* and control mice using potassium selective electrodes. Increases in $[K^+]_0$ were elicited by alvear stimulation of CA1 pyramidal neurons with a train of stimuli (10s, 20Hz) at stimulus intensities to elicit 50% and 100% of maximal population spike amplitude. Maximal field potential amplitudes did not differ significantly between control and *Tsc1^{GFAP}CKO* mice (5.5 ± 0.5 mV, $n=14$ slices from 8 *Tsc1^{GFAP}CKO* mice; 7.0 ± 1.0 mV, $n=12$ slices from 8 control mice). In response to stimulation, an increase in $[K^+]_0$ reached a peak immediately following the end of the stimulus train and decayed over seconds (Fig. 4A). Higher stimulation intensities (to elicit 100% of the maximal population spike) induced larger increases in $[K^+]_0$ compared to lower stimulation (to elicit 50% of the maximal population spike) in both control and *Tsc1^{GFAP}CKO* mice (Fig. 4B). However, with both lower and higher stimulation levels, the peak increase in $[K^+]_0$ was significantly great in *Tsc1^{GFAP}CKO* mice compared to controls (1.05 ± 0.12 mM in 14 *Tsc1^{GFAP}CKO* slices versus 0.64 ± 0.12 mM in 12 control slices for 50% stimulation, $p<0.05$; 2.57 ± 0.17 mM in 14 *Tsc1^{GFAP}CKO* slices versus 1.25 ± 0.12 mM, in 12 control slices for 100% stimulation, $p<0.001$) (Fig. 4B). Since higher stimulation intensities cause greater activation of neurons and more potassium release, the increases in $[K^+]_0$ was normalized to field potential amplitude to evaluate effects on $[K^+]_0$ independent of the degree of neuronal activation. After normalization, there were still significant differences between *Tsc1^{GFAP}CKO* and control mice in normalized $[K^+]_0$ rises at both 50% (0.46 ± 0.06 mM/mV, $n=14$ *Tsc1^{GFAP}CKO* slices; 0.23 ± 0.05 mM/mV, $n=12$ control slices, $p<0.01$) and 100% (0.49 ± 0.04 mM/mV, $n=14$ *Tsc1^{GFAP}CKO* slices; 0.21 ± 0.03 mM/mV, $n=12$ control slices, $p<0.001$)

stimulation intensity (Fig. 4C), indicating a deficiency in the $[K^+]_0$ clearance in the *Tsc1*^{GFAP}CKO mice. While the peak increase in $[K^+]_0$ was larger in hippocampal slices from *Tsc1*^{GFAP}CKO mice, there was no significant differences in decay time in recovery of $[K^+]_0$ back to baseline following stimulation (50% stimulation: $t_{1/2} = 4.9 \pm 2.6$ s, $n=14$ *Tsc1*^{GFAP}CKO slices; $t_{1/2} = 3.5 \pm 1.6$ s, $n=12$ control slices; 100% stimulation: $t_{1/2} = 8.1 \pm 4.6$ s, $n=14$ *Tsc1*^{GFAP}CKO slices; $t_{1/2} = 7.6 \pm 3.7$ s, $n=12$ control slices).

Although the deficiency in astrocyte gap coupling could be responsible for the increased $[K^+]_0$ following stimulation in the *Tsc1*^{GFAP}CKO mice, there are other possible mechanisms contributing to potassium buffering that could also account for this difference. Thus, we used a pharmacological gap junction inhibitor, carbenoxolone, to determine the contribution of gap junctions to K^+ buffering in control and *Tsc1*^{GFAP}CKO mice. Peak increases in $[K^+]_0$ at 50% and 100% intensity stimuli were compared before and after application of 100 μ M carbenoxolone for 20 minutes. In control mice, carbenoxolone treatment resulted in a significant, additional increase in peak $[K^+]_0$ in response to both 50% and 100% stimulation in control mice that was similar to peak $[K^+]_0$ in *Tsc1*^{GFAP}CKO mice without carbenoxolone (Fig. 4D), indicating that potassium buffering through gap junctions was active in control mice. In contrast, there was no significant difference in $[K^+]_0$ rises for 50% and 100% stimulus intensities comparing before and after carbenoxolone treated in *Tsc1*^{GFAP}CKO mice (Fig. 4D), suggesting that the impaired potassium buffering in *Tsc1*^{GFAP}CKO mice is due, at least in part, to the deficient astrocyte gap junction coupling.

To provide evidence for a mechanistic link between this impaired gap junction potassium buffering, deficient Cx43 expression, and *Tsc1* gene inactivation, we again tested the effect of mTOR inhibition. Similar to the effect on Cx43 expression, rapamycin treatment (3 mg/kg/d for 3 weeks) reversed the impairment of potassium buffering in *Tsc1*^{GFAP}CKO mice (Fig. 5). With both lower and higher stimulation levels, the peak increase in $[K^+]_0$ was significantly decreased in rapamycin-treated *Tsc1*^{GFAP}CKO mice compared to untreated *Tsc1*^{GFAP}CKO mice (Fig. 5A). After normalization to population spike amplitude, there were still significant differences between untreated and rapamycin-treated *Tsc1*^{GFAP}CKO mice in normalized $[K^+]_0$ rises at both 50% and 100% stimulation intensity (Fig. 5B), indicating that the mTOR pathway does mediate the effects of *Tsc1* gene inactivation on Cx43 expression and potassium buffering.

Discussion

Recent clinical and basic science studies are beginning to provide insights into the mechanisms of neurological deficits and epileptogenesis in TSC (Holmes et al., 2007; Wong, 2008). Consistent with modern trends recognizing the importance of astrocytes in brain function and neurological disease, pathological studies of brains from TSC patients document a number of histological abnormalities in astrocytes (Crino, 2004; Sosunov et al., 2008; Talos et al., 2008), suggesting that astrocyte dysfunction may be central to the pathophysiology of neurological deficits and epilepsy in TSC. We have explored the role of astrocytes in a mouse model of TSC involving *Tsc1* gene inactivation primarily in astrocytes (GFAP+ cells) (Uhlmann et al., 2002). In the present study, we demonstrate that *Tsc1*^{GFAP}CKO mice are deficient in the astrocyte-specific gap junction protein, connexin 43, and have an associated impairment in gap junction coupling between astrocytes. Furthermore, we have provided evidence that this deficiency in gap junction coupling may contribute to altered potassium buffering and increased extracellular potassium levels in *Tsc1*^{GFAP}CKO mice. The abnormal connexin 43 expression and impaired potassium buffering in *Tsc1*^{GFAP}CKO mice were reversed by rapamycin, indicating that the mTOR pathway mediates these effects. Thus, given the known effects of extracellular potassium on neuronal excitability, we propose that abnormal

astrocyte-controlled buffering of potassium may promote epileptogenesis and other neurological deficits in TSC.

The role of astrocytic gap junction coupling in the spatial buffering of potassium and other neuroactive compounds has long been suspected, but has only recently been directly tested. Although pharmacological inhibitors of gap junctions have lacked specificity to differentiate the contribution of different cell types, cell-specific differences in the molecular components of gap junctions, the connexin (Cx) proteins, have allowed the contribution of astrocytes to homeostatic mechanisms to be isolated. Astrocytic gap junctions consist primarily of Cx43, as well as Cx30 and Cx26 (Nagy and Rash, 2000). Genetically-engineered mice deficient in Cx43 and Cx30 completely eliminates astrocyte coupling within hippocampus (Theis et al., 2003; Wallraff et al., 2006). Furthermore, as evidence of the physiological importance of astrocyte coupling, these mice exhibit reduced potassium buffering capabilities and increased susceptibility to epileptiform activity (Wallraff et al., 2006).

The significance and relationship of astrocytic gap junction coupling to epilepsy per se is more complex and controversial. While astrocytic gap junctions may inhibit neuronal excitability and seizures by buffering extracellular potassium, it has also been proposed that the coupling of astrocyte networks might promote seizures, such as by non-synaptic synchronization of neuronal activity via glial calcium waves or other intracellular signals propagated between astrocytes (Steinhauser and Seifert, 2002; Samoilova et al. 2008). Variable changes (primarily increases, but also decreases) in astrocytic connexin proteins have been documented in human epilepsy brain specimens and animal models, and it is difficult to determine whether these changes represent causative or compensatory mechanisms for epileptogenesis (Naus et al., 1991; Elisevich et al., 1997; Sohl et al., 2000; Aronica et al., 2001; Fonseca et al., 2002). Finally, the distinction between astrocytic and neuronal gap junctions is also important, as they may have different physiological roles related to epilepsy. While spatial buffering by astrocytes may reduce neuronal excitability, it is likely that neuronal gap junctions contribute directly to the spread of seizures among neuronal networks and thus that gap junction inhibitors may be effective anti-convulsant drugs (Jahromi et al., 2002; Gajda et al., 2003; Gigout et al., 2006; Medina-Ceja et al., 2008).

In the present study, the functional and pathophysiological significance of impaired astrocyte gap junction coupling in relation to epilepsy and other neurological deficits in *Tsc1*^{GFAP}CKO mice was not directly addressed. However, several lines of evidence from this and previous studies suggest that the deficient astrocyte coupling is detrimental and promotes epileptogenesis in this mouse model of TSC. First of all, the initial genetic defect in *Tsc1*^{GFAP}CKO mice primarily affects astrocytes, and progressive astrogliosis slightly precedes the onset of epilepsy in these mice (Uhlmann et al., 2002; Erbayat-Altay et al., 2007), suggesting that astrocyte dysfunction is responsible for epileptogenesis in this model. Furthermore, previous studies have found that hippocampal slices from *Tsc1*^{GFAP}CKO mice are more sensitive to elevated potassium-induced epileptiform activity (Jansen et al., 2005), which would be consistent with the impaired potassium buffering in hippocampal slices of these mice in the present study. Finally, the decreased expression of Cx43 and impaired potassium buffering could be reversed by rapamycin, a treatment that has also been shown to prevent epilepsy in *Tsc1*^{GFAP}CKO mice (Zeng et al. 2008), suggesting that the impaired astrocyte gap junction coupling may participate in epileptogenesis due to mTOR hyperactivation and mechanistically linking these astrocyte defects to *Tsc1* gene inactivation in these mice.

As mTOR can affect a variety of other downstream effectors through gene regulation, the abnormalities in Cx43 expression could represent just one component of a spectrum of brain abnormalities that promote epileptogenesis and other neurological deficits in *Tsc1*^{GFAP}CKO

mice. This might include more global effects on brain metabolism, homeostasis, or blood brain maintenance (Marchi et al., 2007) or other specific molecular defects in astrocytes or neurons. For example, although the decreased connexin expression and gap junction coupling of astrocytes could account for the excessive potassium concentrations following stimulation in the *Tsc1*^{GFAP}CKO mice, other mechanisms may also contribute to differences in potassium buffering. Both inward-rectifying potassium channels and sodium-potassium pumps of astrocytes and neurons have been implicated in absorbing extracellular potassium (Haglund and Schwartzkroin, 1990; Xiong and Stringer, 2000; D'Ambrosio et al., 2002), and defects in these molecules may promote seizure generation (Janigro et al., 1997; Bordey and Sontheimer, 1998; Gabriel et al., 1998; Xiong and Stringer, 1999; Hinterkeuser et al., 2000). In fact, decreased expression of specific inward-rectifying potassium channels have been previously documented and proposed to contribute to epileptogenesis in *Tsc1*^{GFAP}CKO mice (Jansen et al., 2005). However, the effects of carbenoxolone in the present study in causing a further increase in $[K^+]_0$ in control mice, but not in *Tsc1*^{GFAP}CKO mice, suggest that gap junction mechanisms account, at least in part, for the observed differences in potassium buffering between control and *Tsc1*^{GFAP}CKO mice.

Assuming that defects in astrocyte gap junction coupling and potassium buffering do contribute to epileptogenesis in *Tsc1*^{GFAP}CKO mice, these findings have potentially significant clinical and therapeutic implications for epilepsy in human TSC. Although to our knowledge, abnormalities in astrocytic connexin expression or gap junction function have not yet been reported in human TSC, there have been several recent examples where initial findings in animal models of TSC have been subsequently confirmed in human TSC (Wong, 2007). As the role of astrocytes in epilepsy in general has been receiving more attention recently (Binder and Steinhauser, 2006; Jabs et al., 2008; Wetherington et al., 2008), future anti-epileptic therapies may be targeted specifically for astrocytes. As exemplified by the potentially opposite effects of neuronal versus astrocytic gap junctions on brain excitability, designing therapies that modulate cell-specific gap junctions will be important. While there is already much excitement and promise for developing novel anti-epileptogenic treatments for epilepsy in TSC (Zeng et al., 2008), further understanding of astrocytic mechanisms of epileptogenesis should lead to safer, more effective treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Astrocytic expression of the gap junction protein Cx43 is downregulated in *Tsc1*^{GFAP}CKO mice. (A, B) Representative western blots from neocortex and hippocampal extracts of brains from 4–5 week old *Tsc1*^{GFAP}CKO mice and littermate controls showed a decrease in Cx43 protein expression. Neuronal specific gap junction protein Cx36 is unchanged. β -actin is included as an internal control for protein loading. (C) After normalization to β -actin levels, quantitative summary of all experiments shows that Cx43 expression was decreased by ~40% in both neocortex and hippocampus in *Tsc1*^{GFAP}CKO mice compared with controls. The Cx43/ β -actin ratio was normalized to the control group. (D) Although there appeared to be a slight increase in Cx36 expression in neocortex and hippocampus of *Tsc1*^{GFAP}CKO mice, there was no significant difference between the two groups. Con – control mice, KO - *Tsc1*^{GFAP}CKO mice. ** $p < 0.01$, *** $p < 0.001$, by t-test ($n = 9$ mice/group).

Fig. 2.

The decrease in Cx43 expression in *Tsc1*^{GFAP}CKO mice is mTOR-dependent. (A,B) Representative western blots show Cx43 expression in *Tsc1*^{GFAP}CKO mice and control mice administered 3mg/kg rapamycin or vehicle for 3 weeks starting at P14. While vehicle-treated *Tsc1*^{GFAP}CKO mice have significantly decreased Cx43 levels in both neocortex and hippocampus compared with control mice, rapamycin prevented this decrease in Cx43 expression in *Tsc1*^{GFAP}CKO mice. (C,D) Quantitative summary of all experiments demonstrates that rapamycin treatment of *Tsc1*^{GFAP}CKO mice increased Cx43 levels back to control levels. The Cx43/ β -actin ratio was normalized to the vehicle-treated control group. Con_Ve – vehicle-treated control mice, Con_Ra – rapamycin-treated control mice, KO_Ve – vehicle-treated *Tsc1*^{GFAP}CKO mice, KO_Ra – rapamycin-treated *Tsc1*^{GFAP}CKO mice. ****p* < 0.001, by ANOVA (*n* = 8 mice/group).

Fig. 3. Hippocampal astrocytic gap junction coupling is impaired in *Tsc1*^{GFAP}CKO mice. (A, B) Representative hippocampal sections showing the extent of biocytin-stained astrocytes from control mice and *Tsc1*^{GFAP}CKO mice following injection of a single astrocyte in stratum radiatum. Scale bars, 50 μ m. (C, D) Representative hippocampal sections showing density of GFAP-positive astrocytes in control and *Tsc1*^{GFAP}CKO mice. Scale bars, 20 μ m. (E) Typical "passive" current profile of recorded astrocytes. A range of depolarizing and hyperpolarizing voltage steps between -160 to +70 mV (holding potential -70mV) activated large time- and voltage-independent membrane currents. Scale bars, 500 pA, 10 ms. (F) Summarized data from all experiments showed reduced numbers of biocytin stained astrocytes in hippocampal slices

from *Tsc1*^{GFAP}CKO mice. *** $p < 0.001$, by t-test ($n = 20$ injections, *Tsc1*^{GFAP}CKO mice; $n=16$ injections, control mice). (G) After injection of a single astrocyte in the stratum radiatum along the superficial surface of the hippocampal slice, 40 μ m thick sections obtained from the original, injected 400 μ m slice were processed and counted individually for biocytin-positive cells. The number of biocytin-positive cells decreased in sections more distant from the injected astrocyte (in Section 1 or 2) in both *Tsc1*^{GFAP}CKO and control mice and was lower in *Tsc1*^{GFAP}CKO mice in all sections. Dark triangle, control mice; open triangle, *Tsc1*^{GFAP}CKO mice. (H) When comparing the distribution of biocytin-positive cells in different strata within the hippocampus after injection of a single astrocyte in the stratum radiatum, control mice exhibited an expected decrease in coupled cells in the more distal stratum lacunosum-moleculare compared to the stratum radiatum. In contrast, *Tsc1*^{GFAP}CKO mice had equal numbers of coupled cells in these two strata. Thus, compared to control mice, the number of coupled cells in *Tsc1*^{GFAP}CKO mice was only decreased in the stratum radiatum, not the stratum lacunosum-moleculare. Con – control mice, KO – *Tsc1*^{GFAP}CKO mice, s.r – stratum radiatum, s.l.m – stratum lacunosum-moleculare. *** $p < 0.001$ by ANOVA.

Fig. 4. Potassium buffering is impaired in *Tsc1*^{GFAP}CKO mice. (A) Representative traces generated from a reference electrode for field potential (f.p.) recording (showing an antidromic population spike immediately following the stimulation artifact) and potassium-selective electrode in response to a 20Hz, 200 pulse train (starting at the arrow) at 100% stimulation intensity in hippocampal slices from control mice and *Tsc1*^{GFAP}CKO mice. Scale bars: for field potential 5mV, 25ms; for [K⁺]_o 2mM, 5s. (B) Summary of maximal absolute rises in [K⁺]_o (delta [K⁺]_o) evoked at 50 and 100% stimulation intensity. With both levels of stimulation, the peak increase in [K⁺]_o was significantly great in *Tsc1*^{GFAP}CKO mice compared to controls. (C) Summary of maximal evoked [K⁺]_o elicited by stimuli at 50 and 100% stimulation intensity, normalized to amplitudes of respective population spikes. With both levels of stimulation, the normalized peak increase in [K⁺]_o was significantly great in *Tsc1*^{GFAP}CKO mice compared to controls. (D) Absolute rises in stimulation-induced [K⁺]_o changes in the hippocampal slices before and after perfusion with the gap junction inhibitor, carbenoxolone. Carbenoxolone treatment induced a significant, additional increase in peak [K⁺]_o in control mice, but had no effect in *Tsc1*^{GFAP}CKO mice. Black bars, before carbenoxolone; grey bars, after carbenoxolone. *p < 0.05, **p < 0.01, ***p < 0.001, by ANOVA (n = 6 mice/group).

Fig. 5. The impairment in potassium buffering in *Tsc1*^{GFAP}CKO mice is mTOR-dependent. (A,B) Summary of maximal rises in [K⁺]_o evoked at 50 and 100% stimulation intensity in control mice, and untreated or rapamycin-treated *Tsc1*^{GFAP}CKO mice. nAs previously, with both levels of stimulation, the absolute rise in [K⁺]_o (delta [K⁺]_o) (A) and the normalized peak increase in [K⁺]_o (B) were significantly greater in *Tsc1*^{GFAP}CKO mice compared to controls. Rapamycin treatment of *Tsc1*^{GFAP}CKO mice decreased [K⁺]_o levels back to control levels. *p < 0.05, **p < 0.01, ***p < 0.001, by ANOVA (n = 6 mice/group).