Reduced expression of I_A channels is associated with post-ischemic seizures

Zhigang Lei[1], **Hui Zhang**[2], **Yanling Liang**[3], and **Zao C. Xu**[1]

[1]Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202, USA

[2]Department of Pediatrics, the Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong 510150, People's Republic of China

[3]Department of Neurology, the Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong 510150, People's Republic of China

Abstract

Purpose—Post-stroke seizures are considered as a major cause of epilepsy in adults. The pathophysiologic mechanisms resulting in post-stroke seizures are not fully understood. The present study attempted to reveal a new mechanism underlying neuronal hyperexcitability responsible to the seizure development after ischemic stroke.

Methods—Transient global ischemia was produced in adult Wistar rats using the 4-vessel occlusion (4-VO) method. The spontaneous behavioral seizures were defined by the Racine scale III – V. The neuronal death in the brain was determined by hematoxylineosin staining. The expression levels of A-type potassium channels were analyzed by immunohistochemical staining and western blotting.

Results—We found that the incidence of spontaneous behavioral seizures increased according to the severity of ischemia with 0% after 15-min ischemia and ~50% after 25- min ischemia. All behavioral seizures occurred with 48 hrs after ischemia. Morphological analysis indicated that brain damage was not correlated with behavioral seizures. Immunohistochemical staining showed that the expression levels of the A-type potassium channel subunit Kv4.2 was significantly reduced in ischemic brains with behavioral seizures, but not in ischemic brains without seizures. In addition, rats failing to develop spontaneous behavioral seizures within 2 days after ischemia were more sensitive to bicuculline-induced seizures at 2 months after ischemia than control rats. Meanwhile, Kv4.2 expression was decreased in brain at 2 months after ischemia.

Correspondent: Zhigang Lei, Ph.D., Department of Anatomy and Cell Biology, Indiana University School of Medicine, 635 Barnhill Drive, MS507, Indianapolis, IN 46202, USA, Tel: +1-317-274-0547, Fax: +1-317-278-2040, zhlei@iupui.edu.

Conclusion—Our results demonstrated the reduction of Kv4.2 expression might contribute to the development of post-ischemic seizures and long-term increased seizure susceptibility after ischemia. The mechanisms underlying post-stroke seizures and epilepsy is unknown so far. The down-regulation of I_A channels may explained the abnormal neuronal hyperexcitability responsible for the seizure development after ischemic stroke.

Keywords

A-type potassium channels; Kv4.2; epilepsy; excitability; epileptogenesis

Introduction

Seizures are one of the most frequent complications in stroke patients and stroke is considered as a major cause of epilepsy in the adults (Hauser, 1992). The reported incidence of post-stroke seizures and epilepsy varies widely between studies due to many factors that include differences in study design, the patient population, diagnostic criteria, and the duration of follow-up. A large international prospective multicenter study reported that seizures occur in 8.9% of patients with stroke (10.6% with hemorrhagic and 8.6% with ischemic stroke) (Bladin et al., 2000). According to the temporal relation with the stroke onset, seizures are commonly classified as early- and late-onset, but there is no consistent definition how to separate early from late seizures. In most studies, early-onset is defined as the first seizure occurring within 2 weeks after stroke onset and late-onset is defined as the first seizure occurring more than 2 weeks after stroke (Bladin et al., 2000). Most early-onset seizures are likely to occur during the first 1 to 2 days after stroke onset. The risk of late-onset seizures and epilepsy is believed to increase when early-onset seizures occur (So et al., 1996). It is widely accepted that post-stroke seizures are harmful and can exacerbate the injury processes, leading to higher morality and poor functional disability (Szaflarski et al., 2008). Therapeutic treatments for post-stroke seizures and epilepsy have been limited, because of the lack of a clear understanding of the pathophysiological mechanisms involved.

Although the relationship between a stroke and seizures has been described in both clinical studies and animal models (Kelly, 2002), the number of studies investigating the underlying pathophysiologic mechanisms leading to post-stoke seizures is sparse. This is partly due to the fact that animal models of post-stroke seizures is not well-established so far. The 4-vessel occlusion (4-VO) ischemia model described by Pulsinelli is the most used technique to produce transient global ischemia (Pulsinelli and Brierley, 1979). The percentage of animals developing behavioral seizures following 10, 20, or 30 minutes of four-vessel occlusion is 0, 8, and 40%, respectively (Pulsinelli et al., 1982). Unfortunately, ischemic animals with behavioral seizures are often died or excluded from experimental groups. Therefore, the pathologic changes in these post-ischemic animals with seizures have not been fully investigated.

Increase of neuronal excitability is a recognized cause of seizures generation. Spontaneous interictal epileptiform discharges in the hippocampus have been reported from ischemic rat brain (Epsztein et al., 2006). There are several mechanisms that may be associated with the neuronal hyperexcitability and the generation of epileptiform discharges in post-ischemic

brain, one of which is the changes in intrinsic membrane properties (Congar et al., 2000). The transient A-type potassium currents (I_A currents) are critical to modulate of intrinsic membrane properties of neurons through the regulation of resting membrane potential, action potential (AP) half-width, frequency-dependent AP broadening and dendritic action potential propagation (Kim et al., 2005). In neurons, Kv4 subunits, together with, KChIPs and DPPX, give rise to the majority of somatodendritic I_A currents (An et al., 2000; Nadal et al., 2003; Sheng et al., 1992). In our previous studies, we found that the reduction of I_A currents and channels contributes to the seizure development after hyperglycemic ischemia (Lei et al., 2014). However, the involvement of I_A currents and channels in the post-ischemic seizures in rats with normal glucose levels has not been elucidated.

In the present study, we explored the association of I_A channels with the earlyonset behavioral seizures and the long-term changes in seizure susceptibility after 4-VO ischemia. In addition to a description of the incidence and the characteristics of ischemia-induced behavioral seizures, we found that the down-regulation of I_A channels might be involved in the pathological process leading to post-ischemic seizures in both early and late stage.

Methods

The rat model of transient global ischemia

Transient global ischemia in rat was induced using the 4-VO occlusion as described in our previous study with modifications (Ruan et al., 2009). On the first day, adult male Wistar rats (150–200 g, Charles River) were anesthetized with 2% isoflurane via a nasal mask. Both vertebral arteries were electrocauterized. A length of silicone tubing (0.025″ I.D., 0.047″ O.D.) will be placed loosely around each common carotid artery and passed through two holes in a small teflon button before being tied in a loop. A suture line was tied at the end of the loop. The incision was closed with wound clips. The rats were then allowed to recover from anesthesia overnight. The next day, the carotid arteries were occlusion in the unanesthetized rats. The 2% lidocaine-HCl (Sparhawk Lab., Lenexa, KS, USA) was applied as a local anesthetic in the region of incision. The awake rats were hand-held in a simple restraint, the ventral neck suture removed. The silicone tubing was then threaded and drawn through a 2-cm plastic cylinder, compressing the artery against the Teflon button. During occlusion, the rectal temperature was maintained at 37°C with a heating lamp via a feedback system, and the completeness of global ischemia was confirmed by testing the loss of righting and pupil reflexes. After the termination of 15, 20, 25 or 30-min occlusion, the silicone tubings, teflon buttons and sutures was removed and the incision was closed. The rats were then allowed to recover in a standard cage. Behavioral (i.e., convulsive) seizures include symptoms in a scale from III to V using the Racine scale (Racine, 1972). A class III seizure was characterized by forelimb clonus, an erect tail and lordotic posturing. A class IV was characterized by continued forelimb clonus and rearing on hindlimbs. Rats showing all of these behaviors in combination with a fall were defined as having a class V seizure. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Immunohistochemical staining

The rats were deeply anesthetized, perfused with PBS and fixed with 4% paraformaldehyde in PBS (Lei et al., 2012; Lei et al., 2010). After being postfixed overnight, six sets of coronal sections of the hippocampus were cut (40 μm) with a vibratome (Technical Products International) and collected in PBS. One set of sections will be stained with hematoxylin & eosin (H&E). Then, the sections from control and ischemia groups were stained together in each immunohistochemical session. The sections were incubated for 30-min in 0.3% H_2O_2 (Sigma) to quench the endogenous peroxidase activity. Subsequently, the sections were blocked and permeabilized in permeabilization solution (5% goat or horse serum, 0.1% Triton X-100 in PBS) for 1 hr at room temperature. Thereafter, the sections were incubated with an antibody against Kv4.2, Kv4.3, or KChIP2 in permeabilization solution overnight at 4 °C. After being washed, the sections were incubated with biotinylated horse anti-mouse or goat anti-rabbit IgG (1:200; Vector) in blocking solution (5% horse or goat serum in PBS) for 1 hr at room temperature. After three washes, the sections were processed with ABC and DAB reaction. All sections within the reaction were exposed to DAB for the exact same time. The sections were mounted onto slides, air dried, dehydrated in graded series of ethanol, infiltrated in xylene, and embedded in paraffin. The slides were then examined with a microscope (BX50; Olympus). Images were acquired with a digital camera coupled to control software (DP70-BSW; Olympus) at 4 and 20 X magnification. The settings were kept constant throughout all experiments.

Western blotting

Brain slices were prepared using procedures similar to those previously described (Deng et al., 2009). Briefly, the animals were anesthetized with overdose of isoflurane and decapitated. The brains were quickly removed and immersed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 130 NaCl, 3 KCl, 2 CaCl$_2$, 2 MgCl$_2$, 1.25 NaH$_2$PO$_4$, 26 NaHCO$_3$, and 10 glucose (pH 7.4, 295 – 305 mOsm/L). Transverse brain slices containing the hippocampus (400 μm) were cut using a vibratome (VT 1000; Leica). Subsequently, the hippocampus was microdissected under a surgical microscope (Bausch & Lomb, Rochester, NY, USA) and frozen in liquid nitrogen. Tissues were lysed with ice-cold RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS; 5 mM EDTA, Boston BioProducts, Worcester, MA, USA) supplemented with a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and incubated an additional 30 min on ice. After brief sonication on ice, cell lysates were centrifuged at 12,000 X g for 20 min at 4 °C to pellet nuclei and debris, and the resulting supernatants were collected for analysis. Protein concentration was determined by BCA protein assay (Bio-Rad, Hercules, CA, USA). Protein samples were boiled in 2 X SDS gel-loading buffer (Invitrogen, Carlsbad, CA, USA) prior to SDS-PAGE. Proteins (20 μg) were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were rinsed with distilled water, blocked with 1% bovine serum albumin (BSA; Sigma) in TBS-0.1% Tween20 (TBST) for 1 hr, and then incubated with primary antibodies overnight in blocking buffer at 4 °C. We used rabbit polyclonal anti-Kv4.2 (1:1,000; Chemicon), mouse monoclonal anti-β-actin (1:20,000; Sigma), mouse monoclonal anti-Kv4.2 (1:1,000; NeuroMab), mouse monoclonal anti-Kv4.3 (1:1,000; NeuroMab), or mouse monoclonal anti-KChIP2 (1:1,000; NeuroMab) antibodies. The membranes were

washed with TBST, and incubated at room temperature for 1 hr with HRP-conjugated anti-rabbit (1:15,000; Chemicon) or anti-mouse secondary antibodies (1:10,000; Chemicon). Bands were detected by the enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ, USA) and visualized by exposing the membrane to X-ray films (Fuji, Tokyo, Japan). Band densitometry analysis of the membrane was performed using scanned images of unsaturated immunoblot films, using NIH ImageJ 1.37 analysis software (Lei et al., 2010).

Bicuculline-induced seizures

(+)-bicuculline (Sigma) was dissolved in 0.1 N HC1 and adjusted to pH 6 – 6.5 with 1 N NaOH. Rats were injected with (+)-bicuculline (4.0 mg/kg, i.p.). After injection of bicuculline, rats were placed in a clear cage. The rats' behavior was videotaped and observed for a period of 30 min. Bicuculline-induced seizures started with rhythmic movements of the head and forelimbs, followed by wild running and jumping, and then developed into a short-lasting tensing of all muscles (tonus) and a long-lasting clonus (Lei et al., 2012).

Statistical Methods

The values were presented as mean ± SEM. The results were analyzed using one-way ANOVA followed by *post hoc* Scheffe's test, Student's *t*-test, Chi-square test, one sample *t*-test, or Mann-Whitney U-test (StatView 5.0; Abacus Concepts, Berkeley, CA, USA). Changes were considered significant if $P < 0.05$.

Results

Early-onset spontaneous behavioral seizures after ischemia

In our previous studies (Ruan et al., 2009), male Wistar rats were fasted overnight and ischemia duration were 10 min. The spontaneous behavioral seizures were barely observed in ischemic rats. We made a couple of modifications on our 4-VO model. Firstly, rats were subjected to the ischemic insult without overnight fasting, because overnight fasting could significantly reduce blood glucose levels from 116.6 ± 4.0 mg/dL (N = 10) to 78.0 ± 2.8 mg/dL (N = 10, $P < 0.01$, Fig. 1A). We found that the occurrence rate of behavioral seizures in 30-min ischemic rats (5 of 10 rats, 50.0%) without fasting were much higher than that (1 of 9 rats, 11.1%) in ischemic rats with fasting (Fig. 1B). Rats suffering 30-min ischemia developed behavioral seizures between 22 to 32 hrs after recirculation. The seizures were status epilepticus involving forelimbs and hindlimbs rigidly pushed away from the body followed by all four limbs tonically flexed. The rats exhibited violent shaking, vibrating or even jumping. Secondly, we changed the ischemia duration. We found that 9 of 22 (40.9%) rats with 25-min ischemia and 5 of 10 (50%) rats with 30-min ischemia had behavioral seizures, while none of 10 rats with 15-min ischemia and 1 of 10 (10%) rats with 20-min ischemia had spontaneous behavioral seizures (Fig. 1C). The ischemia duration had no significant effect on the latency of seizure onset (20-min: 30.0 ± 0.0 hrs hrs, N = 1; 25-min: 29.2 ± 3.2 hrs, N = 9; 30-min: 27.6 ± 1.7 hrs, N = 5; $P > 0.05$ by one-way ANNOVA; Fig. 1D). All seizures occurred within 48 hrs after ischemia. Therefore, 25-min global ischemia on rats without overnight fasting was used as the experimental paradigm in the following studies.

Brain damage

The brain damage was double-blinded scored on a 5-point scale in the CA1, CA3 and DG subregions of the hippocampus and cortex where grade 0 meant no observed damage, grade 1 < 10% damaged neurons, grade 2 50% damage, grade 3 >50% damage, and grade 4 infarction (Li et al., 1994). The ischemic rats with seizures were sacrificed immediately when the seizure activity was first observed, while the ischemic rats without seizures were sacrificed around 24 hrs after ischemia.

Moderate brain damage (grade 2) was observed in the hippocampal CA1 region, while mild brain damage (grade 1) in the CA3, DG, and cortex of non-seizure rats at about 24 hrs after 25-min ischemia (CA1: 1.3 ± 0.4; CA3: 0.4 ± 0.2; DG: 0.3 ± 0.2; cortex: 0.6 ± 0.2, N = 7; Fig. 2). Compared to non-seizure rats exposed to the same duration of ischemia, seizure rats had greater brain damage (Fig. 2). In 25-min ischemia groups, seizure rats exhibited significant greater neuronal damage (grade 3) in the hippocampal DG region than non-seizure rats (DG: 1.6 ± 0.4, N = 5; $P < 0.05$ by Mann-Whitney U-test; Fig. 2D). For the hippocampal CA1 and CA3 regions, and cortex, seizure rats exposed to 25-min ischemia showed numerically greater neuronal damage than non-seizure animals, but there was no statistically significant difference (CA1: 2.0 ± 0.5; CA3: 1.2 ± 0.4; cortex: 1.2 ± 0.4, N = 5; $P > 0.05$ by Mann-Whitney U-test; Fig. 2B, C, and E). It is worthwhile to point out that seizures could occur in rats without obvious brain damage in the cortex and hippocampus (Fig. 2A).

I_A channel subunit Kv4.2 was decreased in rat brain with behavioral seizures after ischemia

Kv channels exhibit diverse patterns of cellular expression and subcellular localization in the normal rat brain (Rhodes et al., 2004). There is high density of immunoreactivity for Kv4.2 and KChIP2 on dendrites of dentate granule cells and pyramidal neurons in the hippocampus. The immunoreactivity for Kv4.3 is also present in the interneurons, as well as in the dendrites of dentate granule cells, and CA3 but not CA1 pyramidal cells. Principal cells and interneurons in the cortex also exhibit specific expression patterns of I_A channels subunits. Pyramidal neurons in layer II exhibit high levels of Kv4.2 staining, and Kv4.2 staining predominates in those throughout layers II–VI. There is a very high density of immunoreactivity for Kv4.2 in pyramidal cells in layers II and III. This pattern of immunoreactivity for Kv4.2 is matched by a similar pattern of immunoreactivity for KChIP2.

I_A currents play important role in controlling neuronal excitability and therefore might contribute to seizure generation after ischemia. To investigate the involvement of I_A channels in post-ischemia seizures, the expression of I_A channel subunit Kv4.2 was compared in rats with and without behavioral seizures after 25-min ischemia. There was an obvious reduction in immunoreactivity for Kv4.2 in the hippocampus and cortex of rats with post-ischemic seizures (Fig. 3A). Such reduction in Kv4.2 expression was not observed in rats that suffered the same duration (25-min) of ischemia but did not develop seizures (Fig. 3A). For quantification of protein expression, Western blotting analysis was performed on the hippocampus and cortex from control and ischemic rats. The Kv4.2 band recognized by the

polyclonal anti-Kv4.2 antibody was at 75 kDa (Lei et al., 2010). Consistent with immunohistochemical results, Western blotting analysis indicated a reduction of total Kv4.2 protein levels in the hippocampus ($65.2 \pm 5.3\%$ of control, N = 3, $P < 0.05$) and cortex ($73.2 \pm 14.2\%$ of control) of the seizure rats, but not in the hippocampus ($103.0 \pm 16.2\%$ of control) and cortex ($101.2 \pm 16.4\%$ of control) of the non-seizure rats after ischemia (Fig. 3B&C).

In comparison to Kv4.2, no obvious changes of immunoreactivity in other I_A channel subunits, such as Kv4.3 and KChIP2, were detected in rats with post-ischemic seizures (Fig. 4A&B). The Kv4.3 (75 kDa) and KChIP2 (35 kDa) protein levels in the hippocampus and cortex of seizure rats were similar to those of the control animals (N = 3, Fig. 4C&D).

These results indicate that Kv4.2 is selectively reduced in ischemic rats developing behavioral seizures.

Long-term increased seizure susceptibility after ischemia

Rats suffered with ischemia (20–30 min) that failed to display behavioral seizures within the first 2 days after ischemia were allowed to survive for 2 months. None of them developed apparent chronic behavioral seizures. To examine whether ischemia changed the susceptibility of rats to seizures, ischemic and age-matched control rats were injected intraperitoneally with 4.0 mg/kg of (+)-bicuculline. Bicuculline induced clonic-tonic seizures in all of ischemic rats (100%, N = 10), but only in 3 of 5 (60%) control rats (Fig. 5A). There was also a tendency of decrease in the latency to onset of bicuculline-induced seizures in ischemic rats (control: 71.4 ± 10.8 s, N = 3; ischemia: 57.0 ± 14.6s, N = 10, $P > 0.05$; Fig. 5B). The results indicate that the 4-VO ischemia is followed by an increased susceptibility to chemical convulsants. H&E staining of hippocampal slices from these rats showed a completely loss of CA1 pyramidal neurons with a good preservation of CA3 pyramidal neurons (Fig. 5C). We then measured Kv4.2 expression in the brain at 2 months after 4-VO using immunohistochemical staining and western blotting. A reduction of Kv4.2 immunoreactivity was found in the ischemic hippocampus and cortex (Fig. 5D). Western blotting analysis indicated a reduction of Kv4.2 levels in the ischemic hippocampus ($60.2 \pm 7.8\%$ of control, N = 4, $P < 0.01$, Fig. 5E) and cortex ($70.0 \pm 12.7\%$ of control, Fig. 5F). The results implicate that the reduction of Kv4.2 in the hippocampus might be associated with the long-term increased seizure susceptibility of ischemic rats.

Discussion

The main findings of this study are the following: (i) seizures are associated with more brain damage and neuronal damage is not the major contributor of seizure generation in ischemic rats; (ii) the expression of I_A channel subunit Kv4.2 is selectively reduced in ischemic rats with spontaneous behavioral seizures; (iii) The reduction of Kv4.2 may be associated with the long-term increased seizure susceptibility of ischemic rats.

Despite that metabolic changes in the surround of the ischemic infarct are assumed as a cause for acute seizures (within 48 hrs) after ischemia, the supporting evidence is still incomplete. Our results indicate that the post-ischemic seizure incidence is increased with

ischemia duration and blood glucose level. In this study, none of normoglycemic rats suffering 15-min global ischemia and about 40% rats suffering 25-min ischemia developed spontaneous seizures. These rates of post-ischemic seizure are consistent with previous studies (Congar et al., 2000; Pulsinelli and Brierley, 1979; Pulsinelli et al., 1982). In our model, global ischemia was induced by the 4-VO. (Reid et al., 1996)(Kawai et al., 1995) (Kawai et al., 1995) Stroke severity is an important determinant of outcome in stroke patients. Clinic studies have reported that stroke severity was independently associated with the development of seizures after ischemic stroke (Bladin et al., 2000; Reith et al., 1997). It is plausible that longer ischemia duration increased ischemic severity, which results in higher seizure incidence. It is well recognized clinically that hyperglycemia (> 108 mg/dL) augments ischemic brain injury (Capes et al., 2001) and hyperglycemia (> 110 mg/dL) has been identified as an independent post-stroke seizure predictor (Procaccianti et al., 2012). The specific mechanisms by which hyperglycemia increase post-stroke seizures is not known, although several have been proposed based on experimental results from rats with pre-ischemic hyperglycemia. The proposed mechanisms include more tissue acidosis (Li et al., 1995), selective damage to GABAergic neurons in the substantia nigra pars reticulata (SNPR) (Smith et al., 1988) greater brain edema (Morimoto et al., 1996; Warner et al., 1987), reduction of potassium channels (Lei et al., 2014), and activation of Toll-like receptor 4 (TLR-4) pathway (Liang et al., 2014).

Transient global ischemia induces selective neuronal death in vulnerable brain regions. The hippocampal CA1 neurons die while CA3 and DG neurons survive, and neocortical layers II, III, and V also selectively degenerate after transient global ischemia (Lipton, 1999). It is well-known that CA1 neurons start to show degenerating signs at 2 days after ischemia and more than 90% neurons die at 7 days after reperfusion. However, post-ischemic seizures usually occur with 48 hrs after ischemia. Therefore, the loss of CA1 neurons is not the major cause of post-ischemic seizures. A major objective of the present study was to test whether seizure rats have the same extent of brain damage as non-seizure rat after ischemic insult. The results demonstrate that seizure rats had consistently higher brain damage than non-seizure in all observed brain regions. Given that more brain damage was found in seizure rats, the question arises of whether the aggravation of brain damage in seizures rats is related to post-ischemic seizure generation. It is well known that transient global ischemia induces delayed cell death. CA1 pyramidal neurons start to show degenerating signs 2 days after ischemia and more than 90% neurons die at 7 days after reperfusion. In consistent with clinical studies, acute behavioral seizures all happened within 48 hrs after ischemia, at when ischemia-induced neuronal damage is not obvious. It is well-known that seizures can cause neuronal damage. The type of post-ischemic seizures is status epileptics. The whole hippocampal regions and cortex are sensitive to status epileptics-induced neuronal damage. Thus, it seems that seizures are the cause of aggravated brain damage. Our recent published results excluded the causal relationship of neuronal damage with the seizure development after hyperglycemic ischemia (Lei et al., 2014). Furthermore, based on the result that seizures could occur in rats without obvious brain damage in the cortex and hippocampus (Fig. 2A), it is tempting to conclude that the exaggerated brain damage is not responsible for the seizure development after ischemia.

The hippocampal CA3 region seems a good candidate to study the mechanisms of post-stroke seizure because this region is one of the most susceptible regions in the brain for the generation of seizure (Hablitz and Johnston, 1981). Therefore, the CA3 region has been studied to elucidate the mechanisms associated with post-stroke seizures and epilepsy. Using chronic *in vivo* EEG recordings and *in vitro* field recordings in hippocampal slices, spontaneous interictal epileptiform discharges in the CA3 region was reported from post-ischemic rats. There are three major mechanisms which may be associated with the neuronal hyperexcitability and the generation of epileptiform discharges in the post-ischemic hippocampus. The first one is changes in intrinsic membrane properties. The whole-cell recordings reveal a permanent depolarization of the post-ischemic CA3 pyramidal and a reduced threshold to generate synchronized bursts (Congar et al., 2000). The second one is the imbalance of excitatory-inhibitory network. Ischemic insult causes a dramatic loss of GABAergic interneurons and terminals together with an increase in glutamatergic terminals in the CA3 area of the hippocampus. CA3 pyramidal neurons show a permanent reduction in the frequency of spontaneous and miniature GABAergic IPSCs and a parallel increase in the frequency of spontaneous and miniature glutamatergic postsynaptic currents (Epsztein et al., 2006). The loss of inhibitory interneurons coupled with excitatory shift in the excitatory-inhibitory balance persists even a year after the ischemic impact (Arabadzisz and Freund, 1999). The last one is the reorganization of hippocampal structure. CA3 region is the projection target of the dentate gyrus cells via the mossy fibers. It has been previously shown the mossy fiber sprouting several months after ischemic stroke (Arabadzisz and Freund, 1999), which leads to an elevation of the frequency of miniature synaptic glutamatergic events.

Since I_A plays a critical role in dampening neuronal membrane excitability, disruptions in I_A are associated with the pathophysiology of epilepsy. It is well known that seizures can be induced by selective pharmacological blockers of I_A (Bagetta et al., 1992). Furthermore, disruptions of I_A have been described in many animal models of epilepsy and in human temporal lobe epilepsy (Bernard et al., 2004). For example, Kv4.2 truncation mutation lacking the last 44 amino acids in the carboxyl terminal has been observed in a patient with temporal lobe epilepsy (Singh et al., 2006). It has been reported that Kv4.2$^{-/-}$ mice have a decreased seizure and status epilepticus latency compared to wild-type mice in the kainic acid model of epilepsy (Barnwell et al., 2009). We recently reported that traumatic brain injury (TBI) causes a downregulation of I_A in hippocampal neurons, which is associated with the hyperexcitability in the post-traumatic hippocampus and leads to an enhanced seizure susceptibility (Lei et al., 2012). In the present study, we compared I_A channel expression between non-seizure and seizure rats following global ischemia. The results indicate a specific reduction of Kv4.2 expression in the hippocampus and cortex of seizure rats. This suggests a causal relationship between Kv4.2 expression and post-ischemic seizures. We tentatively hypothesize that the reduction of Kv4.2 increases the intrinsic membrane excitability of hippocampal neurons and in turn leads to epileptiform discharges in the post-ischemic hippocampus. Additional studies are needed to determine the validity of this hypothesis.

This brings up one more question: what causes the reduction of Kv4.2 expression? Some previous *in vitro* studies might provide clues to answer this question. An *in vitro* model of

stroke-induced epilepsy is established using glutamate-injured hippocampal neurons. After glutamate-induced injury, surviving neurons manifest spontaneous and recurrent epileptiform discharges in neural networks that persist for the life of the culture (Sun et al., 2001). We found that the application of glutamate to primary cultured hippocampal neurons reduces total Kv4.2 levels, diminishes Kv4.2 clusters, and shifts voltage-dependent inactivation of I_A currents in the hyperpolarization direction (Lei et al., 2008). Furthermore, we found that activation of NR2B-containing NMDA receptors, Ca^{2+} influx and calpain-mediated proteolysis contribute to these changes (Lei et al., 2010; Lei et al., 2008). Therefore, it is likely that increased calpain-mediated degradation of Kv4.2 may contribute to the reduction of Kv4.2 expression in ischemic rats with seizures.

Acknowledgments

Abbreviations

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4-VO	4-vessel occlusion
ACSF	artificial cerebrospinal fluid
BSA	bovine serum albumin
ECL	enhanced chemiluminescence
HRP	horseradish peroxidase
I_A	A-type K^+ currents
Kv	voltage-dependent K^+ currents
MCAO	middle cerebral artery occlusion
PBS	phosphate-buffered saline
SDS	sodium dodecyl sulfate
TBS	tris-buffered saline
TBST	TBS-0.1% Tween20

References

An WF, Bowlby MR, Betty M, Cao J, Ling HP, Mendoza G, Hinson JW, Mattsson KI, Strassle BW, Trimmer JS, Rhodes KJ. Modulation of A-type potassium channels by a family of calcium sensors. Nature. 2000; 403:553–556. [PubMed: 10676964]

Arabadzisz D, Freund TF. Changes in excitatory and inhibitory circuits of the rat hippocampus 12–14 months after complete forebrain ischemia. Neuroscience. 1999; 92:27–45. [PubMed: 10392828]

Bagetta G, Nistico G, Dolly JO. Production of seizures and brain damage in rats by alpha-dendrotoxin, a selective K+ channel blocker. Neuroscience letters. 1992; 139:34–40. [PubMed: 1357602]

Barnwell LF, Lugo JN, Lee WL, Willis SE, Gertz SJ, Hrachovy RA, Anderson AE. Kv4.2 knockout mice demonstrate increased susceptibility to convulsant stimulation. Epilepsia. 2009; 50:1741–1751. [PubMed: 19453702]

Bernard C, Anderson A, Becker A, Poolos NP, Beck H, Johnston D. Acquired dendritic channelopathy in temporal lobe epilepsy. Science (New York, N.Y. 2004; 305:532–535.

Bladin CF, Alexandrov AV, Bellavance A, Bornstein N, Chambers B, Cote R, Lebrun L, Pirisi A, Norris JW. Seizures after stroke: a prospective multicenter study. Archives of neurology. 2000; 57:1617–1622. [PubMed: 11074794]

Capes SE, Hunt D, Malmberg K, Pathak P, Gerstein HC. Stress hyperglycemia and prognosis of stroke in nondiabetic and diabetic patients: a systematic overview. Stroke; a journal of cerebral circulation. 2001; 32:2426–2432.

Congar P, Gaiarsa JL, Popovici T, Ben-Ari Y, Crepel V. Permanent reduction of seizure threshold in post-ischemic CA3 pyramidal neurons. Journal of neurophysiology. 2000; 83:2040–2046. [PubMed: 10758114]

Deng P, Pang ZP, Lei Z, Xu ZC. Excitatory roles of protein kinase C in striatal cholinergic interneurons. Journal of neurophysiology. 2009; 102:2453–2461. [PubMed: 19657079]

Epsztein J, Milh M, Bihi RI, Jorquera I, Ben-Ari Y, Represa A, Crepel V. Ongoing epileptiform activity in the post-ischemic hippocampus is associated with a permanent shift of the excitatory-inhibitory synaptic balance in CA3 pyramidal neurons. J Neurosci. 2006; 26:7082–7092. [PubMed: 16807337]

Hablitz JJ, Johnston D. Endogenous nature of spontaneous bursting in hippocampal pyramidal neurons. Cellular and molecular neurobiology. 1981; 1:325–334. [PubMed: 6765736]

Hauser WA. Seizure disorders: the changes with age. Epilepsia. 1992; 33(Suppl 4):S6–14. [PubMed: 1425495]

Kawai K, Penix LP, Kawahara N, Ruetzler CA, Klatzo I. Development of susceptibility to audiogenic seizures following cardiac arrest cerebral ischemia in rats. J Cereb Blood Flow Metab. 1995; 15:248–258. [PubMed: 7860659]

Kelly KM. Poststroke Seizures and Epilepsy: Clinical Studies and Animal Models. Epilepsy Curr. 2002; 2:173–177. [PubMed: 15309107]

Kim J, Wei DS, Hoffman DA. Kv4 potassium channel subunits control action potential repolarization and frequency-dependent broadening in rat hippocampal CA1 pyramidal neurones. The Journal of physiology. 2005; 569:41–57. [PubMed: 16141270]

Lei Z, Deng P, Li J, Xu ZC. Alterations of A-type potassium channels in hippocampal neurons after traumatic brain injury. J Neurotrauma. 2012; 29:235–245. [PubMed: 21895522]

Lei Z, Deng P, Li Y, Xu ZC. Downregulation of Kv4.2 channels mediated by NR2B-containing NMDA receptors in cultured hippocampal neurons. Neuroscience. 2010; 165:350–362. [PubMed: 19857555]

Lei Z, Deng P, Xu ZC. Regulation of Kv4.2 channels by glutamate in cultured hippocampal neurons. Journal of neurochemistry. 2008; 106:182–192. [PubMed: 18363830]

Lei Z, Zhang H, Liang Y, Cui Q, Xu Z, Xu ZC. Reduced expression of IA channels is associated with postischemic seizures in hyperglycemic rats. J Neurosci Res. 2014; 92:1775–1784. [PubMed: 25043828]

Li PA, Shamloo M, Katsura K, Smith ML, Siesjo BK. Critical values for plasma glucose in aggravating ischaemic brain damage: correlation to extracellular pH. Neurobiology of disease. 1995; 2:97–108. [PubMed: 8980013]

Li PA, Shamloo M, Smith ML, Katsura K, Siesjo BK. The influence of plasma glucose concentrations on ischemic brain damage is a threshold function. Neuroscience letters. 1994; 177:63–65. [PubMed: 7824184]

Liang Y, Lei Z, Zhang H, Xu Z, Cui Q, Xu ZC. Toll-like receptor 4 is associated with seizures following ischemia with hyperglycemia. Brain research. 2014; 1590:75–84. [PubMed: 25223904]

Lipton P. Ischemic cell death in brain neurons. Physiological reviews. 1999; 79:1431–1568. [PubMed: 10508238]

Morimoto Y, Morimoto Y, Warner DS, Pearlstein RD. Acute changes in intracranial pressure and pressure-volume index after forebrain ischemia in normoglycemic and hyperglycemic rats. Stroke; a journal of cerebral circulation. 1996; 27:1405–1409. discussion 1410.

Nadal MS, Ozaita A, Amarillo Y, Vega-Saenz de Miera E, Ma Y, Mo W, Goldberg EM, Misumi Y, Ikehara Y, Neubert TA, Rudy B. The CD26-related dipeptidyl aminopeptidase-like protein DPPX is a critical component of neuronal A-type K+ channels. Neuron. 2003; 37:449–461. [PubMed: 12575952]

Procaccianti G, Zaniboni A, Rondelli F, Crisci M, Sacquegna T. Seizures in acute stroke: incidence, risk factors and prognosis. Neuroepidemiology. 2012; 39:45–50. [PubMed: 22777596]

Pulsinelli WA, Brierley JB. A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke; a journal of cerebral circulation. 1979; 10:267–272.

Pulsinelli WA, Brierley JB, Plum F. Temporal profile of neuronal damage in a model of transient forebrain ischemia. Annals of neurology. 1982; 11:491–498. [PubMed: 7103425]

Racine RJ. Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol. 1972; 32:281–294. [PubMed: 4110397]

Reid KH, Young C, Schurr A, Tseng M, Payne RS, Keelen P, Miller J, Iyer V. Audiogenic seizures following global ischemia induced by chest compression in Long-Evans rats. Epilepsy research. 1996; 23:195–209. [PubMed: 8739123]

Reith J, Jorgensen HS, Nakayama H, Raaschou HO, Olsen TS. Seizures in acute stroke: predictors and prognostic significance. The Copenhagen Stroke Study. Stroke; a journal of cerebral circulation. 1997; 28:1585–1589.

Rhodes KJ, Carroll KI, Sung MA, Doliveira LC, Monaghan MM, Burke SL, Strassle BW, Buchwalder L, Menegola M, Cao J, An WF, Trimmer JS. KChIPs and Kv4 alpha subunits as integral components of A-type potassium channels in mammalian brain. J Neurosci. 2004; 24:7903–7915. [PubMed: 15356203]

Ruan YW, Lei Z, Fan Y, Zou B, Xu ZC. Diversity and fluctuation of spine morphology in CA1 pyramidal neurons after transient global ischemia. J Neurosci Res. 2009; 87:61–68. [PubMed: 18709659]

Sheng M, Tsaur ML, Jan YN, Jan LY. Subcellular segregation of two Atype K+ channel proteins in rat central neurons. Neuron. 1992; 9:271–284. [PubMed: 1497894]

Singh B, Ogiwara I, Kaneda M, Tokonami N, Mazaki E, Baba K, Matsuda K, Inoue Y, Yamakawa K. A Kv4.2 truncation mutation in a patient with temporal lobe epilepsy. Neurobiology of disease. 2006; 24:245–253. [PubMed: 16934482]

Smith ML, Kalimo H, Warner DS, Siesjo BK. Morphological lesions in the brain preceding the development of postischemic seizures. Acta Neuropathol. 1988; 76:253–264. [PubMed: 3213428]

So EL, Annegers JF, Hauser WA, O'Brien PC, Whisnant JP. Population-based study of seizure disorders after cerebral infarction. Neurology. 1996; 46:350–355. [PubMed: 8614493]

Sun DA, Sombati S, DeLorenzo RJ. Glutamate injury-induced epileptogenesis in hippocampal neurons: an in vitro model of stroke-induced "epilepsy". Stroke; a journal of cerebral circulation. 2001; 32:2344–2350.

Szaflarski JP, Rackley AY, Kleindorfer DO, Khoury J, Woo D, Miller R, Alwell K, Broderick JP, Kissela BM. Incidence of seizures in the acute phase of stroke: a population-based study. Epilepsia. 2008; 49:974–981. [PubMed: 18248443]

Warner DS, Smith ML, Siesjo BK. Ischemia in normo- and hyperglycemic rats: effects on brain water and electrolytes. Stroke; a journal of cerebral circulation. 1987; 18:464–471.

Highlights

- The incidence of spontaneous behavioral seizures increased according to the severity of ischemia. All behavioral seizures occurred with 48 hrs after ischemia.

- Brain damage was not correlated with behavioral seizures.

- A-type potassium channel subunit Kv4.2 was selectively reduced in ischemic brains with behavioral seizures.

- Rats were more sensitive to bicuculline-induced seizures at 2 months after ischemia. Meanwhile, Kv4.2 expression was decreased in brain at 2 months after ischemia.

- Our results demonstrated the reduction of Kv4.2 expression might contribute to the development of post-ischemic seizures and long-term increased seizure susceptibility after ischemia.
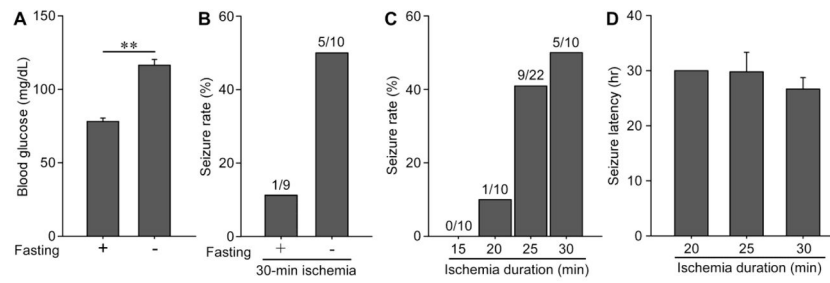
Figure 1.
The incidence rate of the spontaneous behavioral seizures after ischemia is highly correlated to blood glucose levels and ischemia duration. (A) Blood glucose levels of rats with or without overnight fasting (N =10). ** $P < 0.01$ by Student's t-test. (B) Overnight fasting decreased the occurrence of behavioral seizures after 30-min ischemia. (C) The incidence rate of behavioral seizures in rats without overnight fasting increased with ischemia duration. (D) The ischemia duration did not influence the latency of post-ischemia seizures in rats without overnight fasting. Seizure rate (%) is the percentage of rats experiencing seizures within 48 hrs after ischemia.
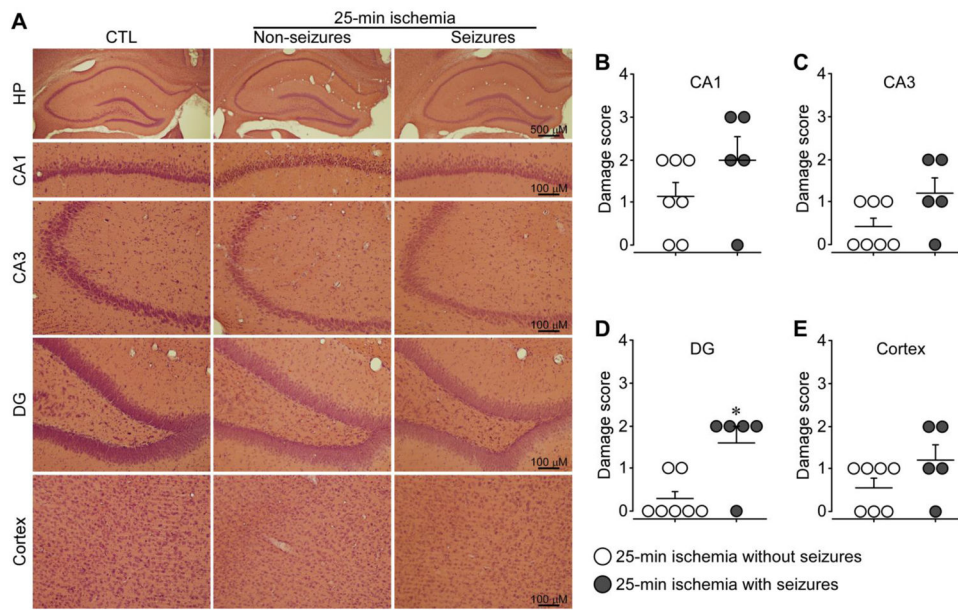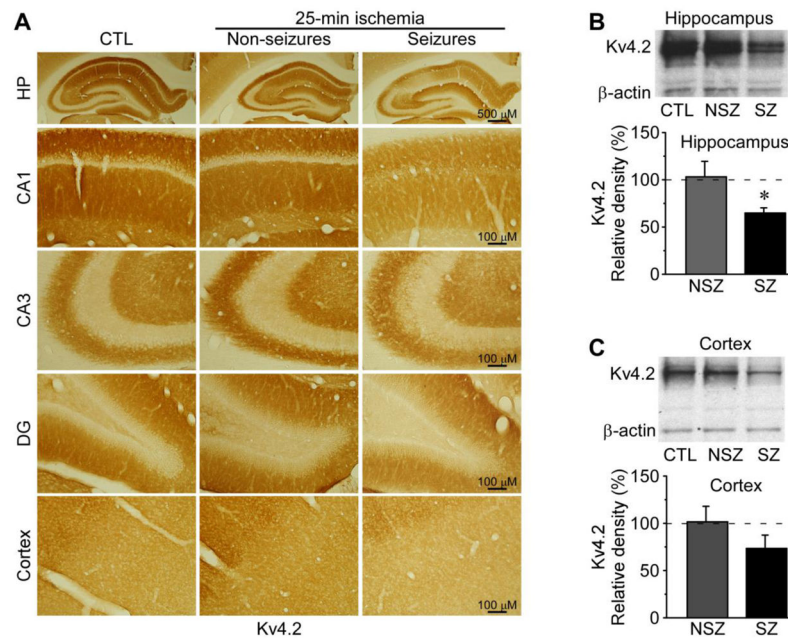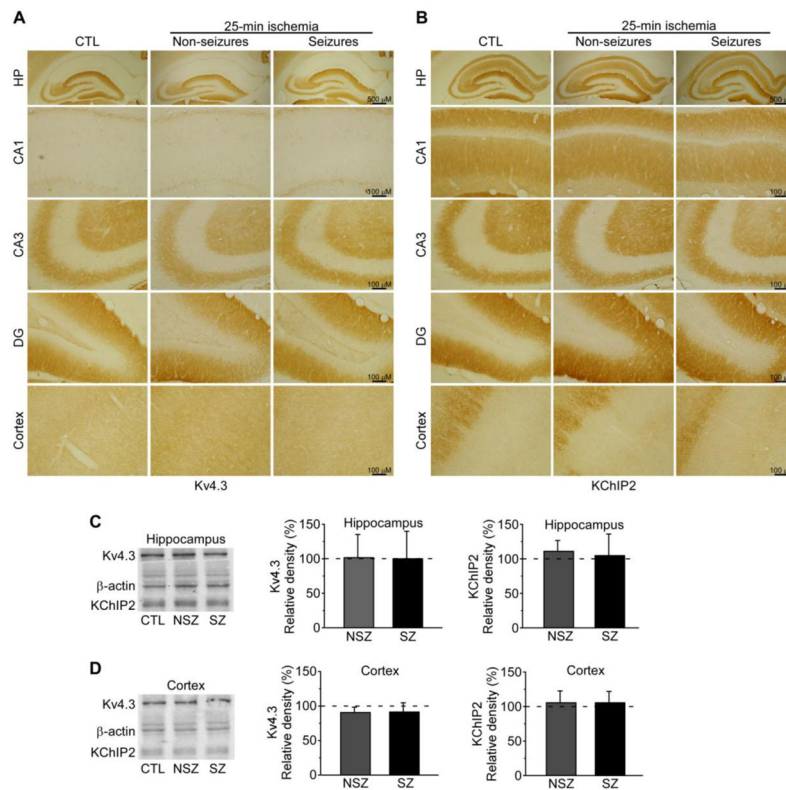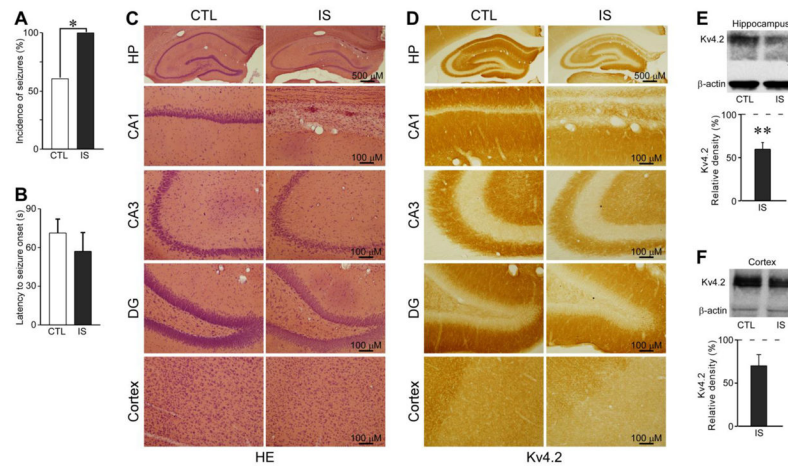
Figure 2.
Seizure rats have slightly more brain damage than non-seizure rats after 25-min ischemia.
(A) Representative photomicrographs showing H&E-stained brain sections at 1 day after 25-min ischemia. (B – E) Pooled data showing the brain damage in hippocampal CA1 (B), CA3 (C), dentate gyrus (DG, D) and cortex (E), which was evaluated on a 5-point scale. Except the DG, seizure rats did not have significantly more brain damage than non-seizure rats in other observed regions. Seizures could occur in rats absent of brain damage in observed regions. * $P < 0.05$ vs non-seizure rats by Mann-Whitney U-test.

Figure 3.
Kv4.2 expression is selectively reduced in rat brains with behavioral seizures after 25-min ischemia. (A) There was a decrease in immunoreactivity for Kv4.2 in the cortex and hippocampus of ischemic rats with seizures at 1 day after ischemia. Such reduction in Kv4.2 expression was not observed in rats that suffered ischemic insults and did not developed behavioral seizures. (B & C) Western blotting showing a reduction of total Kv4.2 protein levels in seizure rats after ischemia. Total Kv4.2 protein levels were reduced in the hippocampus (B) and cortex (C) of the seizure (SZ) rats. There was no obvious decrease in the hippocampus and cortex of the non-seizure (NSZ) rats. * $P < 0.05$ vs CTL by one sample t-test. The dashed line indicates the control value against which the other values are measured. CTL, control; NSZ, non-seizures; SZ, seizures.

Figure 4.

The expression of Kv4.3 and KChIP2 was unchanged after 25-min ischemia. (A & B) In contrast to Kv4.2, no obvious decrease of immunoreactivity for other somatodendritic I_A channel subunits, Kv4.3 (A) and KChIP2 (B), was noticed in the brain of either non-seizure and seizure rats at 1 day after ischemia. (C & D) Western blotting showing that Kv4.3 and KChIP2 protein levels remained unchanged in the hippocampus (C) and cortex (D) in both seizure and non-seizure rats after ischemia. The dashed line indicates the control value against which the other values are measured. CTL, control; NSZ, non-seizures; SZ, seizures.

Figure 5.
The seizure threshold was decreased in ischemic rats, together with the Kv4.2 expression, at two months after transient global ischemia. (A) Occurrence rate of seizures in control and ischemic (20–30 min) rats after injection of 4 mg/kg bicuculline. A marked increase in seizure rate was observed in ischemic rats. * $P < 0.05$ by Chi-square test. (B) Latency to seizure onset in control and ischemic rats after injection of 4 mg/kg bicuculline. (C) Representative H&E photomicrographs showing neuronal death in the hippocampus and cortex after 25-min ischemia. (D) Kv4.2-immunoreactive profiles in the hippocampus of control and ischemic (25-min) rats. Kv4.2 immunoreactivity was decreased in the hippocampus and cortex, compared with the control ones. (E & F) Kv4.2 protein levels were reduced in the ischemic hippocampus (E) and cortex (F) as measured by western blotting. ** $P < 0.01$ vs CTL by one sample t-test. The dashed line indicates the control value against which the other values are measured. CTL, control; IS, ischemia.