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Chronic deficit in the expression of voltage-gated potassium channel Kv3.4 subunit in the hippocampus of pilocarpine-treated epileptic rats

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Abstract

Voltage gated K^+ channels (Kv) are a highly diverse group of channels critical in determining neuronal excitability. Deficits of Kv channel subunit expression and function have been implicated in the pathogenesis of epilepsy. In this study, we investigate whether the expression of the specific subunit Kv3.4 is affected during epileptogenesis following pilocarpine-induced *status epilepticus*. For this purpose, we used immunohistochemistry, Western blotting assays and comparative analysis of gene expression using TaqMan-based probes and delta-delta cycle threshold (Δ ΔCT) method of quantitative real-time polymerase chain reaction (qPCR) technique in samples obtained from age-matched control and epileptic rats. A marked down-regulation of Kv3.4 immunoreactivity was detected in the *stratum lucidum* and hilus of dentate gyrus in areas corresponding to the mossy fiber system of chronically epileptic rats. Correspondingly, a 20% reduction of Kv3.4 protein levels was detected in the hippocampus of chronic epileptic rats. Real-time quantitative PCR analysis of gene expression revealed that a significant 33% reduction of transcripts for Kv3.4 (gene *Kcnc4*) occurred after 1 month of pilocarpine-induced *status epilepticus* and persisted during the chronic phase of the model. These data indicate a reduced expression of Kv3.4 channels at protein and transcript levels in the epileptic hippocampus. Down-regulation of Kv3.4 in mossy fibers may contribute to enhanced presynaptic excitability leading to recurrent seizures in the pilocarpine model of temporal lobe epilepsy.

Keywords

Pilocarpine; *status epilepticus*; mossy fibers; TaqMan; potassium channels; temporal lobe epilepsy

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Introduction

Voltage-gated potassium (K^+) channels (Kv) are a highly diverse group of ion channels that critically modulate neuronal excitability. Compelling data indicate that activation of Kv channels crucially affect intrinsic membrane properties and action potential firing patterns of neurons (e.g. firing frequency) (Christie, 1995; Mathie et al., 1998; Pongs, 2008; Sansom et al., 2002). For instance, their activation limits the firing frequency of action potentials and are important for regulating afterhyperpolarization in central neurons and other types of electrically excitable cells (Pongs, 2008). Moreover, these channels are also expressed at presynaptic terminals playing a pivotal role in controlling neurotransmitter release (Christie, 1995; Edwards and Weston, 1995; Feinshreiber et al., 2009; Pongs, 2008). Kv channels therefore exert profound physiological effects within the nervous system.

Compelling data indicate that seizure-related modifications in expression and function of different ion channels and receptors can lead to enhanced excitability in epilepsy (Bond, 2000; Celesia, 2001; Jung et al., 2007; Kullmann, 2002; Lerche et al., 2001; Li and Lester, 2001; Ptacek and Fu, 2001; Rogawski, 2000; Steinlein and Noebels, 2000). Deficit in potassium (K^+) channels has been implicated in the pathogenesis of neurological disorders characterized by abnormal neuronal excitability including epilepsy.

Accumulating evidence supports K^+ channel activation as an important inhibitory mechanism in epilepsy. Accordingly, it has been acknowledged that depolarization-induced activation of Kv channels may provide a stabilizing influence by hyperpolarizing the membrane upon opening. Hence, blocking K^+ channels and preventing them from opening in response to membrane depolarization will lower seizure threshold and can produce seizures (Frohlich et al., 2008). Supporting the important role that K^+ channels play in controlling neuronal excitability, it was recently discovered that retigabine, a new drug in development for the treatment of epilepsy, activates subtypes of Kv7 K^+ channels, which are responsible for M-current in neurons (Lawrence et al., 2006; Lerche et al., 2001). Accordingly, retigabine has a broad spectrum anticonvulsive profile in both animal seizure and epilepsy models (Luszczki, 2009; Tatulian et al., 2001; Walker and Sander, 1999).

Expression of Kv channels has been explored in different epilepsy models. For instance, the expression of A-type Kv channels (*i.e.* somatodendritic Kv4.2, Kv4.3, KChIPl and KChIP2 and axonal Kv1.4), which control neuronal excitability and regulate action potential propagation and neurotransmitter release, change from being uniformly distributed across the molecular layer of the dentate gyrus to concentrated in just the outer two-thirds in the pilocarpine model of mesial temporal lobe epilepsy (MTLE) (Monaghan et al., 2008), a common neurological disorder characterized by chronic hyperexcitability of hippocampal and parahippocampal neuronal networks (Bertram, 2009; Ojemann, 2001; Pringle et al., 1993; Sloviter, 2008; Swanson, 1995). Moreover, pentylenetetrazole-induced seizures cause a reduction of Kv1.2 and Kv4.2 mRNAs in the dentate granule cells of the hippocampus, indicating that K^+ channel gene regulation may play a role in long-term neuronal plasticity (Tsaur et al., 1992).

Certain subtypes of potassium channels have been found to regulate the release of glutamate from pre-synaptic membranes, and dysfunction of these has been proposed to increase brain excitability. In the present study, we investigated the expression of Kv3.4, a member 4 of the Shaw-related Kv3 subfamily (also known as *Kcnc4*), in the pilocarpine model of MTLE (Cavalheiro, 1995; Turski et al., 1989).

The functional Kv3.4 channels are multimeric complexes of four identical subunits associated to form a symmetric tetramer (each of the subunits has six transmembrane domains) (Christie, 1995). Kv3.4-immunoreacitvity at post- and pre-synaptic structures

(Brooke et al., 2004a) suggests a role both in the control of transmitter release and in regulating neuronal excitability. For instance, at the mouse neuromuscular junction and at the calyx of Held terminal, Kv3 channels directly regulate evoked transmitter release (Brooke et al., 2004b; Ishikawa et al., 2003). In the hippocampus, Kv3.4 is preferentially expressed in the mossy fibers where they may play a pivotal role in regulating transmitter (*i.e.* glutamate) release (Riazanski et al., 2001). Compelling data indicate that the mechanisms controlling the release machinery at mossy fibers are disrupted in epilepsy (Goussakov et al., 2000). For instance, a down-regulation of metabotropic glutamate receptors group II (i.e. mGluR2) and Ca^{2+}-activated large conductance K^+ (BK) channels have been detected in chronic epileptic rats (Ermolinsky et al., 2008a; Ermolinsky et al., 2008b; Garrido-Sanabria et al., 2008; Pacheco Otalora et al., 2008). In addition, Kv3.4 mRNA and protein levels in kainate-epileptic rats were reduced in the subiculum, entorhinal cortex and perirhinal cortex in comparison to controls (Zahn et al., 2008). However, Kv3.4 channels are not down-regulated in the hippocampus of seizure-sensitive gerbil hippocampus, as compared to seizure-resistant gerbils (Lee et al., 2009). Therefore, it remains unclear whether Kv3.4 channels play a role in MTLE. Although their role in mossy fiber-CA3 neurotransmission remains unexplored, considering their hyperpolarizing effect, abnormalities (down-regulation) in the expression of Kv3.4 at granule cells will conceivably enhance mossy fiber excitability and glutamate release.

Results

Gene expression of Kv3.4 is significantly reduced in the chronic phase of the pilocarpine model of epilepsy

Changes in mRNA transcript levels of *Kcnc4* were evaluated by qPCR approach using cDNA libraries obtained from microdissected hippocampus of animals sacrificed at different time points following SE and compared to controls. For this purpose, we implemented the comparative method Δ ΔCT of qPCR with the aid of pre-validated ABI TaqMan gene expression assays for Kv3.4 and the normalizing gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) following described procedure (Ermolinsky et al., 2008a; Ermolinsky et al., 2008b). In a previous study, we demonstrated that *GAPDH* levels are relatively unchanged across all experimental time points following SE, indicating that *GAPDH* expression in our cDNA library of dentate gyrus is not affected by neuronal loss in hippocampus (Ermolinsky et al., 2008b). Thereby, *GAPDH* gene was considered suitable as normalization gene to investigate relative changes in gene expression in our study. For this analysis, data from both control groups were pooled together since no significant changes were observed in relative expression of *Kcnc4* transcripts. Values were expressed as relative quantification (RQ) index (Table 1) and compared to control level of gene expression also as percentage change of control values. Experimental groups included animals sacrificed at 1 day (n=5) and 10 days following SE (n=4), 30–60 days (1 month) (n=3) and more than 60 days (> 2 months) (n=5) following SE. Animals sacrificed at 10 days following SE fail to reveal spontaneous behavioral seizures. This group thereby was considered as representative of the latent period of the pilocarpine model for epilepsy. Interestingly, a significant difference in gene expression for Kv3.4 was detected by one-way ANOVA ($F=6.69$, $P<0.01$, Fig. 1A) at different time points following induction of SE (Table 1). Post-hoc comparisons denoted a significant 33% (RQ=0.53±0.08) and 43% (RQ=0.46±0.13) reduction in RQ value levels at 1 month and >2 months following SE respectively when compared to controls (RQ=0.80±0.12) (Table 1). Moreover, a significant reduction was also detected when compared to 1 day and 10 day following SE in both 1 month and > two month groups (Fig. 1). A 10% non significant reduction was detected between 1 day and 10 days following SE respectively (Table 1, Fig. 1B) when compared to control RQ values. No significant

difference was observed between 1 month and >2 month groups of chronically epileptic rats (Table 1).

Immunostaining for Kv3.4 channels was reduced in the mossy fibers of chronic epileptic rats

To investigate whether down-regulation of *Kcnc4* gene expression in the late chronic phase of the pilocarpine model was accompanied by a corresponding reduction of Kv3.4 protein expression we performed an immunohistochemistry study in hippocampus obtained from 4 sets of control and chronically epileptic rats experiencing recurrent spontaneous seizures. These epileptic rats exhibited at least 4–6 seizures/week when monitored for a period of 4 hours (morning section) similar to rats (>2 months) used for qPCR assays. In the hippocampus of control rats, Kv3.4 immunostaining was intense in the mossy fibers in the hilus of dentate gyrus and in the area of *stratum lucidum* in CA3 area (Fig. 2Aa,b). Intense Kv3.4 immunolabeling was evident in the mossy fiber terminal zone, the *stratum lucidum* (Fig. 2Ab, arrow). Kv3.4 immunoreactivity of moderate to strong intensity was also observed in the lateral portion of the *stratum lacunosum/moleculare* near the CA2–CA3 area (Fig. 2Aa, see *) indicating that Kv3.4 channels are also localized on perforant path axons and terminals. In general, granule cells were almost devoid of somatic staining but moderate to intense staining was observed in the pyramidal cell layer (soma and proximal neuropils). These control animals did not exhibit behavioral seizures or any difference in the pattern and expression level of Kv3.4 channels among them. In marked contrast with control rats, epileptic animals exhibited a reduction in Kv3.4 immunoreactivity in all areas described above. Specifically, a dramatic down-regulation of Kv3.4 expression was detected in the CA3 *stratum lucidum* and in the hilus of dentate gyrus (Fig. 2A c,d) indicating a reduction of expression in axons and terminal areas of the mossy fiber pathway. A reduction of immunostaining for Kv3.4 was also evident in *stratum lacunosum/molecular* and in the pyramidal layer. Adjacent sections were processed for Nissl staining and NeuN immunohistochemistry to reveal a pattern of neuronal loss associated with the chronic phase of the pilocarpine model of MTLE (data not shown). Since immunostaining was observed predominantly in *stratum lucidum* and hilus (corresponding to mossy fibers), immunolabeling data was not normalized to neuronal cell loss. Reduction of Kv3.4 immunolabeling in the *stratum lucidum* of CA3 area of epileptic rats was confirmed using immunofluorescence assays and counterstaining with the fluorescent Nissl staining (NeuroTrace 435/455) which revealed the location of the soma and proximal dendrites of pyramidal neurons in this area (Fig. 2B).

Western blot analysis revealed a down-regulation of Kv3.4 protein expression in chronically epileptic rats

A possible deficit in Kv3.4 channel protein expression in the hippocampus was also investigated by Western blotting analysis of protein extracts obtained from control and epileptic rats during the late chronic phase of the pilocarpine model of epilepsy. Total protein per sample was measured, normalized and the same amount subjected to Western blot analysis using monoclonal mouse antibody against Kv3.4 channels. The experimental group included epileptic animals sacrificed at more than 60 days (> 2 months) following SE. Samples from control and epileptic rat pair sets were simultaneously processed in the) same membrane (n=5). Kv3.4 was identified as an immunopositive band with apparent molecular weight in the 70 kDa range (Fig. 3A). After measuring the optical density, we found that the level of Kv3.4 channel protein was significantly reduced in epileptic samples from the hippocampus (38.6 % reduction, Fig. 3B) (*$p < 0.005$, Student t-test).

Discussion

In this study we demonstrated a reduction in the expression of the voltage-gated K^+ channel subunit Kv3.4 in the chronic phase of the pilocarpine model of temporal lobe epilepsy. Specifically, a marked deficit of Kv3.4 mRNA transcript expression was detected in total RNA extracted from hippocampus after 1 month of pilocarpine-induced self-sustained *status epilepticus*. These data correlate with a marked reduction in the immunoreactivity for Kv3.4 in the mossy fiber pathway of chronically epileptic rats and with a reduction in Kv3.4 protein expression in hippocampus. We cannot rule out that changes in Kv3.4 protein expression may be associated with the massive cell death observed in chronic epileptic hippocampus. However, several studies have confirmed that in contrast to pyramidal neurons and interneurons, granule cells in dentate gyrus are particularly preserved (minimal cell loss) in mesial temporal lobe epilepsy. Hence, since Kv3.4 proteins, which are heavily expressed in mossy fibers (axons of granule cells), may not be significantly affected by SE-induced cell loss, we propose that Kv3.4 down-regulation is not necessarily related to a selective loss of granule cells in the pilocarpine model. In this context, *GAPDH* gene is ubiquitously expressed in all cell types and so the reduction of the controls may be less than the reduction of the signal of the putative neuronal interest thus giving an overall impression of reduced expression. However, in a previous study, we determined that for that cDNA library obtained from microdissected dentate gyrus, *GAPDH* was the most appropriate normalizing gene considering that it exhibited the minimal variation across samples obtained from different control and epileptic rats (Ermolinsky et al., 2008b). In this study, relative quantification was performed using *GAPDH* as endogenous control gene after analyzing the gene expression profile of four candidate reference genes (i.e. *RpL10*, *RpL28*, eukaryotic *18S* and *GAPDH*) in randomly selected twenty different samples (cDNA from control and several epileptic groups). The rationale was to select the more stable gene that varied the least even during the epileptogenic process. *GAPDH* exhibited the less variability among the samples as determined by the analysis of the standard deviation of the threshold cycles (CT) values. Therefore, it appears that *GAPDH* is a suitable normalizing gene in our conditions. Nevertheless, we did not rule out the possibility that a more appropriate normalizing gene can be discovered after further investigation.

The critical role of K^+ channels in the pathogenesis of neurological disorders is highlighted by several human diseases in which K^+ channels are dysfunctional or where modulation of K^+ has been considered as a therapeutic option (Errington et al., 2005; Judge et al., 2007; Kullmann, 2002; Wua and Dworetzky, 2005). Kv channels form a large and diverse family of ion channels that are involved in establishing the resting membrane potential, determining the action potential waveform and duration, regulating neurotransmitter release, as well as modulating rhythmic firing patterns and pacemaking activities of neurons (Rudy et al., 1999). Subunits of the Kv3 subfamily (Kv3.1–Kv3.4) assemble into homotetrameric and heterotetrameric K^+ channels that display unique biophysical properties, namely high thresholds of activation, rapid activation and deactivation kinetics as well as relatively large conductances (Martina et al., 1998; Weiser et al., 1994). Kv channel role as modulator of neuronal excitability is associated with its expression and localization in specific neuronal subtypes, and in its somato-dendritic (post-synaptic) *versus* axonal and synaptic terminal (presynaptic) distribution. In the brain, the Kv3.4 subunits are expressed at both post-synaptic and presynaptic terminals (Brooke et al., 2004a; Brooke et al., 2004b; Ishikawa et al., 2003; Nakamura and Takahashi, 2007), thus suggesting a role both in the control of transmitter release and in regulating neuronal excitability (Brooke et al., 2004a). In mouse motor nerve endings, activation of Kv3 subfamily regulate neurotransmitter release (Brooke et al., 2004b). Abundance of the Kv3.4 subfamily in the mossy fiber pathway in hippocampus indicates that these channels may also control conduction of action potentials and neurotransmitter release. However, in contrast to other members of the Kv3 subfamily

(i.e. KCNC1, KCNC2, and KCNC3) (Ho et al., 1997), knockout mice are not currently available for a Kv3.4 (*Kcnc4* gene). Moreover, mice lacking Kv3.2 channel proteins exhibit impaired fast-spiking, suppressed cortical inhibition, and increased susceptibility to seizures (Lau et al., 2000). Behavioral consequence of Kv3.4 complete deficit has not been determined yet. Hence, reduced expression of Kv3.4 in mossy fiber of chronically epileptic rats may enhance axonal excitability and glutamate release, similarly to down-regulation of BK channels (Ermolinsky et al., 2008a).

In this context, our results are in agreement with those by Zahn et al. (2008), who have shown that Kv3.4 mRNA and protein levels in kainate-epileptic rats were reduced in the subiculum, entorhinal cortex and perirhinal cortex in comparison to control animals (Zahn et al., 2008). However, Kv3.4 channels were not down-regulated in the hippocampus of seizure-sensitive gerbil hippocampus, as compared to seizure-resistant gerbils (Lee et al., 2009), bringing apparently conflicting results. It should be noted, however, that important differences between the epileptogenic process that occurs after pilocarpine- or kainate-induced SE and the mechanisms underlying the increased seizure susceptibility in seizure-prone gerbils may have accounted for such discrepant findings. In what regards the mechanisms underlying the currently described deficit in Kv3.4 epileptic animals, while one may promptly figure out altered gene expression as a cause for such a reduction in Kv3.4 channels, alternative splicing, altered protein processing, trafficking and recycling and post-translational modifications, such as protein phosphorylation may also contribute to this acquired ion channel dysfunction.

As a difference with Ca^{2+} and voltage-activated BK channel subunit (*Kcnma1*), mGluR2 and mGluR3 expression, the deficit in Kv3.4 expression was detected late into the epileptogenenic process (more than 1 month following SE). As a contrast, reduction in BK channel transcript *Kcnma1* expression was noticed as early as 1 day following pilocarpine-induced SE. Such a deficit was permanent but progressively increased reaching a maximum of approximately 50% reduction at 1 month after SE (Ermolinsky et al., 2008a). Interestingly, a partial recovery was observed in late phase (more than 2 months following SE). The patterns SE-mediated changes in mGluR3 and mGluR3 expression were slightly different (Ermolinsky et al., 2008b). As for *Kcnma1*, down-regulation was significant at 1 day following SE, but recovered after 10 days in both transcripts with a further “rebound” reduction only in mGluR2 mRNA levels consistent with immunohistochemical and western blot data (Garrido-Sanabria et al., 2008). All together, these data indicate that deficits in gene expression of molecular modulators for the presynaptic function in hippocampus are dynamic and complex following SE. Consequently, additional single-cell PCR quantification experiments are necessary to provide more accurate information on these changes related to specific neuronal subtypes and pathways. Moreover, other Kv3 channels can also be chronically modified during epileptogenesis, and further experiments should address this issue.

In summary, our data indicate a reduced expression of Kv3.4 channels at protein and transcript levels in the pilocarpine epileptic rat hippocampus. Down-regulation of Kv3.4 in mossy fibers may contribute to enhanced presynaptic excitability leading to recurrent seizures in the pilocarpine model of temporal lobe epilepsy. Additional studies are necessary to evaluate the functional implications of Kv3.4 down-regulations on the pathogenesis of temporal lobe epilepsy.

Experimental Procedure

Animals and rat model of chronic epilepsy

Experiments were developed in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and with the approval of The University of Texas at Brownsville Institutional Animal Care and Use Committee (Protocol# 2004-007-IACUC-1). Sprague Dawley rats were kept in IACUC-approved *vivarium* with water and food *ad libitum*. Chronically epileptic rats were obtained by the pilocarpine model of temporal lobe epilepsy following described procedures (Cavalheiro, 1995; Mello et al., 1993). At the time of performing the model of epilepsy, animals were approximately 35–45 days old (130–200g). A total of 35 animals were used in our experiments. All animals received same dose of 1% methyl-scopolamine nitrate (0.1 mg/kg in saline, s.c.) (Sigma-Aldrich, St. Louis, MO) thirty minutes before pilocarpine administration to minimize the peripheral effects of cholinergic stimulation (Turski et al., 1984). Animals were then injected with 4% pilocarpine hydrochloride (Sigma-Aldrich) (320 mg/kg in saline, i.p.). Controls included (a) animals that received methyl-scopolamine but were injected with saline, instead of pilocarpine and (b) saline-injected control animals. Pilocarpine administration induced *status epilepticus* (SE) in about 70% of injected rats. SE consisted of continuous tremor, rearing, myoclonic jerks, clonic forearms and head movements with eventual side fallings and tonic seizures. Mortality rate of the procedure was reduced by administering diazepam (10 mg/kg, *i.p.*) 3 hours after SE onset to quell behavioral seizures (Danzer and McNamara, 2004; Mello et al., 1993; Pacheco Otalora et al., 2006). All animals that received pilocarpine were given fresh apples and water in an easily-reachable container inside the recovery cage for 48 hr after SE induction. Subcutaneous injections of 20 ml Ringer-lactate were administered to compensate for any liquid lost (*i.e.* salivation, urination) 1 hr after diazepam injection and the following morning after induction of the pilocarpine-mediated SE. SE induction protocol was lethal in about 20% of pilocarpine-treated rats and another 10% of the animals did not enter into SE. After SE, rats were monitored for the appearance of at least two spontaneous seizures using a JVC MiniDV digital video-camera and researcher-assisted SeizureScan software (Clever Sys., Inc, Reston, VA) to detect whether SE-experienced rats exhibit spontaneous seizures. Seizures were confirmed *off-line* by an experienced researcher. SE-suffering rats experienced approximately a range of 4–6 seizures per week during the observation period.

RNA isolation, cDNA library and real-time quantitative PCR analysis

Total RNA was isolated from control group and pilocarpine treated rats that were anaesthetized and killed at 1 day, 10 days, 1 month, and at more than 2 months after SE induction. Total RNA was isolated from the dissected hippocampus. In brief, tissue was collected, weighed (about 30–40 mg), homogenized, and processed for total RNA isolation at 4°C using RNeasy Mini Kit (Cat# 74104, Qiagen, Valencia, CA, USA), following manufacturer's instructions. The concentration and purity of total RNA for each sample was determined by Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The integrity of the extracted RNA was confirmed by electrophoresis under denaturizing condition (Sambrook and Russel, 2001). Trace amount of genomic DNA contamination were minimized by performing DNAse treatment using *DNA*-free™ *DNase Treatment* (Applied Biosystems, ABI, CA, USA) following manufacturer's protocols. RNA samples from each set of control and different groups of pilocarpine-treated rats were processed in parallel under the same conditions. RT was performed on an iCycler Thermal Cycler PCR System (Bio-Rad Laboratories, Hercules, California, USA), the High Capacity cDNA Reverse Transcription Kit (P/N: 4368814; ABI, CA, USA) for synthesis of single-stranded cDNA library. The cDNA synthesis was carried out according to the manufacturer's protocol using random primers for 1 μg of starting RNA. Each RT reaction

contained 1000 ng of extracted total RNA template and RT reagents. The 20 μl reactions were incubated in the iCycler Thermocycler in thin-walled 0.2-ml PCR tubes for 10 min at 25°C, 120 min at 37°C, 5 s at 85°C, and then held at 4°C. The efficiency of the RT reaction and amount of input RNA template was determined by serial dilutions of input RNA. Each RNA concentration was reverse transcribed using the same RT reaction volume. The resulting cDNA template was subjected to real-time quantitative PCR (rtqPCR) using Taqman-based ABI gene expression assays, TaqMan Fast Universal PCR Master Mix (ABI) and the StepOne real-time thermocycler engine (ABI). Gene expression analysis was performed using the TaqMan Gene Expression Assays Rn01748431_m1 (amplicon size=68 bp) for target gene Kv3.4 (*Kcnc4*, Rattus norvegicus potassium voltage gated channel, Shaw-related subfamily, member 4, RefSeq: NM_001122776.1) and the bioassay Rn99999916_s1 for the normalization gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, RefSeq: NM_017008.3, amplicon size=87 bp). Each sample was run in triplicates and non-template control to test for contamination of assay reagents was also included in the plate. After a 94°C denaturation for 10 min, the reactions were cycled 40 times with a 94°C denaturation for 15 s, and a 60°C annealing for 1 min. Three types of controls aimed at detecting genomic DNA contamination in the RNA sample or during the RT or qPCR reactions were always included: a RT mixture without reverse transcriptase, a RT mixture including the enzyme but no RNA, negative control (reaction mixture without cDNA template). The data were collected and analyzed using OneStep Software (ABI). Relative quantification was performed using the comparative threshold (CT) method after determining the CT values for reference (GAPDH) and target (Kv3.4) gene in each sample set according to the 2_ΔΔCt method (Δ ΔCT, delta-delta CT) (Pfaffl, 2006) as described by the manufacturer (Applied Biosystems; User Bulletin 2). Changes in mRNA expression level were calculated after normalization to GAPDH. As calibrator sample we used cDNA from an arbitrarily selected control rat. The Δ ΔCT method provides a relative quantification ratio according to calibrator that allows statistical comparisons of gene expression among samples. Values of fold changes in the control sample versus the post-SE samples represent averages from triplicate measurements. Changes in gene expression were reported as percent changes relative to controls. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD (Honest Significant Difference), with significance *P* value set at <0.05.

Tissue preparation, cresyl violet staining and immunohistochemistry

A set of 4 control and epileptic rats were used for the study. Anaesthetized animals (ketamine, 30 mg/kg, i.p) were transcardially perfused with 0.1 M phosphate-buffered saline (PBS) for 1 min followed by 4% paraformaldehyde in PBS. The brains were removed immediately after perfusion, post-fixed in the same fixative solution overnight. After cryoprotection in 30% buffered sucrose, brains were then sectioned in 50 μm thick-slices using a freezing cryostat, collected, and washed with PBS (pH 7.4). Paired free-floating sections from control and epileptic rats at matched anteroposterior levels (Bregma, −3.2/3.8 mm) were processed in the same vial in order to minimize the intergroup differences during the immunocytochemical procedure.

Sections were Nissl-stained to assess cytoarchitectonic boundaries of hippocampal subfields. In addition, Nissl's staining was used to detect neuronal death and atrophy induced by pilocarpine-mediated SE. For immunohistochemistry, corresponding free-floating sections containing hippocampus were selected from both control and epileptic rat groups and processed simultaneously. All sections were treated with 0.1% H_2O_2 in 0.01 M PBS to quench endogenous peroxidase and then incubated for 30 min in PBS containing 0.3% Triton X-100. After that, the sections were incubated for 2 h in 5% normal goat serum (NGS) in 0.01M PBS and then once again incubated overnight with the primary antibody.

Several concentrations of primary antibodies were used to optimize immunostaining. To assess the expression pattern of Kv3.4, sections were incubated overnight with monoclonal anti-Kv3.4 antibody (1:500, NeuroMab, UC Davis/NIH NeuroMab Facility, CA, USA) in the presence of 1% NGS. In separate assays, homologous sections were incubated in anti-NeuN antibody (1:1000, Millipore, Billerica, MA, USA) to confirm neuronal loss in epileptic rats. After incubating sections in the primary antibody, sections were washed three times in 0.01M PBS. Then, the sections were incubated with biotinylated secondary antibody (1:200, Vector Labs, Burlingame, CA, USA). Immunodetection was performed using the Vectastain ABC Elite Kit (Vector Labs) and the complex antigen-antibody peroxidase was visualized using diaminobenzidine substrate in 0.01M TBS and H_2O_2 (1 μl/ml). Tissue sections were then mounted on glass slides and coverslipped with regular mounting media. The material processed with DAB and sections stained with cresyl violet were studied with a microscope using bright-field illumination. Results were visualized and pictures were acquired using a Zeiss Axioskop 2 microscope, AxioCam HR CCD 24-bit camera and AxioVision software (Zeiss, Oberkochen, Germany). Immunofluorescence for Kv3.4 channels was perfomed using the same primary antibody and secondary antibody conjugated to Alexa Fluor 594 (Invitrogen). Sections were counterstained with NeuroTrace® 435/455 blue fluorescent Nissl stain (Cat. No. N-21479, Invitrogen) following manufacturer's instructions. Double fluorescence staining was visualized and imaged using appropriate laser and filter pairs using an inverted IX81 microscope equipped with Fluoview laser scanning confocal system (Leeds Instruments, Olympus, Japan).

Protein sample preparation and Western blotting

Control and epileptic rats were anesthetized and sacrificed during the chronic phase of the model (>2 months) following SE. After decapitation, brains were removed and submerged in oxygenated (95% O_2/5% CO_2) ice-cold ACSF containing the following (in mM): 125 NaCl, 3.5 KCl, 2 $CaCl_2$, 2 $MgSO_4$, BES, 15 D-glucose, pH 7.4 for dissection of both hippocampi (~ 60 mg). Samples were separately homogenized in Dounce homogenizers in 10 vol of ice-cold standard radioimmunoprecipitation assay (RIPA) buffer (Pierce, Rockford, IL) (in mM): 25 mM Tris-4HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate and 0.1 % SDS, pH 7.4 containing 10 μl of a Halt protease inhibitor cocktail and 10 mM PMSF (phenylmethylsulfonyl fluoride) (Pierce) per 1 ml of buffer. Protein concentration was determined using Pierce BCA™ (bicinchoninic acid) protein assay kit and an Infinity M200 multimode microplate reader (Tecan US, Inc, Durham NC). Bovine serum albumin (BSA) was used as standard. Protein samples were then adjusted to a final protein concentration of 1.5 mg/ml for normalization, boiled for 5 min in Laemmli buffer and resolved on Tris-tricine buffered SDS-PAGE (overnight at 75 V). Proteins were electrotransferred (25 V, 35 min) to a polyvinylidene difluoride (PVDF) membrane using Mini-PROTEAN 3 electrophoresis apparatus (Bio-Rad Laboratories, Mississauga, ON). Membranes were blocked for 2 hrs at room temperature in 0.01M Tris-buffered saline (TBS) containing 5% non-fat dry milk and 0.1% Tween-20. The membranes were then probed overnight at 4°C with the primary antibody (anti-Kv3.4, NeuroMab) diluted 1:500 in 0.01M TBS containing 2% non-fat dry milk and 0.1% Tween-20. As reference (loading control), membranes were probed with primary antibody against Actin (1:20 000) (Sigma Aldrich, Saint Louis, USA). The membranes were washed in TBS with 0.1% Tween-20 and incubated for 2 hr in biotinylated anti-mouse secondary antibody (1:400, Vector Labs). After washing 3 times in 0.01M PBS, membranes were incubated for 90 min in Vectastain ABC Elite Kit (room temperature), rinsed 3 times (15 min each) in PBS and the immunopositive bands visualized by chemiluminescent detection using ECL Plus Western Blotting Kits according to manufacturer's protocols (Pierce) and a Bio-Rad ChemiDoc XRS digital documentation system. Exposure times were adjusted so that the darkest bands did not saturate the film. Levels of protein immunoreactivity were quantified by measuring the

optical density of specific Kv3.4 reactive bands. Background optical density levels were taken for each image of a blot and were subtracted from the optical density obtained for each individual immunoreactive band. Western blot data were compiled from control and epileptic rats for statistical analysis. For each experiment samples were run in duplicate.

Abbreviations

BK	Large conductance calcium- and voltage activated potassium channel
CA1	Corpus Ammonis Area 1
CT	Cycle Threshold
ΔΔCT	Delta-delta Cycle Threshold method of comparative real time PCR
DG	Dentate gyrus
Kcnma1	Gene coding for pore-forming alpha subunit of large conductance calcium-activated potassium channel
Kcnc4	Gene name for a member 4 of the Shaw-related Kv3 subfamily Kv3.4
Kv3.4	Voltage gated K^+ channel subtype 3.4
qPCR	Quantitative real time polymerase chain reaction
mGluR2	Metabotropic glutamate receptor type 2
MTLE	Mesial Temporal Lobe Epilepsy
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
RQ	Relative quantification index
SE	*Status epilepticus*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Literature references

Bond EF. Channelopathies: potassium-related periodic paralyses and similar disorders. AACN Clin Issues. 2000; 11:261–270. [PubMed: 11235435]

Brooke RE, Atkinson L, Batten TF, Deuchars SA, Deuchars J. Association of potassium channel Kv3.4 subunits with pre- and post-synaptic structures in brainstem and spinal cord. Neuroscience. 2004a; 126:1001–1010. [PubMed: 15207333]

Brooke RE, Moores TS, Morris NP, Parson SH, Deuchars J. Kv3 voltage-gated potassium channels regulate neurotransmitter release from mouse motor nerve terminals. Eur J Neurosci. 2004b; 20:3313–3321. [PubMed: 15610163]

Cavalheiro EA. The pilocarpine model of epilepsy. Ital J Neurol Sci. 1995; 16:33–37. [PubMed: 7642349]

Celesia GG. Disorders of membrane channels or channelopathies. Clin Neurophysiol. 2001; 112:2–18. [PubMed: 11137655]

Christie MJ. Molecular and functional diversity of K+ channels. Clin Exp Pharmacol Physiol. 1995; 22:944–951. [PubMed: 8846516]

Danzer SC, McNamara JO. Localization of brain-derived neurotrophic factor to distinct terminals of mossy fiber axons implies regulation of both excitation and feedforward inhibition of CA3 pyramidal cells. J Neurosci. 2004; 24:11346–11355. [PubMed: 15601941]

Edwards G, Weston AH. The role of potassium channels in excitable cells. Diabetes Res Clin Pract. 1995; 28 Suppl:S57–S66. [PubMed: 8529520]

Ermolinsky B, Arshadmansab MF, Pacheco Otalora LF, Zarei MM, Garrido-Sanabria ER. Deficit of Kcnma1 mRNA expression in the dentate gyrus of epileptic rats. Neuroreport. 2008a; 19:1291–1294. [PubMed: 18695509]

Ermolinsky B, Pacheco Otalora LF, Arshadmansab MF, Zarei MM, Garrido-Sanabria ER. Differential changes in mGlu2 and mGlu3 gene expression following pilocarpine-induced status epilepticus: a comparative real-time PCR analysis. Brain Res. 2008b; 1226:173–180. [PubMed: 18585369]

Errington AC, Stohr T, Lees G. Voltage gated ion channels: targets for anticonvulsant drugs. Curr Top Med Chem. 2005; 5:15–30. [PubMed: 15638775]

Feinshreiber L, Singer-Lahat D, Ashery U, Lotan I. Voltage-gated potassium channel as a facilitator of exocytosis. Ann N Y Acad Sci. 2009; 1152:87–92. [PubMed: 19161379]

Frohlich F, Bazhenov M, Iragui-Madoz V, Sejnowski TJ. Potassium dynamics in the epileptic cortex: new insights on an old topic. Neuroscientist. 2008; 14:422–433. [PubMed: 18997121]

Garrido-Sanabria ER, Otalora LF, Arshadmansab MF, Herrera B, Francisco S, Ermolinsky BS. Impaired expression and function of group II metabotropic glutamate receptors in pilocarpine-treated chronically epileptic rats. Brain Res. 2008; 1240:165–176. [PubMed: 18804094]

Goussakov IV, Fink K, Elger CE, Beck H. Metaplasticity of mossy fiber synaptic transmission involves altered release probability. J Neurosci. 2000; 20:3434–3441. [PubMed: 10777806]

Ho CS, Grange RW, Joho RH. Pleiotropic effects of a disrupted K+ channel gene: reduced body weight, impaired motor skill and muscle contraction, but no seizures. Proc Natl Acad Sci U S A. 1997; 94:1533–1538. [PubMed: 9037088]

Ishikawa T, Nakamura Y, Saitoh N, Li WB, Iwasaki S, Takahashi T. Distinct roles of Kv1 and Kv3 potassium channels at the calyx of Held presynaptic terminal. J Neurosci. 2003; 23:10445–10453. [PubMed: 14614103]

Judge SI, Smith PJ, Stewart PE, Bever CT Jr. Potassium channel blockers and openers as CNS neurologic therapeutic agents. Recent Pat CNS Drug Discov. 2007; 2:200–228. [PubMed: 18221232]

Jung S, Jones TD, Lugo JN Jr, Sheerin AH, Miller JW, D'Ambrosio R, Anderson AE, Poolos NP. Progressive dendritic HCN channelopathy during epileptogenesis in the rat pilocarpine model of epilepsy. J Neurosci. 2007; 27:13012–13021. [PubMed: 18032674]

Kullmann DM. The neuronal channelopathies. Brain. 2002; 125:1177–1195. [PubMed: 12023309]

Lau D, Vega-Saenz de Miera EC, Contreras D, Ozaita A, Harvey M, Chow A, Noebels JL, Paylor R, Morgan JI, Leonard CS, Rudy B. Impaired fast-spiking, suppressed cortical inhibition, and increased susceptibility to seizures in mice lacking Kv3.2 K+ channel proteins. J Neurosci. 2000; 20:9071–9085. [PubMed: 11124984]

Lawrence JJ, Saraga F, Churchill JF, Statland JM, Travis KE, Skinner FK, McBain CJ. Somatodendritic Kv7/KCNQ/M channels control interspike interval in hippocampal interneurons. J Neurosci. 2006; 26:12325–12338. [PubMed: 17122058]

Lerche H, Jurkat-Rott K, Lehmann-Horn F. Ion channels and epilepsy. Am J Med Genet. 2001; 106:146–159. [PubMed: 11579435]

Li M, Lester HA. Ion channel diseases of the central nervous system. CNS Drug Rev. 2001; 7:214–240. [PubMed: 11474425]

Luszczki JJ. Third-generation antiepileptic drugs: mechanisms of action, pharmacokinetics and interactions. Pharmacol Rep. 2009; 61:197–216. [PubMed: 19443931]

Martina M, Schultz JH, Ehmke H, Monyer H, Jonas P. Functional and molecular differences between voltage-gated K+ channels of fast-spiking interneurons and pyramidal neurons of rat hippocampus. J Neurosci. 1998; 18:8111–8125. [PubMed: 9763458]

Mathie A, Wooltorton JR, Watkins CS. Voltage-activated potassium channels in mammalian neurons and their block by novel pharmacological agents. Gen Pharmacol. 1998; 30:13–24. [PubMed: 9457476]

Mello LE, Cavalheiro EA, Tan AM, Kupfer WR, Pretorius JK, Babb TL, Finch DM. Circuit mechanisms of seizures in the pilocarpine model of chronic epilepsy: cell loss and mossy fiber sprouting. Epilepsia. 1993; 34:985–995. [PubMed: 7694849]

Nakamura Y, Takahashi T. Developmental changes in potassium currents at the rat calyx of Held presynaptic terminal. J Physiol. 2007; 581:1101–1112. [PubMed: 17331991]

Pacheco Otalora LF, Couoh J, Shigamoto R, Zarei MM, Garrido Sanabria ER. Abnormal mGluR2/3 expression in the perforant path termination zones and mossy fibers of chronically epileptic rats. Brain Res. 2006; 1098:170–185. [PubMed: 16793029]

Pacheco Otalora LF, Hernandez EF, Arshadmansab MF, Francisco S, Willis M, Ermolinsky B, Zarei M, Knaus HG, Garrido-Sanabria ER. Down-regulation of BK channel expression in the pilocarpine model of temporal lobe epilepsy. Brain Res. 2008; 1200:116–131. [PubMed: 18295190]

Pfaffl, MW. Real Time PCR (BIOS Advanced Methods). Vol. New York: Taylor & Francis Group; 2006.

Pongs O. Regulation of excitability by potassium channels. Results Probl Cell Differ. 2008; 44:145–161. [PubMed: 17579818]

Ptacek LJ, Fu YH. Channelopathies: episodic disorders of the nervous system. Epilepsia. 2001; 42 Suppl 5:35–43. [PubMed: 11887966]

Riazanski V, Becker A, Chen J, Sochivko D, Lie A, Wiestler OD, Elger CE, Beck H. Functional and molecular analysis of transient voltage-dependent K+ currents in rat hippocampal granule cells. J Physiol. 2001; 537:391–406. [PubMed: 11731573]

Rogawski MA. KCNQ2/KCNQ3 K+ channels and the molecular pathogenesis of epilepsy: implications for therapy. Trends Neurosci. 2000; 23:393–398. [PubMed: 10941184]

Rudy B, Chow A, Lau D, Amarillo Y, Ozaita A, Saganich M, Moreno H, Nadal MS, Hernandez-Pineda R, Hernandez-Cruz A, Erisir A, Leonard C, Vega-Saenz de Miera E. Contributions of Kv3 channels to neuronal excitability. Ann N Y Acad Sci. 1999; 868:304–343. [PubMed: 10414303]

Sambrook, J.; Russel, JW. Molecular Cloning, a Laboratory Manual. Vol. Cold Spring: Cold Spring Harbor Laboratories Press; 2001.

Sansom MS, Shrivastava IH, Bright JN, Tate J, Capener CE, Biggin PC. Potassium channels: structures, models, simulations. Biochim Biophys Acta. 2002; 1565:294–307. [PubMed: 12409202]

Steinlein OK, Noebels JL. Ion channels and epilepsy in man and mouse. Curr Opin Genet Dev. 2000; 10:286–291. [PubMed: 10826987]

Tatulian L, Delmas P, Abogadie FC, Brown DA. Activation of expressed KCNQ potassium currents and native neuronal M-type potassium currents by the anticonvulsant drug retigabine. J Neurosci. 2001; 21:5535–5545. [PubMed: 11466425]

Turski L, Ikonomidou C, Turski WA, Bortolotto ZA, Cavalheiro EA. Review: cholinergic mechanisms and epileptogenesis. The seizures induced by pilocarpine: a novel experimental model of intractable epilepsy. Synapse. 1989; 3:154–171. [PubMed: 2648633]

Turski WA, Cavalheiro EA, Bortolotto ZA, Mello LM, Schwarz M, Turski L. Seizures produced by pilocarpine in mice: a behavioral, electroencephalographic and morphological analysis. Brain Res. 1984; 321:237–253. [PubMed: 6498517]

Walker MC, Sander JW. New anti-epileptic drugs. Expert Opin Investig Drugs. 1999; 8:1497–1510.

Weiser M, Vega-Saenz de Miera E, Kentros C, Moreno H, Franzen L, Hillman D, Baker H, Rudy B. Differential expression of Shaw-related K+ channels in the rat central nervous system. J Neurosci. 1994; 14:949–972. [PubMed: 8120636]

Wua YJ, Dworetzky SI. Recent developments on KCNQ potassium channel openers. Curr Med Chem. 2005; 12:453–460. [PubMed: 15720253]

Zahn RK, Tolner EA, Derst C, Gruber C, Veh RW, Heinemann U. Reduced ictogenic potential of 4-aminopyridine in the perirhinal and entorhinal cortex of kainate-treated chronic epileptic rats. Neurobiol Dis. 2008; 29:186–200. [PubMed: 17942314]

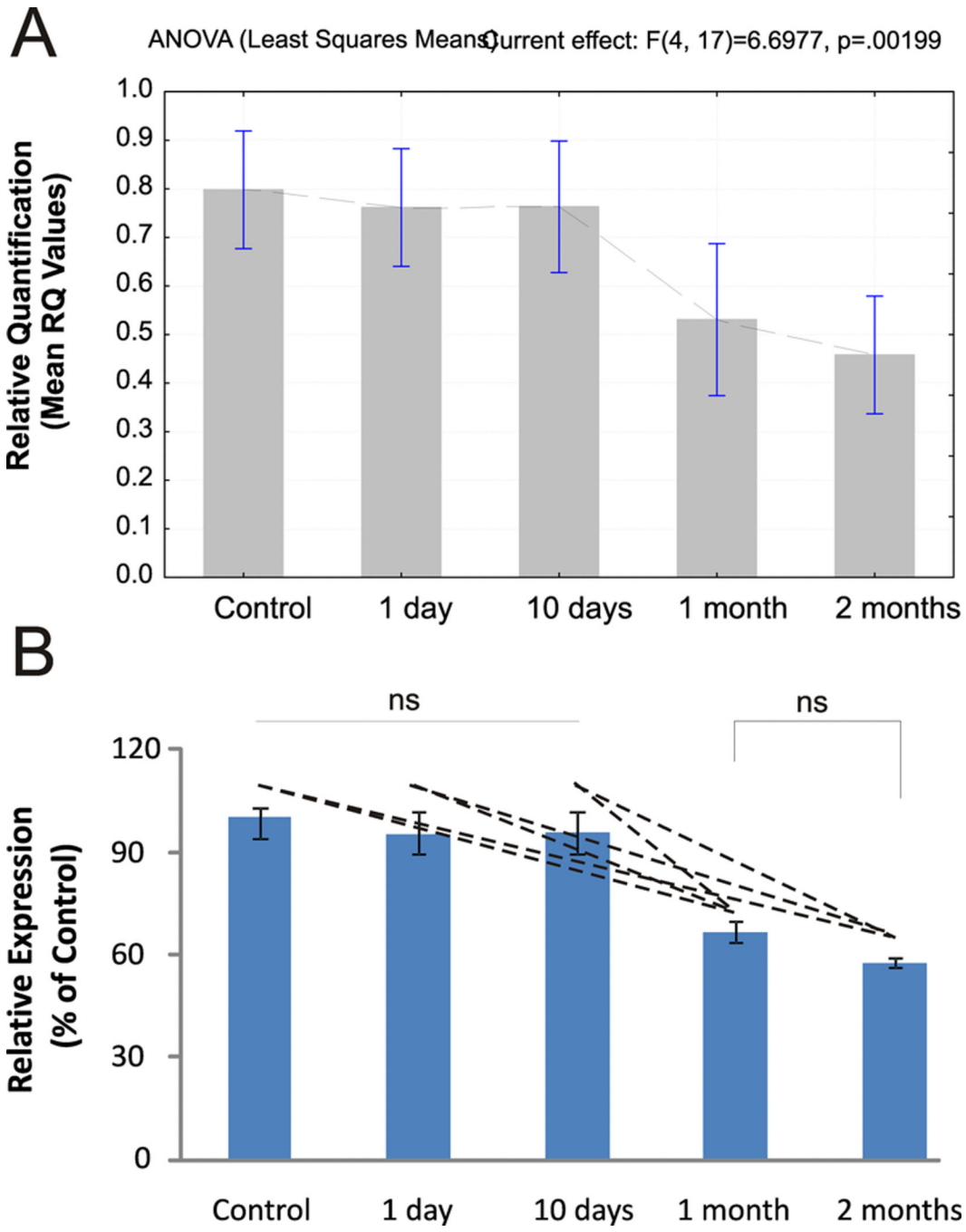

Figure 1.

Analysis of gene expression of Kv3.4 by the comparative delta-delta CT qPCR method using TaqMan assays. A. Analysis was performed in total RNA isolated from hippocampus and converted to cDNA at 1 day, 10 days, 30–60 days (1 month) and at more than 2 months following pilocarpine-induced *status epilepticus*. Statistical analysis by one-way ANOVA revealed a significant difference for Kv3.4 expression among these groups ($F=6.69$, $P<0.01$). Bars represent mean ± SD of normalized relative quantification (RQ) index. B. Representative graph of real-time PCR analysis normalized to control values and expressed as percent of control. Bars represent mean ± SD of normalized data.

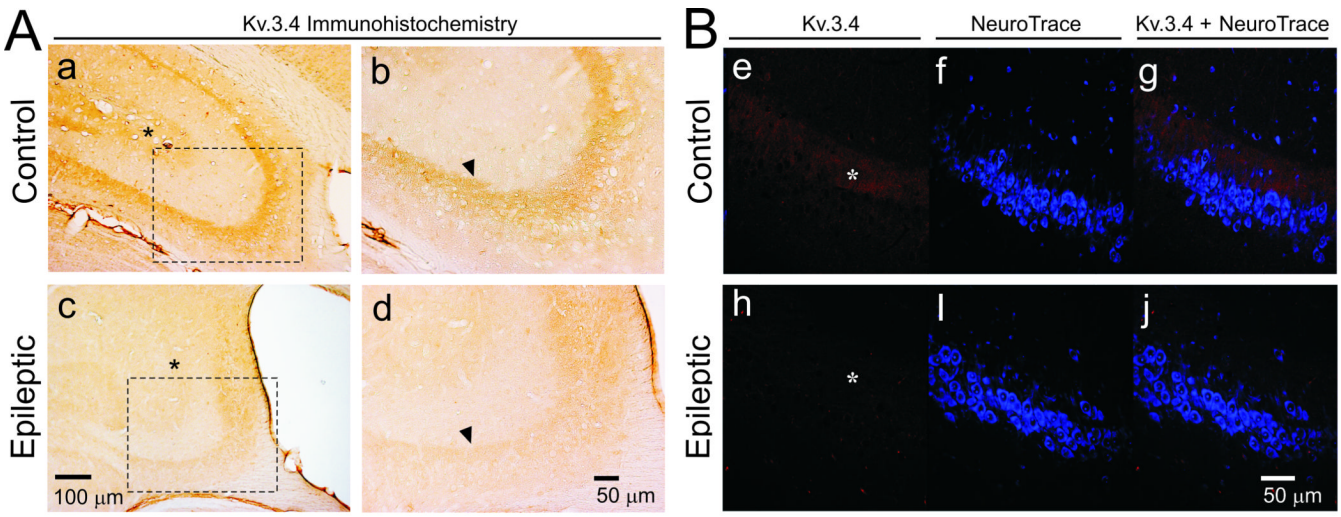

Figure 2.

Kv3.4 immunoreactivity hippocampus of control and chronically epileptic rats. A. Representative images of Kv3.4 immunohistochemistry show intense Kv3.4 immunolabeling in the *stratum lucidum* in CA3 and hilus of dentate gyrus. Notice moderate labeling of *stratum lacunosum/moleculare* (*) in a 75 days-old control rat (a). Marked Kv3.4 immunolabeling was noticed in *stratum lucidum* of CA3 at higher magnification (b). c. In chronic epileptic rat (82 days old), Kv3.4 was markedly reduced in *stratum lucidum* and hilus where a more diffuse staining was detected (*) (c). Reduction of Kv3.4 immunolabeling was evident in the *stratum lucidum* of CA3 at higher magnification (d). B. Laser scanning confocal microscopy images representing Kv3.4 immunofluorescence and counterstaining with fluorescent Nissl staining Neurotrace 435/455 to visualize position of neuronal cell bodies and proximal dendrites in CA3 area at higher magnification. Notice intense Kv3.4 immunofluorescent signal (red channel) above the pyramidal cell layer in an area corresponding to the *stratum lucidum* (* in h) of representative control rat (e–g). In contrast, Kv3.4 immunofluorescence signal was markedly reduced in the corresponding area (* in e) in a chronically epileptic rat (64 days after pilocarpine-induced SE) (h–j).

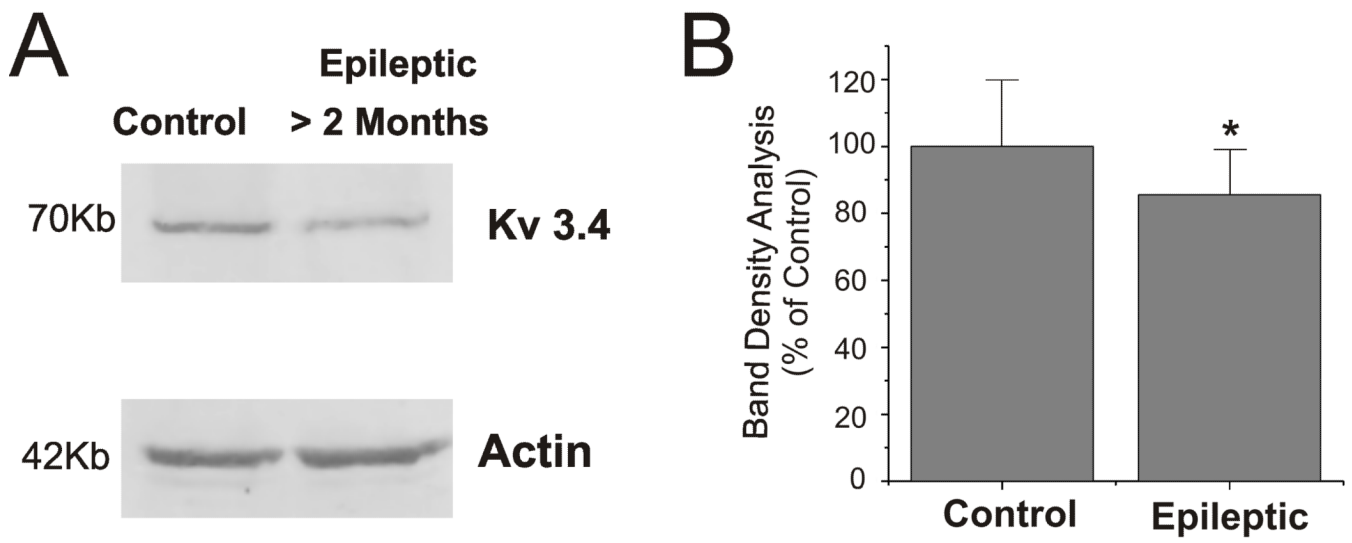

Figure 3.

Down-regulation of Kv3.4 protein expression in chronically epileptic rats. **A**. Representative immunoblot analysis for Kv3.4 expression in hippocampal lysates obtained from control rat (left lane) and in pilocarpine-treated rats more than 2 months (75 days) following *status epilepticus* (right lane). Immunoblot for actin were used as loading control. **B**. Semi-quantitative western blot analysis of density of immunopositive bands for Kv3.4 revealed a significant reduction in protein expression in chronic epileptic rats. *Significantly different ($p<0.005$) on Student t-test analysis compared to Control.

Table 1

Pilocarpine-induced *status epilepticus* decreased Kv3.4 gene expression along time.

	Mean RQ Value	SD
control	0.80	0.12
1 day	0.76	0.13
10 days	0.76	0.12
1 month	0.53*	0.08
2 months	0.46*	0.03

Data presented are mean relative quantification values (RQ) and standard deviation.

*Significantly different ($p<0.05$) on Tukey's HSD post hoc analysis compared to Control, 1 day and 10 days respectively.