3-20-2008

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Recommended Citation

Pacheco Otalora, L. F., Hernandez, E. F., Arshadmansab, M. F., Francisco, S., Willis, M., Ermolinsky, B., Zarei, M., Knaus, H. G., & Garrido-Sanabria, E. R. (2008). Down-regulation of BK channel expression in the pilocarpine model of temporal lobe epilepsy. Brain research, 1200, 116–131. https://doi.org/10.1016/j.brainres.2008.01.017

Published in final edited form as:
Brain Res. 2008 March 20; 1200C: 116–131.

Downregulation of BK channel expression in the pilocarpine model of temporal lobe epilepsy

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Abstract

In the hippocampus, BK channels are preferentially localized in presynaptic glutamatergic terminals including mossy fibers where they are thought to play an important role regulating excessive glutamate release during hyperactive states. Large conductance calcium-activated potassium channels (BK, MaxiK, Slo) have recently been implicated in the pathogenesis of genetic epilepsy. However, the role of BK channels in acquired mesial temporal lobe epilepsy (MTLE) remains unknown. Here we used immunohistochemistry, laser scanning confocal microscopy (LSCM), western immunoblotting and RT-PCR to investigate the expression pattern of the alpha-pore forming subunit of BK channels in the hippocampus and cortex of chronically epileptic rats obtained by the pilocarpine model of MTLE. All epileptic rats experiencing recurrent spontaneous seizures exhibited a significant down-regulation of BK channel immunostaining in the mossy fibers at the hilus and *stratum lucidum* of the CA3 area. Quantitative analysis of immunofluorescence signals by LSCM revealed a significant 47% reduction in BK channel in epileptic rats when compared to age-matched non-epileptic control rats. These data correlate with a similar reduction in BK channel protein levels and transcripts in the cortex and hippocampus. Our data indicate a seizure-related down-regulation of BK channels in chronically epileptic rats. Further functional assays are necessary to determine whether altered BK channel expression is an acquired channelopathy or a compensatory mechanism affecting the network excitability in MTLE. Moreover, seizure-mediated BK down-regulation may disturb neuronal excitability and presynaptic control at glutamatergic terminals triggering exaggerated glutamate release and seizures.

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Classification terms: THEME J: DISORDERS OF THE NERVOUS SYSTEM
Epilepsy: basic mechanisms
Epilepsy: human studies and animal models

Section: **Disease-Related Neuroscience**
Associate Editors: George F. Koob, La Jolla, CA, USA.

Keywords

pilocarpine; epilepsy; presynaptic; granule cells; mossy fibers

Introduction

Previous studies of large conductance calcium (Ca^{2+})-activated potassium (BK) channel physiology (Hotson and Prince, 1980) and immunocolocalization in the hippocampus (Knaus et al., 1996) prompted investigators to suggest that these channels play a major role in the pathogenesis of acquired mesial temporal lobe epilepsy (MTLE). Nonetheless, more than ten years later, this notion remains to be elucidated.

Functional BK channels are multimeric complexes consisting of a pore-formingα subunit and auxiliary β subunits (*i.e.* β1 to β4) that influence channel expression and biophysical properties (Lu et al., 2006; Wu, 2003). BK channel opening yields an unusually large single-channel conductance of 200–400 pS (Ghatta et al., 2006; Salkoff et al., 2006). Accordingly, the activity of a few BK channels can produce a large outward potassium current exerting a robust modulatory action on excitability. The voltage gating of BK channels is modulated by Ca^{2+} (Salkoff et al., 2006), therefore, BK channels are considered neuronal calcium sensors playing an important feedback role in multiple processes including neurotransmitter release and cellular excitability. Interestingly, it has been revealed that BK channels exert a minimal action on normal neuronal function and it is generally believed that its functions may be critical in conditions leading to harmful effects on neurons (*e.g.* ischemia and trauma).

The *in vivo* physiological and pathophysiological role of BK channels remains an enigma. Because of its role in preventing excessive Ca^{2+} buildup and abnormal glutamate release, BK channels have become an attractive pharmacological target for developing neuroprotective agents (Vrudhula et al., 2005; Wu, 2003). Indeed, numerous BK channel openers are currently under investigation to ameliorate ischemic damage and trauma (Cheney et al., 2001; Gribkoff et al., 2001b; Hewawasam et al., 2003). Although potassium channel openers are thought to counteract uncontrolled cellular depolarizations (Lawson, 2000) their therapeutic utility remains unexplored in epilepsy.

Recent genetic studies have implicated enhanced BK channel function in the pathogenesis of genetic epilepsy (Du et al., 2005). In another study, the gene encoding the β3 subunit (*Kcnmb3*) has been associated with idiopathic epilepsy (Lorenz et al., 2006) and increased neuronal excitability (Hu et al., 2003). Supporting this notion, gene-targeted β4 (*Kcnmb4)* null mice display an epileptic phenotype (*i.e*. temporal lobe seizures) due to a gain-of-function for the BK channels (Brenner et al., 2005). Moreover, β4 deficient mice showed little spike frequency adaptation leading to a substantial increase in firing frequency. The dentate gyrus acts as a low-pass filtering gate that limits high-frequency inputs into the hippocampus (Heinemann et al., 1992; Nadler, 2003). Hence, β4 modulation on intrinsic firing properties of granule cells may protect against epileptogenesis (Brenner et al., 2005).

Because of their presynaptic location and function in glutamatergic pathways, BK channels may exert an antiepileptic effect in hippocampal circuits by controlling axonal excitability and glutamate release in chronic epilepsy. Here, we investigated the distribution of the BK channel expression in the hippocampus and cortex of epileptic rats obtained by the pilocarpine model of MTLE (Cavalheiro, 1995). Our data revealed an acquired seizure-dependent BK channel down-regulation in mossy fibers of chronically epileptic rats.

Results

Down-regulation of BK channels in axons and terminal fields in the hippocampus of chronically epileptic rats

The pattern of BK channel immunoreactivity has been previously described in the rodent hippocampus using polyclonal antibodies raised against residue positions 1118–1135 (highly conserved across species) of mouse BK channels (Kaczorowski et al., 1996; Knaus et al., 1996). We used three different antibodies that yielded a similar immunoreactivity pattern in six control rats which include three animals injected with saline instead of pilocarpine and three animals injected with pilocarpine but that did not exhibit seizures. These control animals did not exhibit behavioral seizures or any difference in the pattern and expression level of BK channels. To briefly summarize our findings, immunoreactivity for the α-pore forming subunit of BK channels detected with the polyclonal anti-$BK_{913–926}$ (1 μg/ml) (Wanner et al., 1999) was predominantly concentrated in the mossy fibers (Fig. 1a, clear arrow) which represents axons of granule cells innervating CA3 pyramidal neurons. Accordingly, we observed an intense BK channel staining in the hilus of dentate gyrus (Fig. 1a, arrow 1) of control rats. Immunolabeling of axon bundle-like fibers extended to the CA3 mossy fiber terminal zone, the *stratum lucidum* (Fig. 1c, clear arrow). Diffuse staining of moderate intensity was detected along the middle molecular layer of the dentate gyrus (Fig. 1a, *) and in the lateral portion of the *stratum lacunosum/moleculare* near the CA2-CA3 area (Fig. 1a, 2a) indicating that BK channels may be localized on perforant path axon terminals (Fig.1, 2b) Because the granule cell dendrites extend radially through both inner and outer molecular layers, while the mossy cell axons and terminals are restricted to the middle layer, the BK staining in the inner molecular layer most likely represents presynaptic localization (Cooper et al., 2000). Less intense and widespread staining was detected in the *stratum radiatum* and *stratum oriens* in CA2-CA3 (Fig.1 2a). In general, granule cells and pyramidal cells are devoid of somatic staining. Similar distribution of BK channel staining was previously reported in the mouse hippocampus using either monoclonal anti-$BK_{690–1196}$ (Misonou et al., 2006) or polyclonal anti-$BK_{675–1115}$ antibodies (Sailer et al., 2006).

Patterns of hippocampal BK immunoreactivity was assessed in chronically epileptic rats (n=7) and qualitatively and quantitatively compared to control ones. In order to demonstrate that deficit in BK channel expression in the hippocampus of epileptic rats is not due to seizure-related cell death we performed standard and fluorescent Nissl's staining (for co-localization studies) as presented in Figure 2a and Figure 2b respectively. Accordingly, the granule cell layer was preserved in epileptic rats although granule cell dispersion was noticed (compare Fig. 2a1 and Fig. 2b1 to Fig. 2a3 and Fig. 2b3 respectively) as described elsewhere (Masukawa et al., 1995). Moreover, Nissl's staining revealed moderate to severe neuronal loss in different areas of the brain of chronic epileptic rats experiencing more than one month of SE (Fig. 2) including the *hilus* of the dentate gyrus, hippocampal subfields CA1 and CA3, different layers of the entorhinal cortex, and the amygdale. Extensive gliosis and atrophy were also noticed in the hippocampus as previously reported (Borges et al., 2003; Liu et al., 1994; Tang et al., 2004). In marked contrast with control rats, chronically epileptic rats exhibited a highly significant reduction in the BK channel immunoreactivity intensity in the hilus of dentate gyrus and CA3 *stratum lucidum* (Fig. 1c, 1e and 1b). At high magnification, some BK immunolabeling was sparsely detected on mossy fiber bundles traversing the stratum lucidum close to CA3 pyramidal layer (Fig. 1d). Staining was also reduced in the *stratum lacunosum/moleculare* and dentate gyrus molecular layer (Fig. 1c, 1b) consistent with a depletion of BK channels from glutamatergic axonal system innervating the hippocampus (*i.e.* from enthorinal cortex). Epileptic rats experienced spontaneous recurrent seizures (an average of 5–6 seizures per week) with a mean latent period of 9.5±2 days (ranging 8–15 days). Immunohistochemical procedures were performed on epileptic rats that suffered at least 20 days of post-SE period.

A complex process of epileptogenesis develops as a consequence of SE insult (Leite et al., 2002; Mello et al., 1993). As previously described in the pilocarpine model, granule cells in the dentate gyrus are apparently preserved (Fig. 2, a3,b3) but show signs of dispersion (Leite et al., 2002; Mello et al., 1993). In contrast, a marked cell loss was observed in CA1 (not shown) and CA3 area of epileptic rats in both standard Nissl staining (Fig. 2, a4) and in counterstaining (fluorescent Nissl staining) of BK channel immunolabeled sections (Fig. 2, b4). Intense BK channel staining was detected in the hilus and *stratum lucidum* in control sections contrasting with a marked reduction in chronically epileptic rats (Fig. 2, b3, b4). In order to assess whether BK channel immunolabeling reduction is related to seizures, several animals were sacrificed at different post-SE survival periods. We observed a progressive seizure-related down-regulation of BK channel staining that appeared more evident after a post-SE period of 20–30 days (n=3) (Fig. 2, c3) when compared with early stages of the disease (<10 days post-SE period, n=5) where staining was comparable to control rats (Fig. 2, c1 and c2). Animals suffering a longer survival period and consequentially larger number of spontaneous recurrent seizures (45–65 post-SE period, n=10) exhibited a marked reduction in BK channel immunoreactivity in the hilus and CA3 *stratum lucidum* (Fig. 2, c4).

Moreover, we noticed that the decline in mossy fiber BK immunorectivity progressed with the evolution of the disease since sections from rats sacrificed at more than 55 days post-SE period displayed minimal BK channel expression. Further studies are necessary to confirm this notion.

Immunofluorescence of BK channel and VGlut1 in the hippocampal formation

The reorganization of mossy fibers and reactive neo-synaptogenesis are well-known physiopathogenic phenomena in human and experimental MTLE as confirmed using Timm's histochemistry or immunohistochemistry (growth-associate phosphoprotein or VGluT1 staining) in the pilocarpine model of epilepsy (Gabriel et al., 2004; Mello et al., 1993; Naffah-Mazzacoratti et al., 1999; Pacheco Otalora et al., 2006). Therefore, the decrease in BK immunostaining at *stratum lucidum* and hilus in epileptic rats probably represent a down-regulation in BK channel expression considering that the mossy fiber system is preserved and reorganized in the epileptic brain. We further explored this notion by performing double immunofluorescence analysis of BK channel and VGluT1. These experiments allowed us to determine the relative amount of BK channel staining deficit in epileptic rats by using quantitative confocal imaging analysis (Pacheco Otalora et al., 2006). First, we performed co-localization assays using high-resolution confocal imaging and digital zoom (>300x) to assess whether BK channel immunolabeling was localized at mossy fiber axons or presynaptic boutons (Fig. 3). At high magnification, agglomerates of VGluT1-positive rounded-shaped elements with an immunonegative core in most cases, appeared to be distributed in patterns resembling giant mossy fiber presynaptic *boutons* (Fig. 3A) surrounding thorny excrescences at dendrites of CA3 pyramidal cells (Gonzales et al., 2001). BK immunofluorescent signals were not completely co-localized at these VGluT1-positive structures but appear as densely packed axon bundle-like fibers infiltrating through VGluT1-negative areas in the core of the mossy fiber projection area (Fig. 3B). Co-localization assays indicate that BK channels may be distributed at the periphery of mossy fiber boutons but this notion had to be confirmed in further electron microscopy studies (Fig. 3C). The pattern of BK channel-VGluT1 expression resembled previous data for mGluR2/3-VGluT1 co-labeling (Pacheco Otalora et al., 2006). It has been proposed that mGluR2/3 is localized at the axons and the neck of the synaptic boutons (extrasynaptic locations) (Shigemoto et al., 1997). Additional electron microscopy studies are necessary to confirm whether BK channels are expressed at similar locations.

In control rats, intense VGluT1 immunoreactivity was observed at the mossy fiber pathways (Fig. 4A, upper panel) as described elsewhere (Kaneko and Fujiyama, 2002;Kaneko et al., 2002;Pacheco Otalora et al., 2006). In control and epileptic rats, VGluT1 staining was abundant

in hippocampal *stratum radiatum*, *stratum lucidum* and *stratum oriens* but less intense or almost absent at the *stratum pyramidale* and granule cell layer. VGluT1 labeling was intense in the *hilus* and CA3 where it appeared as a dense c-shaped band at the *stratum lucidum (suprapyramidale region)* (Fig. 4). Animals experiencing pilocarpine-induced SE had abundant VGluT1-positive elements in the CA3 *stratum pyramidale* which extend massively to the *stratum oriens* in a zone immediately surrounding the outer boundaries of *stratum pyramidale* but also in the area corresponding to the infrapyramidal region in *stratum oriens*) (Fig. 4Ad, see arrows) consistent with previous findings of abnormally sprouted mossy fiber projections in epileptic rats (Holmes et al., 1999;Liu et al., 1999).

Although BK channel immunoreactivity was present in the hilus of epileptic rats (Fig. 5e, **) it was less intense than the staining in corresponding area of a control dentate gyrus (Fig. 5b,*) as confirmed in the statistical analysis of the mean fluorescence intensity (Fig. 4. B, C). Quantitative analysis of BK channel immunoreactivity was performed in circled region of interests (ROI=50 μm diameter) positioned in the *stratum lucidum* and in the hilus at corresponding positions for control and epileptic rats. Analysis of fluorescence intensity was simultaneously performed using confocal imaging and same parameters of the photomultiplier tube gain and laser excitation for each set of control and epileptic rats. Two sections were selected from dorsal hippocampus in each animal and measurements of relative fluorescence intensity were performed at three different locations (ROIs) in the hilus and at the *stratum lucidum*. The mean fluorescence intensity for each region was computed and averaged for both sections in each control and epileptic rats. Accordingly, we determined the percent change (%) in immunofluorescence intensity in six pairs (sets) of epileptic (suffering 30–65 days of post-SE period) and age-matched control rats which included three saline-injected rats and three animals that were injected with pilocarpine but did not developed seizures. No differences were noticed between each sub-group of control rats. Analysis of BK immunofluorescence revealed a significant 47% reduction in relative fluorescence intensity in the CA3 *stratum lucidum* of epileptic rats (877.6±139) when compared to controls (1456.5± 167.17) (Fig. 4B, 4C) (Student's t-test, T=2.65, DF=10, *P*=0.024, n=6). Similarly, BK fluorescence intensity was significantly reduced (~60%) at the *hilus* of the dentate gyrus in chronically epileptic rats (900.3 ±134.5) compared to control group (1914.15±366.6) (Fig. 4B and Fig.4C) (Student's t-test, T=2.5, DF=10, *P*=0.026, n=6). All together, data revealed a significant down-regulation of BK channel expression at the CA3 *stratum lucidum* and hilus in the hippocampus of chronically epileptic rats. Additionally, we found a strong VGluT1 immunopositive band in the supragranular region (inner molecular layer) of epileptic rats resembling reactive supragranular mossy fiber sprouting (Fig. 5 d2) as previously reported in pilocarpine-treated epileptic rats (Pacheco Otalora et al., 2006) and elsewhere (Epsztein et al., 2005). This VGlut1-positive band (supragranular sprouting) in the supragranular layer appeared devoid of BK immunostaining (compare Fig. 5b to Fig. 5e). Moreover, BK staining was also reduced in the middle molecular layer and in the hilar region (Fig. 5e1, f**), a major recipient of perforate path axons from the entorhinal cortex layer II neurons. In the dentate gyrus, VGluT1 stained the *hilus* intensely at areas adjacent to granule cells corresponding to mossy fibers initial segments extending towards the CA3 *stratum lucidum* (Fig. 5a and Fig. 5d). As described earlier, VGluT1 immunoreactivity was significantly enhanced in the *hilus* of epileptic rats (Pacheco Otalora et al., 2006). In a separate experiment, co-localization assays of BK channel and synaptic terminal marker synaptophysin were developed revealing that mossy fibers are preserved in epileptic rats while the expression of BK channel is down-regulated (data not shown) which confirms the immunoreactivity of VGluT1 in the mossy fiber pathway.

BK channel protein expression in hippocampus and cortex in chronically epileptic rats.

Western blot analysis was performed to examine alterations in BK channel protein levels in hippocampus and cortex during the chronic phase of the pilocarpine model of epilepsy. Total

protein per sample was measured and same amount subjected to Western blot analysis using two different primary antibodies against BK channels to allow comparisons. Samples from control and epileptic rat pair set were simultaneously processed in same membrane (n=4) using anti-$BK_{690–1196}$ antibodies (L6/60; 1:500-1:1000; UC Davis/NINDS/NIMH). Consistent with previous studies (Chen et al., 2005; Knaus et al., 1996), BK channel protein was identified as a diffuse immunopositive band with apparent molecular weight in the 117–122 kDa range (Fig. 6A) probably consisting of multiple partially resolved components and splice variants (Chen et al., 2005; MacDonald et al., 2006). After measuring the optical density, we found that the levels of BK channel protein (~120 kD bands) were significantly less intense in epileptic samples from cortex (34.5±4.9% reduction) and hippocampus (24.2±3.4% reduction) (*$p<0.05$, Student t-test). Assays were performed on protein extracted from cortex and hippocampus of control (n=4) and epileptic rats (n=4). After protein quantification (BCA method), PVDF membranes and were probed with BK antiserum (1:500, UC Davis). Similar results were obtained when western blot assays were performed using anti-$BK_{1098–1196}$ (Alomone labs). This band was not present when the primary antibody was omitted, or when the primary antibody anti-$BK_{1098–1196}$ was pre-incubated with corresponding control peptide (3μg/μg of antibody; Alomone labs) (data not shown). In order to determine whether down-regulation of BK channel expression is associated with granule cell loss and their markers we developed western blotting for VGluT1 (a synaptic marker highly expressed in mossy fibers) in protein extracted from control and epileptic of hippocampus (30–60 days post-SE period, n=4). These experiments revealed a non-significant reduction of VGluT1 protein expression (~60 kD) at one month of post-SE period (Fig. 6C, E1) and a minor reduction after two month following SE (Fig. 6C, E2). For the statistical analysis, data from all epileptic rats were pooled together and revealed a non-significant reduction on the density of immunopositive VGluT1 band in epileptic rats when compared to control rat (Fig. 6D) (Student t-test, $P=0.43$). These data indicate that BK channel expression is down-regulated in mossy fiber of epileptic rats which still maintain capability to express VGluT1 synaptic marker following recurrent seizures.

BK channel transcripts are down-regulated in micro-dissected granule cells of epileptic rats

To determine whether reduction of BK channel immunoreactivity and protein levels in epileptic hippocampus correlate with a decline in mRNA transcript expression, we developed semi-quantitative RT-PCR assays. Total RNA extracted from micro-dissected dentate gyrus (containing granule cells) of control and epileptic rats was normalized and used as template. BK channel expression data was normalized to the signal obtained from the amplification of the constitutively expressed (“housekeeping”) gene. To discern changes in expression levels, the number of PCR cycles was adjusted so that both signals would be amplified within the exponential phase of the reaction as previously determined (not shown). The primers to amplify rat BK transcripts have been previously characterized and yield a 312 bp product (Muller et al., 1998). Semi-quantitative RT-PCR assays revealed that expression of BK channel transcripts was significantly decreased at 30 cycles (31.1±3.5% reduction of controls, $P<0.01$, Paired *t*-test, n=4) (Fig. 6F). A non significant reduction was observed at and 40 cycles (Student’s t-test, $P=0.1$) when compared to control levels (Fig. 6F). Since in semi-quantitative PCR the differences in signal intensity might not correlate directly to the amount of mRNA present in the respective tissue, further real time quantitative PCR assays will be necessary to monitor seizure-induced changes in transcripts levels for BK channels and splice variants. Primers for the housekeeping gene GAPDH (~274bp) were used for internal control and semi-quantitative analysis.

Discussion

Our findings indicate that BK channel expression is down-regulated at mossy fibers and molecular layer of dentate gyrus in the hippocampus from pilocarpine-treated chronically epileptic rats. Moreover, animals experiencing more seizures or longer post-SE survival period exhibited a more intense decline in BK channel expression indicating seizure-related phenomenon which contrasted with increased VGluT1 expression in the reorganized epileptic hippocampus. Changes in expression levels for BK and VGluT1 are most probably associated with the sustained epileptogenic process after SE and not due to the acute treatment with the chemical pilocarpine considering that these abnormalities were not detected in control animals that received pilocarpine but did not developed seizures. It remains to be elucidated whether such dysregulation on a subset of ion channels is cause by a seizure-induced long-lasting deficiency of the transcriptional/translational machinery and whether these changes may lead to enhanced excitability of neuronal networks. The notion that acquired channelopathies may develop in the course of temporal lobe epilepsy is supported for recent findings in the pilocarpine model of epilepsy (Bernard et al., 2004; Dyhrfjeld-Johnsen and Soltesz, 2004; Hirose, 2006; Jung et al., 2007; Poolos, 2005; Richichi et al., 2007). For instance, it was recently reported that a progressive transcriptional channelopaty ("downregulation") of hyperpolarization-activated cation (HCN) channels occurs in dendrites of CA1 hippocampal pyramidal neurons after pilocarpine-induced SE (Jung et al., 2007). These authors suggest that such deficit in HCN expression and function may increase neuronal excitability and may be associated with both the process of epileptogenesis and maintenance of the epileptic state. The mechanisms responsible for changes in expression levels (*i.e.* down-regulation) remains to be determined, but it may probably involve changes in the transcription or translational machinery. For instance, it was proposed that the mechanisms for HCN1 reduction involved calcium-permeable AMPA receptor-mediated calcium influx, and subsequent activation of Calcium/calmodulin-dependent protein kinase II (Richichi et al., 2007). Seizure-related changes in components of the transcriptional and translational machinery may contribute to dysregulation of genes and proteins in epilepsy. For instances, it has been reported an abnormal histone acetylation after SE induced by kainic acid, a modification that was considered to play an important role in the development of epilepsy (Taniura et al., 2006). Furthermore, the investigation of seizure-related modifications on the expression levels of biologically-relevant molecules such as ion channels, transporters, enzymatic pathways, etc is being considered a promising strategy to uncover molecular mechanisms of epilepsy as well as potential new antiepileptogeneic targets (Aronica and Gorter, 2007; Crino and Becker, 2006; Jamali et al., 2006; Lukasiuk et al., 2006; Majores et al., 2004; Oscarson et al., 2006). For instances, a large number of down-regulated and up-regulated genes were detected using gene expression profiling by the Affymetrix Gene Chip (RAE230A) in samples (total RNA) isolated from CA3 area and entorhinal cortex at different periods (acute, latent and chronic) following electrically-induced SE (Gorter et al., 2006).

In the hippocampus, the majority of BK channels are preferentially located at glutamatergic presynaptic terminals especially at the mossy fibers and Schaffer's collateral where they are thought to act as a homeostatic mechanism ("emergency break") for regulating synaptic transmission in conditions of enhanced excitability. Several acquired channelopathies have been recently described in experimental and human MTLE including a loss of A-type potassium ion channels (Bernard et al., 2004) and abnormalities of sodium channels (Celesia, 2001; Lerche et al., 2001; Noebels, 2002; Remy et al., 2003). Here, we described, for the first time, an acquired, most probably presynaptic BK channel deficit in experimental MTLE.

Multivesicular glutamate release from mossy fiber boutons can reliably fire several CA3 pyramidal cells ("detonator synapses") (Henze et al., 2002). Hence, down-regulation of BK channels and impaired feedback regulation of Ca^{2+} concentration may trigger hyperactivity of

mossy fibers, exaggerate glutamate release and cell death in the epileptic hippocampus (Nadler, 2003). Upon activation, the large outward BK current hyperpolarizes the preterminal limiting Ca^{2+} influx and transmitter release. BK channels are in close proximity to Ca^{2+} channels (Robitaille et al., 1993). BK channels are positioned to react to action potential-triggered Ca^{2+} influx. Electrophysiological approaches indicate that BK channels can control glutamate release under conditions of excessive neuronal activity (Gribkoff et al., 2001a; Hu et al., 2001; Yamashita et al., 2006). Functionally, a deficit in axonal or presynaptic BK channels might render mossy fibers hyperexcitable during epileptogenesis. Insulin inhibits pyramidal neurons through a similar mechanism that was proposed as a potential therapeutic target for the treatment of epilepsy (O'Malley et al., 2003). Moreover, it was proposed that during periods of enhanced synaptic activity excessive activation of group I and II metabotropic glutamate receptors (mGluR I and II) inhibits the apamin-insensitive slow Ca^{2+}-activated K^+ current in hippocampal CA1 pyramidal neurons that in turn can cause epileptogenesis (Martin et al., 2001). Indeed, several presynaptic modulators of mossy fiber presynaptic machinery have been implicated in the pathophysiology of MTLE including mGluR II (Pacheco Otalora et al., 2006), neuropeptide Y (Tu et al., 2005) and kainate receptors (Epsztein et al., 2005; Feng et al., 2003). If the pro-epileptic role of such disturbances ultimately can be demonstrated in the experimental ground it will lead to a new concept of acquired presynaptic multi-channelopathies in MTLE.

The exact role of BK channel in controlling hippocampal neuronal bursting activity remains to be elucidated. For example, iberiotoxin (IbTX) completely inhibited bursting activity induced by pentylenetetrazol (PTZ), caffeine, 1,4,5-inositol triphosphate (IP3) and direct forced increase of intracellular calcium (REF). In this study, spontaneous bursting activity in the cerebral cortical neurons of the El mouse, which shows a high susceptibility to convulsions, was also completely inhibited by IbTX (Jin et al., 2000). However, insulin and leptin that inhibit epileptiform-like bursting activities in rat hippocampal neurons might involve in activation of BK channels (O'Malley et al., 2003; Shanley et al., 2002). Interestingly, BK channel knockout mice do no apparently exhibit seizures but cerebellar-related neurological problems (*i.e.* ataxia) (Sausbier et al., 2004). Hence, a functional study in parallel with changes in expression of BK channels associated with pilocarpine-induced epilepsy will certainly contribute to a better understanding of the role of this acquired channelopathy on the pathogenesis of temporal lobe epilepsy.

The *in vivo* pro-epileptic or antiepileptic effect of BK channel modulation is obscured by recent findings indicating that mossy fibers co-release GABA with glutamate in MTLE (Gutierrez and Heinemann, 2006). In such scenario, and considering that "gain-of-function" of BK channels has been proposed to increased granule cell excitability, seizure-mediated down-regulation of BK channels at mossy fibers may hypothetically reduce mossy fiber excitability and consequentially reduce the release of GABA which will result in a indirect disinhibition in CA3 area. Further studies are necessary to tackle this dilemma. If presynaptic BK channels also block presynaptic GABA release at mossy fibers or interneurons that may results in enhanced CA3 excitability characterized by recurrent or continuous seizure-like activity and spreading depression in some cases (Debanne et al., 2006; Wong and Traub, 1983).

Recent genetic studies indicate that a gain-of-function BK channel mutation (increased open probability) is responsible for generalized epilepsy in humans (Du et al., 2005). In this study, several mechanisms were proposed to explain the paradoxical increase in excitability at thalamo-cortical circuits by a mutated BK channels exhibiting increased open-channel probability. For instance, it was suggested that enhanced BK channel function can accelerate action potential repolarization leading to removal of the inactivation of sodium channels and thus allowing higher firing frequencies. An alternative explanation was that mutated channels are differentially expressed at GABAergic neurons, then provoking inhibition of these neurons

that could lead to network disinhibition and seizures. In MTLE, seizures originate in the hippocampus para-hippocampal areas (partial seizures) with secondary generalization in some cases(Arabadzisz et al., 2005; Bernasconi et al., 2005; Cavazos and Cross, 2006; Kutlu et al., 2005). Therefore, the role of BK channel dysfunction will probably be different in generalized epilepsy with thalamo-cortical involvement. Interestingly, β4-knockout mice with associated gain-of-function of BK channels in granule cells were reported to exhibit distinctive temporal lobe seizures emanating from hippocampus (Brenner et al., 2005). In this study β4 deficit enhanced the activity of BK channels that, in turn, altered the intrinsic properties of granule cells by sharpening the action potential and sustaining higher firing rates. Hence, these authors hypothesized that β4 expression represents a unique nonsynaptic mechanism for gate control of hippocampal synchronization leading to temporal lobe epilepsy. Supporting this hypothesis, a fraction of granule cells in the sclerotic hippocampus of MTLE patients are hyperexcitable displaying a weak spike frequency adaptation and an enlarged fast afterhyperpolarization (Selke et al., 2006). Furthermore, a recent microarray study revealed variations in the down-regulation of *Kcnmb4* in CA3 and entorhinal cortex during acute and latent periods after SE (Gorter et al., 2006). Our study focused on the α-pore-forming BK channel subunit instead of other auxiliary subunits but it will be interesting to further explore the role of β4 in acquired MTLE. Expression of β4 RNA has been reported in granule cells but it is not clear whether this subunit is expressed at BK channel complexes in mossy fibers or at somatodendritic regions. Curiously, in granule cells, BK channels are mainly targeted to axons with minimal expression at somatic compartments. Moreover, BK channel immunoreactivity at the middle molecular layer has been related to perforant path preterminals but not with expression at dendrites of granule cells (Knaus et al., 1996). Despite the exact role of BK channel dysfunction on enhancing presynaptic versus postsynaptic excitability of granule cells away further elucidation in MTLE, the dramatic down-regulation describe here may be part of a protective compensatory mechanism to further reduce seizures (according to the "β4 down-regulation hypothesis").

BK channel complexes, especially formed by β4 subunits, are widely distributed throughout the mammalian brain (Behrens et al., 2000; Brenner et al., 2000; Ha et al., 2004). The precise distribution and preferential trafficking of BK channels to axon terminals and somatodendritic regions can ultimately determine the function of BK channels in neurons and circuits. For instance, in hippocampal pyramidal neurons, BK channels are trafficked to presynaptic membranes where they are thought to modulate glutamatergic neurotransmission in conditions of excessive excitability (Hu et al., 2001). Nevertheless, in many neurons, BK channels also contribute to the action potential repolarization and to the fast afterhyperpolarization thereby influence additionally the neuronal firing frequency (Shao et al., 1999). Such functional evidences of somatodendritic localization lead to additional electron microscopy and immunocolocalization studies that confirmed the expression of BK channels in dendritic membranes at hippocampal glutamatergic synapses, in addition to their well-established presynaptic location (Sailer et al., 2006). The abundance of BK channels on such subcellular locations is differentially regulated across several neuronal phenotypes. In the hippocampus, for instance, BK channels are highly expressed at the Schaffer's collateral pathway and mossy fibers of granule cells (Hu et al., 2001; Knaus et al., 1996; Misonou et al., 2006; Sailer et al., 2006).

Hyperactive neuronal circuits in the limbic system (*e.g.* hippocampus) are the major contributors to chronic hyperexcitability states leading to spontaneous recurrent seizures in MTLE. Accordingly, as a result of anomalous neuronal firing, neurons in the epileptogenic zone are constantly challenged by sudden and dramatic increases in intracellular calcium (Ca^{2+})(Delorenzo et al., 2005). For instance, it has been reported that large transient elevations in neuronal Ca^{2+}levels correlate with epileptiform discharge onset (Pisani et al., 2004). Altered Ca^{2+} homeostasis is thought to underlie some aspects of the epileptic phenotype and contribute

to the persistent neuroplasticity changes associated with acquired MTLE (Delorenzo et al., 2005). For instance, epileptic neurons in the pilocarpine model have been shown to maintain consistently higher baseline intracellular Ca^{2+} levels in the range of 250 to 400 nM that peak up to high micromolar ranges (20–50 μM) after stimulation (Pal et al., 2001; Raza et al., 2001). Moreover, these neurons show a delayed recovery of increased Ca^{2+} homeostasis leading to abnormal baseline Ca^{2+} levels in epileptic neurons. Deficit in feedback regulation via BK channels may contribute to persistent impairment of neuronal Ca^{2+} homeostasis in MTLE.

The reduction in BK channel protein and mRNA transcript levels suggest that the most probable mechanism for a reduction in BK channel expression is related to a transcriptional down-regulation of the BK channel genes instead of post-transcriptional defects including abnormal trafficking of channels to the membrane. It has been recently reported that pilocarpine-triggered SE can induced changes in expression of ion channels and transporters that might be associated with the pathogenesis of mesial temporal lobe epilepsy. For instances, a recent report by Jung et al revealed progressive dendritic HCN channelopathy during epileptogenesis in the rat pilocarpine model of epilepsy (J Neurosci. 27:13012–21, 2007).

In summary, since epileptogenesis is linked to excessive glutamate release, we hypothesize that BK channel dysregulation may render neuronal circuits hyperexcitable and contribute to epileptogenesis in MTLE. Nonetheless, these changes in BK expression can also be interpreted as a compensatory mechanism that reduces hyperexcitability if the putative pro-epileptic role of BK channels can be indeed demonstrated. A better understanding of the molecular mechanisms as well as the electrophysiological consequences of acquired presynaptic BK channel deficit in chronic epileptogenesis may result in novel therapeutics (*e.g.* BK channel openers) for the treatment of MTLE.

Experimental Procedure

Animals and rat model of chronic epilepsy

All experiments were performed 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). Wistar rats were kept in acclimatized temperature-controlled *vivarium* with water and food *ad libitum*. All efforts were made to minimize the number of animals in the study. Chronically epileptic rats were obtained by the pilocarpine model of temporal lobe epilepsy following described procedures which is characterized by bilateral hippocampal pathology (Cavalheiro, 1995; Mello et al., 1993). Therefore, one hemisphere was used for RNA isolation and the other for protein extraction. At the time of performing the model of epilepsy animals were approximately 20–30 days (180–250g). A total of forty animals were used in our experiments. Pilocarpine was administered between 9 A.M. and 12 A.M in an acclimatized room set at 20° C. 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) (350 mg/kg in saline, i.p.). Controls included (a) animals that received methyl-scopolamine but were injected with saline instead of pilocarpine**,** (b) pilocarpine-injected animals that did not exhibit seizures and (c) saline-injected control animals that also received diazepam. The control group that, despite being injected with pilocarpine, does not develop SE more effectively allow assessment of the effects associated with SE as opposed to the effects associated with the mere administration of pilocarpine. As previously described, these animals do not develop spontaneous recurrent seizures and the brain is as normal as in saline-injected rats (i.e. no detectable neuronal death, etc). Several studies have demonstrated that molecular, structural

and functional changes observed during the chronic phase, an even early after SE, on the pilocarpine model of epilepsy are strictly related to self-sustained seizures (SE) but not to the administration of pilocarpine (Cavalheiro et al., 1991; Mello et al., 1993; Mello et al., 1996). It has been reported that more than 30 min of SE are necessary to trigger long-term modifications in neuronal circuits and chronic epileptogenesis (Lemos and Cavalheiro, 1995). Likewise, no significant differences or alterations on BK channel expression have been observed among those control groups. Therefore, data from those groups was grouped together as a single control group. In addition to facilitate the analysis, grouping together control animals allow us to minimize the number of animals necessary for the study. Systemic pilocarpine injection induced *status epilepticus* (SE) in 70% of injected rats consisting of continuous tremor, rearing, myoclonic jerks, clonic forearms and head movements with eventual side fallings. Only animals that experienced at least 3 hr of self-sustained SE (up to 4 hr duration) were included in this study. To minimize the mortality rate of the procedure, animals were allowed to remain in SE for 3 hr rats by administering diazepam (10 mg/kg, *i.p.*) to quell behavioral seizures (Danzer and McNamara, 2004; Mello et al., 1993; Pacheco Otalora et al., 2006). All animals that received pilocarpine were given Nutra-Gel® soft food (Bio-Serv, Frenchtown, NJ), fresh apples and water in an easily-reachable container inside the recovery cage for 48 hr after SE induction. Several subcutaneous injections of 20 ml Ringer-lactate was administered to compensate for any liquid lost (*i.e.* salivation, urination) every 8 hr after SE termination for 24 hr. SE induction protocol was lethal in about 20% of pilocarpine-treated rats. After SE, rats were monitored for detection of at least two spontaneous seizures using a JVC MiniDV digital video-camera and researcher-assisted SeizureScan software (Clever Sys., Inc, Reston, VA) to define the beginning of the chronic phase (on average 2–4 weeks after SE induction). Seizures were confirmed *off-line* by an experienced researcher. Only seizures graded ≥3 in the Racine's scale (Racine, 1972) per week were computed (according to maximal sensitivity of the detection system). In average, SE-suffering rats experienced approximately 5–8 seizures per week regularly during the observation period.

Tissue preparation and immunohistochemistry

Age-matched control (see below) and epileptic rats were anesthetized with 100 mg/kg ketamine and transaortically perfused with 4% formaldehyde in 0.1 M sodium phosphate buffer containing 0.9% NaCl (PBS), pH 7.4. Brains were carefully removed and postfixed by overnight immersion in the same fixative at 4° C. After a brief wash in PBS, brains were cryoprotected in a solution of 30% sucrose in PBS for ~48 hr at 4° C. Brains were then immersed in OCT medium and frozen (−20° C) and 40 μm-thick coronal sections were sliced using cryostat (HM500 Microm by Zeiss, Waldorf, Germany) and stored in 150mM Tris, pH 7.4, in 150mM NaCl (TBS) containing 0.1% NaN_3. Coronal sections correspond to Paxinos's atlas at the level of Bregma −2.57 up to −3.8 (Paxinos and Watson, 2004). Several sections were Nissl-stained to assess amount of seizure-mediated neuronal loss as well as cytoarchitectonic boundaries and characteristics of hippocampal subfields. Corresponding free-floating sections were selected from both control and epileptic groups and processed simultaneously as described previously (Sailer et al., 2004). Free floating sections were permeabilized in TBS with 0.4% Triton X-100 (T-TBS) for 90 min. After blocking the endogenous peroxidase with 25% methanol and 1% H_2O_2 in TBS, nonspecific binding sites were blocked with 2% normal goat serum (NGS), 2% bovine serum albumin (BSA) and 0.2% milk powder in TBS-T. Brain sections were incubated with BK antibodies overnight, followed by a peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibody (1:400; Dako, Glostrup, Denmark) for 150 min. Immunoreaction products were visualized by addition of 0.06% 3,3′-diaminobenzidine and 0.003% H_2O_2 for exactly 30 min. Images were captured into Adobe Photoshop from a Zeiss Axiocam CCD 24-bit colour digital camera mounted on a Zeiss Axioplan 2 microscope (Zeiss, Oberkochen, Germany). For detection BK channels we used different primary antibodies as follows: a mouse monoclonal anti-$BK_{690–1196}$ (L6/60; 1–2 μg/ml, UC Davis/NINDS/NIMH

NeuroMab Facility), rabbit polyclonal anti-$BK_{1098–1196}$ (APC-021, 1–2 μg/ml; Alomone Labs, Inc) and affinity purified rabbit polyclonal anti-$BK_{913–926}$ (1 μg/ml) (Wanner et al., 1999).

Immunofluorescence

In order to assess the integrity of the mossy fiber pathway and whether BK channels are expressed at mossy fiber axon and terminals, double immunofluorescence assays were developed using anti-$BK_{690–1196}$ (2 μg/ml; L6/60) and guinea pig polyclonal anti-vesicular glutamate transporter 1 (anti-VGluT1; 1:10000, AB5905; Chemicon, Temecula, CA) on sections (40 μm) that were processed simultaneously from a pair of control and epileptic rats. Bound primary antibodies were detected by using a cocktail of fluorescence goat secondary anti-mouse or anti-rabbit IgG conjugates (1:200-1:1000; Invitrogen, Carlsbad, CA) tagged with Alexa Fluor 488 and 568 for green and red fluorescence respectively. Omitting primary antibodies from the immunohistochemistry processing steps eliminated fluorescence or peroxidase labeling of cells. Sections were mounted in histological slides, coverslipped using FluorSave™ reagent (Calbiochem, San Diego, CA), and stored at 4° C for analysis. All of the immunohistochemical procedures were performed at room temperature (~25°C) except for the incubation with primary antibodies (4°C). Fluorescent signals were captured by using computerized acquisition system Fluoview FV300 and inverted confocal laser scanning microscope (Leeds Precision Instrument Inc., MN) equipped with argon (488 nm) and krypton (568 nm) lasers for Alexa Fluor 488 (emission by 505–550 nm bandpass filter) and Alexa Fluor 568 (emission by 585 nm long-pass filter) imaging respectively. The two fluorophores were acquired separately and combined in Fluorview. For immunofluorescence quantification, several corresponding sectors (~50 um diameter) were selected for analysis in mossy fiber termination zone (*stratum lucidum*) and in hilus of dentate gyrus. To allow direct comparison of different data sets, imaging was performed using same parameters for laser excitation (*i.e*. intensity) and photomultiplier tube detection (*i.e*. sensitivity) in each set of control and epileptic samples. *For quantification of immunofluorescence intensity*, sections at equivalent coronal levels (3.0 to −3.6 mm from bregma) (Paxinos and Watson, 2004) were analyzed, and Nissl-stained adjacent sections were used to verify the identity of structures. For quantitative analysis, two sections from each pair of simultaneously processed control and epileptic rat set were analyzed by measuring the relative fluorescence intensity in Fluoview. Four hippocampi per animal (one slide per animal containing two adjacent sections, each with two hippocampi) were analyzed. Data from same animal were pooled together, averaged and presented as a percentage of change in fluorescence intensity when compared to related controls as previously described (Pacheco Otalora et al., 2006). Data were expressed as relative fluorescence intensity in arbitrary units ± SD.

Sample preparation and Western blotting

Paired four sets of age-matched control and epileptic rats were processed simultaneously. After decapitation, brains were removed and submerged in oxygenated (95% O_2/5% CO_2) ice-cold artificial cerebrospinal fluid (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 hippocampus and parietal cortex (~ 50 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-HCl 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 Cocktails 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 SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA) and bovine serum albumin (BSA) was used as standard. Samples from paired control and epileptic brain were adjusted to a final protein concentration of 1.5 mg/ml, boiled for 5 min in Laemmli buffer and resolved on Tris-glycine buffered SDS-PAGE (95V, 60 min). Proteins were electrotransferred (25 V, overnight)

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 40C with the primary antibody (anti-$BK_{690–1196}$, anti-$BK_{1098–1196}$ or anti-VGlut1, anti-neuron-specific nuclear protein, NeuN, Chemicon) diluted at 1:500 and 1:5000 in TBS containing 2% non-fat dry milk and 0.1% Tween-20. The membranes were washed in TBS with 0.1% Tween-20 then incubated for 2 hr in biotinylated secondary antibody (goat anti-mouse, anti-rabbit or anti-guinea pig) as recommended by vendor (Vector labs). After washing 3 times in 0.01M TBS, membranes were incubated 90 min in ABC (room temperature), rinsed 3 times (15 min each) in PBS and the proteins 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 BK and VGluT1 reactive bands using Quantity One 1-D Analysis Software (Bio-Rad). 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. Stripped membranes were reprocessed once for detecting VGluT1-positive bands using similar protocol. Western blot data were compiled from four independent control and epileptic rats for statistical analysis. For each experiment samples were run in duplicate. Samples from cortex and hippocampus for each pair of control and epileptic rats where processed in parallel on a single gel and subjected to same immunoblotting procedure.

Dentate gyrus RNA isolation

A set of a control and epileptic rats were anesthetized as above and rapidly decapitated to prepare 600 μm hippocampal slices in ACSF (2–4° C). The region of dentate gyrus was microdissected with 27 gauge needles assisted via stereomicroscope (10–20x). Sections were collected, weighed (~20 mg), homogenized, (Dounce tissue grinder) and processed for total RNA isolation using the RNeasy Mini Kit (Qiagen, Valencia, CA) following manufacture protocol. The concentration and purity of total RNA for each sample was determined by optical density measurements at 260 and 280nm using a BioMate 5 UV-visible spectrophotometer (Thermo Spectronic, Waltham, Mass.).The RNA integrity was assessed for clear, sharp 28S: 18S rRNA bands and electrophoresed on a 1% Agarose gel stained with ethidium bromide (Invitrogen).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA concentration was normalized for all samples. RNA samples from a pair of control and epileptic rats where processed in parallel under the same conditions. RT and PCR reactions were performed in iCycler Thermal Cycler PCR System (Bio-Rad) using SuperScript First Strand Synthesis reaction for RT-PCR System (Invitrogen) and Platinum Taq DNA Polymerase (Invitrogen). Three types of controls aimed at detecting genomic DNA contamination in the RNA sample or during the RT or PCR reactions were always included: a RT mixture without reverse transcriptase, a RT mixture including the enzyme but no RNA, and a PCR mixture with no cDNA template were subjected to PCR. First-Strand cDNA Synthesis reaction was performed using oligodeoxythymidine primer, 5μg total RNA, and 50 units of SuperScript II Reverse Transcriptase in 20μl of total volume. Specific primers for the rat BK channel related sequences were sense 5′-GGCTGGAAGTGAATTCTGTAG-3′ and antisense 5′-TGAGTAAGTAGACACATTCCC-3′. These primer sequences are based on the rat brain BK channel sequence rslo in regions that correspond in mslo to 1063-1083 bases and produced a product of 312 base pairs (bp) as reported before (Muller et al., 1998). Housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control and ran parallel in separate reaction tubes for the semi-quantitative relative

comparison purposes. GAPDH is a catalytic enzyme involved in glycolysis and is constitutively expressed in almost all tissues at high levels. Primers for the GAPDH sequence were as follows: GAPDH sense, 5′-CAGCACCAGCATCACCCCATTT-3′ and antisense 5′-CAAGATGGTGAAGGTCGGTGTGAA and produced a product of 200 bp. The BK channel reactions used the following steps: 1 cycle of 94 C for 3 min, denaturation at 94° C for 30 sec, annealing at 57° C for 30 sec, and polymerization at 72° C for 1 min for 20 cycles, 30 cycles, and 40 cycles; ending with 7 min at 72° C and storage at 4° C. The GAPDH reactions used the following steps: 1 cycle of 94 C for 3 min, 35 cycles of 94° C for 1 min, 59° C for 1.5min, 72° C for 1.5min, and a final 7 min extension at 72° C and stored at 4° C. Each PCR amplification (50 μl of final volume) contained 10μM sense and antisense primer, 10mM deoxynucleotides (dNTP), 10X PCR buffer, 50mM MgCl2, 5 units of Platinum Taq DNA Polymerase, and 2μl of cDNA template. For gel electrophoresis, 10 μl of the PCR reaction was electrophoresed on an 2% agarose gel at 80 V for 3.5 hrs. Gels were stained with ethidium bromide, visualized under U.V. light and images digitally acquired using Bio-Rad ChemiDoc XRS. Bands were identified according to DNA ladders and reported product size. To determine the expression BK channel mRNAs in control versus epileptic tissue, we used a semi-quantitative method based on the amplification of the constitutively expressed GAPDH as internal control. Thus, the signal from the internal control can be used to normalize the sample data. Optical density was quantified using UN-SCAN-IT software (Silk Scientific, Inc, Orem, UT) and expressed as arbitrary units to calculate BK mRNA/GAPDH mRNA ratio.

Statistical analysis of data

For means comparisons the statistical significance of the difference was assessed using paired, non-paired Student's *t*-test or one-way ANOVA, as indicated. Statistics and graphs were prepared using the software package Statistica (Statsoft, Inc) and Sigmaplot (Systat Software, Inc. San Jose, CA). The level of statistical significance was set as $p<0.05$.

Abbreviations

BK
: MaxiK Large conductance calcium-activated potassium channels

β
: BK channel accessory β subunits

β4
: BK channel accessory β subunit

MTLE
: Mesial temporal lobe epilepsy

iml
: inner molecular layer

I
: Relative fluorescence intensity

mml
: middle molecular layer

LSCM
: Laser scanning confocal microscopy

SE
: status epilepticus

gcl
granule cell layer

mf
mossy fibres

ACSF
artificial cerebrospinal fluid

sl
stratum lucidum

dT
oligodeoxythymidine

dNTP
deoxynucleotides

GAPDH
gene for glyceraldehyde-3-phosphate dehydrogenase

dNTP
deoxynucleotides

CA
Cornus amonnis

DG
dentate gyrus

slm
stratum lacunosum/moleculare

sr
stratum radiatum

so
stratum oriens

sp
stratum pyramidale

VGluT1
vesicular glutamate transporter 1

RFI
relative fluorescence intensity

PBS
phosphate-buffered saline

PVDF
polyvinylidene difluoride

mGluR I and II
group I and II metabotropic glutamate receptors

IF
immunofluorescence

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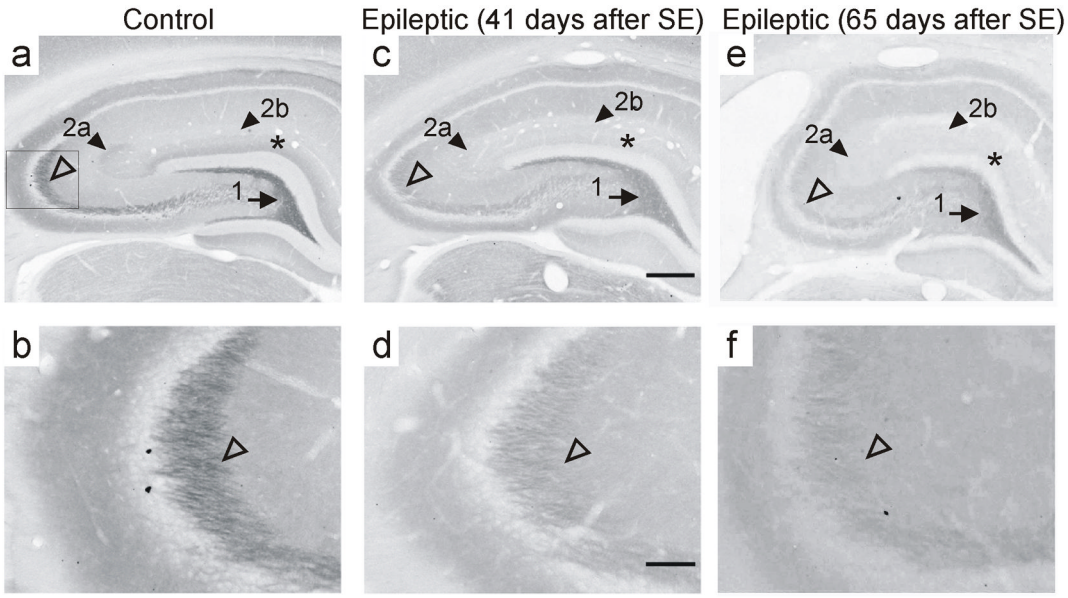

Figure 1.

Photomicrographs illustrating pattern of immunoperoxidase BK channel staining in a control (a, b) *versus* a pilocarpine-induced epileptic rats (41 days after SE induction, 2–3 seizures per week) (c, d) and (65 days after SE induction, 6–8 seizures per week) (e, f). In controls (a, b) robust BK channel labeling was localized at the mossy fiber terminals in the CA3 *stratum lucidum* (sl) (clear arrows) and hilus of dentate gyrus (black arrows 1). A less intense BK staining was seen in the terminal zones of perforant path including *stratum lacunosum/ moleculare* and middle molecular layer of the dentate gyrus (2b,*). At higher magnification, axon bundle-like profiles in CA3 *stratum lucidum* appeared positive for BK channels (b). Granule cell layer lacks BK channel immunoreactivity that was mostly distributed in hilus and middle molecular layer (1 arrow, *). In epileptic rats suffering 2–3 seizures a week, a marked deficit of BK channel immunoreactivity was detected in the CA3 *stratum lucidum*, hilus, *stratum/lacunosum moleculare* and middle molecular layer of dentate gyrus (c). At higher magnification, only few fibers were found positive for BK channels in the *stratum lucidum* (d: clear arrow). BK channel staining was reduced in hilus and middle molecular layer (c: clear arrow, 1arrow and 2b). In epileptic rats suffering 6–8 seizures per week, the hippocampus exhibited signs of extensive atrophy and BK channel immunoreactivity was markedly reduced in hilus of dentate gyrus (e: clear arrow, 1arrow and 2b) and *stratum lucidum* of the CA3 area (f). Scale bar 200μm in a, c, e; 100μm in b, d, f.

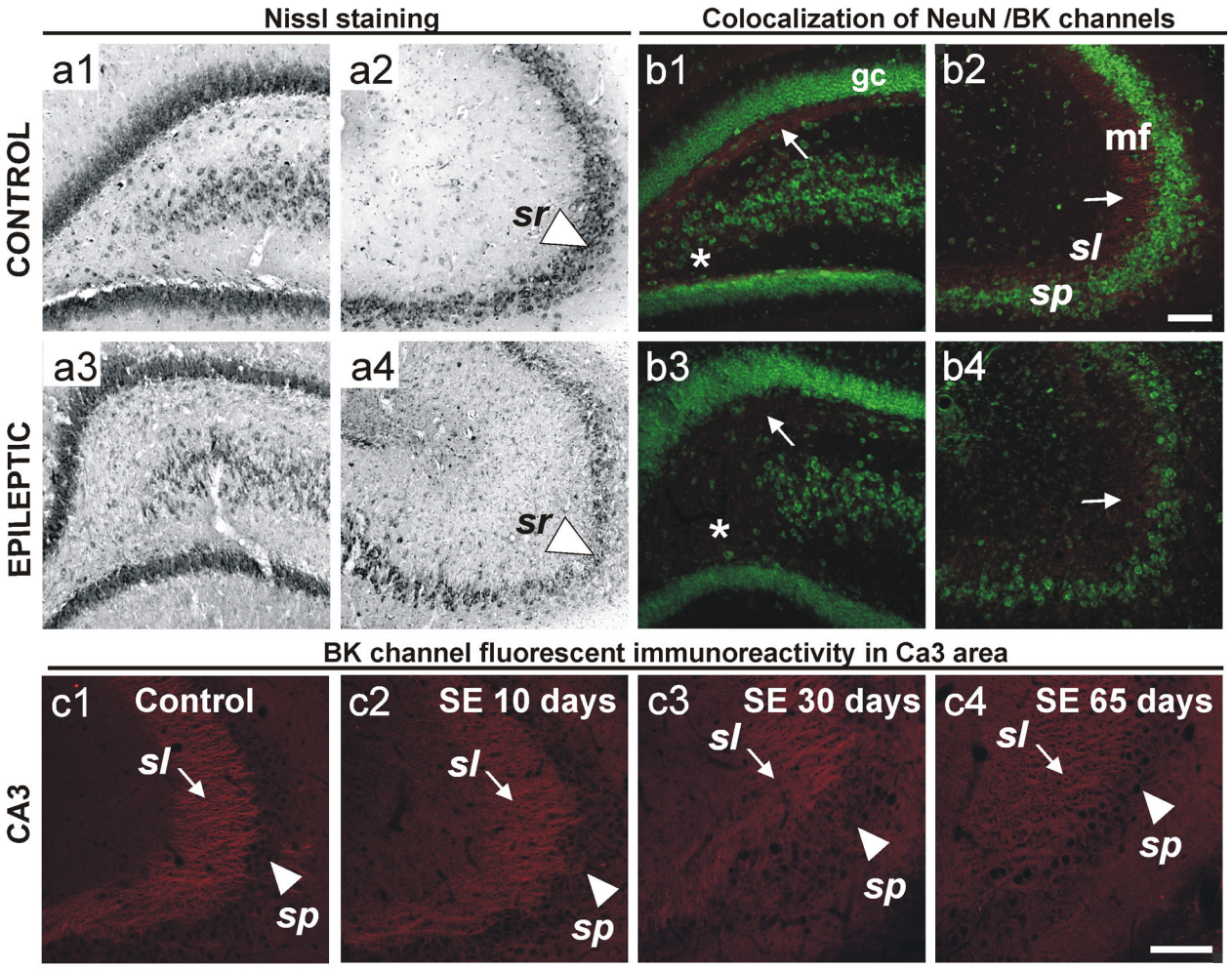

Figure 2.

Seizures induce neuronal loss and reduction of BK channel expression in the chronically epileptic hippocampus. Representative Nissl staining of hippocampal dentate gyrus and Ca3 area in control rat (a1, a2) and epileptic rat sacrificed at 55 days after s*tatus epilepticus* (SE) (a3, a4). Notice a reduction in hilar cells in DG and neurons in the stratum pyramidale (sp) and stratum lucidum (sl) of the epileptic rats and apparent preservation of granule cells in dentate gyrus. Fluorescent Nissl counterstaining in sections processed for BK channel immunofluorescence shows an intense BK channel immunolabeling in the hilus of dentate gyrus (b1) and mossy fiber projection area (*i.e*. sl) in Ca3 area of control rat (b2). In contrast, sections from epileptic rat show signs of granule cell dispersion in dentate gyrus, preserved granule cell layer, and neuronal loss in Ca3 area in addition to a severe down-regulation of BK channel immunofluorescence in hilus (b3) and sl (b4). c1–4. Progressive reduction in BK channel IF following SE. c1. BK channel IF in the Ca3 area sl from age-matched saline-injected control rat (c2), animal sacrificed 10 days after SE (c2), chronically epileptic rat suffering 30 days post-SE survival period (c3) and epileptic rat sacrificed at 65 days after SE (c4). Scale bars= 100 mm.

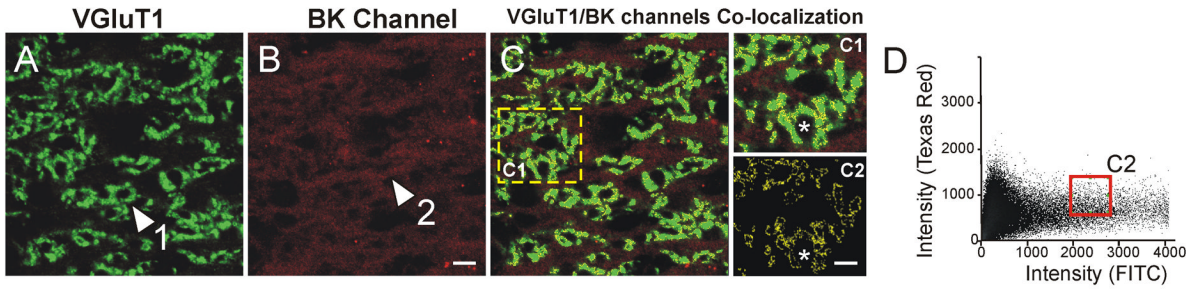

Figure 3.

Double immunofluorescence and co-localization assays for vesicular glutamate transporter 1 (VGluT1) and BK channels in the *stratum lucidum* of CA3 area. High magnification images revealing that VGluT1 immunolabeling was localized in clusters of rounded structures resembling mossy fiber boutons (A) while BK channel immunofluoresence signals were localized in axon-like fibers and bundles (B) that passed surrounding VGluT1-positive structures (C) with some degree of co-localization (C1). (D). Co-localization plot for both signals revealed that a portion of VgluT1-positive signals strongly co-localize with BK channels mainly in a perimeter of VGluT1-positive bouton-like structures (C2). Notice that the hollow center of VGluT1-positive structures are devoid of BK channel staining (* in C1 and C2), thus representing, most probably, postsynaptic spines (thorny excrescences) of CA3 pyramidal cell dendrites. Scale for A-C = 5 μm and Scale for C1 and C2 = 2.5 μm.

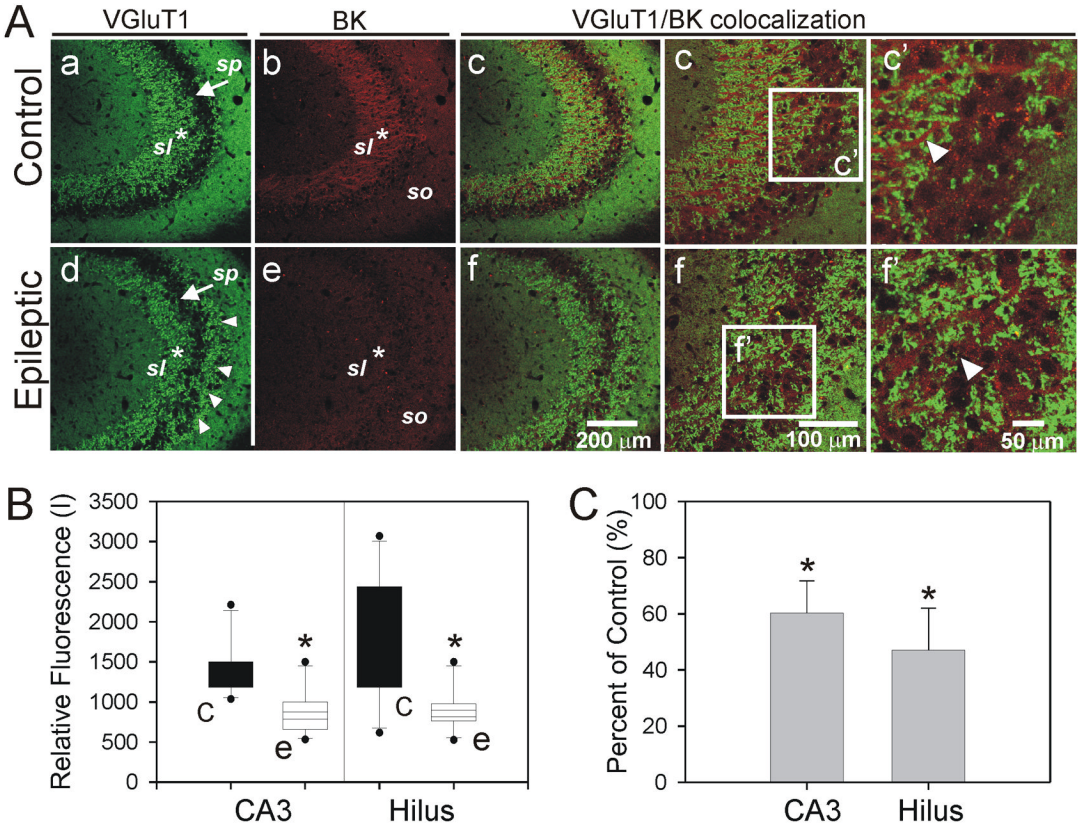

Figure 4.

(A). Double immunofluorescence assay for VGluT1 and BK channels in the CA3 area of controls (a-c) *versus* epileptic rats (d-f). In representative saline-injected control rat, prominent fiber-like BK channel staining (b) apparently co-localized with VGluT1 staining (a) at the CA3 *stratum lucidum* (*sl*) (*i.e.* suprapyramidal zone) (c). Notice that pyramidal layer (*) was almost completely devoid of both VGluT1 and BK channel labeling. At high magnification, strong BK immunofluorescence appeared at axon bundle-like fibers (c') intermixed and slightly co-localized with VGluT1-positive figures resembling giant mossy fiber boutons. In the epileptic rat (40 days post-SE period), VGluT1 staining was disorganized and appeared crossing across the pyramidal layer (*) top the infrapyramidal area in *stratum oriens* (d). A significant reduction in BK immunofluorescence was noticed in *stratum lucidum* CA3 (e, f). At higher magnification, few BK channel-positive fiber-like staining was observed in CA3 *stratum lucidum*.(f') (B). Analysis of relative fluorescence intensity (I) was performed in two areas (hilus) and *stratum lucidum* CA3 of control (c, black box, C) versus epileptic rats (e, white box, E) that experienced more than 30 days of post-SE period (up to 65 days of post-SE period) revealed a significant reduction in BK channel signals at CA3 area and in the hilus of chronically epileptic rats (white boxes) of approximately 40 and 50% respectively (* Student *t*-test, $P<0.01$) (C).

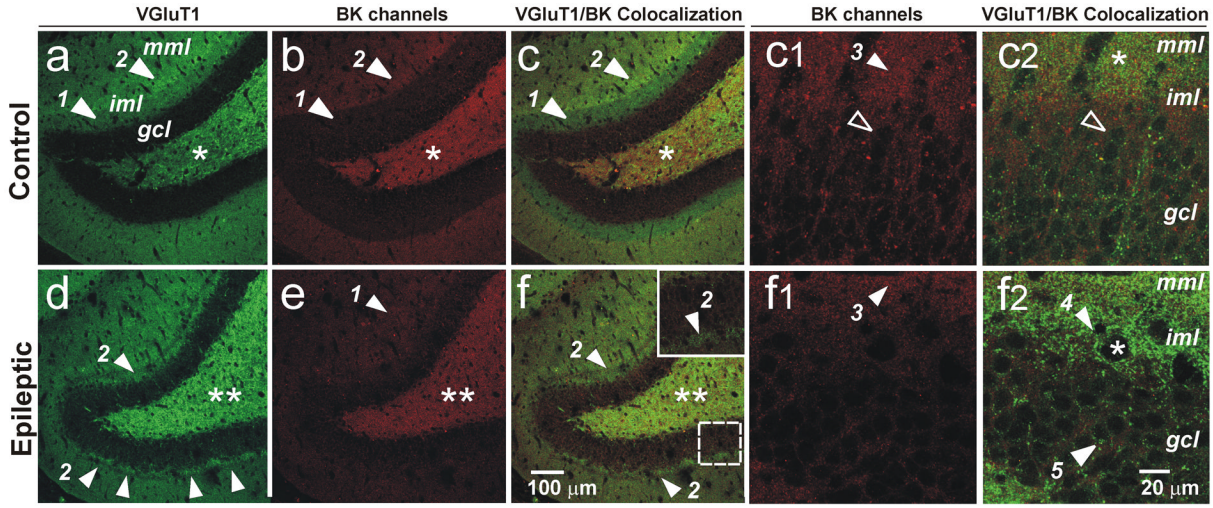

Figure 5.

Double immunofluorescence staining for VGluT1 and BK channels in the dentate gyrus of saline-injected control rat (a - c) *versus* epileptic rats (d-f). Representative confocal microscopy images from a control rat revealed abundant VGluT1 immunoreactivity (green channel) (a) in colocalization with strong BK channel staining (red channel) in the hilus (*) (b and c). Notice less intense signal for both BK and VGluT1 immunofluorescence staining in a band corresponding to the inferior and superior blade of the inner molecular layer (iml) region (b, arrow 1). Moderately intense BK staining was observed in the middle molecular layer (mml) (b, arrow 2) contrasting with a intense VGluT1 staining in the same region (a and c, arrow 2). At higher magnification, BK channel immunolabeling punctuate appeared in the supragranular area (iml) (c1 and f1arrows 3) and throughout the granule cell layer (gcl) whereas somas (immunonegative rounded shapes) were mostly devoid of BK channel staining (open arrows in c1 and c2). Notice that VGluT1 immunolabeling appear distributed as a punctuate labeling in the supragranular region (mml) and scattered throughout the granule cell layer (c2). In a representative chronic epileptic rat (45 days post-SE period), an intense band of VGlut1 staining was observed in the supragranular region resembling mossy fiber sprouting (d and f, arrows 2). This VGluT1-positive band is better illustrated at higher magnification (Inset box in f). VGluT1 immunoreactivity was very intense in the hilus prevailing over BK immunosignals (f, **). Notice that BK channel immunostaining was present in the hilus (e, **) but signal intensity was reduced when compared to the corresponding area in the control dentate gyrus (b,*). A higher magnification (f1 and f2), a weak punctuate BK immunolabeling appeared in iml and mml regions (arrow 3) and was almost completely absent from the gcl. In contrast, increased VGluT1 immunoreactive punctuate was observed in the supragranular region (arrow 4) in the granule cell region (arrow 5) (f2).

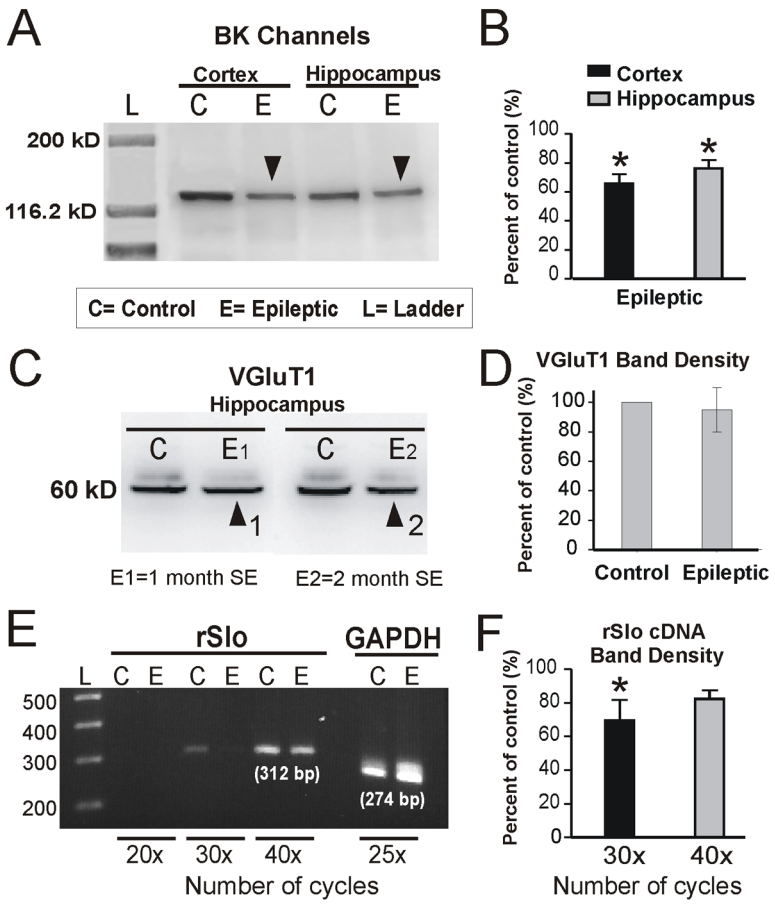

Figure 6.

BK protein expression and transcript levels are reduced in chronically epileptic rats. A, E. Western blot analysis revealed a reduction in the expression of BK channel proteins (~125 kD immunopositive diffuse band) was detected in total proteins extracted from cortex and hippocampus by the chemiluminiscence ECL detection system. Statistical analysis revealed a significant reduction of about 34.5±4.9% in cortex and 24.2±3.4% in the hippocampus of epileptic rats (n=4, *Student *t*-test, $P<0.05$). C. Western blotting to detect an excitatory synaptic marker VGluT1 (immunopositive-band at 52 kDa) revealed no evident changes in VGluT1 expression (arrow 1) in proteins extracted from the hippocampus of an epileptic rat suffering one month of post-SE period (E1). Slight reduction (arrow 2) on the density of VGluT1-positive band was observed in an epileptic rat that suffered 2 month of post-SE period (E2) when compared to control rat (C). D. Graph representing statistical analysis of VGluT1-positive band in control versus epileptic rats (one month + 2 month post-SE, total n=4). Notice a non significant reduction on the density of immunopositive band for VGluT1 protein expression in the hippocampus (Student t-test, $P=0.43$, t=2.9). E. BK transcript levels in total mRNA was isolated from microdissected dentate gyrus of control and epileptic rat. F. Semi-quantitative RT-PCR analysis revealed a down-regulation in BK channel transcripts (rSlo, 312 bp bands) that was evident at 30x and 40x PCR cycles. GAPDH primers were used as internal controls. Statistical analysis revealed a significant 31.1±3.5% reduction in density of band corresponding to BK channel cDNA at 30 cycles in epileptic rats (*Student *t*-test, $P<0.01$).