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Altered expression and function of small-conductance (SK) Ca^{2+}-activated K^+ channels in pilocarpine-treated epileptic rats

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Abstract

Small conductance calcium (Ca^{2+}) activated SK channels are critical regulators of neuronal excitability in hippocampus. Accordingly, these channels are thought to play a key role in controlling neuronal activity in acute models of epilepsy. In this study, we investigate the expression and function of SK channels in the pilocarpine model of mesial temporal lobe epilepsy. For this purpose, protein expression was assessed using western blotting assays and gene expression was analyzed using TaqMan-based probes and the quantitative real-time polymerase chain reaction (qPCR) comparative method delta-delta cycle threshold (ΔΔCT) in samples extracted from control and epileptic rats. In addition, the effect of SK channel antagonist UCL1684 and agonist NS309 on CA1 evoked population spikes was studied in hippocampal slices. Western blotting analysis showed a significant reduction in the expression of SK1 and SK2 channels at 10 days following *status epilepticus* (SE), but levels recovered at 1 month and at more than 2 months after SE. In contrast, a significant down-regulation of SK3 channels was detected after 10 days of SE. Analysis of gene expression by qPCR revealed a significant reduction of transcripts for SK2 (*Kcnn1*) and SK3 (*Kcnn3*) channels as early as 10 days following pilocarpine-induced SE and during the chronic phase of the pilocarpine model. Moreover, bath application of UCL1684 (100 nM for 15 min) induced a significant increase of the population spike amplitude and number of spikes in the hippocampal CA1 area of slices obtained control and chronic epileptic rats. This effect was obliterated by co-administration of UCL1684 with SK channel agonist NS309 (1 μM). Application of NS309 failed to modify population spikes in the CA1 area of slices taken from control and epileptic rats. These data indicate an abnormal expression of SK channels and a possible dysfunction of these channels in experimental MTLE.

Keywords

pilocarpine; epilepsy; hippocampus; temporal lobe epilepsy

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1. Introduction

Mesial temporal lobe epilepsy (MTLE) is 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). Despite major advances in deciphering pathogenic mechanisms in MTLE, about 20–30% of patients continue experiencing seizures after treatment with antiepileptic drugs (Andrade et al., 2006; Berg et al., 2003; Jones and Andermann, 2000). Therefore, basic research remains critical to gain additional insights on the pathogenic mechanisms and to develop novel therapeutics to overcome hyperexcitability and pharmacoresistance in MTLE.

Compelling data indicate that critical changes in expression and function of ion channels and receptors are associated with enhanced excitability leading to persistent abnormalities of both intrinsic firing properties and synaptic circuits in MTLE. Accordingly, Ca^{2+}-activated potassium (K^+) channels have been implicated in the pathogenesis of epilepsy (Blank et al., 2004; Ermolinsky et al., 2008a; Jin et al., 2000; Pacheco Otalora et al., 2008; Sheehan et al., 2009; Wang et al., 2009). Small conductance calcium (Ca^{2+})-activated K^+ channels (SK channels) are a subfamily of Ca^{2+}-activated K^+ channels (also known as KCa2 family) and are key regulators of neuronal excitability (Faber, 2009; Pedarzani and Stocker, 2008). However, it remains unclear whether SK channels play a role in MTLE. SK channel family contains 4 members – SK1 (KCa2.1.), SK2 (KCa2.2.), SK3 (KCa2.3.) and SK4 (KCa3.1 considered as intermediate conductance channel), which are expressed by different genes *i.e. Kcnn1*, *Kcnn2*, *Kcnn3* and *Kcnn4* respectively (Wulff et al., 2007). SK channels consist of heterotetrameric subunit assembly of four identical subunits that associate to form a symmetric tetramer (each of the subunits has six transmembrane domains) (Ishii et al., 1997). In addition, SK channels are tightly associated with the protein calmodulin, which accounts for the Ca^{2+} sensitivity of these channels (Lee et al., 2003; Maylie et al., 2004).

Despite their small unitary conductance, SK channels are powerful modulators of electrical excitability by acting in close proximity of their Ca^{2+} sources to locally modulate membrane currents (Faber and Sah, 2007). Their activation limits the firing frequency of action potentials and are important for regulating afterhyperpolarization (AHP) in central neurons and other types of electrically excitable cells (Faber and Sah, 2007). Three components of AHP have been identified: fast (fAHP), medium (mAHP), and slow (sAHP). The cloning of the SK channel family revealed that mAHP is mediated by SK channels (Kohler et al., 1996). For instance, SK1, SK2 and SK3 channels as well as mAHP are selectively blocked by pharmacological application of the bee venom toxin apamin (Wulff et al., 2007). Moreover, neurons in transgenic mice lacking functional SK channel activity exhibited profound inhibition of mAHP in cortical pyramidal cells (Villalobos et al., 2004). Importantly, it has been shown that mice knockout for SK2 channel completely lack the apamin-sensitive component of the mAHP in CA1 pyramidal neurons (Bond et al., 2004).

Based on the fact that mAHP determines the interval between action potentials and consequentially firing frequency, SK channels and mAHP have been implicated in the pathogenesis of epilepsy (Alger and Nicoll, 1980; Blank et al., 2004; Chamberlin and Dingledine, 1989; Fernandez de Sevilla et al., 2006; Garduno et al., 2005; Knowles et al., 1984; Kobayashi et al., 2008; Lappin et al., 2005). In this context, it has been shown that the SK channel opener 1-EBIO (1-Ethyl-2-benzimidazolinone) showed potent antiepileptic effect similar to conventional antiepileptic drugs on epileptiform activity induced in vitro by 4-aminopyridine (4-AP) in cultured rat hippocampal neurons (Kobayashi et al., 2008). In addition, it has been shown that 1-EBIO decreases seizures in mice following maximal electroshock as well as increases the threshold to electrically- and pentylenetetrazole-induced seizures (Anderson et al., 2006). Despite these studies using *in vitro* and acute *in*

vivo models, it remains unknown whether SK channels play a role in epileptogenesis. To address this issue, we examined whether pharmacological modulation of SK channels affects excitability in the CA1 area of hippocampal slices taken from age-matched control and pilocarpine-treated chronic epileptic rats as a model of MTLE (Cavalheiro, 1995).

2. Results

Electrophysiological analysis was performed in chronically epileptic rats experiencing at least 4–5 seizures per week during post-SE period and age-matched control rats (saline instead of pilocarpine). All the animals used in this study experienced seizures grade 3–5 on the Racine's scale. The seizure severity between the 1 month group and more than 2 month group was not qualitatively different, neither the seizure number range during the monitoring period.

Stimulation of the Schaffer collaterals evoked a single population spike in control slices (Fig. 1Aa1), whereas all slices from epileptic rats exhibited signs of hyperexcitability (> 2 population spikes) (Fig. 1Aa3).

2.1. SK channel blocker increases excitability of the CA1 area in hippocampal slices from chronically epileptic rats

To assess whether SK channels-mediated modulation of CA1 responses were affected in chronic epilepsy, we first evaluated the effects of the selective SK channel blocker UCL1684 on evoked population spikes in the CA1 area of hippocampal slices taken from age-matched control and pilocarpine-treated chronic epileptic rats. As depicted in Figure 1, bath application of UCL1684 (100 nM for 15 min) induced a significant increase of the population spike amplitude in the hippocampal CA1 area of control slices (Fig. 1Aa2 compared to a1). The increase in population spike amplitude persisted for about 45–60 min, and was resistant to washout, even for long periods. Interestingly, UCL1684-mediated increases in population spike amplitude were more pronounced in slices from age-matched control (71.1 ± 27.8 % increase of baseline responses, n=7, paired t-test $P<0.01$) than in slices from chronic epileptic rats (32.3 ± 9.3 % increase of baseline response, n=6, paired t-test $P<0.01$) (Fig. 1B). However, bath application of UCL1684 increased the number of spikes in slices from epileptic rats (from 4.4 ± 0.8 to 5.3 ± 0.7, 31.2% increase, paired t-test $P<0.05$), whereas it did not modify this parameter in the control group (no change was observed in 7 slices). UCL1684 did not cause secondary population spike in slices from control rats (Fig. 1Aa2).

To confirm if UCL1684-elicited increases in slice excitability was related to an effect on SK channels, we tested whether the selective SK channel opener NS309 prevented the excitatory effect of the SK channel blocker UCL1684. We found that bath application of UCL1684 (100 nM) in the presence of NS309 (1 μM) did not elicit any increase in population spike amplitude in controls (4.8 ± 4.7% increase of baseline responses, n=8, paired t-test $P>0.05$) (Fig. 2A, a1 compared to a2, B). Similar effect was noticed in slices from epileptic rats (15.1 ± 3.1% increase of baseline responses, n=3, paired t-test $P>0.05$) (Fig. 2Aa3, a4, B). The SK channel opener NS309 also occluded UCL1684-induced increase in the number of population spikes. Accordingly, no significant changes were detected in the number of population spikes after incubation in NS309 and UCL1684 in slices taken from epileptic rats (n=3, baseline 5.95 ± 0.46 versus 8.13 ± 1.48, paired t-test, $P>0.05$).

2.2. Activation of SK channels does not significantly reduce epileptiform activity in CA1 area of chronically epileptic rats

Since we found that SK channels blockade increased excitability in hippocampal slices of epileptic rats, and that this effect was prevented by the SK channel opener NS309, we investigated whether this compound decreased the epileptiform activity in the CA1 area of pilocarpine-treated epileptic rats. We found that bath application of NS309 (1 μM) did not alter population spike amplitude and number in slices from control animals (no change was observed in 8 slices). Similarly, incubation with NS309 (1mM) failed to significantly change the number of population spikes in slices from epileptic animals (from 6.75 ± 0.89 spikes to 6.92 ± 0.97 spikes, paired t-test P>0.05) (Figure 3).

2.3. Abnormal SK channel protein expression in hippocampus of chronically epileptic rats

Western blot analysis was performed to examine alterations in SK channel protein levels in hippocampus at different time points following pilocarpine-induced *status epilepticus* (SE). Seizure severity (3–5 Racine's scale) and frequency (4–5/week) in the 1 month and >2 month groups were similar to seizure pattern observed in animals sacrificed for electrophysiology and for total RNA extraction (qPCR assays). Total protein per sample was measured, normalized and the same amount subjected independently to Western blot analysis with three different primary antibodies against SK1, SK2 and SK3 channels. These antibodies were previously characterized and detect specific bands for these SK proteins (Obermair et al., 2003; Sailer et al., 2004). To allow for comparisons, sets of samples from control (n=7) and groups of animals subjected to different survival times after *SE* - 1 day (n=3), 10 days (n=3), 30–60 days (1 month group) (n=3), and more than 61 days (>2 months) *after* SE *(n=7)* - were simultaneously processed in the same membrane. Consistent with previous studies, SK channel subtypes were identified as an immunopositive band with apparent molecular weight in the 65–70 kDa range (Fig. 4A). Using statistical analysis (ANOVA) on semi-quantitative data from band optical densities, we detected significant changes in the protein expression levels of SK1 channels ($F=5.4$, $P=0.003$), SK2 channels ($F=4.5$, $P=0.005$, and SK3 channels ($F=10.5$, $P=0.001$). Specifically, comparisons performed by the Honest Significant Difference (HSD) tests revealed a significant 33.9% down-regulation of SK1 channels at 10 days after SE when compared to control group and other experimental groups ($p< 0.05$, Fig. 4B). Levels of SK1 returned to baseline levels in experimental groups at 1 month and after 2 months following SE. No significant differences in SK1 were detected between 1 month and more than 2 months after SE. Moreover, a significant 20% increase in SK2 protein expression (compared to controls by HSD post-hoc test, $P<0.05$) was followed by a significant down-regulation in expression at 10 days (46.2% reduction compared to 1 day, HSD Post-Hoc $P<0.01$ and 26.2% reduction respective to control group, HSD Post-Hoc $P<0.05$) (Figure 4C). A partial recovery of expression was noticed at 1 month (10% reduction) and normalized to control levels after 2 months of SE (no significant changes were detected compared to control. In addition, SK3 channel protein expression increased 1 day after SE (14.2% compared to control group, HSD Post Hoc test, $P<0.05$) and then progressively decreased after 10 days (25.05% reduction), 1 month (29.7% reduction) and more than 2 months (32.5% reduction) after SE (Post Hoc HSD tests compared to control, $P<0.05$ respectively). No significant difference was noticed among 10 days, 1 month, and more than 2 months (Figure 4D).

2.4. Gene expression of SK1, SK2 and SK3 channels after pilocarpine-induced *SE*

Analysis of fold changes in transcript levels in total RNA extracted from different experimental groups was performed using the comparative CT (ΔΔCT) relative quantification method of qPCR and pre-validated ABI TaqMan gene expression assays for SK1, SK2 and SK3 genes (*Kcnn1*, *Kcnn2* and *Kcnn3* respectively) and the normalizing gene *Gapdh*. Values were expressed as relative quantification (RQ) index and compared to

control level of gene expression. Experimental groups included animals sacrificed at 10 days following SE, 30–60 days (1 month) and more than 60 days (> 2 month) following SE. Interestingly, a significant difference in gene expression was observed for SK2 channels (ANOVA $P<0.01$) and SK3 channels (ANOVA $P<0.05$, Fig. 5B and Fig. 5C). Post-hoc analysis by *Tukey's Honest* Significant Difference test revealed a 39% reduction ($P=0.0003$) in RQ value levels of SK2 at 10 days (n=5) following SE compared to controls (n=7), whereas no difference was detected between control and 24h following SE (n=5). Moreover, SK2 gene expression levels were significant reduced relative to control values in the 1 month group (21% reduction, $P<0.05$, n=5) and more than 2 month group following SE (20% reduction, $P<0.05$, n=5). A similar pattern of gene expression was observed for SK3 channel transcripts, although changes were less pronounced. For instance, a significant reduction in the content of SK3 channel transcripts was observed at 10 days (25% reduction, $P<0.05$), 1 month (19.5% reduction, $P<0.05$) and 2 month (21.4% reduction, $P<0.05$) following SE. No significant differences were observed in the transcript levels of SK1 channels.

3. Discussion

In the present study we showed a significant down-regulation of protein and transcript expression of SK2 and SK3 channels in chronic pilocarpine-treated epileptic rats. Real-time quantitative PCR analysis of gene expression revealed that a significant reduction of transcripts for SK2 and SK3 channels occurred as early as 10 days following pilocarpine-induced *SE* and persisted during the chronic phase of the model. Moreover, bath application of the SK channel blocker UCL1684 in control and epileptic slices induced a significant increase of the population spike amplitude and number of spikes in the hippocampal CA1 area. This effect was prevented by co-administration of UCL1684 with the SK channel opener NS309, but application of NS309 failed to modify population spikes in the CA1 area of slices taken from control and epileptic rats.

SK channels are Ca^{2+}-activated K^+ channels of small conductance (10–20 pS) that are widely expressed in vertebrate neurons and other tissues (Kohler et al. 1996). These channels are composed of proteins of the KCa2 family, the three members (KCa2.1–2.3, also called SK1–3) of which are all expressed in the mammalian brain, including the hippocampus (Kohler et al., 1996; Sailer et al., 2002; Stocker and Pedarzani, 2000). SK channels therefore exert profound physiological effects within the nervous system. In normal conditions, SK channels are thought to be involved in synaptic plasticity and therefore play important roles in memory and learning (Stackman et al., 2002). For instance, the SK channel blocker, apamin, a bee venom peptide toxin, has been reported to improve learning and memory retention of mice, facilitate the induction of hippocampal long-term synaptic potentiation (LTP), and increase immediate early gene expression (Behnisch and Reymann, 1998; Heurteaux et al., 1993; Kramar et al., 2004; Messier et al., 1991; Norris et al., 1998; Stackman et al., 2002). Dysfunction of SK channels may be linked to psychiatric disease, including schizophrenia and bipolar disorder (Chandy et al. 1998), and these channels may also be targets of antipsychotic drugs (Terstappen et al., 2001).

In pyramidal neurons, SK channel conductances are particularly important because they supply strong negative feedback control of excitability (Borde et al., 2000; Madison and Nicoll, 1984). Owing to their activation by increased intracellular Ca^{2+} levels following burst firing, and the resultant hyperpolarization and dampening of neuronal excitability, SK channels have been proposed as a potential target for novel antiepileptic drugs (Anderson et al., 2006). In this context, it has been shown that bath application of 1-EBIO, a SK channel opener, suppresses epileptiform activity, induced by 4-aminopyridine (4-AP) or kainate-Mg^{2+}-free solutions in CA3 hippocampal pyramidal neurons (Garduño et al., 2005). In

addition, the SK channel openers NS309, DCEBIO, and 1-EBIO suppress epileptiform activity induced by 4-aminopyridine in cultured rat hippocampal neurons, with efficacy similar to conventional antiepileptic drugs (Kobayashi et al., 2008), further suggesting that SK channel openers are potential drugs to control abnormal epileptiform activity. Additional evidence for an anticonvulsant effect of SK channel openers came from the study of Anderson et al. (2006), which has shown the anticonvulsant properties of 1-EBIO in a series of *in vivo* seizure models. For instance, 1-EBIO was found to be effective at reducing seizure incidence in mice following maximal electroshock as well as increasing the threshold to electrically- and pentylenetetrazole-induced seizures, suggesting that selective enhancers of SK channels function could be effective antiepileptic medications. However, to our knowledge, the role of SK channels in the hippocampal hyperexcitability in an animal model of chronic epilepsy has not been investigated to date.

In the current study we showed that bath application of UCL1684 induced a significant increase excitability of the hippocampal CA1 area of slices taken from pilocarpine-treated chronic epileptic rats and age-matched control rats, as characterized by an increase in the population spike amplitude in both control and epileptic animals and by an increase in the number of spikes in the epileptic rats. These results are in agreement with those of Lappin et al. (2005), who have shown that bath application of apamin increases burst duration in hippocampal slices. Altogether, these results suggest a role for SK channels in the control of hippocampal pyramidal neurons excitability, and that loss of SK channels functionality may lead to increased excitability, as seen in epileptic states.

Importantly, we detected a significant reduction in the content of SK2 and SK3 channel subtypes in the hippocampus of pilocarpine-induced epileptic rats. Specifically, we found a decrease in the protein expression of the SK2 and SK3 channels in chronically epileptic rats, which correlates with a down-regulation of transcript levels for SK2 and SK3 by qPCR analysis using the ΔΔCT comparative method. A potential pitfall from this approach is that the analysis of protein and mRNA expression was performed from total hippocampus including other areas in addition to CA1 (*i.e.* dentate gyrus, CA3 area) and many different neuronal subtypes. Accordingly, we should cautiously associate these data with electrophysiological results. Further studies should determine whether electrophysiological abnormalities on SK channel pharmacology and function are also present in other hippocampal or extra-hippocampal areas.

Gene expression analysis indicated that reduction in transcript levels of SK2 and SK3 initiate as early as 10 days following SE (latent period) and persist during the process of epileptogenesis in rats experiencing recurrent spontaneous seizures. Moreover, SK1 transcripts were not altered at 10 days following SE while protein expression was reduced indicating that other mechanisms are involved in down-regulation (*i.e.* increased proteosomal degradation of channels). In our study, no behavioral spontaneous seizures were detected in rats sacrificed at 10 days following SE. However, previous studies using continuous video-electroencephalographic (EEG) recordings following SE reported that the first spontaneous seizures occurred 7.2 ± 3.6 days after pilocarpine-induced SE (Goffin et al., 2007). Hence, down-regulation of SK2 and SK3 channels during early or “latent” period (10 days) following SE may be related to early expression of seizures either as cause or consequence. Since these channels regulate excitability in the hippocampus and pharmacological suppression of SK channel activity with the selective blocker UCL1684 elicited an increase in hippocampal electrophysiological responses in control and epileptic animals, downregulation of SK channels in hippocampus may represent a pro-epileptic change leading to exacerbated excitability during epileptogenesis. Accordingly, UCL1684-mediated increases in population spike amplitude were more pronounced in age-matched control than in pilocarpine-treated chronic epileptic rats, further suggesting a decrease in

function of SK channels in pilocarpine-epileptic animals. In this context, enhancement of SK channel function may constitute an interesting new approach to epilepsy treatment.

In order to test this possibility, we tested whether the selective SK channel opener NS309 would decrease the epileptiform activity in the hippocampus of epileptic rats. However, activation of SK channels with NS309 did not alter the number of population spikes or their amplitude in slices from epileptic animals. The lack of effect of a SK opener on the excitability of slices from epileptic animals is probably due to the currently reported deficit in the expression of the pharmacological targets (*i.e.* SK2 and SK3 channel proteins), which may render SK channels-targeted drugs inefficient to control hyperexcitability and ultimately seizures in epilepsy. In this context, an interesting possibility to be tested in the future is that transfer of SK channel gene expressing system may offer a new opportunity for therapeutic intervention (gene therapy) of patients with drug-refractory epilepsy. In addition, SK channels are gated by Ca^{2+} ions via calmodulin that is constitutively bound to the intracellular C- terminus of the channels and serves as the Ca^{2+} sensor (Xia et al., 1998), and NS309 is a SK channels activator which increases channel sensitivity to Ca^{2+} without affecting maximally activated SK channels (Hougaard et al., 2007). Thus, NS309 is not a true SK channel opener, but a kind of positive allosteric modulator. In addition, it has been shown that SK2 channel Ca^{2+} sensitivity can be decreased by phosphorylation of CAM threonine-80 via protein kinase CK2, and conversely increased by dephosphorylation via protein phosphatase 2A (Bildl et al., 2004). Therefore, SK channels may, under some regulatory conditions, be too insensitive to intracellular Ca^{2+} or to a positive modulator, such as NS309.

SK channels play a critical role controlling excitability in other brain areas such as thalamus and reticular nucleus. It was recently reported that partial blockade of SK channels by subnanomolar concentrations of apamin combined with picrotoxin can synergistically induce epileptiform oscillations spreading throughout thalamo-cortical circuits (Kleiman-Weiner et al., 2009). At the cellular level, apamin enhanced the intrinsic excitability of reticular nucleus neurons suggesting that regulation of reticular nucleus excitability by SK channels can influence the excitability of thalamocortical networks (Kleiman-Weiner et al., 2009). These authors propose SK channel modulation as a possible pharmacological treatment for absence epilepsy. Based on these findings above, it is possible that abnormalities of GABAergic inhibitory function, as vastly reported in human and experimental MTLE (Bernard et al., 1999; Bernard et al., 2000; Engel, 1996; Magloczky and Freund, 2005), can potentiate the pro-epileptic effect of the defective SK channel expression described here.

MTLE is a complex acquired channelopathy characterized by complex changes in expression and function of ligand-gated and intrinsic conductances (voltage-gated and Ca^{2+}-gated channels) (Becker et al., 2008; Bernard et al., 2004; Pacheco Otalora et al., 2008; Pitkanen and Lukasiuk, 2009; Richichi et al., 2008; Shin et al., 2008). In addition to multiple channeloapthies, hyperexcitability in CA1 area in epilepsy is also related to different mechanisms, including synaptic reorganization, GABA receptor deficits, disinhibition, and enhanced glutamatergic responses. For instance, a previous study has implicated an up-regulation of intrinsic bursting capabilities of CA1 surviving principal cells in the hyperexcitability of this area in the pilocarpine model of epilepsy by complex mechanisms that depends on up-regulation of T-type Ca^{2+} channel activation (Becker et al., 2008; Sanabria et al., 2001; Su et al., 2002; Yaari and Beck, 2002). It is possible that multiple mechanisms overlap or interact, ultimately leading to epileptogenesis. For instance, in normal cells, an increase in intracellular Ca^{2+} can activate compensatory mechanisms including Ca^{2+}-activated K conductances (*i.e.* SK and BK channels) to limit the potential increases in excitability (*e.g.* bursting). However, in epileptic neurons, deficits in SK

expression and function may affect this potential "inhibitory break" mechanism, resulting in neuronal excitability and ultimately epileptiform activity. In fact, this complexity in the underlying mechanisms of MTLE may be a contributing factor for pharmaco-resistance.

In summary, in the present study we present original data showing a decrease in SK channel expression and function in the hippocampus of pilocarpine-treated epileptic animals, a widely used animal model of temporal lobe epilepsy. Although more studies are necessary to fully evaluate the anticonvulsant potential for SK channel pharmacological or genetic modulation in epilepsy, SK channels may represent new targets for drug development for this neurological disorder.

4. Experimental procedures

4.1. 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). Male Sprague Dawley rats were maintained in a temperature- and humidity-controlled *vivarium*, with water and standard laboratory chow *ad libitum*. All efforts were made to minimize the number of animals in the study. A subset of the animals were made chronically epileptic by the systemic injection of pilocarpine, as described elsewhere (Cavalheiro, 1995; Mello et al., 1993). This experimental model of temporal lobe epilepsy is characterized by bilateral hippocampal pathology (Cavalheiro, 1995; Mello et al., 1993). *Status epilepticus* (SE) was induced at postnatal days 30–45 days. 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 solution, *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. Systemic pilocarpine injection induced SE in 70% of injected rats consisting of continuous tremor, rearing, myoclonic jerks, clonic forearms and head movements with eventual side fallings. To minimize the mortality rate of the procedure, animals were allowed to remain in SE for approximately 3 hrs 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 hrs after SE induction. Several subcutaneous injections of 20 ml Ringer-lactate were administered to compensate for any liquid lost (*i.e.* salivation, urination) as needed for the next 24–30 hrs following SE termination. 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 grade 3–5 on the Racine's scale (Racine, 1972) using a JVC MiniDV digital video-camera researcher-assisted SeizureScan software (Clever Sys., Inc, Reston, VA). Animals sacrificed after 30 days following SE and used for electrophysiology and molecular studies exhibited recurrent spontaneous seizures (grade 3–5 of the Racine's scale).

Seizures were confirmed *off-line* by an experienced researcher. After a silent period of 10–20 days, SE-suffering rats experienced approximately 4–5 seizures per week regularly during the observation period.

4.2. Hippocampal slices preparation and electrophysiology

Animals used for electrophysiology experienced a survival period of at least 35 days following SE (post-SE period range= 35–75 days, 65–105 day-old rats). Horizontal slices (350 μm thick) of hippocampi and associated entorhinal cortices were prepared from anesthetized (50 mg/kg ketamine) chronic epileptic and age-matched control rats. Slicing procedures were performed in cold (4 °C) sucrose-based artificial cerebrospinal fluid (ACSF) containing (in mM): 124 sucrose, 3 KCl, 0.5 $CaCl_2$, 5 $MgSO_4$, 0.125 Na_2HPO_4, $NaHCO_3$, and 25 D-glucose, aerated with 95% O_2/5% CO_2 (pH 7.4; 290–310 mOsm) using a vibratome OTS-4000 sectioning device (Electron Microscopy Sciences, Fort Washington, PA). Slices were allowed to recover in standard ACSF at room temperature for at least 1 hour before being placed in the recording chamber. The total number of animals and slices used was as follows:

Experiment 1 (UCL1684): 7 slices from control (n=2 animals) and 6 slices from epileptic rats (n=3 animals).

Experiment 2 (NS309): 8 slices from control (n=3 animals) and 6 slices from epileptic rats (n=3 animals).

Experiment 3 (UCL1684 + NS309): 8 slices from control (n=2 animals) and 3 slices from epileptic rats (n=3 animals).

Recordings were carried out in a modified Haas-type interface chamber at 32 °C on slices continuously perfused with pre-warmed (34°C) ACSF (in mM): 125 NaCl, 26 $NaHCO_3$, 0.15 BES (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid), 3 KCl, 2 $CaCl_2$, 2 $MgSO_4$, and 15 D-glucose, gassed with 95% O_2/5% CO_2 (pH 7.3–7.4) at 1.5 ml/min, as previously described (Sanabria et al., 2004). Extracellular field potential were recorded from the *stratum pyramidale* of the CA1 region of the hippocampus (population spike recordings). Recording pipettes pulled from borosilicate glass (1.5 mm diameter) with filament had a resistance of 5–8 MΩ when filled with 1 M NaCl. To elicit population spike responses, electrical stimuli were delivered using concentric bipolar tungsten stimulating electrodes (World Precision Instruments, Inc., Sarasota, FL) positioned in the Schaffer collaterals in the *stratum radiatum*. Constant square-current pulses (50 μs, 0.05 Hz) were delivered through a constant-current isolation unit (Master-8, AMPI, Jerusalem, Israel). Signals were amplified and recorded with an active SmartProbe (AI 402 x50 ultra-low-noise differential amplifier) coupled to programmable signal conditioner CyberAmp 320, digitized at 10 kHz with a DIGIDATA 1300 A/D board (Axon Instruments, Molecular Devices, CA, USA), and responses were analyzed off-line using the Clampfit 8.0 software (Axon Instruments, Inc.). Stimuli were adjusted to evoke reproducible response amplitudes (50–60 % of the maximal amplitude). Control traces were recorded at test stimuli under standard ACSF for at least 15 minutes in order to establish baseline responses. Slices with more than 10% drift in baseline recordings were excluded from the analysis. After establishing a stable baseline the SK blocker UCL1684 (Campos Rosa et al., 2000; Malik-Hall et al., 2000; Strobaek et al., 2000) (100 nM) and/or the SK channel opener NS309 (Hougaard et al., 2007; Pedarzani et al., 2005; Strobaek et al., 2004) (1 μM) were added to ACSF and applied to slices for at least 15 minutes to assess SK-mediated modulation of population spike amplitude and number in control and epileptic rats. SK ligands were dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted with ACSF, in such a way that DMSO concentration did not exceed 0.01%. In pilot experiments we found that this concentration had no effect in any of the variables measured on this study. Concentrations of SK ligands used in the present study were selected based in the literature and in pilot concentration-response experiments. UCL1684 and NS309 were purchased from Tocris (Ellisville, MO).

The amplitude of the second population spike was measured from the peak negativity to the next positive peak. The maximum negative signal was measured relative to the mean and the maximum positive signal is measured relative the maximum negative signal (the peak-to-peak amplitude) and then divided by two. Population spike amplitude from the last 10 minutes of baseline period was averaged and served as control. Population spike amplitude from the last 10 minutes of drug application was averaged, normalized to control values, and expressed as percent of control. Data from electrophysiological experiments such as amplitude of population spikes (mV) and number of population spikes are presented as mean ± SEM. For mean comparisons, the statistical significance of the difference was assessed using paired, non-paired Student's *t*-test 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 $P<0.05$.

4.3. Protein sample preparation and Western blotting

Protein extracts were obtained from control rats and animals sacrificed at different time periods (groups) following SE (*i.e.* 24 h, 10 days, 1 month, and more than 2 months). The age range for animals sacrificed in the different groups after *status epilepticus* was as follows: 24 hrs, 31–40 days; 10 days, 42–50 days; 1 month, 65–70 days; and more than two months, 92–140 days. 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 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 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 multimode microplate reader? Infinity M200 (Tecan US, Inc, Durham NC) 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-tricine buffered SDS-PAGE (2 hrs at 75 V). Proteins (20 μg/lane) 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 4°C with the primary antibody (anti-SK1, anti-SK2, or anti-anti-SK1) diluted 1:1000 in TBS containing 2% non-fat dry milk and 0.1% Tween-20. As a reference, we probed membranes with primary antibody against Actin (1:20 000) (Sigma Aldrich, Saint Louis, USA). Primary antibodies against SK channels (SK1, SK2 and SK3) were developed by Dr. Hans-Günther Knaus at the Medical University Innsbruck, Austria. These affinity-purified antibodies (IgG concentration 100 ng/ml) have been previously validated (Obermair et al., 2003; Sailer et al., 2002; Sailer et al., 2004). The membranes were washed in TBS with 0.1% Tween-20 then incubated for 2 hrs 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 PBS, membranes were incubated 90 min in ABC (room temperature), rinsed 3 times (15 min each) in PBS and the proteins immunopositive bands visualized by enhanced chemiluminescent (ECL Plus) Kit 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 SK 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 different control and experimental groups for statistical analysis performed in the optical density of SK immunopositive band by ANOVA test. No apparent difference was observed in SK1, SK2 and SK3 expression levels among samples from control animals (n=7) sacrificed at different ages (ranging 32–140 days). Thereby, control samples were pooled together for statistical analysis. Sets of samples were run in duplicate. Protein extracts from hippocampus for each set of control and experimental epileptic rats where processed in parallel on a single gel and subjected to same immunoblotting procedure.

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

Total RNA was isolated from control rats and pilocarpine-treated rats that were anaesthetized and sacrificed at 24 hrs, 10 days, 1 month, and at more than 2 months after SE induction. Total RNA was isolated from dissected hippocampus. In brief, tissue was collected, weighed (about 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 denaturating condition [17]. Traces amounts of genomic DNA contamination were minimized by performing DNAse treatment using *DNA*-free™ *DNase Treatment (*Applied Biosystems, ABI, CA, USA*).* RNA samples from each set of control and epileptic rats were processed in parallel under the same conditions. RT were 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 manufacturer's protocol using random primers for 1 mg 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 (qPCR) 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 Rn00570904_m1 for target gene SK1 (*Kcnn1*, RefSeq: NM_019313.1, amplicon size=68 bp), Rn01268575_m1 for target gene SK2 (*Kcnn2*, RefSeq: NM_019314.1 amplicon size=69 bp), Rn00570912_m1 for SK3 (*Kcnn3*, RefSeq: NM_019315.2, amplicon size=65 bp) and Rn99999916_s1 for the normalization gene glyceraldehyde-3-phosphate dehydrogenase GAPDH (*Gapdh*, RefSeq: NM_017008.3, amplicon size=87 bp). In previous study we investigated 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 less even during the epileptogenic process. *Gapdh* exhibited the least variability among the samples as determined by the analysis of the standard deviation of the threshold cycle values (Ermolinsky et al., 2008b). We concluded that *Gapdh* can be used as a reliable housekeeping gene (internal control gene) to analyze gene expression in epileptic rats since expression throughout the different phases of the model is minimal. Each sample was run in triplicates and a no-template control to test for contamination of assay reagents was also included. 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 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 (ΔΔCT, delta-delta CT) after determining the CT values for reference (*Gapdh*) and target genes (*Kcnn1*, *Kcnn2* or *Kcnn3*) in each sample sets as described by the manufacturer (Applied Biosystems; User Bulletin 2). Fold changes in target mRNA expression level were calculated after normalization to *Gapdh*. As calibrator sample we used cDNA from arbitrarily selected control rat. The ΔΔCT 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 fold changes relative to controls. Statistical analysis was performed using Statistica 7.0 (StatSoft, Inc, Tulsa, OK). The Shapiro-Wilk W test was used in testing for normality. Data were analyzed by analysis of variance (ANOVA) followed by post-hoc test *Tukey's Honest* Significant Difference (HSD), the significance *P* value was set at <0.05.

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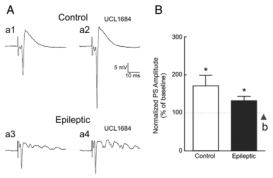

Figure 1.

Block of SK channels with selective antagonist UCL 1684 enhance hippocampal excitability in slices from control and epileptic rats. **A**. Representative signal traces of population spikes recorded in CA1 area of hippocampus indicating an increase in responsiveness upon stimulation of the Schaffer collateral pathway in slices from control rat after bath application of UCL1684 (a2). Notice normal response characterized by a single population spike in control slice. In slices from epileptic rat exhibiting enhanced excitability (more than one population spike) (a3), application of UCL1684 (100 nM) triggered an increase in the amplitude and number of population spikes (a4). Scale bar depicted for all traces. **B**. Graph represents changes in amplitude of the first population spike (PS) compared to normalized baseline responses (indicated by arrow, b: baseline) in control and epileptic rats following UCL1684.

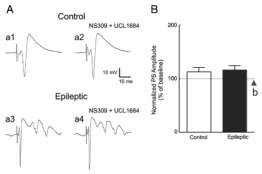

Figure 2.

Application of SK channel opener NS309 occlude the effect of UCL1684 on population spikes recorded in CA1 area of hippocampus. **A**. Representative experiments showing that co-application of UCL1684 (100 nM) and NS309 (1 μM) induce no changes in amplitude of evoked population spikes in control slices (a2) or slices from epileptic rat sacrificed 72 days after *status epilepticus* (a4) when compared to pre-drug baseline responses (a1) and (a3) respectively. No changes were noticed in the number of population spikes in epileptic slices. Scale is shown for all traces. **B**. Graph depicting no changes in the amplitude of population spikes compared to normalized baseline responses in control and epileptic rats following co-application of UCL1684 (100 nM) and NS309 (1 μM).

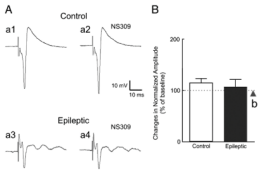

Figure 3.

Effect of application of SK channel opener NS309 on population spike responses evoked in CA1 area of hippocampal slices. **A**. Representative recordings in slice from control animal revealed no effect of NS309 (1 μM) in amplitude (a2) when compared to baseline response (a1). No effect of NS309 was detected in slices from chronically epileptic rat (78 days following *status epilepticus*) (a4) when compared to baseline (a3) (n=3). Scale is shown for all traces. **B**. Graph depicting no changes in the amplitude of population spikes compared to normalized baseline responses in control and epileptic rats following co-application of UCL1684 (100 nM) and NS309 (1 μM).

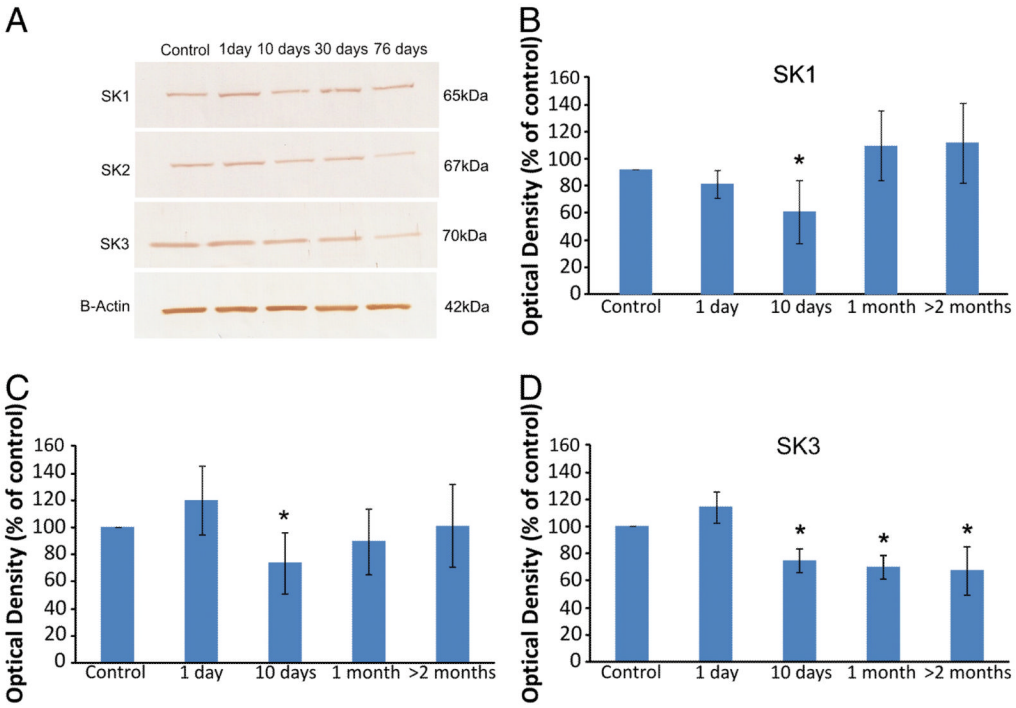

Figure 4.

Down-regulation of SK2 and SK3 protein immunoreactivity in chronically epileptic rats. **A**. Representative immunoblot analysis for SK1, SK2 and SK3 channels performed on hippocampal lysates obtained from representative age-matched control and rats sacrificed 1 day, 10 days, 30 days and 76 days after pilocarpine-induced status *epilepticus* (SE). Notice down-regulation of SK2 and SK3 protein expression at 76 days after SE. Immunoblot for actin were used as loading control. **B**. Semi-quantitative western blot analysis of density of immunopositive bands for SK1, SK2 and SK3 revealed a significant reduction in protein expression for SK1, SK2 and SK3 at 10 days after SE compared to control values. Levels of SK1 and SK2 recover in the 1 month group following SE (no significant changes compared to controls) but SK2 expression was significantly reduced again in animals sacrificed at more than 2 months following SE. SK3 expression was persistently at 10 days, 1 month group and > 2 months after pilocarpine induced SE.

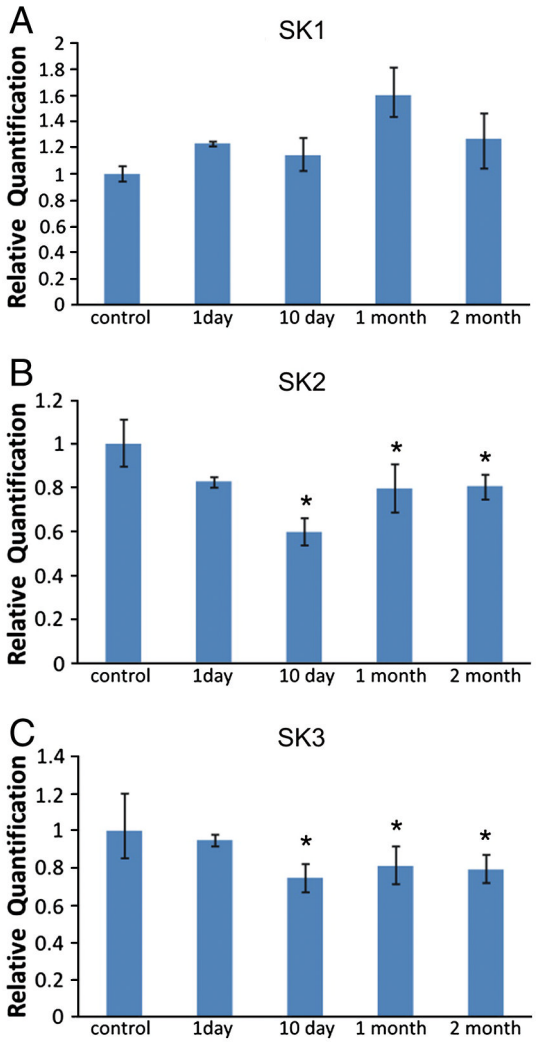

Figure 5.

Relative gene expression analysis of SK1 channels (*Kcnn1*), SK2 channels (*Kcnn2*) and SK3 channels (*Kcnn3*) (A, B and C, respectively) was performed by the comparative delta-delta CT (ΔΔCT) qPCR method using TaqMan probes. Data (fold changes) is represented as arbitrary units normalized relative to gene expression in control group. Analysis was performed in total RNA isolated from hippocampus at 1 day, 10 days, 1 month, and at more than 2 months following pilocarpine-induced *status epilepticus*. Data represented as mean ±SD of normalized relative quantification index. Statistical analysis by ANOVA revealed no significant difference for SK1 channel transcripts ($F=0.35$, $P=0.89$) and a significant difference for SK2 channels ($F=4.5$, $P=0.008$) and SK3 channels ($F=2.84$, $P=0.048$). * $P<0.05$, significant difference compared to control group by the *Tukey's* HSD post-hoc test.