

Published in final edited form as:

*Brain Res.* 2009 May 13; 1270: 107–111. doi:10.1016/j.brainres.2009.02.034.

## Protein expression of small conductance calcium-activated potassium channels is altered in inferior colliculus neurons of the genetically epilepsy-prone rat

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### Abstract

The genetically epilepsy-prone rat (GEPR) exhibits inherited predisposition to sound stimuli-induced generalized tonic-clonic seizures (audiogenic reflex seizures) and is a valid model to study the physiopathology of epilepsy. In this model, the inferior colliculus (IC) exhibits enhanced neuronal firing that is critical in the initiation of reflex audiogenic seizures. The mechanisms underlying IC neuronal hyperexcitability that leads to seizure susceptibility are not as yet fully understood. The present report shows that the levels of protein expression of SK1 and SK3 subtypes of the small conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels were significantly decreased, while SK2 channel proteins were increased in IC neurons of seizure-naive GEPR-3s (SN-GEPR-3), as compared to control Sprague-Dawley rats. No significant change was found in the expression of BK channel proteins in IC neurons of SN-GEPR-3s. Single episode of reflex audiogenic seizures in the GEPR-3s did not significantly alter the protein expression of SK1-3 and BK channels in IC neurons compared to SN-GEPR-3s. Thus, downregulation of SK1 and SK3 channels and upregulation of SK2 channels provide direct evidence that these Ca<sup>2+</sup>-activated K<sup>+</sup> channels play important roles in IC neuronal hyperexcitability that leads to inherited seizure susceptibility in the GEPR.

### Keywords

Seizure susceptibility; Calcium-activated potassium channel; Protein; Inferior colliculus

### 1. Introduction

Defective ion channels are thought to play important roles in the mechanisms underlying neuronal hyperexcitability that leads to inherited epilepsies in human (Reid et al. 2009). The genetically epilepsy-prone rat (GEPR) exhibits inherited susceptibility to generalized tonic-clonic seizures elicited by auditory stimulus (reflex audiogenic seizures) and is a relevant model to study the physiopathology of generalized seizures and epilepsy (Faingold 1999). In this model, the inferior colliculus (IC) exhibits an enhanced neuronal firing that is critical in the

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initiation of reflex audiogenic seizures (Faingold 1999). Two sub-strains of the GEPR have been characterized including the GEPR-3 that exhibits generalized clonic seizures, while the GEPR-9 displays generalized tonic-clonic seizures following exposure to the same sound stimulus (Dailey et al. 1989). Evidence suggests that both GEPR-3 and GEPR-9 exhibit similar pharmacological sensitivity suggestive of similar mechanisms, at least, in the initiation of reflex audiogenic seizures (Dailey and Jobe 1985). Although ion channels play an important role in the control of IC neuronal excitability, their involvement in the development of inherited seizure susceptibility in the GEPR is not yet fully understood (Evans et al. 1994, 2006; Verma-Ahuja et al. 1995; Molnar et al. 2000; Faingold 2002). We have recently reported that the current density of high threshold voltage-gated  $\text{Ca}^{2+}$  channels was markedly enhanced in IC neurons of the GEPR-3 (N'Gouemo et al. 2009). Such increase of  $\text{Ca}^{2+}$  currents is suggestive of a massive  $\text{Ca}^{2+}$  influx resulting in abnormal levels of cytosolic  $\text{Ca}^{2+}$  and disturbance of  $\text{Ca}^{2+}$  homeostasis, which in turn can alter  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from intracellular stores and deregulated  $\text{Ca}^{2+}$ -dependent mechanisms including  $\text{K}^{+}$  channels.  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels are classified into large (BK), intermediate (IK), and small (SK) conductance channels on the basis of their electrophysiological, pharmacological and molecular profiles (Köhler et al. 1996; Sah 1996; Stocker and Pedarzani 2000). SK subtype of  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels are encoded by three different genes (SK1, SK2, and SK3) resulting in distinct channels, which are expressed in many brain loci including the IC (Köhler et al. 1996; Stocker and Pedarzani 2000). Evidence suggests that BK channels play important roles in the induction of fast repolarization and afterhyperpolarization (AHP) associated with the action potential and bursting, while SK1 channels as well as SK2 and SK3 channels contribute to the slow AHP and medium AHP, respectively (Sah 1996; Pedarzani et al. 2001). BK channels are activated by both voltage and in increases in intracellular  $\text{Ca}^{2+}$ , while SK channels are exclusively activated by submicromolar increases in intracellular  $\text{Ca}^{2+}$  interacting with a cytosolic calmodulin-binding domain of the channel (Xia et al. 1998). Electrophysiological studies reported that IC neurons expressed both BK and SK channels and these  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  conductances exhibited both fast and slower AHP (Li et al. 1998; Sivaramakrishnan and Oliver 2001). Since  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels are thought to play important roles in control of neuronal excitability, altered expression of these channels in the IC may contribute to reflex audiogenic seizure susceptibility in the GEPR. Interestingly, the amplitude of both slow and fast AHP potentials was reduced in hippocampus CA3 neurons of the GEPR and in human epilepsy suggesting a downregulation of these channels (Verma-Ahuja et al. 1995; Williamson et al. 1993). In addition, pharmacological studies reported that blockade of apamin-sensitive channels within the IC elicited spontaneous seizures and decreased the threshold for electrically-induced seizures from the IC in normal rats; these findings therefore highlighted the role of apamin-sensitive SK channels in generation of non-audiogenic seizures (McCown and Breese 1990). Abnormal function of BK channels also has been implicated in the mechanisms of seizures and genetic epilepsy (Brenner et al. 2005; Lorenz et al. 2007; Ermolinsky B., Arshadmansab et al. 2008, Du et al., 2005; Shruti et al. 2008; Pacheco et al. 2008). Here, we sought to determine the levels of protein expression of BK, SK1, SK2, and SK3 subtypes of  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels in IC neurons obtained from normal Sprague-Dawley rats and quantify their changes in seizure-naïve GEPR-3 (SN-GEPR-3) and seizure-experienced GEPR-3 (SE-GEPR-3). A preliminary report of these studies has already appeared (N'Gouemo et al., 2003).

## 2. Results

Each GEPR-3 tested for seizure susceptibility only exhibited one episode of reflex audiogenic clonic seizures. SN-GEPR-3 and control Sprague-Dawley rats were not tested and did not exhibit seizures. The presence of  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channel protein in IC neurons was determined by using rabbit polyclonal antibodies to BK, SK1, SK2 and SK3 subtypes of  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channels. BK channels were identified as an immunoreactive band with a

molecular mass of 125 kDa while SK1, SK2 and SK3 channels were observed at 75 kDa, 60 kDa and 80 kDa, respectively. Western blot analysis showed that IC neurons expressed all subtypes of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (Figure 1). Quantification shows that levels of protein expression of SK1 subtype of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels were significantly ( $F = 7.3$ ,  $P = 0.004$ ) decreased in IC neurons of SN-GEPR-3, as compared to control SD rats (Figure 1). The levels of protein expression of SK3 subtype of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels also were significantly ( $F = 10.8$ ,  $P = 0.001$ ) decreased in IC neurons of SN-GEPR-3 ( $n = 8$ ), as compared to control SD rats ( $n = 8$ ) (Figure 1). In contrast to SK1 and SK3 channels, the levels of protein expression of SK2 subtype of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels were significantly ( $F = 5.3$ ;  $P = 0.01$ ) increased in IC neurons of SN-GEPR-3 ( $n = 8$ ), as compared to control SD rats ( $n = 8$ ) (Figure 1). No change in the levels of protein expression of SK1-3 channels was found in SE-GEPR-3 ( $n=8$ ) compared to SN-GEPR-3 ( $n=8$ ) (Figure 1); in fact, seizure episodes appear to non-significantly restore the levels of protein expression of SK1 channels to nearly control levels. Seizure episodes also non-significantly increased the levels of protein expression of SK2 channels. The data also shows that the levels of protein expression of BK subtype of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels were non-significantly ( $F = 0.2$ ;  $P = 0.8$ ) decreased in IC neurons of SN-GEPR-3 ( $n = 8$ ) as compared to control SD rats ( $n = 8$ ), as well as in SE-GEPR-3 ( $n = 8$ ) compared to SN-GEPR-3 ( $n = 8$ ).

### 3. Discussion

The main finding of this study is that levels of protein expression associated with SK1 and SK3 subtypes of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels are significantly reduced in IC neurons of SN-GEPR-3. In contrast, the levels of protein expression of SK2 channels are significantly increased, while the levels of BK channels are not significantly altered in IC neurons of SN-GEPR-3. Seizure episodes do not significantly alter the expression of SK1-3 and BK subtypes of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels. These findings indicate that downregulation of SK1 and SK3 channels as well as upregulation of SK2 channels in IC neurons are seizure susceptibility-related phenomena in the GEPR-3.

Multiple lines of evidence indicated that IC neurons are critical in the initiation of reflex audiogenic seizures in the GEPR-9 (Browning 1986; Faingold 1999). At the transition to seizures, unlike other brain sites, the IC exhibits sustained increases in neuronal firing, which support the role of this brain site in the initiation of reflex audiogenic seizures (Faingold 1999; N'Gouemo et al 1998). Such changes in IC neuronal firing may also occur in the GEPR-3. In addition to audiogenic seizures, both GEPR-9 and GEPR-3 also exhibited enhanced propensity to seizures induced by non-audiogenic stimuli including limbic kindling (Savage et al. 1986). In line with this report, functional ion channel abnormalities were reported in the hippocampus of the GEPR-9, a brain site that is not implicated in the initiation of reflex audiogenic seizures (Evans et al. 1994, Verma-Ahuja and Pencek, 1994; Verma-Ahuja et al. 1995). The mechanisms underlying the increases in IC neuronal firing and inherited seizure susceptibility in the GEPR-9 and GEPR-3 are not as yet well understood. The large (BK) and small (SK) conductance,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels are known to play important roles in the control of IC neuronal excitability, and altered expression of these channels therefore may play important roles in seizure susceptibility and seizure generation (Li et al 1998; Sivaramakrishnan and Oliver 2001). In support of this idea, electrophysiological study reported that the amplitude of slow AHP was smaller in hippocampal neurons of the GEPR-9 (Verma-Ahuja and Pencek, 1994; Verma-Ahuja et al. 1995; 1998). Such altered slow AHP also was found in human with epilepsy suggesting that abnormal AHP may be a general mechanism underlying neuronal hyperexcitability across species (Williamson et al. 1993).  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels also may play an important role in the generation of non-audiogenic seizures, since pharmacological blockade of apamin-sensitive  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels, including SK2 and SK3 channels, within the IC triggered spontaneous seizures and electrically-evoked

seizures in normal rats (McCown and Breese 1990). Whether the blockade of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels within the IC induces reflex audiogenic seizure susceptibility in normal rats remains unknown. Nevertheless, the levels of protein expression of SK1 and SK3 channels were downregulated in IC neurons of the GEPR-3 suggesting that these channels may play an important role in inherited audiogenic seizure susceptibility. BK subtype of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels also may play an important role in neuronal hyperexcitability that leads to seizures. Indeed, the amplitude of fast AHP associated with BK channels was smaller in hippocampal neurons of the GEPR-9 suggesting that down regulation of these channels may contribute to neuronal hyperexcitability associated with non-audiogenic seizure susceptibility. In line with this idea, the expression of BK channel protein was only slightly reduced in IC neurons of the GEPR-3, indicative of a minor role of these channels in the mechanisms underlying audiogenic seizure susceptibility.

In contrast to SK1 and SK3 channels, the levels of protein expression of SK2 channels were significantly enhanced in IC neurons of the GEPR-3. These findings suggested that upregulation of SK2 channels may represent a compensatory mechanism to the downregulation of SK1 and SK3 channels in IC neurons. Alternatively, upregulation of SK1 channels may contribute to enhanced seizure susceptibility in the GEPR-3. In support of this concept, recent reports indicated that a gain-of-function of BK channels was associated with picrotoxin-induced seizures and inherited epilepsy, while blockade of these channels suppressed chemically-induced seizures (Brenner et al. 2005; Du et al. 2005; Shruti et al. 2008; Sheehan et al 2008).

We conclude that altered levels of expression of SK1-3 subtypes of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels provide direct evidence that these channels contribute to IC neuronal hyperexcitability that leads to reflex audiogenic seizures in the GEPR. Functional studies are required to determine whether altered expression these channels is a critical step for seizure generation in the GEPR.

#### 4. Experimental Procedure

Male GEPR-3 (8–12 week-old) were obtained from the colony maintained at Southern Illinois University, Springfield, IL. This animal strain derives from Sprague-Dawley rats by selective breeding for audiogenic seizure susceptibility. Age-matched Sprague-Dawley rats purchased from Taconic (MD) were used as controls. Spontaneous seizures are rare in the GEPR-3 and were not monitored in this study. Two groups of GEPR-3 were used: seizure naive GEPR-3 (SN-GEPR-3) refer to animals that have never been exposed to sound stimulus-induced seizures, while seizure exposed-GEPR-3 (SE-GEPR-3) refer to animals that have been exposed to loud sound stimulus-induced seizures. SN-GEPR-3 and SE-GEPR-3 were selected randomly from the colony. In order to elicit seizures, GEPR-3s were exposed to an electrical bell (120 dB SPL, re: 0.0002 dynes/cm<sup>2</sup>) until seizure onset or for a maximum of 60 sec. In this study, all SE-GEPR-3 exhibited one episode of generalized seizures consisted of wild running that evolved into clonic seizures. The severity of seizures was scored according to the scale for audiogenic seizures (Jobe et al. 1973). SE-GEPR-3 were used 4–6 weeks after seizure induction; this time interval avoids the effects of acoustic stimulation and acute effects of seizures themselves on the protein expression of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels. All experimental procedures were approved by the Institution Animal Care and Use Committee, and all efforts were made to minimize the number of animals used and their suffering.

Animals were deeply anesthetized with pentobarbital (100 mg/kg; i.p.). The brains were removed and colliculi were quickly dissected (the periaqueductal grey was discarded) and stored at  $-70^{\circ}\text{C}$  until processed. Tissue homogenates were prepared according to the method described by Twombly et al. (1979). Briefly, IC homogenates from each animal were prepared in TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA) and centrifuged at 30,000 X  $g_{\text{max}}$  for

16 min at 4°C. The crude membrane pellets were resuspended in TE buffer. Protein concentrations in the crude membrane suspensions were determined using Bicinchoninic acid (BCA) Assay Reagent (Pierce, Rockford, IL) and each sample was diluted to a protein concentration of 10 mg/ml in TE buffer. The optimal Ca<sup>2+</sup>-activated K<sup>+</sup> channel concentration of 15 µg/lane used in this study was determined by testing several concentrations: 10, 15, and 30 µg of protein per lane. The membrane proteins were separated using sodium dodecyl sulfate-polyacrylamide gel (containing 7.5% polyacrylamide; Invitrogen, Carlsbad, CA) electrophoresis and transferred to the polyscreen polyvinylidene fluoride (New England Nuclear, Boston, MA) membranes in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol). The blots were processed using methods similar to those described by Towbin et al. (1979). Briefly, the blots were blocked in TBST buffer (20 mM Tris-HCl, pH 7.4; 140 mM NaCl and 0.1% v/v Tween 20; TBST) containing 5% nonfat dry milk (BLOTTO buffer) then incubated with Ca<sup>2+</sup>-activated K<sup>+</sup> channel BK, SK1, SK2, and SK3 antibodies (Alomone labs, Jerusalem, Israel) at concentrations of 1–1.5 µg/ml, overnight at 4°C. The following day, the blots were washed with a TBST buffer and incubated with a donkey anti-rabbit antibody (Amersham, Arlington Heights, IL) at a dilution of 1:5000 in BLOTTO buffer for 30 min at room temperature. After several washes of the blots, individual bands were visualized on the Hyperfilm (Amersham, Piscataway, NJ) using the enhanced chemiluminescence Super Signal West Pico and/or Femto (Pierce, Rockford, IL).

To quantify the protein levels of each Ca<sup>2+</sup>-activated K<sup>+</sup> subtype, the exposed films were analyzed and individual bands quantified by computer-assisted densitometry using a BioImage Analysis System (Ann Arbor, MI), which calculated the integrated intensity of a band using both the density and the area of the band. In each experiment, the integrated intensity of a specific protein band was compared to a standard curve of known protein concentration from rat hippocampus as done previously in our laboratory (N'Gouemo et al., 2006; Wang et al., 1995). The integrated intensities of the bands from experimental tissues determined at the same time and on the same piece of film were compared with the interpolated standard curve to yield a value of subunit expression relative to the standard hippocampus tissue. The utilized integrated intensities were always in the linear part of the standard curve. To determine differences in the protein expression between groups, one way analysis (ANOVA) was performed followed by Student-Newman-Keuls post hoc test. Data fulfill to the normality test (Shapiro-Wilk test) and tests for homogeneity of variance (Levene and Brown-Forsthe tests). Data obtained from SN-GEPR-3 were compared to control SD rats to determine changes associated with seizure susceptibility whereas findings in SE-GEPR-3s were compared to SN-GEPR-3s to assess the effects of seizure episodes. Data are presented as mean ± S.E.M.

## Acknowledgments

This publication was made possible by Public Health Service grants (NS047193 to P.N, AA11284 to R.P.Y. and AA11628 to C.L.F.) from the National Institutes of Health (NIH) and its contents are responsibility of the authors and do not necessarily represent the official views of NIH.

## Abbreviations

<b>AHP</b>	afterhyperpolarization
<b>BK</b>	large conductance, Ca <sup>2+</sup> -activated K <sup>+</sup> channel
<b>GEPR</b>	genetically epilepsy-prone rat



<b>IC</b>	inferior colliculus
<b>IK</b>	intermediate conductance, Ca <sup>2+</sup> -activated K <sup>+</sup> channel
<b>SE-GEPR-3s</b>	seizure-experienced GEPR-3s
<b>SK</b>	small conductance, Ca <sup>2+</sup> -activated K <sup>+</sup> channel
<b>SN-GEPR-3s</b>	seizure-naive GEPR-3s

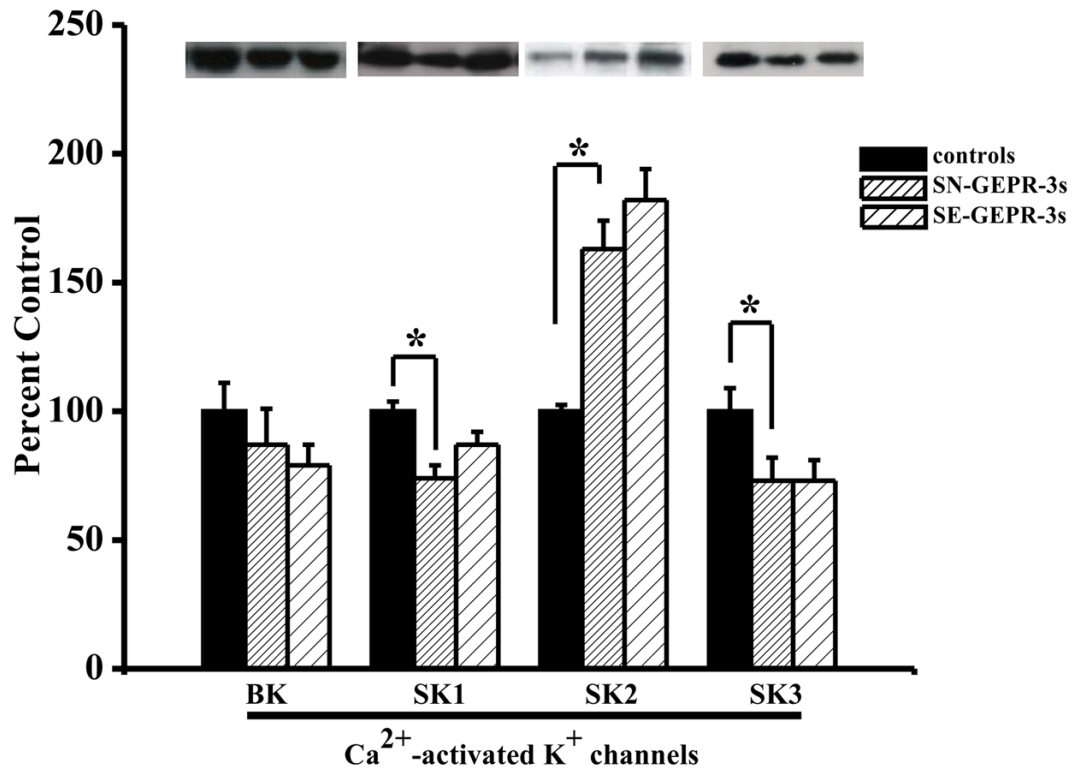
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**Figure 1.** Quantification of the protein expression of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels. Representative immunoblots of BK, SK1, SK2 and SK3 subtypes of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels obtained from IC neurons of control SD rats, SN-GEPR-3s and SE-GEPR-3s are showed in insets. The levels of protein expression of SK1 and SK3 subtypes of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels were significantly decreased in IC neurons of SN-GEPR-3s compared to control SD rats. In contrast, the levels of proteins associated with SK2 channels were significantly increased in IC neurons SN-GEPR-3s compared to control SD rats. Seizure episodes slightly increased the protein expression of SK1 and SK2 channels in IC neurons compared to SN-GEPR-3s, but did not alter expression of SK3 channels. The levels of protein expression of BK channels were slightly reduced in SN-GEPR-3s compared to control SD rats as well as in SE-GEPR-3s compared to SN-GEPR-3s. Each column represents the mean  $\pm$  S.E.M. ( $n = 8$ ). \* $P < 0.05$  (ANOVA followed by post hoc test).