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Seizure susceptibility is associated with altered protein expression of voltage-gated calcium channel subunits in inferior colliculus neurons of the genetically epilepsy-prone rat

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Abstract

The inferior colliculus (IC) is the consensus site for seizure initiation in the genetically epilepsy-prone rat (GEPR). We have previously reported that the current density of high threshold voltage-activated (HVA) calcium (Ca^{2+}) channels was markedly enhanced in IC neurons of the GEPR-3 (moderate seizure severity substrain of the GEPR). The present study examines whether subunit protein levels of HVA Ca^{2+} channels are altered in IC neurons that exhibit enhanced Ca^{2+} current density. Quantification shows that the levels of protein expression of the Ca^{2+} channel pore-forming $\alpha 1\text{D}$ (L-type) and $\alpha 1\text{E}$ subunits (R-type), were significantly increased in IC neurons of seizure-naive GEPR-3s (SN-GEPR-3s) compared to control Sprague-Dawley (SD) rats. Significant increases and decreases in the levels of protein expression of Ca^{2+} channel regulatory $\beta 3$ and $\alpha 2\delta$ subunits occurred in IC neurons of SN-GEPR-3s compared to control SD rats, respectively. No changes occurred in the protein expression of Ca^{2+} channel pore-forming $\alpha 1\text{A}$ (P/Q-type), $\alpha 1\text{B}$ (N-type) and $\alpha 1\text{C}$ (L-type) subunits in IC neurons of SN-GEPR-3s compared to control SD rats. A single seizure selectively enhanced protein expression of Ca^{2+} channel $\alpha 1\text{A}$ subunits in IC neurons of GEPR-3s. Thus, up-regulation of Ca^{2+} channel $\alpha 1\text{D}$ and $\alpha 1\text{E}$ subunits may represent the molecular mechanisms for the enhanced current density of L- and R-type of HVA Ca^{2+} channels in IC neurons of the GEPR, and may contribute to the genetic basis of their enhanced seizure susceptibility. The up-regulation of Ca^{2+} channel $\alpha 1\text{A}$ subunits induced by seizures may contribute to the increasing IC neuronal excitability that results from repetitive seizures in the GEPR.

Keywords

Ca^{2+} ; channel; L-type; R-type; protein; $\beta 3$; $\alpha 2\delta$; audiogenic seizure

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1. Introduction

Acoustically evoked seizures represent a heterogeneous group of reflex epilepsies in which seizures mostly tonic-clonic seizures are consistently triggered by sound stimuli in humans (Dreifuss 1998; Rosenow and Luder 2000). Genetic determinants are likely to underlie such seizures (Berkovic et al., 1998; Prasad et al., 1999). The pathophysiology of acoustically evoked seizures is not completely understood. Nevertheless, acoustically evoked seizures appear to respond well to antiepileptic drugs that suppress Ca^{2+} channel currents (Dreifuss 1998). The genetically epilepsy-prone rat (GEPR) is a model of acoustically evoked seizures (audiogenic) epilepsy that exhibits inherited susceptibility to generalized tonic and clonic seizures induced by auditory stimuli, and both these reflex audiogenic seizures are completely suppressed by Ca^{2+} channel blocker (Dailey et al., 1989; De Sarro et al., 1990). Ca^{2+} signaling therefore appears to play an important role in the underlying mechanisms of neuronal hyperexcitability that leads to acoustically evoked seizures in both the GEPR and human. Thus, the GEPR provides unique opportunities to study the mechanisms underlying seizure susceptibility and epilepsy in a model that, unlike normal brains, does not require exposure to convulsive treatments, such as convulsive drugs or electrical stimulation. There are two separate substrains of the GEPR based on the behavioral expression of seizure severity. The GEPR-9 exhibits generalized tonic seizures, while the GEPR-3 displays generalized clonic seizures in response to the same auditory stimuli (Dailey et al., 1985). The neuronal networks for reflex audiogenic seizures is mainly located in the brainstem and multiple lines of evidence indicate that inferior colliculus (IC) is critical in the initiation of these seizures in the GEPR (Browning 1986; Faingold 1999; Raisinghani and Faingold 2003). The mechanisms underlying IC neuronal hyperexcitability that leads to seizures are not yet fully understood in the GEPR (Faingold et al., 1986; Evans et al., 2006; Li et al., 1994; Faingold 2002). We have recently reported that the current density of L-, N-, and R-type high threshold voltage-activated (HVA) Ca^{2+} channels was significantly elevated in IC neurons of seizure-naïve GEPR-3s (SN-GEPR-3s), and seizure experience further enhanced the current density (N'Gouemo et al., 2009a). The molecular mechanisms underlying the enhancement of HVA Ca^{2+} currents in IC neurons of GEPR-3s are not completely elucidated. In an attempt to determine the underlying molecular mechanisms of the increased HVA current density, we used Western blot analysis to determine whether the levels of protein expression of various subunits of HVA Ca^{2+} channels are altered in IC neurons of SN-GEPR-3s and seizure-experienced GEPR-3s (SE-GEPR-3s), as compared to control Sprague-Dawley (SD) rats. Here we report that seizure susceptibility and seizure experience are associated with significant alterations of some HVA Ca^{2+} channel subunits in IC neurons of GEPR-3s.

2. Results

All GEPR-3s (n=9) tested for seizure susceptibility exhibited one episode of reflex clonic audiogenic seizures. SN-GEPR-3s (n=9) and control SD rats (n=9) were not stimulated acoustically and did not exhibit seizures. Western blot analysis using specific antibodies readily detected Ca^{2+} channel pore-forming $\alpha 1A$ (P/Q-type, $\text{Ca}_v2.1$), $\alpha 1B$ (N-type, $\text{Ca}_v2.2$), $\alpha 1C$ (L-type, $\text{Ca}_v1.2$), $\alpha 1D$ (L-type, $\text{Ca}_v1.3$), and $\alpha 1E$ (R-type, $\text{Ca}_v2.3$) as well as the regulatory Ca^{2+} channel $\beta 3$ and $\alpha 2\delta$ subunit proteins in IC neurons (Figs. 1, 2). Quantification analysis shows significant differences among groups in the levels of protein expression of Ca^{2+} channel $\alpha 1D$ (control SD rats, n=9; SN-GEPR-3s, n=8; SE-GEPR-3s, n=8; $F=5.7$, $P=0.009$), $\alpha 1E$ (control SD rats, n=8; SN-GEPR-3s, n=8; SE-GEPR-3s, n=8; $F=4.9$, $P=0.018$) and $\alpha 1A$ (control SD rats, n=9; SN-GEPR-3s, n=9; SE-GEPR-3s, n=9; $F=7.9$, $P=0.002$) subunits. Post hoc test reveals that the levels of Ca^{2+} channel $\alpha 1D$ subunits were significantly ($P<0.05$) increased in IC neurons of SN-GEPR-3s (n=8) compared to control SD rats (n=9; Fig. 1). Similarly, the levels of Ca^{2+} channel $\alpha 1E$ subunits were also significantly ($P<0.05$) increased in IC neurons of SN-GEPR-3s (n=8) compared to control SD rats (n=8; Fig. 1). No significant

changes were found in the protein expression of Ca^{2+} channel $\alpha 1\text{A}$, $\alpha 1\text{B}$, and $\alpha 1\text{C}$ subunits in IC neurons of SN-GEPR-3s ($\alpha 1\text{A}$, $n=9$; $\alpha 1\text{B}$, $n=8$; $\alpha 1\text{C}$, $n=9$) compared to control SD rats ($\alpha 1\text{A}$, $n=9$; $\alpha 1\text{B}$, $n=9$; $\alpha 1\text{C}$, $n=9$). Inferior colliculus tissues obtained from SE-GEPR-3s ($n=9$) showed a significant ($P<0.05$) enhanced expression of Ca^{2+} channel $\alpha 1\text{A}$ subunit proteins compared to SN-GEPR-3s ($n=9$; Fig. 1). No change was found in the protein expression of $\alpha 1\text{B}$, $\alpha 1\text{C}$, and $\alpha 1\text{D}$ subunits of HVA Ca^{2+} channels in IC neurons of SE-GEPR-3s ($\alpha 1\text{B}$, $n=9$; $\alpha 1\text{C}$, $n=9$; $\alpha 1\text{D}$, $n=8$) compared to SN-GEPR-3s ($\alpha 1\text{B}$, $n=8$; $\alpha 1\text{C}$, $n=9$; $\alpha 1\text{D}$, $n=8$). Nevertheless, the levels of protein expression of Ca^{2+} channel $\alpha 1\text{A}$, $\alpha 1\text{D}$, and $\alpha 1\text{E}$ subunits were significantly ($p<0.05$) enhanced in IC neurons of SE-GEPR-3s ($\alpha 1\text{A}$, $n=9$; $\alpha 1\text{D}$, $n=8$; $\alpha 1\text{E}$, $n=8$) compared to control SD rats ($\alpha 1\text{A}$, $n=9$; $\alpha 1\text{D}$, $n=9$; $\alpha 1\text{E}$, $n=8$; Fig. 1). Analysis of HVA Ca^{2+} channel auxiliary subunits also showed significant differences among groups in the expression of $\beta 3$ (control SD rats, $n=8$; SN-GEPR-3s, $n=8$; SE-GEPR-3s, $n=9$; $F=8.7$, $P=0.001$) and $\alpha 2\delta$ (control SD rats, $n=8$; SN-GEPR-3s, $n=8$; SE-GEPR-3s, $n=8$; $F=6.3$, $P=0.007$) subunits. Post hoc test indicates that levels of protein expression of Ca^{2+} channel $\beta 3$ subunits were significantly ($P<0.05$) increased, whereas a significant ($P<0.05$) decrease was found in the expression of $\alpha 2\delta$ subunits in IC neurons of SN-GEPR-3s ($\beta 3$, $n=8$; $\alpha 2\delta$, $n=8$) compared to control SD rats ($\beta 3$, $n=8$; $\alpha 2\delta$, $n=8$; Fig. 2). Seizure experience did not significantly alter the levels of protein expression of Ca^{2+} channel $\alpha 2\delta$ and $\beta 3$ subunits in IC neurons of SE-GEPR-3s ($\beta 3$, $n=8$; $\alpha 2\delta$, $n=9$), as compared to SN-GEPR-3s ($\beta 3$, $n=8$; $\alpha 2\delta$, $n=8$). Nevertheless, the expression of Ca^{2+} channel $\alpha 2\delta$ and $\beta 3$ subunit proteins were significantly decreased and elevated in IC neurons of SE-GEPR-3s ($\beta 3$, $n=8$; $\alpha 2\delta$, $n=9$) compared to control SD rats ($\beta 3$, $n=8$; $\alpha 2\delta$, $n=8$), respectively (Fig. 2).

3. Discussion

The major findings of the present study are that HVA Ca^{2+} channel pore-forming $\alpha 1\text{D}$ (L-type; $\text{Ca}_v1.3$) and $\alpha 1\text{E}$ (R-type, $\text{Ca}_v2.3$) subunits, as well as the regulatory $\beta 3$ subunit are up-regulated, whereas the auxiliary $\alpha 2\delta$ subunit is down-regulated in IC neurons of SN-GEPR-3s. A single seizure experience also selectively enhanced the expression of Ca^{2+} channel $\alpha 1\text{A}$ (P/Q-type, $\text{Ca}_v2.1$) subunits in IC neurons of GEPR-3s. Since the IC is critical for seizure initiation in the GEPR (Browning 1986; Faingold 1999; Raisinghani and Faingold 2003), these findings suggest that altered expression of L-, R- and P/Q-type of HVA Ca^{2+} channels may contribute to seizure susceptibility and seizures in the GEPR-3.

Ca^{2+} channels are thought to play an important role in seizure susceptibility in GEPR-3s (De Sarro et al., 1990). We previously reported that HVA Ca^{2+} channel current density was markedly elevated in IC neurons of SN-GEPR-3s and that seizure experience further enhanced the current density (N'Gouemo et al., 2009a). The fractions of HVA currents pharmacologically identified to be carried by L-, N- and R-type Ca^{2+} channels appear to contribute to the increased current density in IC neurons of SN-GEPR-3s. Interestingly, the levels of total protein expression of Ca^{2+} channel pore-forming $\alpha 1\text{D}$ and $\alpha 1\text{E}$ subunits associated with L- and R-type channels, respectively, were significantly elevated in IC neurons of SN-GEPR-3s compared to control SD rats. Thus, increases in the levels of total protein expression of Ca^{2+} channel $\alpha 1\text{D}$ and $\alpha 1\text{E}$ subunits may provide the molecular determinants for enhancement of L- and R-type currents associated with seizure susceptibility in GEPR-3s. In this study, the levels of total protein expression of Ca^{2+} channel $\alpha 1\text{B}$ (N-type) subunit was not significantly altered in IC neurons of SN-GEPR-3s and SE-GEPR-3s. These data suggest that other factors such as altered trafficking may contribute to the enhanced current density of N-type Ca^{2+} channels in IC neurons of SN-GEPR-3s. Interestingly, enhanced cell surface density of N-type Ca^{2+} channel was reported in the hippocampus following kindling epileptogenesis (Bernstein et al., 1999). Such changes may also occur in IC neurons of SN-GEPR-3s. We also reported that seizure experience further enhanced total current density of HVA Ca^{2+} channels in IC neurons of SE-GEPR-3s (N'Gouemo et al., 2009a). Although no

pharmacological studies were performed to determine which type of HVA Ca^{2+} channels accounted for the enhanced current density, the up-regulation of Ca^{2+} channel $\alpha 1A$ subunits found in IC neurons of SE-GEPR-3s suggests that P/Q-type of HVA Ca^{2+} channels may contribute to the enhanced current density following seizure experience in SE-GEPR-3s. The enhanced HVA Ca^{2+} current density and Ca^{2+} channel $\alpha 1A$ subunit proteins in SE-GEPR-3s may also contribute to the elevated IC neuronal firing associated with repetitive reflex audiogenic seizures in the GEPR-9 (N'Gouemo and Faingold, 1996; N'Gouemo et al., 2009a).

Ca^{2+} channels are comprised of the pore-forming, voltage-sensing $\alpha 1$ subunits and auxiliary subunits including $\alpha 2\delta$, β , and γ , which modulated the expression of Ca^{2+} currents (Catterall 2000). In this study, significant increases in the levels of expression of Ca^{2+} channel regulatory $\beta 3$ subunit proteins were found in IC neurons of SN-GEPR-3s. Evidence suggests that Ca^{2+} channel $\beta 3$ subunits were associated with N- and L-type of HVA Ca^{2+} channels in the brain (Scott et al., 1996). Consistent with this report, L- and N-type Ca^{2+} channel currents were significantly reduced in the Ca^{2+} channel $\beta 3$ subunit-deficient mice and the present study shows up-regulation of Ca^{2+} channel $\beta 3$ subunit proteins that is associated with increased current density of L- and N-type Ca^{2+} channels (Namkung et al., 1998; N'Gouemo et al., 2009a). Thus, up-regulation of Ca^{2+} channel $\beta 3$ subunits may contribute to seizure susceptibility, probably by enhancing the current density of L- and N-type of HVA Ca^{2+} channels in IC neurons of GEPR-3s. The increased amount of Ca^{2+} channel $\beta 3$ subunit proteins was accompanied by significant reduction in the levels of protein expression of Ca^{2+} channel $\alpha 2\delta$ subunits in IC neurons of SN-GEPR-3s. The down-regulation of Ca^{2+} channel $\alpha 2\delta$ subunit proteins in IC neurons also may contribute to seizure susceptibility in the GEPR-3. Evidence shows that Ca^{2+} channel $\alpha 2\delta$ -2 knockout mice exhibited enhanced seizure susceptibility to pentylenetetrazol-induced clonic seizures (Ivanov et al., 2004). Such altered Ca^{2+} channel $\alpha 2\delta$ -2 gene may also occur in IC neurons and contributed to enhanced seizure susceptibility to acoustically evoked clonic seizures in the GEPR-3.

The mechanisms how the up-regulation of Ca^{2+} channels contributes to IC neuronal hyperexcitability and enhanced susceptibility to seizures is not fully understood in the GEPR. The up-regulation of Ca^{2+} channels is suggestive of a massive Ca^{2+} influx which can disturb Ca^{2+} homeostatic mechanisms including Ca^{2+} -induced Ca^{2+} release from internal stores and activation of downstream K^{+} channels. We recently reported that levels of expression of SK1 and SK3 subtype of small conductance Ca^{2+} -activated K^{+} channels were significantly reduced in IC neurons of SN-GEPR-3s (N'Gouemo et al., 2009b). Thus, up-regulation of Ca^{2+} channel $\alpha 1D$ and $\alpha 1E$ subunits associated with down-regulation of SK1 and SK3 subtype of Ca^{2+} -activated K^{+} channels may represent cohesive mechanisms for seizure susceptibility in the GEPR-3. Additional *in vivo* studies are needed to determine whether modulation of these channels alters seizure susceptibility in the GEPR.

4. Experimental Procedures

Animals

Male, GEPR-3s (8-12) were obtained from the colony maintained at Southern Illinois University at Springfield, IL. This animal strain derives from Sprague-Dawley (SD) rats by selective breeding for audiogenic seizure susceptibility. Age-matched SD rats purchased from Taconic (Germantown, MD) were used as controls, which were housed for at least 14 days prior to experimentation. The animals were placed in plastic cages with free access to food and water, and housed in an environment with controlled temperature and humidity, as well as a standard day/night cycle. Spontaneous seizures are rare in the GEPR-3s and were not monitored in this study. Two groups of the GEPR-3 were used. The seizure-naïve GEPR-3s (SN-GEPR-3s) refer to animals that have never been exposed to sound stimulus-induced seizures,

while seizure experienced GEPR-3s (SE-GEPR-3s) refer to animals that have been exposed to loud sound stimulus-induced seizures. SN-GEPR-3s and SE-GEPR-3s were selected randomly from the colony. In order to elicit reflex audiogenic seizures, GEPR-3s were exposed to an electrical bell (120 dB SPL, re: 0.0002 dynes/cm²) until seizure onset or for a maximum of 60 sec. In this study, all SE-GEPR-3s exhibited one episode of generalized clonic seizures (Jobe et al., 1973). SE-GEPR-3s were used 4–6 weeks after seizure induction for molecular studies; this time interval avoids the effects of acoustic stimulation and acute effects of seizures themselves on protein expression of HVA Ca²⁺ channel subunits. The experimental procedures using animals were reviewed and approved by the Georgetown University Institutional Animal Care and Use Committee. Efforts were made to minimize the number of animals used and their suffering.

Gel electrophoresis and immunoblotting

Animals were deeply anesthetized with pentobarbital (100 mg/kg; i.p.). The brains were removed and the IC was quickly dissected (the area corresponding to the periaqueductal grey was discarded) and stored at –70°C until processed (N'Gouemo et al., 2003). Tissue homogenates from each animal were prepared in TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA) and centrifuged at 30,000xg_{max} for 16 min at 4°C. The crude membrane pellets were resuspended in TE buffer. Protein concentrations in the crude membrane suspension were determined using Bicinchoninic acid Assay Reagent (Pierce, Rockford, IL) and each sample was diluted to a protein concentration of 5 or 10 mg/ml in TE buffer. The optimal Ca²⁺ channel concentration of 15 µg of protein per 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 polyscreen polyvinylidene fluoride membrane (New England Nuclear, Boston, MA) 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 Ca²⁺ channel subunit antibodies α_{1A} , α_{1B} , α_{1C} , α_{1D} , α_{1E} , β_3 , and $\alpha_2\delta$ (Alomone labs, Jerusalem, Israel) at concentrations of 0.5–1.5 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 1:4000 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 or Femto (New England Nuclear, Boston, MA) and analyzed.

Data analysis

To quantify the expression of each HVA Ca²⁺ channel subunit proteins, the exposed films were analyzed and individual bands quantified by computer-assisted densitometry using a BioImage Analysis System software (Ann Arbor, MI), which calculated the integrated intensity of a band using both the density (darkness) and the size (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; 2009b; Wang et al., 1995). The hippocampus was used because of its higher levels of HVA Ca²⁺ channel protein compared to the IC. 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 on the linear part of the standard curve. All statistical analyses were performed on raw data. Data fulfill to both the normality test (Shapiro-Wilk test) and tests for homogeneity of variance (Levene and

Brown-Forsythe tests). A one-way analysis of variance (ANOVA), where F test statistics is the ratio of variance of the group mean and mean of the within-group variance, was used to determine if there were significant differences among groups in the levels of protein expression of HVA Ca^{2+} channel subunits. The Student-Newman-Keuls post hoc test was performed to identify the origin of any significant differences. Data obtained from SN-GEPR-3s 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 were normalized as a percentage ($\pm$ S.E.M.) of the mean control value.

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Abbreviations

GEPR	genetically epilepsy-prone rat
HVA	high threshold voltage-activated
IC	inferior colliculus
SE-GEPR-3s	seizure-experienced GEPR-3s
SN-GEPR-3s	seizure-naïve GEPR-3s

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Figure 1.

Protein expression and representative individual immunoblots (inset) of α_{1A} (190kDa), α_{1B} (240 kDa), α_{1C} (207 kDa), α_{1D} (180 kDa), and α_{1E} (220 kDa) subunits of HVA Ca^{2+} channels. The levels of protein expression of α_{1D} , and α_{1E} subunits of HVA Ca^{2+} channels were significantly increased in IC neurons of SN-GEPR-3s (α_{1D} , n=8; α_{1E} , n=8) and SE-GEPR-3s (α_{1D} , n=8, α_{1E} , n=8) compared to control SD rats (α_{1D} , n=9; α_{1E} , n=8), whereas the expression of HVA Ca^{2+} channel α_{1A} subunit was significantly elevated in IC neurons SE-GEPR-3s (n=9) compared to SN-GEPR-3s (n=9) and control SD rats (n=9). Each column represents a percentage fold ($\pm$ S.E.M) change over mean control value. * $P < 0.05$ (ANOVA followed by Student-Newman-Keuls test).

Figure 2.

Protein expression and representative individual immunoblots (inset) of $\alpha 2\delta$ (140 kDa) and $\beta 3$ (60 kDa) subunits of HVA Ca²⁺ channels. The levels of protein expression of Ca²⁺ channel $\beta 3$ subunits was significantly increased in IC neurons of SN-GEPR-3s (n=8) and SE-GEPR-3s (n=9) compared to control SD rats (n=8). The expression of Ca²⁺ channel $\alpha 2\delta$ subunits was significantly decreased in IC neurons SN-GEPR-3s (n=8) and SE-GEPR-3s (n=9) compared to control SD rats (n=8). Each column represents percentage fold change ($\pm$ S.E.M) over mean control value. * $P < 0.05$ (ANOVA followed by Student-Newman-Keuls test).