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Genetic absence epilepsy rats from Strasbourg have increased corticothalamic expression of stargazin

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ABSTRACT

Stargazin is membrane bound protein involved in trafficking, synapse anchoring and biophysical modulation of AMPA receptors. A quantitative trait locus in chromosome 7 containing the stargazin gene has been identified as controlling the frequency and duration of absence seizures in the Genetic Absence Epilepsy Rats from Strasbourg (GAERS). Furthermore, mutations in this gene result in the Stargazer mouse that displays an absence epilepsy phenotype. GAERS stargazin mRNA expression is increased 1.8 fold in the somatosensory cortex and by 1.3 fold in the thalamus. The changes were present before and after the onset of absence seizures indicating that increases are not a secondary consequence of the seizures. Stargazin protein expression was also significantly increased in the somatosensory cortex after the onset of spontaneous seizures. The results are of significant importance beyond the GAERS model, as they are the first to show that an *increase* in stargazin expression may be pro-epileptic.

Introduction

The generalised epilepsies are a common group of diseases that are believed to be largely hereditary, but with more than one gene involved in causing the epileptic phenotype. Absence seizures are a common type of seizure in patients with generalised epilepsies characterised by staring, loss of facial expression and unresponsiveness electrographically characterised by spike-and-wave discharges (SWDs). Linkage analysis on Genetic Absence Epilepsy Rats from Strasbourg (GAERS) double crossed with Brown Norway (BN) rats identified three quantitative trait loci (QTL) on chromosomes 4, 7 and 8 associated with various components of the expression of the seizures (i.e. number, duration, amplitude and frequency of SWDs) (Rudolf et al., 2004). The locus on chromosome 7 is particularly interesting because it contains the gene for *Cacng2* (stargazin) (Letts et al., 1998). This interest stems from the finding that a mutation in this gene causes an absence seizure phenotype in the Stargazer mouse model (Letts et al., 1998).

Voltage-gated calcium (Ca^{2+}) channels are believed to play a critical role in the generation of the hypersynchronous oscillatory thalamocortical activity that underlies absence seizures (Tsakiridou et al., 1995). The key determinant of Ca^{2+}channel subtypes character is their $\alpha 1$ pore forming subunit. However, additional ancillary subunits significantly influence the kinetics of Ca^{2+}channels. For instance, the α_2-δ subunit increases Ca^{2+}currents, the β-subunit modifies activation and inactivation, while the γ-subunit increases inactivation (Catterall, 2000; Black, 2003; Dolphin, 2003). It is defects in this ancillary role that are believed to underlie the epilepsy phenotype in the Stargazer mouse (Letts et al., 1998). However, recent research has shed light on a new role for γ subunits in the trafficking and anchoring of AMPA receptors to the synaptic membrane, leading to the designation of a new family of proteins termed transmembrane AMPA receptor regulatory proteins (TARPs) of which several γ subunits are members (Tomita et al., 2003; Yamazaki et al., 2004; Vandenberghe et al., 2005; Ziff, 2007). AMPA receptors are ionotropic transmembrane receptors for glutamate that mediate fast synaptic transmission in the CNS (Dingledine et al., 1999). TARPs also influence electrophysiological properties of AMPA receptors including the slowing of deactivation and reducing desensitization rates (Yamazaki et al., 2004; Priel et al., 2005; Tomita et al., 2005; Turetsky et al., 2005; Ziff, 2007) as well as enhancing the plasma membrane expression of the AMPA receptor subtype, GluR1, in HEK cells (Bedoukian et al., 2008). These newly

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identified TARP roles for stargazin could have major functional implications on the homeostatic balance of neuronal excitation, and potentially for the pathophysiology of epilepsy.

We hypothesised that abnormalities of stargazin sequence and/or expression may contribute to the epileptic phenotype in GAERS. This study compared the genetic sequence, and thalamic and cortical mRNA and protein expression of stargazin between GAERS and their control strain, Non-Epileptic Control rats (NEC). GAERS and NECs were selectively bred from the same original colony according to the presence or absence of the epileptic phenotype, thus any differences identified between them has a strong a priori case for being aetiologically involved in the epileptic phenotype.

Methods

Animals

The experimental procedures on GAERS and NEC rats were approved by the Department of Medicine, Royal Melbourne Hospital (AEC #2004.019) animal ethics committee. GAERS and NEC rats had their phenotype confirmed by analysis of two 90-minute EEG recording on separate days using six extradural scalp electrodes (implanted one week previously under ketamine and xylazine anaesthetic) (Stroud et al., 2005; Jones et al., 2008). Rats were culled by a lethal dose of Pentobarbital, (Lethabarb™) anaesthetic (Virbac, Sydney, Australia) followed by rapid extraction of the brain. The thalamic, somatosensory cortical (S1) and motor cortical (M1) regions were rapidly dissected and stored in RNALater (Applied Biosystems), and frozen at −80 °C. Liver tissue was also collected and frozen at −80°C for genomic DNA isolation.

Gene sequencing

RNA for sequencing was isolated from the cortex, thalamus and whole brain samples from both GAERS and NEC rats using Trizol reagent (Invitrogen). Isolation of genomic DNA from liver tissue was performed as described previously (Laird et al., 1991). Cortical RNA sample from each rat was reverse transcribed with Oligo dT_{12-18}, using Superscript III reverse transcriptase kit (Invitrogen). Polymerase chain reaction (PCR) amplification was performed on the resultant cDNA and genomic DNA, using Amplitaq Gold™ DNA polymerase (Applied Biosystems). Reactions followed a touchdown algorithm where annealing temperature began at 70 °C and reduced by 1 °C every repeat until 63 °C, 60 °C or 55 °C was reached depending on the Tm of the primers. Then annealing was held at that temperature for the remainder of the repeats. The PCR products were purified using QIAquick© spin-column PCR purification kits (QIAGEN). Cycle sequencing reactions were performed on PCR products using Big Dye Terminator (Applied Biosystems) and 3.2 μM final concentration of primers. Subsequently, samples were run on 4.8% polyacrylamide/6 M urea sequencing gel on an ABI Prism™ 377 DNA sequencer (Applied Biosystems). Sequences were analysed using ABI Autoassembler™ program v2.1 (Perkin Elmer). The sequence from GAERS rats, NEC rats and *Rattus norvegicus* database were compared. Any ambiguous sequences or variations identified were verified by another repeat sequencing procedure.

Northern blot hybridisation

Messenger RNA (mRNA) was isolated from total RNA using Oligotex mini kit (QIAGEN). RNA ladder (Sigma) and mRNA (1.5 μg per lane) isolated from cortex, thalamus and whole brain was separated on a 1% agarose gel containing 0.4M formaldehyde and transferred to Hybond-N+membrane (Amersham) by capillary blot. Gene specific probes ~500 bp PCR products from stargazin and β-actin were labelled with α^{32}P-dCTP. Hybridisation was performed using RapidHyb hybridisation buffer (Amersham).

Quantitative polymerase chain reaction (qPCR)

RNA was extracted using RNeasy mini kit (QIAGEN) and treated with DNase I (QIAGEN) to remove any contaminating genomic DNA and stored at −80 °C. Spectrophotometric readings were taken with the NanoDrop Spectrophotometer (NanoDrop Technologies) to determine RNA concentration and purity. 500 ng of RNA was reversed transcribed to cDNA with random primers using the Omniscript Reverse Transcription kit (QIAGEN) and stored at −20 °C. Quantitative real time PCR (qPCR) was performed on 25 ng cDNA using custom designed gene expression assays for stargazin (Assay ID Rn00584355_m1, Applied Biosystems). Stargazin mRNA levels were compared to mRNA levels of the housekeeping gene ribosomal 18S RNA using a custom designed gene expression assay for this gene (Assay ID Hs99999901_s1, Applied Biosystems). Analysis was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

Western blotting

Proteins were extracted from rat somatosensory cortex by hand-held homogenisation in RIPA lysis buffer (10% glycerol, 20 mM Tris, pH7.5, 137 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.5% IGEPAL and 1% Triton X-100), supplemented with trasylol, leupeptin and vanadate. Cell lysates were incubated at 4 °C, for 10 min with rotation. Cell debris was cleared by centrifugation at 13,000 rpm for 10 min at 4 °C. The cleared lysates were transferred to clean Eppendorf tubes and stored at −70 °C until use. Protein concentrations were determined using the BCA protein assay reagent (Pierce). Immuno-blotting was performed using 50 μg of cleared lysate. Samples were resolved by SDS-polyacrylamide electrophoresis. Prior to loading Laemmli Buffer was added to each sample, followed by heating to 100 °C for 2 min. Proteins were transferred onto Hybond C Super membrane (Amersham) for western blotting. Membranes were blocked in 5% BSA, dissolved in Tris-buffered-saline pH 7.5/0.1%Tween20 (TBST, Sigma) and probed with the stargazin primary antibody (1:3000, Santa Cruz (sc-18284)) at 4 °C overnight or the β-tubulin primary antibody (1:50,000, Sigma) at room temperature for 1 h followed by incubation with a rabbit anti-goat (for stargazin) or goat anti-mouse (for β-tubulin) HRP secondary antibodies (both at 1:10,000; DAKO) for 45 min at room temperature. Proteins were visualised using the enhanced chemiluminescence (ECL) reagent (Perkin Elmer) and band intensity analysis was done using ImageJ software (NIH).

Statistical analysis

All data is expressed as mean±S.E.M. Statistical significance was determined using the non-parametric Mann Whitney *U* test with significance level set at $p<0.05$.

Results

No mutations were detected in the coding region of the Cacng2 gene

The details for *Cacng2*, according to the *Rattus norvegicus* genome database from Ensembl genome browser 2004, are shown in Table 1. All 1120 bp of *Cacng2* coding region was sequenced in 4 NEC and 6

Table 1
Gene information for *Cacng2* according to the *Rattus norvegicus* genome database from Ensembl genome browser

Gene name	*Cacng2*
Entrez protein ID	NP_445803.1
Chromosome	7
Location	115,913,440 to 116,037,891 bp
Transcript length	1120 bp
Exons	4
Translation length	323 residues
Known splice variants	Nil

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GAERS rats. The entire length of this gene was sequenced from cDNA and genomic DNA. No differences in sequence were detected between GAERS, NEC and *R. norvegicus* sequences.

GAERS have increased stargazin expression in Corticothalamic structures

Seizures in GAERS start to develop from 7–8 weeks of age in our colony and by 13 weeks all GAERS are having spontaneous absence seizures (Jones et al., 2008). For the 6 week group the average age of NEC rats was 41.6±0.7 days (n=11) and GAERS was 41.3±0.2 days (n=10). For the >13 week group the average age of NEC rats was 21±0.3 weeks (n=8) and GAERS was 19.7±0.3 weeks (n=12). We measured mRNA expression of stargazin in the somatosensory cortex and thalamus in juvenile pre-epileptic (6 weeks) and adult epileptic GAERS (>13 weeks) and NEC rats. Stargazin mRNA expression increased in a region and age specific manner (Fig. 1). Specifically, stargazin mRNA expression was significantly increased (1.8 fold) in the somatosensory cortex of GAERS (n=8–10) compared to NEC (n=7–8) rats before (6 weeks; p<0.05, Fig. 1A) and after (>13 weeks; p<0.001, Fig. 1B) the onset of spontaneous absence seizures. Additionally a smaller but significant increase (1.3 fold) in stargazin mRNA expression was observed in the thalamus of GAERS (n=10) compared to NEC (n=8) rats at the 6 week time point (p<0.05; Fig. 1A). After the onset of spontaneous absence seizures a similar increase (1.2 fold) in stargazin mRNA expression was observed in the thalamus although it did not reach statistical significance (Fig. 1B). Stargazin protein expression was significantly increased in the somatosensory cortex of adult epileptic GAERS (n=4) compared to NEC rats (n=4, p<0.05, Fig. 2) demonstrating a concordance with the mRNA results. We also measured stargazin mRNA expression in the motor cortex (M1), a region of the brain not primarily involved in the generation of the absence seizures in GAERS (Polack et al., 2007). Here a 1.5 fold increase in stargazin mRNA expression in both juvenile pre-epileptic and adult epileptic GAERS (6 week group n=10;>13 week group n=12) was demonstrated compared to NEC rats (6 week group n=11, p<0.01;>13 week group n=7, p<0.05; Figs. 1A and B).

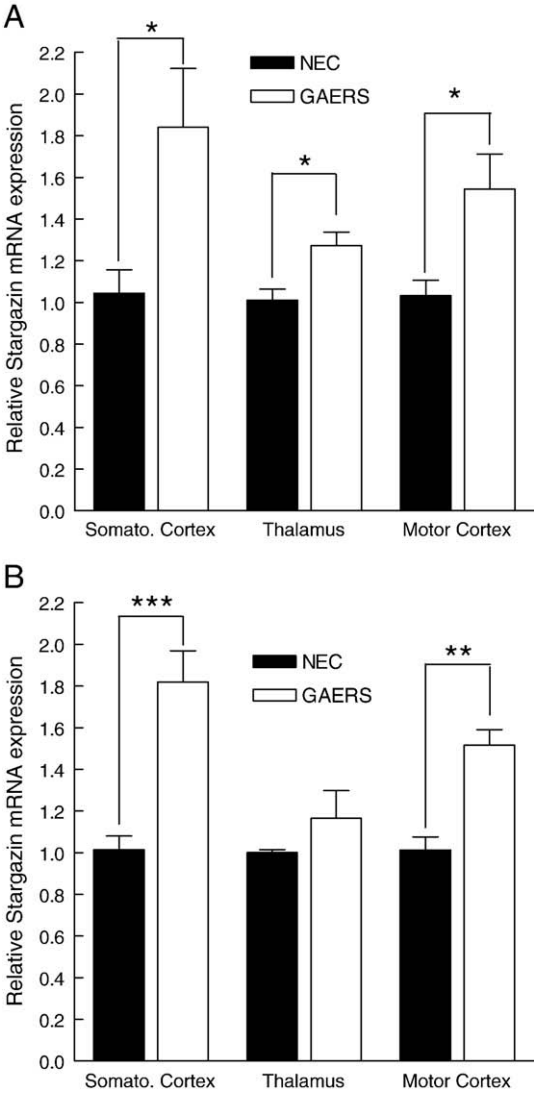

Fig. 1. Stargazin mRNA expression is increased in a region and age specific manner. Stargazin mRNA expression is significantly increased in the somatosensory cortex (S1) and motor cortex (M1) of GAERS by approximately 1.8 fold and 1.5 fold respectively before (6 weeks; A) and after (>13 weeks; B) the onset of spontaneous absence seizures. A smaller but significant increase (1.3 fold) in stargazin mRNA was observed in the thalamus of GAERS at the 6 week time point (A). A similar trend was observed at the >13 week time point although it did not reach significance (B). Data is expressed as mean±S.E.M. and has been normalised to Ribosomal 18S RNA mRNA expression levels. *p<0.05, **p<0.01, ***p<0.001.

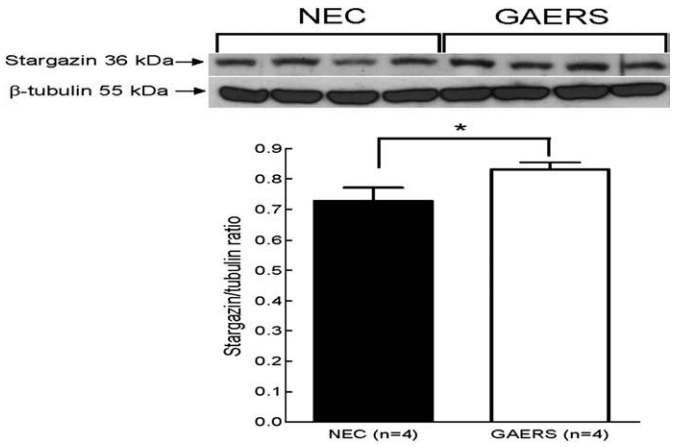

Fig. 2. Western Blot results show a 14% increase in stargazin protein expression in the somatosensory cortex of adult epileptic GAERS (n=4) compared to NEC rats (n=4). Stargazin protein levels were normalized to β-tubulin protein levels. Upper panel: Western Blot; lower panel: Western blot analysis. *p<0.05.

Increased expression of stargazin in GAERS is not caused by splice variation in Cacng2 gene

The stargazin probe showed a thick band at approximately 5.5 kb in each sample. No splice variations were detected in cortex, thalamus or whole brain for either GAERS or NEC rats (Fig. 3). Ensembl genome

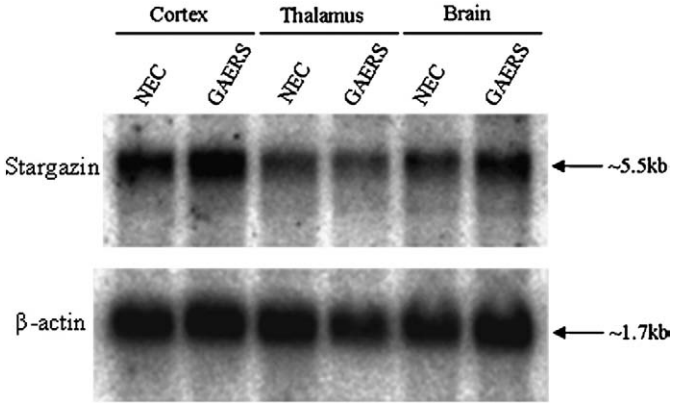

Fig. 3. Northern blot analysis of stargazin. Each lane contains 1.5 μg of mRNA isolated from each region. Samples were probed with α32P labelled PCR product for each gene. β-actin was used as loading standard. Band sizes were determined by RNA ladder (Sigma).

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database suggests *Cacng2* transcript length to be 1.1 kb. However, stargazin northern blot revealed only a single band at 5–6 kb, suggesting the actual transcript to be much longer than noted in the database. No rat stargazin northern blot data has previously been published. Northern blots in human and mouse homologues of stargazin revealed the transcripts to be 6–7 kb in length (Letts et al., 1998; Moss et al., 2003), similar to that found in this study. The *Cacng2* gene in human and mice have long 5ô and 3ô UTR, and based on our results this may also to be true for the rat transcript.

Discussion

Stargazin (γ_2, *Cacng2*) acts to decrease the current activity of HVA Ca^{2+}channels. It was initially thought that a loss of this function was responsible for the absence seizure phenotype in the *stargazer* mouse in which a mutation in the stargazin gene results in markedly *reduced* expression of the stargazin protein (Letts et al., 1998). However, more recent data has demonstrated that stargazin also plays a critical role in AMPA receptor expression and function, being involved in trafficking, anchoring AMPA receptor to post-synaptic membranes by binding them to PSD-95, and modulating their biophysical properties (Priel et al., 2005; Ziff, 2007). Given that AMPA receptors are the primary receptors involved in fast excitatory neurotransmission in the brain, changes in the level of expression or function of stargazin may potentially also contribute to altered excitability in the brain. Here we demonstrate an *increase* in mRNA expression and protein expression of stargazin in an epileptic phenotype.

The study results demonstrated a marked increased (1.8 fold) in mRNA expression for stargazin in the somatosensory cortex of GAERS compared their NEC counterparts, opposite to that found in the *Stargazer* mouse (Letts et al., 1998). It should be noted that these two animal models of absence epilepsy are very different. The *Stargazer* mouse is a monogenic condition with a complex phenotype of absence seizures and ataxia, whereas, the GAERS model is believed to be a polygenic condition with no other neurological deficits (Vergnes and Marescaux, 1992). Human absence epilepsy is also known to be a polygenic condition without other neurological deficits, and thus the GAERS model may more validly model the human condition. The increase stargazin mRNA expression was present both in adult, fully epileptic animals, as well as young animals before the development of spontaneous seizures. This indicates that the increased stargazin expression is not merely a secondary consequence of the seizure activity. Intriguingly the increased expression was significantly more marked in the somatosensory cortex than in the thalamus. This is pertinent given the growing evidence in support of a cortical origin for the seizures in GAERS (Pinault, 2003; Polack et al., 2007) and WAG/Rij rats (Meeren et al., 2002). It is important to note that stargazin expression was also increased in the motor cortex (M1) of pre-epileptic and epileptic GAERS. The motor cortex is not primarily involved in the generation of seizures in these rats (Polack et al., 2007), and therefore this indicates that increase in stargazin expression in the cortex is not restricted to epileptogenic regions.

While the mechanism by which increased stargazin expression may promote absence seizures is unknown, we hypothesize that this may occur via its TARP-related role, enhancing AMPA receptor expression and function and therefore cortical excitability. Increased stargazin would be anticipated to result in more AMPA receptors anchored at the post-synaptic membrane and decreased degradation of these receptors. Additionally, stargazin has recently been shown to allosterically reduce AMPA receptor desensitization in response glutamate exposure, slow their deactivation and accelerate recovery from desensitization (Priel et al., 2005), shifting the equilibrium away from desensitized confirmations to the open channel state (Tomita et al., 2005). Thus the net effect of both the increased AMPA receptor expression and enhanced biophysical responsiveness to glutamate could be a hyperexcitable, epileptogenic somatosensory cortex.

The increased cortical stargazin expression by itself is clearly not sufficient to result in seizures, because it was also present to the same magnitude in the young pre-epileptic rats. Therefore, additional factors are required to allow the expression of the epileptic phenotype. While the nature of these factors is uncertain, one possibility is that there may be a change in the subunit composition of the AMPA receptors that more strongly associate with the stargazin protein, a possibility that requires further exploration. Indeed evidence in the literature has shown that different AMPA receptor channel composition determines their interactions with various trafficking proteins (Zamanillo et al., 1999; Shi et al., 2001).

The explanation for the increased stargazin expression in GAERS is also uncertain. Given that the *stargazin* gene is in the locus identified as being a QLT associated with the expression of seizures in GAERS, it is tempting to speculate that a primary genetic defect in this region may drive the increased stargazin expression (Rudolf et al., 2004). We did not identify any differences in the genetic sequence of the *stargazin* gene in GAERS and NEC rats. However, sequencing was only performed on the 1.1 kb of coding region present in the database and not the 3ô UTR. cDNA or genomic DNA sequences downstream from the coding region were not available on the database. Therefore, it is possible that single nucleotide polymorphisms (SNPs) in the untranslated regions may have been missed and may contribute to the increases in stargazin mRNA that we have found. There is increasing evidence that 3ô-UTRs of mRNAs contain regulatory elements that have important roles in post-transcriptional control of gene expression and mutations or SNPs in these regions can lead to disease (Hesketh, 2004; Russo et al., 2006; Lopez de Silanes et al., 2007).

The increases in stargazin mRNA expression in absence epilepsy-related brain structures reported here warrant further research to understand how this increase may be responsible for an increase in neuronal excitability. For example; does an increase in membrane expression of specific AMPA receptor subunits accompany the increase in stargazin expression? Is the increased stargazin mRNA correctly localized to the plasma membrane? Does the increase in stargazin expression primarily act to enhance absence seizures via an effect on voltage-dependant Ca^{2+}channels or AMPA receptor function, or are both important? Would down-regulation of stargazin function act to decrease the number of seizures in GAERS? Are there SNPs or mutations in the 3ô untranslated region?

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