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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 $\alpha_2$-$\delta$ subunit increases $Ca^{2+}$currents, the $\beta$-subunit modifies activation and inactivation, while the $\gamma$-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 $\gamma$ 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 $\gamma$ 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 $\alpha^{32}$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 |