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Stargazin and AMPA receptor membrane expression is increased in the somatosensory cortex of Genetic Absence Epilepsy Rats from Strasbourg

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ABSTRACT

Absence-like seizures in the Genetic Absence Epilepsy Rats from Strasbourg (GAERS) model are believed to arise in hyperexcitable somatosensory cortical neurons, however the cellular basis of this increased excitability remains unknown. We have previously shown that expression of the Transmembrane AMPA receptor Regulatory Protein (TARP), stargazin, is elevated in the somatosensory cortex of GAERS. TARPs are critical regulators of the trafficking and function of AMPA receptors. Here we examine the developmental expression of stargazin and the impact this may have on AMPA receptor trafficking in the GAERS model. We show that elevated stargazin in GAERS is associated with an increase in AMPA receptor proteins, GluA1 and GluA2 in the somatosensory cortex plasma membrane of adult epileptic GAERS. Elevated stargazin expression is not seen in the epileptic WAG/Rij rat, which is a genetically distinct but phenotypically similar rat model also manifesting absence seizures, indicating that the changes seen in GAERS are unlikely to be a secondary consequence of the seizures. In juvenile (6 week old) GAERS, at the age when seizures are just starting to be expressed, there is elevated stargazin mRNA, but not protein expression for stargazin or the AMPA receptor subunits. In neonatal (7 day old) pre-epileptic GAERS there was no alteration in stargazin mRNA expression in any brain region examined. These data demonstrate that stargazin and AMPA receptor membrane targeting is altered in GAERS, potentially contributing to hyperexcitability in somatosensory cortex, with a developmental time course that would suggest a pathophysiological role in the epilepsy phenotype.

Introduction

The majority of excitatory fast synaptic neurotransmission in the mammalian central nervous system is mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which are heterotetramers consisting of different combinations of GluA1–4 subunits (Dingledine et al., 1999; Hollmann and Heinemann, 1994). Changes in the functional properties of AMPA receptors, and the extent of their synaptic recruitment, play a critical role in various forms of activity-dependent synaptic plasticity such as long term potentiation, long term depression and models of learning and memory (Derkach et al., 2007; Malenka and Bear, 2004; Malinow and Malenka, 2002; Shepherd and Huganir, 2007). Stargazin is a member of a family of proteins known as Transmembrane AMPA receptor Regulatory Proteins (TARPs) which act as AMPA receptor auxiliary proteins modulating AMPA receptor trafficking, deactivation and desensitization kinetics and pharmacology (Bats et al., 2007; Bedoukian et al., 2006; Cho et al., 2007; Kott et al., 2007; Milstein et al., 2007; Priel et al., 2005; Schnell et al., 2002; Tomita et al., 2003; Tomita et al., 2005).

The generalised epilepsies are a common group of diseases that are believed to be largely hereditary, but with more than one gene contributing to the epileptic phenotype. Absence seizures are a common type of seizure in patients with generalised epilepsies, characterised behaviourally by staring, loss of facial expression and unresponsiveness, and electrographically by generalised spike-and-wave discharges (SWDs). The Genetic Absence Epilepsy Rats from Strasbourg (GAERS) are a well validated genetic rat model of absence epilepsy that exhibit spontaneous SWDs on a normal electroencephalogram (EEG) background, closely resembling the human condition (Marescaux et al., 1984). Spontaneous seizures begin to manifest in GAERS from around 6 to 7 weeks of age and by 3 months all GAERS are having seizures (Danober et al., 1998). The thalamocortical network is critically involved with SWD propagation in human absence epilepsy and many animal models, with SWDs demonstrated in both GAERS

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and another model of absence epilepsy, the Wistar Albino Glaxo from Rijswijk (WAG/Rij) rat, to be initiated in the somatosensory cortex (Meeren et al., 2002; Polack et al., 2007). We have previously shown that stargazin mRNA and protein expression is increased in somatosensory cortex and thalamus of GAERS compared to non-epileptic control (NEC) rats (Powell et al., 2008). A key role of TARPs, including stargazin, is to regulate AMPA receptor trafficking and function (Nicoll et al., 2006; Priel et al., 2005; Tomita et al., 2003; Turetsky et al., 2005; Yamazaki et al., 2004). AMPA receptors localize at synapses by forming protein complexes with transmembrane AMPA receptor regulatory proteins (TARPs) and PSD-95-like membrane-associated guanylate kinases. Therefore, we hypothesised that AMPA receptor membrane expression will be increased in the somatosensory cortex of GAERS. Supporting this idea is the finding that there is an increased firing frequency in the deep layers of the somatosensory cortex in these animals, suggesting that these neurons are hyperexcitable (Polack et al., 2007). In this study we examined the mRNA and protein expression of stargazin and AMPA receptors in somatosensory cortex tissue in GAERS and age-matched NEC rats at different developmental ages. Subcellular fractionation was performed to allow for discrimination between protein expression in plasma membrane and cytosolic compartments. Stargazin expression was also examined in a second, distantly related, GAERS colony and in WAG/Rij rats.

Methods

Animals and brain tissue extraction

The experimental procedures on GAERS and NEC rats were approved by The Royal Melbourne Hospital Animal Ethics Committee (AEC #2004.019). Two distinct colonies of GAERS rats were used for this study: the first were the colony used in our previous publication (Powell et al., 2008) which were sourced from Hull, UK in 2002, having been originally sourced from the Strasbourg colony several years prior to this, and have since been bred in our facility in Melbourne; and the second colony was sourced directly from Strasbourg in 2008 and bred in our facility separate to the first colony. These two colonies are therefore separated by >20 generations, and therefore represent a way to investigate whether molecular changes observed are true to the GAERS model, or were colony specific. Both colonies are homozygous for the $Ca_v3.2$(R1584P) mutation in the low threshold calcium channel *Cacna1h* gene that our group has recently reported (Powell et al., 2009). To assess whether the increases in stargazin expression were specific for GAERS, or a more ubiquitous change associated with absence-like seizures in rat strains, this was also examined in the somatosensory cortex of another polygenic rat model of absence epilepsy, WAG/Rij rats (Coenen and Van Luijtelaar, 2003; Meeren et al., 2002), and the Wistar control strain (30–31 weeks old), from colonies bred in the John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia, a generous gift from Professor Greg Stuart and Dr Maarten Kole.

Rats were culled by a lethal dose of anaesthetic (150 mg/kg ketamine and 20 mg/kg xylazine) at one of three ages: neonatal (7 days old, pre-epileptic), juvenile (6 weeks old—early epileptic), and adult (>13 weeks—when the epilepsy is fully expressed). The animals' brains were rapidly extracted and the somatosensory cortex, motor cortex and thalamus were dissected, snap-frozen over liquid nitrogen and stored at −80 °C.

Quantitative polymerase chain reaction (qPCR) for stargazin mRNA expression

RNA was extracted from somatosensory cortical tissue using RNeasy mini kit (QIAGEN), 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. Five hundred nanograms 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).

Tissue fractionation for Western blotting

Somatosensory cortex tissue was fractionated into cytosol and membrane fractions using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) following the manufacturer's protocol. Protein concentrations were determined using the BCA protein assay reagent (Pierce).

Western blotting for stargazin and AMPA receptor protein expression

Immunoblotting was performed using 50 μg of membrane or cytosolic lysate fractions. Samples were denatured at 95 °C for 5 min and resolved by SDS-polyacrylamide electrophoresis. Proteins were transferred onto Hybond C Super membrane (Amersham) for Western blotting. Membranes were blocked in 3% skim milk dissolved in Tris-buffered-saline pH 7.5/0.1% Tween-20 (TBS-T, Sigma) overnight at 4 °C. Membranes were probed with one of the following primary antibodies: stargazin (1:1000, Santa Cruz sc-18284, 36 kDa), GluA1 (1:3000, Santa Cruz sc-13152, 106 kDa), GluA2 (1:3000, Santa Cruz sc-7611, 100 kDa), PSD-95 (1:1000, BD Biosciences 610496, 95 kDa), GluN1 (NMDA receptor subunit 1, 1:1000, Invitrogen 32-0500, 103 kDa) at 4 °C overnight or β-tubulin (loading control, 1:20,000 Sigma T4026, 55 kDa) at room temperature for 1 h followed by incubation with the appropriate conjugated-HRP secondary antibody (1:10,000; DAKO) for 45 min at room temperature. Proteins were visualised using the enhanced chemiluminescence (ECL) reagent (Perkin Elmer) and band optical density (OD) analysis was performed using Quantity One software (BioRad).

Data analysis

Relative expression of stargazin, GluA1 and GluA2 was determined using β-tubulin as a loading control by calculating the ratio of ODs for each protein of interest versus β-tubulin OD in each individual subject. The mean of all NEC values on individual gels was calculated and each NEC and GAERS value was expressed relative to that mean, to account for inter-gel variability. All data are expressed as mean ± standard error of the mean for these ratios. Statistical significance was determined using the non-parametric Mann–Whitney *U* test (two-tailed unless stated) with significance level set at $p<0.05$.

Results

Stargazin mRNA expression is increased in the somatosensory cortex in adult epileptic GAERS from two different colonies, but not in WAG/Rij rats

We have previously demonstrated that stargazin mRNA is increased in the somatosensory cortex from adult epileptic (>13 weeks) GAERS from a colony that had been bred in our institution since 2002 (Powell et al., 2008). Here, we have replicated these findings in adult epileptic GAERS from a second colony that was sourced directly from the Strasbourg colony in 2008, and is separated by >20 generations from our