SELECTIVE LOSS OF AMPA RECEPTORS AT CORTICOTHALAMIC SYNAPSES IN THE EPILEPTIC STARGAZER MOUSE

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Abstract—Absence seizures are common in the stargazer mutant mouse. The mutation underlying the epileptic phenotype in stargazers is a defect in the gene encoding the normal expression of the protein stargazin. Stargazin is involved in the membrane trafficking and synaptic targeting of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) at excitatory glutamatergic synapses. Thus, the genetic defect in the stargazer results in a loss of AMPARs and consequently, excitation at glutamatergic synapses. Absence seizures are known to arise in thalamocortical networks. In the present study we show for the first time, using Western blot analysis and quantitative immunogold cytochemistry, that in the epileptic stargazer mouse, there is a global loss of AMPAR protein in nucleus reticularis (RTN) and a selective loss of AMPARs at corticothalamic synapses in inhibitory neurons of the RTN thalamus. In contrast, there is no significant loss of AMPARs at corticothalamic synapses in excitatory relay neurons in the thalamic ventral posterior (VP) region. The findings of this study thus provide cellular and molecular evidence for a selective regional loss of synaptic AMPAR within the RTN that could account for the loss of function at these inhibitory neuron synapses, which has previously been reported from electrophysiological studies. The specific loss of AMPARs at RTN but not relay synapses in the thalamus of the stargazer, could contribute to the absence epilepsy phenotype by altering thalamocortical network oscillations. This is supported by recent evidence that loss of glutamate receptor subunit 4 (GluA4) (the predominant AMPAR-subtype in the thalamus), also leads to a specific reduction in strength in the cortico-RTN pathway and enhanced thalamocortical oscillations, in the Gria4$^{-/-}$ model of absence epilepsy. Thus further study of thalamic changes in these models could be important for future development of drugs targeted to absence epilepsy. © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: AMPA receptors, stargazin, thalamus, absence epilepsy, Western blot, immunogold.

INTRODUCTION

Absence epilepsy is classified as one of the genetic generalized forms (GGE) of epilepsy (Berg et al., 2010) according to the Commission on Classification and Terminology of the International League Against Epilepsy (ILAE), and is responsible for about 10% of epileptic seizures in humans, predominantly affecting children. Absence seizures are characterized by sudden, brief losses of consciousness accompanied by spike-wave discharges (SWD) at approximately 2–4 Hz, usually lasting just a few seconds on electroencephalogram (EEG) recordings.

Several rodent models have proved invaluable in studying the cellular and molecular mechanisms underlying absence epilepsy. Of the mouse models with this form of spontaneous generalized epilepsy, many have mutations associated with voltage-dependent calcium channel (VDCC) subunits (Noebels et al., 1990). The stargazer mouse, in particular, has been the focus of studies to understand how mutations in VDCC regulatory subunits contribute to absence seizures. Absence seizures, in the stargazer (Letts, 2005), seem to originate in thalamic networks consisting of excitatory thalamocortical relay neurons and inhibitory nucleus reticularis (RTN) neurons. The mutation underlying the epileptic phenotype in stargazers is a defect in the VDCC gamma subunit gene γ2 (Letts et al., 1998). This severely reduces the normal expression of the γ2 subunit protein called stargazin. Stargazin is involved in the membrane trafficking and synaptic targeting of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors at excitatory glutamatergic synapses (Hashimoto et al., 1999; Chen et al., 2000; Tomita et al., 2003, 2004, 2005; Nicoll et al., 2006) and hence is referred to as a transmembrane AMPA receptor regulatory protein (TARP). The genetic defect in the stargazer results in a loss of AMPARs at synapses (Chen et al., 2000) and thus leads to a *loss* of excitation at excitatory synapses.

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However, one of the central concepts in epilepsy is that seizures result from *excessive* excitation in neuronal networks. Absence seizures are thought to arise from abnormal hypersynchronous activity in the thalamocortical–corticothalamic circuitry (Snead, 1995; Huntsman et al., 1999; McCormick, 1999; McCormick and Contreras, 2001; Crunelli and Leresche, 2002; Beyer et al., 2008). So how does a loss of AMPA receptors at excitatory glutamatergic synapses in the stargazer contribute to the absence epileptic phenotype? A recent study by Menuz and Nicoll (2008), demonstrated that inhibitory thalamic RTN neurons, but not excitatory relay neurons have strongly reduced excitatory postsynaptic currents (mEPSCs) in stargazer mice. This suggests that there could be a selective loss of AMPARs at RTN synapses, which would cause decreased excitability of the inhibitory neurons in RTN and thus lead to disinhibition within the thalamocortical network, which is associated with absence-like seizures. However, to date there have been no quantitative studies to determine if reduced AMPAR function at RTN synapses in stargazers is caused by a selective loss of AMPARs at RTN neurons.

The aim of the current study was: first to quantify AMPAR subunit levels in the RTN and ventral posterior (VP) relay regions of the thalamus using Western blot analysis; and second to determine the relative density of AMPAR subunits at synapses on inhibitory and excitatory neurons in these regions of the thalamus in epileptic stargazers compared to non-epileptic littermates using quantitative immunogold cytochemistry.

EXPERIMENTAL PROCEDURES

Animals

Stargazer mice were raised from breeding stock (Heterozygous for $Cacng2st^g$ × Heterozygous for $Cacng2st^g$) obtained from Jackson Laboratory (Bar Harbor, ME, USA). For Western blot experiments, heterozygous males (+/stg) were mated with either homozygous (stg/stg) or heterozygous (+/stg) females to produce epileptic stg/stg mutants and non-epileptic control littermates that were a mixture of +/stg and wild type (+/+). As there are no reported differences between +/+ and +/*stg* mice (Qiao et al., 1998; Hashimoto et al., 1999), published studies routinely combine +/+ and +/*stg* materials for control experiments (Payne et al., 2006, 2007). For ultrastructural analyses in the present study, we used only epileptic stg/stg mutants and non-epileptic +/stg littermate controls from crosses between heterozygous males (+/stg) and homozygous females (stg/stg), to confirm that any loss of AMPAR subunit expression at thalamic synapses was associated with the epileptic phenotype. In all experiments, only male littermate siblings aged 8–12 weeks old were used. Animals were maintained on a 12 h light/dark cycle, with access to water and food *ad libitum*. The University of Otago Animal Welfare and Ethics Committee approved all animal procedures.

Primary antibodies

The AMPA receptor subunits selected for analyses were glutamate receptor subunit 4 (GluA4), as this is the predominant subunit in RTN region, and GluA2/3 which is expressed in equal proportions to GluA4 in the VP thalamus (Mineff and Weinberg, 2000). Although GluA1 is virtually absent from normal thalamus, tissue levels were also analysed for this subunit to check for any

compensatory changes in the epileptic thalamus. Antibodies against GluA4, GluA2/3 and GluA1 subunits were rabbit polyclonals purchased from Millipore (Billerica, MA, USA; AB1508, AB1506 and AB1504, respectively). The immunogens in all cases were synthetic peptides; sequences were derived from the C terminus of rat GluA4 and GluA2/3 and human GluA1. Preadsorption controls were carried out using the respective control peptides GluA4 (AG306), GluA2/3 (AG305) and GluA1 (AG360). The antigens were used to block antibody binding to confirm antibody specificity. The β-actin-specific antibody, used as the loading control for Western blot, was a polyclonal rabbit antibody obtained from Abcam (ab8227, Cambridge, UK). It was raised against a 375 amino acid long peptide corresponding to residues 1–100 of human β-actin that is highly conserved in vertebrates. Antibody dilutions for both Western blot and EM immunogold cytochemical applications were optimized in initial experiments, using the manufacturer's recommendations as guidelines.

Brain tissue extraction and sample preparation for Western blot

Animals were decapitated, the brains rapidly extracted and snap frozen on dry ice. Frozen whole brains were stored at −80 °C until further processing. To collect samples, brains were frozen onto a specimen holder with Tissue-Tek (Sakura, Finetek, USA) embedding compound. Coronal sections of 300 μm thickness were cut in a cryostat at a constant temperature of −10 °C; and the sections were collected and thaw-mounted on glass microscope slides. The slides were then placed on a chilled block under dissecting microscope. The RTN and the VP regions of the thalamus were identified on sections corresponding to plates 42–44 in the Franklin and Paxinos (2001) mouse brain atlas. Tissue samples were obtained by micropunching from both nuclei. The technique was first described in the rodent brain by Palkovits and Brownstein (1983) and has been routinely used since then (Polli and Kincaid, 1992; LeSauter et al., 1996; Meyer-Luehmann et al., 2003; Kerns et al., 2005; Sava et al., 2006; Karuppagounder et al., 2007; Blouet et al., 2009; Schwindinger et al., 2009; Tung et al., 2010). 24 G blunted needle was used to punch RTN and VP. Punch samples were blown into microtubes containing homogenization buffer (0.5 M Tris, 100 mM EDTA, 4% SDS, pH 6.8, supplemented with PMSF and protease inhibitor cocktail (Sigma-Aldrich Co., Saint Louis, MO, USA; P8340)). Punches from two age-matched animals were pooled together in order to have enough protein for subsequent Western blot experiments. Samples were homogenized by gentle freeze-thawing and sonication and subsequently centrifuged for 5 min at 14,000 rpm. The supernatants were transferred into clean microtubes and stored at −80 °C. The protein concentration of the samples was measured using detergent-compatible protein assay (DC Protein assay, 500-0116, Bio-Rad, Hercules, CA, USA), following the provider's instructions.

Western blotting for GluA4 and GluA2/3

Protein extracts were used to study the expression of each of the AMPA receptor subunits in RTN and VP. The accuracy of micropunching was assessed by probing for GluA4 as this subunit is enriched in RTN compared to other surrounding regions (Mineff and Weinberg, 2000). For each sample, 20 μg of protein was loaded on 8.5% SDS–PAGE gel. Proteins were separated in XCell SureLock Mini-Cell system (Invitrogen) and transferred onto PVDF membranes (Roche, Auckland, NZ) using XCell Blot Module (Invitrogen, Victoria, Australia). Membranes were blocked with 5% BSA in Tris-buffered saline pH 7.6, 0.1% Tween at room temperature for 30 min, followed by incubation with the primary antibodies, anti-GluA4 (AB1508, Millipore, 1:1000) or anti-GluA2/3 (AB1506, Millipore, 1:1000) or anti-GluA1 (AB1504, Millipore, 1:500) and β-actin (ab8227,