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COMPENSATORY NETWORK ALTERATIONS UPON ONSET OF EPILEPSY IN SYNAPSIN TRIPLE KNOCK-OUT MICE

M. KETZEF,[a,b,c] J. KAHN,[a] I. WEISSBERG,[a,b,c] A. J. BECKER,[d] A. FRIEDMAN[a,b,c,e] AND D. GITLER[a,c]*

[a]*Department of Physiology and Neurobiology, Ben-Gurion University of the Negev, Beer-Sheva, Israel*

[b]*Department of Neurosurgery, Ben-Gurion University of the Negev, Beer-Sheva, Israel*

[c]*Zlotowski Center for Neuroscience, Ben-Gurion University of the Negev, Beer-Sheva, Israel*

[d]*Department of Neuropathology, University of Bonn Medical Center, Sigmund-Freud Street 25, D-53105 Bonn, Germany*

[e]*Department of Biomedical Engineering, Ben-Gurion University of the Negev, Beer-Sheva, Israel*

Abstract—Adult synapsin triple-knockout mice exhibit epilepsy that manifests as generalized tonic-clonic seizures. Because *in vitro* recordings have shown a reduction in quantal release from inhibitory neurons, an inherent excitation-inhibition imbalance has been hypothesized as the direct culprit for epilepsy in these mice. We critically assessed this hypothesis by examining neurotransmission during the emergence of epilepsy. Using long-term video and telemetric EEG monitoring we found that synapsin triple-knockout mice exhibit an abrupt transition during early adulthood from a seizure-free presymptomatic latent state to a consistent symptomatic state of sensory-induced seizures. Electrophysiological recordings showed that during the latent period larger field responses could be elicited in slices from mutant mice. However, only after the transition to a symptomatic state in the adult mice did evoked epileptiform activity become prevalent. This state was characterized by resistance to the epileptiform-promoting effects of 4-aminopyridine, by marked hypersensitivity to blockage of $GABA_A$ receptors, and by the emergence of unresponsiveness to NMDA receptor antagonism, all of which were not observed during the latent period. Importantly, enhancement in inhibitory transmission was associated with upregulation of GAD67 expression without affecting the number of inhibitory neurons in the same brain areas where epileptiform activity was recorded. We therefore suggest that while deletion of the synapsins initially increases cortical network activity, this enhanced excitability is insufficient to elicit seizures. Rather, compensatory epileptogenic mechanisms are activated during the latent period that lead to an additional almost-balanced enhancement of both the excitatory and inhibitory components of the network, finally culminating in the emergence of epilepsy.

Key words: epileptogenesis, GABAergic, genetic epilepsy, synapsin, extracellular electrophysiology, compensatory modifications.

When studying epileptogenesis, or how epilepsy is initiated, several caveats arise, the most problematic of which is the fact that epilepsy is a self-reinforcing condition (Sutula, 2004). This means that once seizures commence, it is not practical to discern whether a pathological state is a cause rather than a result of epilepsy. To get a handle on this problem, it is essential to measure brain activity close to the onset of epilepsy, but before the first seizure has occurred. Another common problem for studying epileptogenesis is that in many acute experimental models of epilepsy, for example, those induced by pharmacological agents, there might be significant variability in the onset of epilepsy or in its incidence (Hellier et al., 1998; Glien et al., 2001; Sharma et al., 2007; Curia et al., 2008). This issue is critical when performing *in vitro* electrophysiological studies before the onset of symptoms, because the eventual fate of the neural tissue remains uncertain. Finally, tissue damage in many models is observed, but its role in epileptogenesis is not clear (Curia et al., 2008).

The synapsins are abundant phosphoproteins associated with the surface of synaptic vesicles. They are postulated to bind synaptic vesicles to the reserve pool of vesicles, allowing their mobilization in an activity-dependent manner (Li et al., 1995; Hilfiker et al., 1999). Although it is clear that the synapsins control certain forms of short-term plasticity (Rosahl et al., 1995; Gitler et al., 2004a), the whole range of their functions has not been fully revealed (Evergren et al., 2007; Cesca et al., 2010). Deletion of the synapsins has been shown to differentially affect excitatory and inhibitory neurotransmission, and specifically to tone down basal inhibitory responses (Gitler et al., 2004a; Baldelli et al., 2007). This deficit in inhibitory transmission has been credited with the epileptic nature of synapsin knockout mice (Rosahl et al., 1995; Chiappalone et al., 2008; Boido et al., 2010). Other evidence points to the involvement of synapsins in epilepsy, like the fact that both kindling and kainate injections, two experimental manipu-

*Correspondence to: D. Gitler, Department of Physiology and Neurobiology, Ben-Gurion University, Beer-Sheva 84105, Israel. Tel: +972-8-6477345; fax: +972-8-6477628.
E-mail address: gitler@bgu.ac.il (D. Gitler).

Abbreviations: ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; APV, 2-amino-5-phosphovaleric acid; BMI, bicuculline methoidide; BSA, bovine serum albumin; CNQX, 6-cyano-7-nitroquinoxyline-2, 3-dione; DDW, double distilled water; DEPC, diethyl pyrocarbonate; EEG, electroencephalogram; GABA, gamma amino butyric acid; $GABA_A$, GABA (receptor) type A; GAD65, glutamic acid decarboxylase of 65 kd; GAD67, glutamic acid decarboxylase of 67 kd; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GluR1, AMPA receptor subunit 1; HRP, horseradish peroxidase; KO, knock out; NEM, N-ethylmaleimide; NMDA, N-methyl-D-aspartate; NR2A, NMDA receptor subunit 2A; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RT-PCR, real time/reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; TBST, Tris–buffered-saline-tween-20; TKO, triple knock out; WT, wild type; 4-AP, 4-aminopyridine.

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lations that induce seizures in mice, upregulate the expression of synapsin I (Morimoto et al., 1998; Sato and Abe, 2001; Fournier et al., 2009). Indeed, the synapsin genes *SYN1* and *SYN2* have been found to be of clinical interest based on their linkage to epilepsy susceptibility in humans (Garcia et al., 2004; Cavalleri et al., 2007; Lakhan et al., 2010). Finally, heightening of network activity in slices by 4-aminopyridine, a potassium channel blocker, has revealed a higher frequency of ictal events in synapsin triple knock out (TKO) slices (Boido et al., 2010), confirming their hyperexcitability.

Here we propose the synapsin knockout mice as a valuable model to facilitate the study of epileptogenesis. Although it has been noted that the onset of epilepsy is delayed in several lines of synapsin knock out (KO) mice (Rosahl et al., 1995; Gitler et al., 2004a; Boido et al., 2010), the timing or progression of the epileptic condition has not been determined in detail. Therefore, assisted by telemetric electroencephalogram (EEG) recordings, we first precisely established the timeline for the development of epilepsy in the synapsin TKO mice. Based on the delay in seizure onset we hypothesized that epileptogenesis in the synapsin TKO mice involves compensatory developmental network changes. To examine this hypothesis, we examined the changes in intrinsic excitability of slices by gauging field responses to electrical stimulation in the absence of pharmacological agents, focusing on changes occurring close to symptom onset *in vivo*. Next, pharmacological intervention served to examine the contribution of the main excitatory and inhibitory components of neurotransmission. We present evidence that presymptomatic mice exhibit an increased excitatory tone, followed by a further increase in both excitatory and inhibitory transmission upon the onset of seizures.

EXPERIMENTAL PROCEDURES

Animals

The synapsin TKO mice have been described previously (Gitler et al., 2004b), their genetic background is C57Bl6/SV129. Homozygous *SYN* 1/2/3 (+/+) mice (WT), that are the progeny of litter mates of the original mutant mice, served as controls. The mice were raised in a temperature and humidity controled environment, under a 12/12 h light/dark cycle and were fed *ad libitum*. Pups were separated at the age of 21–28 days. Experiments were conducted in accordance to protocols IL-55-11-2007 and IL-32-03-2009 approved by the Ben-Gurion University Institutional Committee for Ethical Care and Use of Animals in Research, in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Telemetric EEG recording and video monitoring

Implantation and recordings were performed as described (Pitsch et al., 2007). Briefly, 6–7-week-old WT and TKO mice were anesthetized by isoflurane (0.8–1%, Minrad, Bethlehem, PA, USA) inhalation for the duration of the operation. The transmitter (TA10EA-F20, Data Science International, St. Paul, MN, USA) was implanted s.c. on the animals' back. Reference and lead electrodes were placed above the dura through holes drilled in the skull and affixed with bone-cement (Unifast Trad, GC America, Alsip, IL, USA). Electrodes were located 1.5 mm posterior and 1.5 mm lateral to bregma. Antibiotic treatment (5 mg/kg, p.o. enrofloxacin, Bayer, Germany) was given during recovery (7 days). EEG recordings (at 0.5 or 1 kHz) and video monitoring were performed from 7 days post operation till the age of 4 months for WT mice and until a month after seizures could be provoked by cage opening in the TKO mice. Recordings were performed also from adult WT and TKO mice (5–7 months old) for a period of 1–2 months each. Power spectra were calculated using Matlab 7.5 (The Mathworks Inc. Natick, MA, USA)

Histology and immunochemistry

Standard histological procedures were used (Schick et al., 2006; Ivens et al., 2007; Pitsch et al., 2007). Briefly, mice were anesthetized with isoflurane and decapitated. Brains were quickly removed and fixed in 4% paraformaldehyde (PFA) at 4 °C for 48 h, followed by paraffin embedding and sectioning (5–10 μm thick; Microm, Waldorff, Germany). Paraffin sections were cleared with xylene, rehydrated in graded alcohols, and washed in Tris–buffer. Alternate sections were stained with antisera for NeuN (1:200, Millipore, Billeria MA, USA) or glutamic acid decarboxylase of 67 kd (GAD67) (1:400, NeoMarkers, Fremont, CA, USA). NeuN slides were incubated with AB complex (1:200, Vector Laboratories, USA) in phosphate-buffered saline (PBS) including 10% fetal calf serum for 2 h at 37 °C and stained by diaminobenzidine application (1:50 in 0.05 M Tris/HCl pH 7.4 and 1:2000 H_2O_2) followed by Hematoxylin counterstain. GAD67 slides were labeled with NorthernLights-637 secondary antibody (1:400, R&D Systems, Minneapolis, MN, USA).

Imaging, microscopy, and cell counting

Brain slices were imaged using a TiE inverted microscope (Nikon, Japan) equipped with a cooled 14 bit CCD camera (Coolsnap HQ2, Photometrics, Tucson, AZ, USA), using the NIS-elements software (Nikon). For fluorescence images, the Cy5 Brightline filter set (Semrock, Rochester, NY, USA) was used. Neuronal counts were performed on coronal NeuN-stained sections between −1.58 and −2.46 mm posterior to bregma in the temporal association and in the auditory and ectorhinal cortices (Paxinos and Franklin, 2001) while inhibitory interneurons were identified in GAD67 stained sections from the same animals (n=4–5 images from three animals per each genotype). Comparisons were performed using the Student's t-test for independent samples, after confirming their normal distribution (Shapiro-Wilk test, $P<0.05$).

Electrophysiological recordings *in vitro*

The acute slice preparation was used (Pavlovsky et al., 2003; Seiffert et al., 2004; Ivens et al., 2007). Briefly, mice were deeply anesthetized with isoflurane and decapitated. Brains were quickly removed, and 400 μm thick horizontal corticohippocampal slices corresponding to plates 143–157 (Paxinos and Franklin, 2001) were prepared (Dreier and Heinemann, 1991) using a vibroslice (WPI, Berlin, Germany) and incubated at room temperature for at least 1 h in carbogen-bubbled (5% CO_2 and 95% O_2) artificial cerebral spinal fluid (ACSF; in mM: 124 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 3 KCl, and 10 glucose, pH=7.4±0.1). Slices were then placed in a humidified standard interface chamber at 36±1 °C under continuous ACSF perfusion (0.5–0.75 ml/min). Field potentials were recorded using extracellular glass microelectrodes (1.3–1.5 MΩ, Science Products, Germany) containing (in mM): 140 NaCl, 3 KCl, 2 $MgCl_2$, 10 glucose, 2 $CaCl_2$, and 10 HEPES, pH 7.4. Slices were stimulated with 0.1 ms pulses using bipolar concentric tungsten electrodes (WPI, Sarasota, FL, USA). Stimulation frequency was 0.1 Hz in the input-output experiments and 1/min during drug perfusion. When recording from layer 4 of the occipital and temporal cortices, stimulation was at the white/gray matter border. When recording