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Short Communication

Severity of Kainic Acid-Induced Seizures is not Aggravated in the Maternal Immune Activation Mouse Model of Gestational Poly (I:C) Exposure

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Abstract

Epilepsy is a common complication of autism spectrum disorders (ASDs). Clinical studies have estimated that the rate of epilepsy in ASD patients is approximately 30%. To examine the cellular and molecular links between ASD and epilepsy, proper animal models are necessary. Here, we investigated whether seizure severity is increased in the poly (I:C) model, a mouse model of maternal immune activation (MIA). MIA is a risk factor for ASD in offspring, and ASD-like features, including deficits in social interactions, have been observed in the mouse poly (I:C) model. The poly (I:C) mice were administered kainic acid (KA) at postnatal day 15 (P15) and P30 to induce limbic seizures. We found that there was no difference in seizure severity between poly (I:C) and control mice at P15 and 30. Further, immunohistochemical analysis at P15 revealed that the density of excitatory and inhibitory synapses was largely unchanged in the hippocampus of poly (I:C) mice, except for an increase in inhibitory synapses in CA1. Thus, our results indicate that KA-induced seizure severity is not increased in poly (I:C) mice and that the structural synapse E/I balance in the hippocampus is not largely impaired.

ABBREVIATIONS

ASD: Autism Spectrum Disorder; MIA: Maternal Immune Activation; Poly (I:C): Polyinosine:Cytosine; KA: Kainic Acid; P: Postnatal Day; E/I: Excitatory/Inhibitory; EEG: Electroencephalogram; SR: Stratum Radiatum; SL: Stratum Lucidum; ML: Molecular Layer

INTRODUCTION

The incidence of epilepsy in the individuals with autism spectrum disorder (ASD) is estimated to be approximately 30% [1]. The prevalence of epilepsy in ASD increases depending on the severity of ASD symptoms [2] and is higher in older children than younger children [1]. In patients with ASD and epilepsy, common mutations in genes such as *Fmr1*, *MeCP2*, *Shank3* and *SCN2A* have been reported [3]. Importantly, many of the genetic mutations shared by ASD and epilepsy are synapse-related genes. Therefore, it has been suggested that the disruption of excitatory and inhibitory balance (E/I balance) of synaptic transmission underlies the co-occurrence of ASD and epilepsy. E/I balance is regulated by proteins related to synapse anchoring [4] and synaptic vesicle release [5]. Indeed, an E/I imbalance induces both epileptic and ASD phenotypes in mice with mutations in *Fmr1* or *MeCP2*, which are necessary for proper synaptic structures and transmission [3].

In the present study, we examined whether seizure severity is increased in the maternal immune activation (MIA) mouse model of gestational exposure to synthetic double-stranded RNA polyinosine: cytosine (poly (I:C)), which is often used as an environmental trigger-induced ASD model. To prepare the poly (I:C) mouse model, pregnant mice were subjected to an antiviral-like immune challenge by the injection of poly (I:C). MIA is an environmental factor for ASD in offspring, and ASD-like features, including deficits in social interaction, have been observed in the mouse poly (I:C) model [6]. The induction of IL-6 and IL-1β triggered by MIA is suggested to increase propensity to epilepsy in offspring [7]. Here, we investigated whether the severity of kainic acid (KA)-induced limbic seizures is increased in a mouse poly (I:C) model at postnatal day 15 (P15) and P30. We additionally

conducted immunohistochemical analysis to examine the E/I balance of synapse structures in the hippocampus.

MATERIALS AND METHODS

Animals and husbandry

C57BL/6J mice (SLC, Shizuoka, Japan) were used. Animal experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo (approval number: 24-70) and according to the University of Tokyo's guidelines for the care and use of laboratory animals. C57BL/6J male mice (SLC, Shizuoka, Japan) were housed in cages under standard laboratory conditions (a 12-h light/dark cycle, free access to food and water). All efforts were made to minimize the animals' suffering and the number of animals used.

Poly (I:C) preparation and gestational exposure

A maternal immune activation (MIA) model in mice was established according to Naviaux et al., 2013 [6]. Briefly, pregnant dams received intraperitoneal (i.p.) injections of poly (I:C) (Potassium salt; Sigma-Aldrich, St. Louis, MO, USA, Cat#P9582) of two doses (0.25 U/g [3 mg/kg] on E12.5 and 0.125 U/g [1.5 mg/kg] on E17.5).

Seizure induction

For kainic acid (KA)-induced status epileptics experiments, control and poly (I:C) mice were administered kainic acid (Sigma, 5mg/kg s.c. at P15 or 25mg/kg i.p. at P30). We decided the dose of KA based on the previous research [8]. In the previous study KA was administrated intra peritoneally, but we administered KA subcutaneously to mice at P15 to avoid severely injuring mouse pups by inserting a needle to the stomach. Animals were observed and classified according to the following scale: (0) normal behavior; (1) immobility; (2) repetitive scratching, gustatory movement; (3) rigid posture, pivoting, limb clonus; (4) rearing, falling; (5) clonic seizure, jumping, running; (6) tonic-clonic seizure, jumping (interval); and (7) death.

Sample preparation and immunohistochemistry

Experimental mice at P15 were anesthetized with isoflurane and perfused transcardially with cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Coronal hippocampal sections (40 μm thick) were prepared with a cryostat (Leica) at -25°C. The primary antibodies used for immunohistochemistry were as follows: mouse anti-VGlut1 (1:1000; Synaptic Systems), rabbit anti-Homer1 (1:500; Synaptic Systems), mouse anti-VGAT (1:1000; Synaptic Systems), and rabbit anti-Gephyrin (1:1000; Synaptic Systems). The secondary antibodies conjugated with Alexa fluor dyes (1:500; Invitrogen, Gaithersburg, MD, USA) were used.

Quantification of synapses

The samples were analyzed with a FV1200 (Olympus) confocal system under 10× and 60× magnifications. The stacked images were prepared using ImageJ. Z-series images were collected at 0.33-μm steps, and 4 Z-sections (1 μm thick) were stacked using Image. Densities were analyzed from 8 fields each for molecular layers and CA1, and 4 fields for CA3 per mouse.

Statistical analyses

The differences in the rates between the groups were analyzed by Fisher's exact test. The latency to seizure was analyzed by Log-rank test. The density of synapses was analyzed by Mann-Whitney U test.

RESULTS AND DISCUSSION

Clinical studies have reported that some ASD patients start exhibiting seizures as early as 2 years old [1]. Thus, we first tested whether poly (I:C) mice at P15 have a lowered convulsant threshold by administering kainic acid (KA; 5mg/kg subcutaneously) and compared the severity of seizures between control and poly (I:C) mice. We found no significant differences between control and poly (I:C) mice in KA-induced seizure severity for 90 minutes (Figure 1A, see Methods for the scoring of seizure stages), in the development of tonic-clonic seizures (Figure 1B) or in death following tonic-clonic seizures (Figure 1B). In addition, the Survival curve of the latency to tonic-clonic seizures did not show differences between control and poly (I:C) mice (Figure 1C).

The incidence of epilepsy in ASD patients increases from infancy through adolescence [1]. To test the possibility that adolescent poly (I:C) mice exhibit increased susceptibility to KA-induced seizures, KA (25mg/kg, intraperitoneally) was injected at P30, and seizure severity was compared between control and poly (I:C) mice. We found no significant differences between control and poly (I:C) mice in KA-induced seizure severity for 90 minutes (Figure 1D) rearing/falling or tonic-clonic seizure (Figure 1E). In addition, the Survival curve of the latency to rearing/falling did not show differences between control and poly (I:C) mice (Figure 1F). These results suggested that poly (I:C) administration does not lower the seizure threshold during infancy and adolescence in mice.

Accumulating evidence has suggested that altered synapses' E/I balance is the shared mechanism of ASD and epilepsy [9-12]. Therefore, we immunohistochemically examined the synapse E/I balance in the brain at P15. We quantitatively measured the density of excitatory (Figure 2) and inhibitory (Figure 3) synapses in the hippocampus, which is an epileptic focus in KA-induced limbic seizures [13] (Figure 2,3). In this study, colocalized puncta of the pre-synaptic marker VGlut1 (vesicular glutamate transporter 1) and the post-synaptic marker Homer1 were defined as excitatory synapses (Figure 2) and those of pre-synaptic marker VGAT (vesicular GABA transporter) and the post-synaptic marker Gephyrin as inhibitory synapses (Figure 3). We found a significant increase in the density of inhibitory synapses only in the stratum radiatum of the CA1 region, where CA1 pyramidal cell dendrites receive synaptic inputs of poly (I:C) mice compared to controls. Though there was some increase in the density of inhibitory synapses in the molecular layer, where the granule cell dendrites receive synaptic inputs and excitatory synapses in the molecular layer and in CA1 in poly (I:C) mice, the increase was not statistically significant. The density of both excitatory and inhibitory synapses in CA3 was comparable between control and poly (I:C) mice. Our results suggest that the susceptibility to KA-induced limbic seizures is not increased in the poly (I:C) mouse model, which is often used

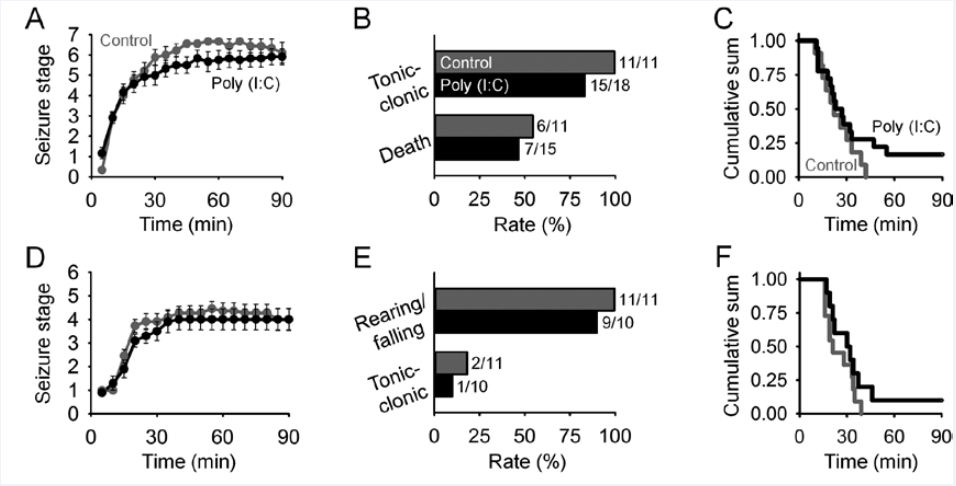

Figure 1 Kainic acid (KA)-induced seizures in mice at P15 (A-C) and P30 (D-E).(A, D) The average of seizure stages (see Methods for scoring)every 5 minutes from 0 to 90 minutes after KA injection at P15 (A) and P30 (D). $p > 0.05$; Mann-Whitney U test, n = 9 for control and 12 for poly (I:C) at P15, n = 11 for control and 10 for poly (I:C) at P30.Data represent mean ± SEM. (B) The rate of mice that exhibited tonic-clonic seizures and died during tonic-clonic seizures at P15. $p > 0.05$; Fisher's exact test, n = 11 for control and 18 for poly (I:C). (C) Survival curves representing latency to tonic-clonic seizures after KA injection at P15. $p > 0.05$; Log-rank test, n = 11 for control and 18 for poly (I:C). (E) The rate of mice that exhibited rearing/falling or tonic-clonic seizure at P30. $p > 0.05$; Fisher's exact test, n = 11 for control and 10 for poly (I:C). (F) Survival curves representing latency to rearing/falling after KA injection at P30. $p > 0.05$; Log-rank test, n = 11 for control and 10 for poly (I:C).

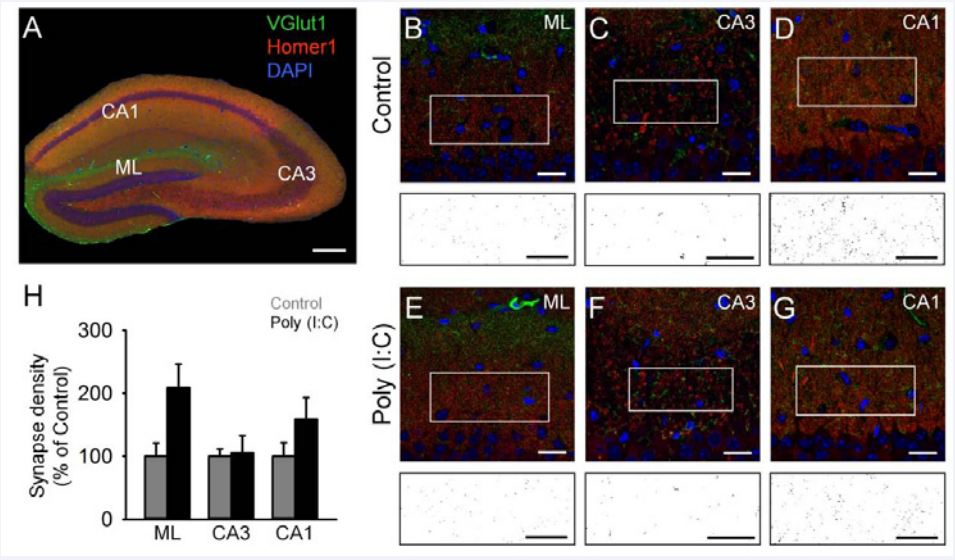

Figure 2 Immunohistochemistry for excitatory synapses in the hippocampus at P15. (A) A representative image of the P15 mouse hippocampus immune stained for the pre-synaptic marker VGlut1 and the post-synaptic marker Homer1. Scale bar = 200 μm.(B-G) Magnified images of the molecular layer (ML), CA3, and CA1 areas of immune stained hippocampal slices. Upper panels show merged signals of VGlut1 (green), Homer1 (red) and DAPI (blue). Lower panels show colocalized puncta of VGlut1 and Homer1. Scale bars = 20 μm. (H) Bar graphs showing the density of excitatory synapses in ML, CA3, and CA1 in control and poly (I:C) mice.Data are normalized by control in each region. $p > 0.05$; Mann-Whitney U test, n = 4 for control and 4 for poly (I:C). Data represent mean ± SEM.

as animal model of ASD. However, our results do not exclude the possible occurrence of spontaneous seizures in poly (I:C) mice when observed by long-term video monitoring accompanied by an EEG recording. Further, although we administered multiple injections of a relatively low dose of poly (I:C) to pregnant mice (3 mg/kg on E12.5 and 1.5 mg/kg on E17.5) according to Naviaux et al., 2013 [6], some researchers used a single injection of 20mg/kg poly (I:C) on E14.5 [14], which suggests the possibility that different methods of poly (I:C) administration may increase seizure susceptibility in offspring.

CONCLUSION

Our findings suggest that the offspring from poly (I:C)-injected pregnant dams do not show increased seizure susceptibility during infancy and adolescence. In addition, immunohistochemical analysis revealed that a poly (I:C)

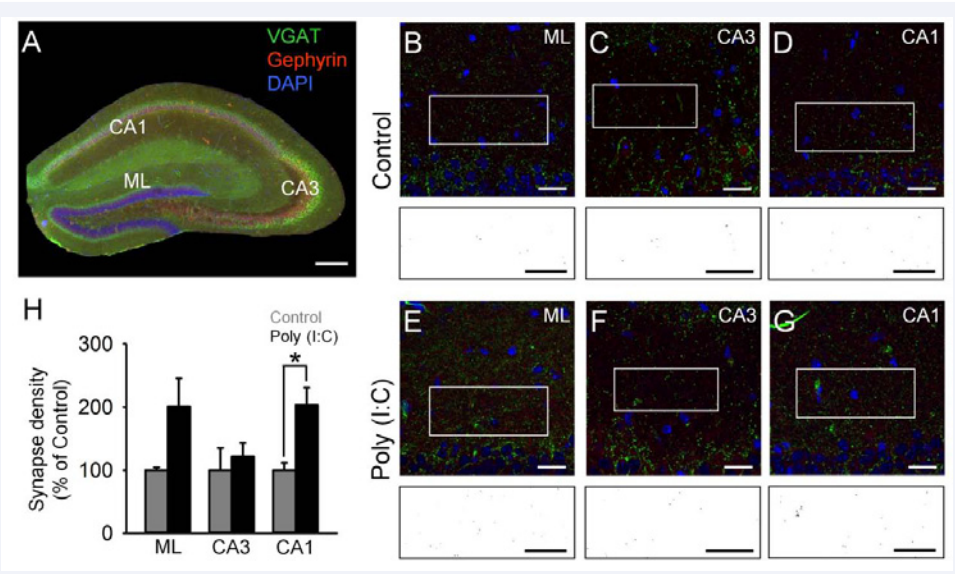

Figure 3 Immunohistochemistry for inhibitory synapses in the hippocampus at P15. (A) A representative image of the P15 mouse hippocampus immune stained for the pre-synaptic marker VGAT and the post-synaptic marker Gephyrin. Scale bar = 200 μm. (B-G) Magnified images of the molecular layer (ML), CA3, and CA1 areas of immune stained hippocampal slices. Upper panels show merged signals of VGAT (green) and Gephyrin (red) and DAPI (blue). Lower panels show colocalized puncta of VGAT and Gephyrin. Scale bars = 20 μm. (H) Bar graphs showing the density of excitatory synapses in ML, CA3, and CA1 in control and poly (I:C) mice. Data are normalized by control in each region.*p < 0.05; Mann-Whitney U test, n = 5 for control and 5 for poly (I:C). Data represent mean ± SEM.

administration does not affect structural synapse E/I balance in the hippocampus.

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