Cytokine-dependent bidirectional connection between impaired social behavior and susceptibility to seizures associated with maternal immune activation in mice

James Washington III a, Udaya Kumar a, Jesus-Servando Medel-Matus a, Don Shin a, Raman Sankar a,b,c, Andrey Mazari a,b,c

a Department of Pediatrics, David Geffen School of Medicine at UCLA, USA
b Department of Neurology, David Geffen School of Medicine at UCLA, USA
c UCLA Children’s Discovery and Innovation Institute, USA

ABSTRACT

Maternal immune activation (MIA) results in the development of autism in the offspring via hyperactivation of IL-6 signaling. Furthermore, experimental studies showed that the MIA-associated activation of interleukin-1β (IL-1β) concurrently with IL-6 increases the rate and the severity of hippocampal kindling in mice, thus, offering an explanation for autism–epilepsy comorbidity. We examined whether epileptic phenotype triggered by prenatal exposure to IL-6 and IL-1β combination is restricted to kindling or whether it is reproducible in another model of epilepsy, whereby spontaneous seizures develop following kainic acid (KA)-induced status epilepticus. We also examined whether in mice prenatally exposed to IL-6 and IL-6 + IL-1β, the presence of spontaneous seizures would exacerbate autism-like features. Between days 12 and 16 of pregnancy, C57BL/6J mice received daily injections of IL-6, IL-1β, or IL-6 + IL-1β combination. At postnatal day 40, male offspring were examined for the presence of social behavioral deficit, and status epilepticus was induced by intrahippocampal KA injection. After 6 weeks of monitoring for spontaneous seizures, sociability was tested again. Both IL-6 and IL-6 + IL-1β offspring presented with social behavioral deficit. Prenatal exposure to IL-6 alleviated, while such exposure to IL-6 + IL-1β exacerbated, the severity of KA-induced epilepsy. Increased severity of epilepsy in the IL-6 + IL-1β mice correlated with the improvement of autism-like behavior. We conclude that complex and not necessarily agonistic relationships exist between epileptic and autism-like phenotypes in an animal model of MIA coupled with KA-induced epilepsy and that the nature of these relationships depends on components of MIA involved.

1. Introduction

Common bidirectional connection between autism and epilepsy [1–3] has prompted numerous studies of autism–epilepsy comorbidity using animal models. Like in clinics, the association between autism-like and epileptic phenotypes varies significantly in experimental systems. For example, SCN1a haploinsufficient mice, which serve as a model of Dravet syndrome, present with both autism-like behavior and spontaneous seizures [4]. Conversely, mice which carry a missense mutation of a gene encoding neuroligin-3 (a mutation that has been implicated in autism [5]) and display autism-like behavior [6], have increased resistance to primary generalized seizures [7]. At the same time, inbred BTBR mice, which are characterized by many behavioral and anatomical abnormalities consistent with autism [8], show no epileptic phenotype [9]. Identifying animal models appropriate for exploring autism–epilepsy connections and reflecting a variety of etiologies and mechanisms of both disorders are important for understanding the mechanisms of the comorbidity and for the development of its effective therapies.

Maternal immune activation (MIA) may represent a system suitable for exploring autism–epilepsy comorbidity. Epidemiological studies suggest that MIA, particularly when triggered by viral infections, represents a risk factor for the development of autism in the offspring [10,11]. Congruent with clinical findings, the offspring of mice in which viral infection has been mimicked during pregnancy by means of polyinosinic-polycltyridic acid (Poly I:C), present with a spectrum of behavioral, anatomical, and physiological perturbations consistent with autism [12, 13]. Concurrently, these animals show increased susceptibility to epilepsy in the hippocampal rapid kindling paradigm [14]. Furthermore, components of MIA liable for the evolution of autism-like and epileptic phenotypes have been identified: while autism-like impairments stem from...
solely from the activation of interleukin-6 (IL-6) [15], parallel propensity to epilepsy requires simultaneous induction of IL-6 and interleukin-1β (IL-1β) [14].

In the present study, we further examined the autism–epilepsy connection in the MIA system. We deemed it important to establish that epileptogenicity in the MIA offspring is not model-specific, that is, not restricted to rapid kindling. Such studies appear even more warranted considering somewhat limited relevance of the rapid kindling model, in which no spontaneous recurrent seizures are observed. We chose a model of chronic epilepsy where spontaneous recurrent seizures develop following status epilepticus (SE) induced by intrahippocampal administration of kainic acid (KA) [16,17]. Further, in order to expand on our earlier findings [14], we examined whether the autism–epilepsy connection in the MIA offspring is bidirectional, that is whether the presence of spontaneous seizures in KA-injected mice would exacerbate the severity of autism-like impairments.

2. Material and methods

2.1. Animals

The experiments were performed in C57BL/6j mice. Breeding pairs were obtained from The Jackson Laboratory (Sacramento, CA). Breeding was performed at the UCLA Department of Laboratory Medicine. The procedures complied with the policies of the National Institutes of Health and were approved by the UCLA Office of Research Administration.

The presence of vaginal plug was considered as embryonic day (E) 0. The offspring were weaned at postnatal day (P) 28. Considering the higher prevalence of autism among males [18], and that in the MIA model, autism-like behavior is reserved for male offspring exclusively [19], the experiment proper was conducted in male mice. Animals were maintained at 12-hour light–dark cycle, with free access to food and water. Mice were housed individually, which was necessary for monitoring spontaneous seizures.

2.2. Modeling MIA

Based on earlier findings [14,15], we used recombinant cytokines IL-6 and IL-1β to mimic MIA in pregnant mice. This offered a cleaner approach as compared with the use of Poly I:C, as the MIA system was limited to factors specifically responsible for the evolution of autism-like and epileptic phenotypes in the offspring and, thus, allowed avoiding wider variability on both seizure and behavioral responses inherent to the Poly I:C protocol. Between E12 and E16, mice received daily intrauterine injections of saline (n = 8), recombinant IL-6 (20 μg/kg, n = 11), recombinant IL-1β (20 μg/kg, n = 10), or recombinant IL-6 + IL-1β (10 + 10 μg/kg, n = 13). Both cytokines were manufactured by R&D systems (Minneapolis, MN) [14].

Cytokine treatment had no observable effects on pregnant mice. Body weight gain was similar to those in saline-treated animals (across all experimental groups, body weight was 30–33 g at E12 and 42–45 g on E16, with no differences among the groups). We did not measure core temperature in these animals, as the insertion of a rectal probe may lead to premature termination of pregnancy. However, in our earlier studies [14], we reported that the applied treatment regimens did not induce hyperthermia, when the temperature was measured in a specially allocated group of mice. After giving birth, cytokine-treated dams did not reject pups at a rate higher than saline-treated ones and did not refuse nursing (occasional rejections and subsequent offspring death occur even in the absence of any manipulations and handling).

From each saline-treated mouse, between 1 and 3 male offspring were selected at random from all offspring belonging to the same litter underwent the same postnatal procedure. For cytokine treatments, the number of male offspring reaching P28, one of them received intrahippocampal injection of saline, another received intrahippocampal injection of kainic acid, and the third one was not used in the experiments. This principle was applied to all groups.

Kainic acid injection resulted in the development of limbic SE, the latter consisting of repeated clonic–tonic generalized seizures (rearing and/or rearing and falling; stages 4–5 on the Racine scale [25]) intermittently with focal seizures (motor arrest and/or facial clonus; stage 1 on the Racine scale). Status epilepticus lasted between 3 and 5 h. Only those animals which developed repeated stage 4–5 convulsions were used for further studies.

Three to four weeks after intrahippocampal KA or saline injection, the animals were prepared for seizure monitoring. Under isoflurane anesthesia, wireless transmitter model ETA-F10 (Data Science International, DSI, St. Paul, MN) was placed inside a subcutaneous pocket on the back; its two leads were fed under the skin to the skull surface and fixed with skull screws. The leads were fixed to the skull with dental cement.

Video and EEG monitoring of spontaneous seizures began 1 week after surgery and continued for 6 weeks. Behavioral seizures were recorded using digital cameras focusing on individual cages; data were saved on the digital video recorder. For the acquisition of electrophysiological seizures, home cages with individually housed animals were placed on top of wireless receivers RPC-1 (DSI) connected to a computer equipped with Harmonie acquisition software (Stellate Systems, Montreal, QC). In order to unambiguously establish the presence of chronic epilepsy, the only seizures considered were secondarily generalized complex partial seizures (stages 4–5 on the Racine scale [25]) with clearly identifiable
2.5. Second behavioral testing

After the end of seizure monitoring (i.e., at the age of 16 weeks), animals underwent a second sociability test, following the procedure described under Section 2.3.

2.6. Histology

After the second behavioral test, the animals were anesthetized with pentobarbital and transcardially perfused with 4% paraformaldehyde, and embedded in paraffin. Coronal 10-micron-thick sections of the hippocampus were cut for immunohistochemistry. In order to characterize neurodegeneration, primary rabbit polyclonal anti-NeuN antibodies (1:300, Abcam, Cambridge, MA) were used in conjunction with secondary biotinylated goat antirabbit antibodies (1:200, Abcam). Immunostaining was visualized using 3,3-diaminobenzidine (Sigma) with nickel intensification. The numbers of NeuN-positive cells (i.e., neurons) in the CA1 and CA3 areas of both left and right hippocampi were counted in 6 consecutive sections, using an eyepiece graticule with an indexed grid under 20× magnification on a Leica DLM microscope (McBain Instruments, Simi Valley, CA). The infusion of KA was visually verified by locating the injection cannula track.

2.7. Data analysis

Data were analyzed using Prism 6 software (Graphpad, San Diego, CA). Sample sizes, treatments, and group names are described in Table 1. Statistical tests are noted in figure legends.

3. Results

3.1. First sociability test (Fig. 2, before KA)

Consistent with earlier findings [12,14,15], the offspring of mice injected during pregnancy with saline showed a high degree of sociability, which was evident as preferential exploration of the conspecific as opposed to the unanimated object (e.g., sociability index 25 translates into 3:1 conspecific vs. object ratio). Also in agreement with reported data [14,15], the offspring of IL-6 and of IL-6 + IL-1β-treated animals exhibited diminished sociability, as they displayed no preference towards either the conspecific, or the object. Prenatal exposure to IL-1β alone did not impair social behavior.

3.2. Spontaneous seizures (Fig. 3)

Over the observation period, all animals of all KA-treated groups displayed spontaneous seizures. Saline/KA mice developed on average one seizure per day (minimal one seizure over 2 days, and maximal 3 seizures per day). In IL-6/KA animals, the occurrence of spontaneous seizures was significantly less frequent than in Saline/KA mice and did not exceed one seizure per 2 days. In contrast, animals of IL-6 + IL-1β/KA group developed significantly more frequent seizures than the Saline/KA counterparts (at least two seizures and up to 5 seizures per day). Prenatal exposure to IL-1β did not affect the frequency of seizures in KA-injected mice.

Occasional spontaneous seizures were observed in mice which were injected with saline in lieu of KA, with daily seizure frequency not exceeding one seizure over 10 days of observation.

![Fig. 1](image-url) An example of a spontaneous seizure after kainic acid (KA)-induced status epilepticus. Spontaneous seizure was captured in a 12-week-old mouse 5 weeks after intrahippocampal KA injection (50 ng). Top two rows: Snapshots of a stage 4 spontaneous seizure. Time is indicated from the seizure onset. Note the progression from tonic phase (3 s) to clonic–tonic phase (“kangaroo” posture, 9–15 s). Also, note postictal depression lasting for over 40 s (22 s and 66 s samples), evident as retaining same position without movements. Bottom row: Electrographic seizure from the same mouse. Note postictal depression.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Experimental groups.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant mouse treatment</td>
<td>Offspring treatment</td>
</tr>
<tr>
<td>Saline</td>
<td>Saline</td>
</tr>
<tr>
<td>Saline</td>
<td>Kainic acid (KA)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Saline</td>
</tr>
<tr>
<td>IL-6</td>
<td>KA</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Saline</td>
</tr>
<tr>
<td>IL-1β</td>
<td>KA</td>
</tr>
<tr>
<td>IL-6 + IL-1β</td>
<td>Saline</td>
</tr>
<tr>
<td>IL-6 + IL-1β</td>
<td>KA</td>
</tr>
</tbody>
</table>
4. Discussion

Our studies showed that complex and sometimes unexpected relationships exist between epileptic and autism-like phenotypes in an animal model of MIA coupled with KA-induced chronic epilepsy and that the nature of these relationships depends on components of MIA involved.

Firstly, it should be noted that mechanisms directly responsible for developmental deficits in the offspring following MIA, by and large, remain unknown. It has been suggested that cytokines induced by an infectious agent can cross the blood–placental barrier and activate the fetal immune system, which in turn precipitates behavioral impairments later in life [26]. It has also been shown that MIA (specifically prenatal exposure to Poly I:C) results in the overexpression of various cytokines in the brain of the offspring possibly throughout the life-span [27].

4.1. Spontaneous seizures

Our earlier studies suggested that prenatal exposure to the recombinant IL-6 – IL-1β combination, while increasing the propensity to hippocampal kindling, by itself produced no spontaneous seizures in the offspring [14]. This finding was, by and large, confirmed in the present experiments: while rare occasional seizures were documented following intrahippocampal saline administration, their occurrence was comparable among animals of the Saline/Saline and Cytokines/Saline groups and was far lower than in all KA-injected mice. These seizures may be attributed to mechanical trauma inflicted to the hippocampus by the saline infusion. In terms of KA-induced epilepsy which in itself produced measurable spontaneous recurrent seizures, in animals of the IL-6 + IL-1β/KA group, the frequency of seizures was significantly higher than in Saline/KA mice. This observation is in line with the earlier established proepileptic effects of prenatal exposure to the IL-6 and IL-1β combination, which accelerated the rate and the severity of hippocampal rapid kindling in the offspring [14] and, therefore, confirms that the reported effects were not model–specific. Also in agreement with the earlier report is the lack of effects of prenatal treatment with IL-1β alone, which again suggests the specificity of IL-6 + IL-1β combination in producing the epileptic phenotype.

At the same time, the offspring of IL-6-treated mice developed significantly fewer seizures than Saline/KA animals. This was an unexpected finding, as the IL-6 offspring did not show increased resistance to kindling epileptogenesis [14]. More importantly, this observation appears to contradict the concept of autism–epilepsy comorbidity although, as it has been discussed in Section 1, it is not unprecedented [7].

The role and the involvement of IL-6 in epilepsy have not been firmly established. Some studies implicate IL-6 signaling in epilepsy. Thus, increased plasma levels of IL-6 have been reported in patients with epilepsy [28,29], and following status epilepticus in rats [30]; kindling of basolateral amygdala increased IL-6 mRNA in the rat hippocampus [31]; IL-6 knockout mice showed decreased incidence of seizures induced by Thieier’s murine encephalomyelitis virus [32]. However, it is not certain whether IL-6 contributes to epileptogenesis, represents a mere surrogate marker of epileptic process, or serves as a compensatory

3.3. Second sociability test (Fig. 2, after KA)

After the establishment of chronic epilepsy, Saline/KA and IL-1β/KA mice displayed normal levels of sociability, with the sociability indices statistically similar to those before intrahippocampal KA injection. The IL-6/KA mice remained impaired; sociability deficit was not further exacerbated in the presence of spontaneous seizures, as the sociability index was statistically similar to the one before the induction of epilepsy. At the same time, IL-6 + IL-1β/KA mice showed significant improvement in social behavior: in these animals, sociability index was significantly higher than that prior to the induction of epilepsy and was comparable to this parameter in Saline/Saline mice. Data are shown as mean ± SEM. *p < 0.05 vs. Saline + KA. Kruskal–Wallis followed by Mann–Whitney test.

3.4. NeuN immunostaining (Fig. 4)

Consistent with our earlier observations [14], prenatal exposure to IL-6, IL-1β, and IL-6 + IL-1β produced no discernible neuronal degeneration in the hippocampus (Fig. 4A). Unilateral intrahippocampal injection of KA produced bilateral neuronal hippocampal degeneration, the extent of which widely varied from animal to animal from very mild to moderate in all KA-treated groups (examples are on Fig. 4B). We failed to find any connection between prenatal exposure to a specific cytokine or cytokine combination, and the extent of KA-produced neuronal cell loss (Fig. 4C).
Various degrees of neurodegeneration in the CA1, CA3, and hilar areas are observed in the hippocampi, and in KA1- and KA3-hippocampi contralateral to the injection. In Saline and KA2 animals, left lateral CA1 and hilar injury. Scale bar 500 μm. C. NeuN-positive cell counts after intrahippocampal KA administration. Administration of neither of the cytokines, nor of their combination led to more severe hippocampal neurodegeneration than in the IL-6/KA mice, which showed no discernible cell loss in the hippocampus in this and earlier studies.

4.2. Social interaction

The present experiments have confirmed earlier reports that prenatal exposure to IL-6 produces autism-like behavioral impairments in the offspring [13,15,26,36,37] and that the addition of IL-1β to IL-6 does not further exacerbate autism-like abnormalities [14]. Increased IL-6 signal- ing and specifically MIA-triggered IL-6 activation have been strongly implicated in autism [13,15,26,38,39]. Mechanisms remain poorly understood, but dysfunctional neuronal circuitries in somatosensory cortex and the hippocampus [37], hyperactivation of STAT3 signaling pathway [26], and disrupted long-range connectivity [36] have been contemplated.

In addition, looking into effects of the cytokines proper, in order to address the known bidirectional connection between epilepsy and autism [3], we examined whether and how chronic epilepsy, once established, affects animals’ social behavior. Here, we found that in animals of the Saline/KA group, sociability was not impaired. Not only did KA alone not produce autism-like phenotype, but also preexisting autism-like impairments in the IL-6 offspring were not further exacerbated by the KA-induced chronic epilepsy. These observations seemingly contradict other reports on the evolution of autism-like behavioral abnormalities, including impaired social interaction following epileptogenic insults. For example, a single episode of KA-induced seizures in P7–P10 rats [40], seizures induced by the injection of bicuculline into prefrontal cortex in P21 rats [41], as well as hypoxia-induced convulsions in P10 rats [42], all resulted in impaired social behavior later in life. An obvious difference between the cited studies and our study is the age of epileptogenic insults, whereby in our experiments, KA SE was induced in young adults, and in other studies—between neonatal and preadolescent ages. Thus a reasonable explanation may be that seizure-induced malplasticity leads to autism only when the insult occurs during a critical window of brain maturation. However, social behavioral deficits were reported in rats, in which pilocarpine SE was induced during adulthood [43]; the difference between this and our study is the mode of seizure induction (i.e., systemic pilocarpine vs. intrahippocampal KA). Another difference between all cited studies on the one hand, and our experiments on the other hand, is species (i.e., rats vs. mice), which may also contribute to the observed differences.

Probably the most surprising finding was the improvement of social behavior in IL-6 + IL-1β/KA mice. Prenatal exposure to the IL-6 + IL-1β combination led to autism-like impairments similar to those produced by IL-6 alone. The major difference between the outcomes of the two treatments (i.e., IL-6 vs. IL-6 + IL-1β) is more severe epilepsy in the IL-6 + IL-1β/KA than in IL-6/KA mice. On the surface, this finding

anticonvulsant mechanism as our findings seem to suggest. Indeed, several studies have found potential antiepileptic effects of IL-6. Interleukin-6 knockout mice showed increased seizure susceptibility to a variety of convulsant stimuli, including pentylentetrazole, KA, NMDA, and AMPA, as compared with normal B6 mice [33], as well as increased susceptibility to audiogenic seizures [34], thus, inferring anticonvulsant effects of the cytokine. Interleukin-6 exerted neuroprotective effects under conditions of hypoxia in hippocampal cell cultures and mitigated the severity of pentylentetrazole convulsions in mice, via increasing the expression and function of the adenosine A1 receptor [35].

Taken together with the discussed data, differential modulation of seizure phenotype by prenatal IL-6 exposure in our kindling experiments [14] vs. present studies performed in the KA model shows that the cytokine involvement in epilepsy depends strongly on the employed epilepsy model.

It should also be mentioned that the observed exacerbation of chronic epilepsy in the IL-6 + IL-1β/KA mice, as well as the mitigation of epilepsy in the IL-6 offspring, was not apparently related to the increased neurodegeneration and neuroprotection, respectively, as the extent of neuronal cell loss was similar across all KA-treated groups. Nor did preexisting hippocampal neurodegeneration contribute to more severe epilepsy in the IL-6 + IL-1β offspring, which showed no discernible cell loss in the hippocampus in this and earlier studies.

**Fig. 4.** NeuN-positive cell counts in the hippocampus. NeuN positive cell counts after prenatal cytokine exposure only. Administration of neither of the cytokines, nor of their combination produced neurodegeneration in the hippocampus. Ipsilateral and Contralateral refer to the site of saline injection. Data are shown as mean ± SEM percent of NeuN-positive cell counts in the offspring of saline-treated mice. 2-Way ANOVA + Sidak’s multiple comparison test. Interaction F = 1.346, p > 0.05; effects of prenatal treatment F = 0.703, p > 0.05. B. Examples of NeuN immunostaining in a Saline/Saline (noted as Saline) and three different Saline/KA (noted as KAα) mice. In Saline and KA2 animals, left (injected) hippocampi, and in KA1- and KA3-hippocampi contralateral to the injection are shown. Various degrees of neurodegeneration in the CA1, CA3, and hilar areas are observed. KA1 = very mild CA1 and CA3 cell loss; KA2 = moderate CA3 injury; KA3 = moderate CA1 and hilus injury. Scale bar = 500 μM. C. NeuN-positive cell counts after intrahippocampal KA administration. Administration of neither of the cytokines, nor of their combination exacerbated KA-induced neuronal cell loss in the hippocampus. Ipsilateral and Contralateral refer to the site of KA injection. Data are shown as mean ± SEM percent of NeuN-positive cell counts in the offspring of Saline/KA mice. 2-Way ANOVA + Sidak’s multiple comparison test. Interaction F = 0.69, p > 0.05; effects of prenatal treatment F = 1.02, p > 0.05; effects of seizures F = 0.41, p > 0.05.
shows that once the frequency of seizures reaches a certain critical level (and indeed, the cytokine combination increased KA-induced seizure frequency 2–3 folds), autism-like impairments improve. This observation contradicts the concept of autism–epilepsy connection and requires further studies and explanation. Whether recurrent seizures have similar effects on other autism-like behaviors in the MIA system (e.g., restricted behavior and impaired social interaction [12,15]), also remains to be explored.

4.3. Conclusions

Overall, our data show that bidirectional relationships between autism and epilepsy do not necessarily follow a straightforward path of comorbidity. Under certain circumstances, bidirectional mitigation, rather than mutual exacerbation of the comorbidity, may be observed. The nature of the autism–epilepsy connection strongly depends on the system used. In our case, on the one hand, prenatal exposure to IL-6 produces autism-like behavior later in life, but at the same time seems to mitigate seizure phenotype upon the introduction of postnatal epileptogenic insult. On the other hand, the presence of spontaneous recurrent seizures induced by KA reverses, rather than exacerbates, preexisting autism-like behavioral deficit. The existing literature may provide reasonable explanation for the antagonism and provide a blueprint for further studies (e.g., reported anticonvulsant effects of IL-6). In addition to explaining for the antagonism and provide a blueprint for further studies, our findings emphasize the importance of choosing an appropriate system for examining the comorbidity between the two disorders.

Acknowledgments

This work was supported by the Research and Training Grant from the Division of Neuroanatomy. Dept. of Pediatrics David Geffen School of Medicine at UCLA to JWIII; by research grants R01NS065783 and R21NS089396 from the National Institutes of Health to AM; and by the Today and Tomorrow Children’s Fund to AM. Dedicated to the memory of Paul H. Patterson.

Conflicts of interest

Dr. Sankar reports receiving research support from Bluebird Bio and NIH. He has also served as a consultant and/or served on the speakers bureau for UCB, Lundbeck, Sunovian, Upsher-Smith, Cydronics, and Supernus. He has received book royalties from Demos Medical Publishers and the CRC Press. Dr. Mazari reports receiving research support from the NIH and the Today and Tomorrow Children’s Fund.

References