Investigating the Role of Microglia in the Regulation of Excitation/Inhibition Balance in the Mouse Brain

A Major Qualifying Project Report
Submitted to the Faculty of
WORCESTER POLYTECHNIC INSTITUTE
in partial fulfillment of the requirements for the
Degree of Bachelor of Science
In Biology and Biotechnology

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Date: April 26, 2018

ABSTRACT

In the healthy brain, excitation and inhibition (E/I balance) governs normal brain function by regulating the on/off state of neural circuits. A number of neurological diseases such as epilepsy, schizophrenia, and autism spectrum disorder (ASD) are correlated with an E/I imbalance, underscoring the clinical significance of E/I balance. Microglia, the resident macrophages of the central nervous system (CNS), are intricately involved in brain homeostasis through debris clearance, communication with neurons, and synapse remodeling. However, it is not yet known if or how microglia regulate E/I balance. In this project, the role of microglia in the regulation of E/I balance was investigated using chemically induced seizures on two mouse models, one lacking microglia and one with abnormal microglia. If microglia are necessary for E/I balance, then we hypothesize that mice with defective microglia will have altered seizure severity. The results showed that both knockout models experienced an increase in seizure severity, suggesting that microglia play a role in modulating E/I balance. A better understanding of the mechanisms behind E/I balance is essential for future treatments of neuropsychiatric disorders.

ACKNOWLEDGEMENTS

I would like to thank Philip Feinberg, MD/PhD candidate, for going above and beyond as my mentor throughout this project. His continual teachings, guidance, and patience were essential for the completion of my project.

I would also like to thank Dr. Dorothy Schafer and the entire Schafer lab for their help and support, in addition to providing a welcoming and productive lab environment.

Lastly, I would like to thank my advisor, Dr. Jagan Srinivasan for his support and enthusiasm, and for editing my project.

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INTRODUCTION

E/I Balance

Throughout the nervous system, neurons transmit information via electrochemical impulses in a process known as neurotransmission. Following sufficient stimulation, an electrical signal is generated, called an action potential, which travels down the neuron's axon to its terminal branches where it forms synaptic connections with downstream cells. There, the action potential leads to release of neurotransmitters that bind to specific receptors on the post-synaptic cell, which can either activate (excitatory) or inactivate (inhibitory) it. Release of glutamate, the canonical excitatory neurotransmitter, into the synaptic junction, binds to ionotropic receptors on the postsynaptic neuron and excite it, continuing the spread of the signal. In this system, the excitatory neurons must be kept in check in order to prevent overstimulation. Inhibitory neurons function by regulating the level of stimulation from the excitatory neurons through the opposing neurotransmitter GABA, which hyperpolarizes the postsynaptic neuron, making it harder to generate an action potential.

In the healthy brain, a balance between excitation and inhibition (E/I balance) is essential for maintaining appropriate neuronal activation and spreading of information (2). As such, the brain ensures precision through tight control over this "on/off switch". Altered E/I balance, such as net hyperexcitabaility or hypoexcitability, can be caused by a variety of defects. Too many excitatory synaptic connections or too few inhibitory synaptic connections result in increased overall excitation whereas too few excitatory synapses or too many inhibitory synapses lead to increased inhibition (Fig. 1). The importance of E/I balance is underscored by disorders such as autism spectrum disorder (ASD), epilepsy, and schizophrenia that are correlated with disruptions in E/I balance (1,3). These disruptions are believed to give rise to the hallmark clinical features

of the conditions including seizures, hallucinations and impaired sociability. However, the mechanisms in which equilibrium is established and controlled are not well understood. One method of investigating E/I balance is to examine synaptic connectivity. Initially, excess synapses are formed and need to be remodeled by pruning to establish mature connections. Microglia play a significant role in pruning synapses during development (4,5).

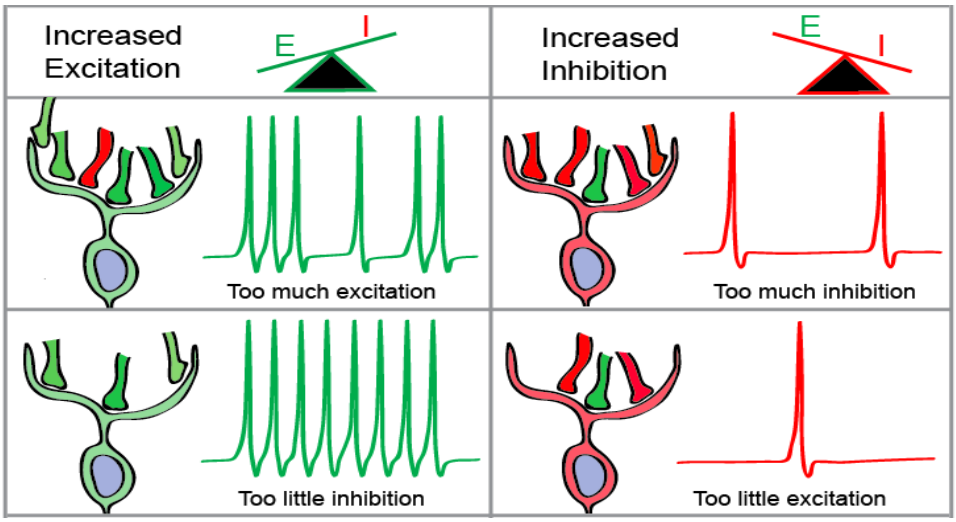

Figure 1. Excitatory inhibitory balance is necessary for normal brain function

Microglia

Microglia, the resident immune cells of the CNS, are critical for maintaining normal brain homeostasis. Canonically, microglia have been known for their role in the injured or diseased brain, surveying tissues and phagocytosing pathogens and other cellular debris. More recent studies have demonstrated the diverse roles of microglia in the healthy brain as well. In 2011, Paolicelli et al revealed that microglia are actively involved in pruning synapses during development (4). In 2012, Schafer et al went on to describe that microglial synapse elimination is directed by neuronal activity and executed by the complement system, an innate immune pathway for clearing material from tissue (5). Given the diverse roles of microglia in the healthy

and diseases brain, researchers have begun to investigate whether defects in microglial function contribute to the susceptibility and severity of neuropsychiatric disease. One study showed that a reduction in the number of microglia during early development leading to an expected reduction in synapse pruning resulted in decreased brain connectivity (6). Interestingly, deficits in social interaction and repetitive behaviors characteristic of ASD and other neuropsychiatric disorders were also observed (6,7). Altogether, this suggests that microglia may play a role in regulating E/I balance, however the underlying mechanisms remain unknown. If microglia are necessary for E/I balance, then we hypothesize that mice with defective microglia will have altered seizure severity.

Experimental Design

Two mouse models with defects in microglia were utilized in order to examine the role of microglia in E/I balance. In the first model, the Csf1r gene was knocked out (Fig. 2). The colony stimulating factor 1 receptor is necessary for microglial population of the brain (9). Knocking out the gene results in an absence of microglia. In the second model, the Irf8 gene was knocked out. Interferon regulatory factor 8 is a transcription factor necessary for microglia maturation and function (10). The knocked out gene results in defective microglia.

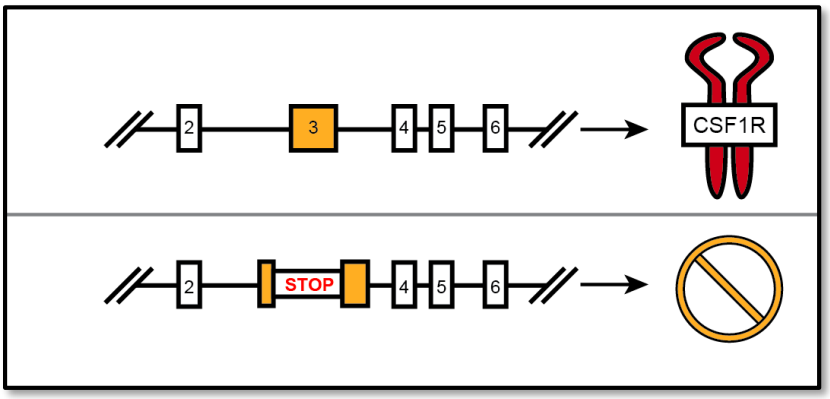

Figure 2. Schematic showing knockout strategy for CSF1R deletion

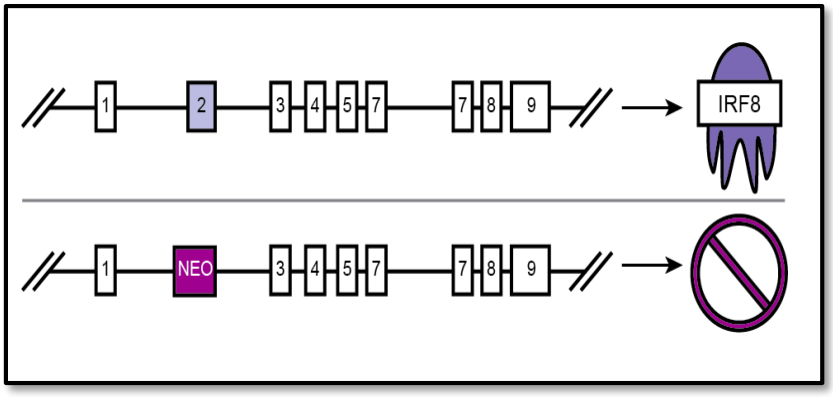

Figure 3: Schematic showing knockout approach for IRF8 deletion

To test E/I balance on the mouse models, seizures were chemically induced using pentylenetetrazol (PTZ), a $GABA_A$ receptor antagonist. PTZ blocks the receptor and prevents inhibition (Fig. 4) resulting in an increase in excitatory signaling, neural overstimulation and the induction of seizures (11). Seizures were chosen as the method for examining E/I balance because they are easily inducible and produce a direct measurable outcome. Additionally, many neuropsychiatric disorders such as ASD are comorbid with seizures.

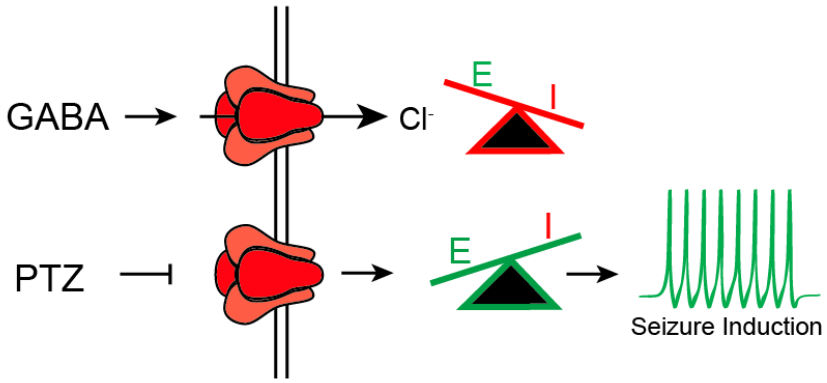

Figure 4: Experimental paradigm

Preliminary Data

Preliminary results showed a clear increase in seizure severity in the CSF1R KO mice. However, the IRF8 KO mice did not display a significant difference in maximum seizure

severity compared to the wild type controls. They did experience more seizure events. More trials are required to gain a more accurate picture of the trends. Altogether, the results suggest a role of microglia in the regulation of E/I balance. Further research can begin to look at how microglia affect E/I balance, by examining structural connectivity of the synapses and neural plasticity.

MATERIALS AND METHODS

Mice

Experiments were performed with male and female mice in accordance to guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School. CSF1R and IRF8 strains from the Schafer colony were bred and maintained in house.

PCR genotyping

Polymerase chain reaction (PCR) genotyping was used to confirm the genotypes of the mice. Ear punches or tail clippings were taken from the mice and digested in 25mM NaOH/0.2mM EDTA buffer and heated in the thermocycler for 1 hour at 98˚C. To stop digestion, 40 mM Tris HCl was added to each sample and stored at 4˚C until use. PCR was conducted using the necessary primers and samples were separated via gel electrophoresis.

PTZ injections

To induce seizures in the IRF8 KO cohort, intraperitoneal (IP) injections of pentylenetetrazol (PTZ) were performed approximately every-other day for a total of 8 injections at 40 mg/kg PTZ. Two days after the last 40 mg/kg injection, the mice were given IP injections at 50 mg/kg PTZ. Six days later, more injections were administered at 60 mg/kg PTZ. The final injections were given four days later at the same dosage. The CSF1R KO cohort was given a one-time IP injection at 40 mg/kg.

Seizure Scoring

Mice were scored at 5-minute intervals for one hour post-injection according to the following stages (8):
Stage 1: hypoactivity

Stage 2: partial clonus

Stage 3: generalized clonus

Stage 4: tonic-clonic seizure

Preparation of tissue samples

Mice were anesthetized with 2.5% Avertin (2,2,2- tribromomethanol) followed by perfusion with 1xPBS through the left ventricle. Brains were fixed in 4% paraformaldehyde overnight and dehydrated in 30% sucrose solution. Brain tissues were then embedded in optimum cutting temperature compound (OCT), cut, and mounted onto slides in 14μm coronal sections using a cryostat.

Immunohistochemical staining

Tissue slices were blocked for an hour in PBTGS (10% goat serum, 0.3% triton X, 89.7% phosphate buffer). The PBTGS was removed and the primary antibodies (for the desired stain) were added and left to incubate overnight. The primary antibody was removed and the slices were washed three times with phosphate buffer. The secondary antibodies were then added and left to incubate for one house. The secondary antibody was removed and the slices were washed three times again. The slices were then cover slipped using FluoroshieldTM. Images were taken using an SD confocal microscope.

RESULTS AND DISCUSSION

In order to test whether microglia play a role in regulating E/I balance, seizures were chemically induced in two mouse models. It was hypothesized that if microglia play a role in modulating E/I balance, then mice with defective microglia will have altered seizure behavior. In the first model, the Csf1r gene was knocked out, yielding mice completely devoid of microglia. Immunostaining for the microglia marker IBA-1 confirms the complete loss of microglia in the brain (Fig. 5).

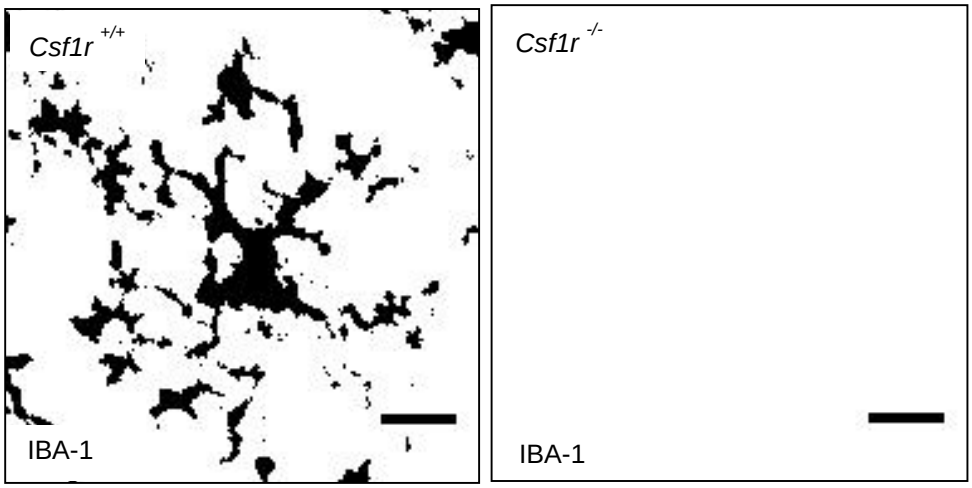

Figure 5. Immunostaining for the microglia marker IBA-1 showing complete loss of microglia in CSF1R null mice

For the CSF1R mice, PTZ was administered in a one-time intraperitoneal injection of 40 mg/kg. The mice were observed for one hour and scored at 5-minute intervals using the 4 stage scoring system. First, animals were assessed for the maximum seizure stage experienced during the one-hour trial (Figure 6A). While wild –type animals primarily had stage 2 partial clonus events, CSF1R KO animals all had stage 4 tonic-clonic seizures. The difference in seizure severity between the KO and control animals is statistically significant ($p<0.05$) Next, the animals were assessed for number of tonic-clonic episodes (Figure 6B). There is a trend of more episodes in the knockout mice compared to the control. The knockout mice experienced 1, 2,2, 3, and 5 seizures,

respectively, while the control mouse only seized once. It is important to note that the graph only displays the mice that reached a stage 4 seizure. Only one control mouse is shown because it is the only control that fully seized. Lastly, the latency to seize was assessed., The knockout animals first seized at 4, 5, 6, 8, and 17 minutes, respectively, while the control seized at 4 minutes. This may be reflective of a difference between sexes. It has been observed that female mice generally seize earlier than males. There seems to be little difference in latency to seize between the knockout and control mice, although this is a tentative observation as only one control mouse experienced a stage 4 seizure.

The CSF1R gene is essential for immune cells in the periphery, so the knockout animals are sickly. Therefore, while it provides evidence, a second model is needed to more precisely assess the functional role of microglia.

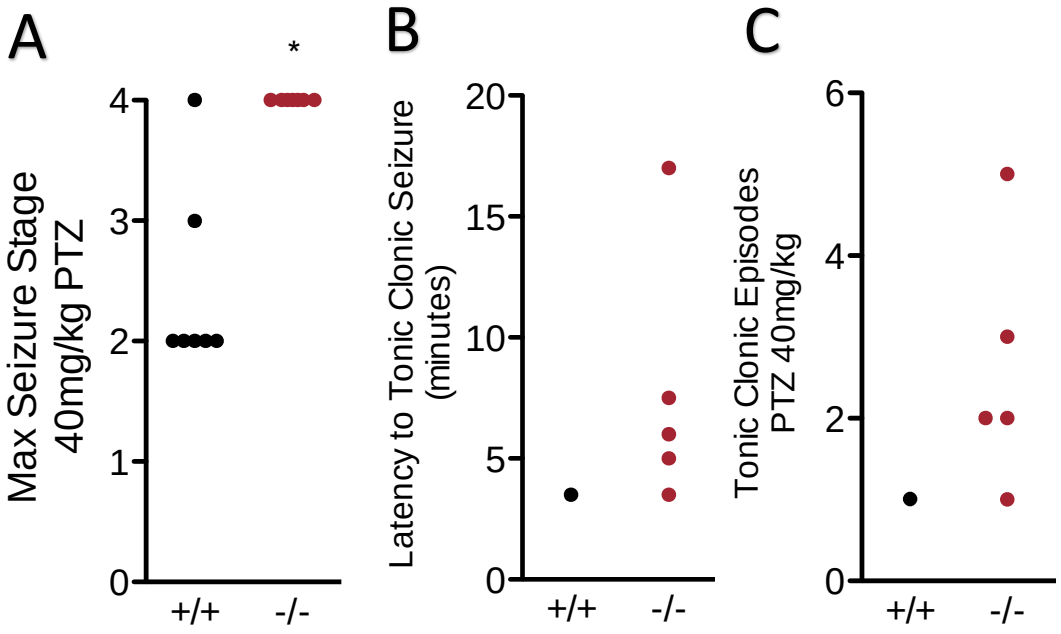

Figure 6. Maximum seizure stage after 40mg/kg PTZ injection (left) latency to tonic clonic seizure (middle) number of tonic-clonic seizure events (right) * $p<0.5$ Mann-Whitney

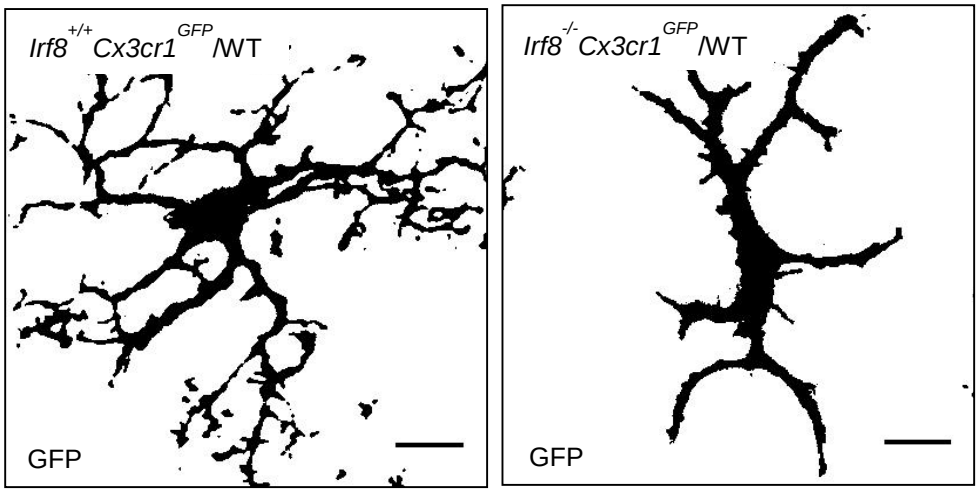

Figure 7. Visualization of microglia cells with GFP showing altered microglia in IRF8 null mice

In the second mouse model, the Irf8 gene was knocked out, resulting in defective microglia. These knockout microglia appear to be more amoeboid in morphology, with less processes and branching. The Irf8 null microglia were visualized with GFP driven from the CX3CR1 promoter (Fig. 7). For the IRF8 mice, PTZ was administered approximately once every two days for a total of 8 injections at 40 mg/kg. Due to a minimal reaction to the drug, the dosage was increased to 50 mg/kg for one injection, then increased once more to 60 mg/kg for the final two injections. The data presented represents the final injection trial at 60 mg/kg (Figure 8). First, maximum seizure score was assessed for the one-hour trial (Figure 8A). The data does not show a difference in seizure severity between the knockouts and controls. Similarly, there is no difference between the knockouts and controls in seizure susceptibility, measured by latency to seize (Figure 8B). There is, however, a trend towards an increased number of seizure events (stage 3 or 4) in the mice with defective microglia compared to the wild type controls. The knockout mice experienced 3 and 13 seizure events, respectively, compared to the 1 event experienced by each of the two controls (Figure 8C). The IRF8 data differed from the expected increase in seizure severity. However, this can be attributed to the relatively small cohort of mice, which can be improved upon be repeating

the experiment. Future experiments will also pay attention to sex distributions, as there is a suspected difference in severity and susceptibility between the sexes.

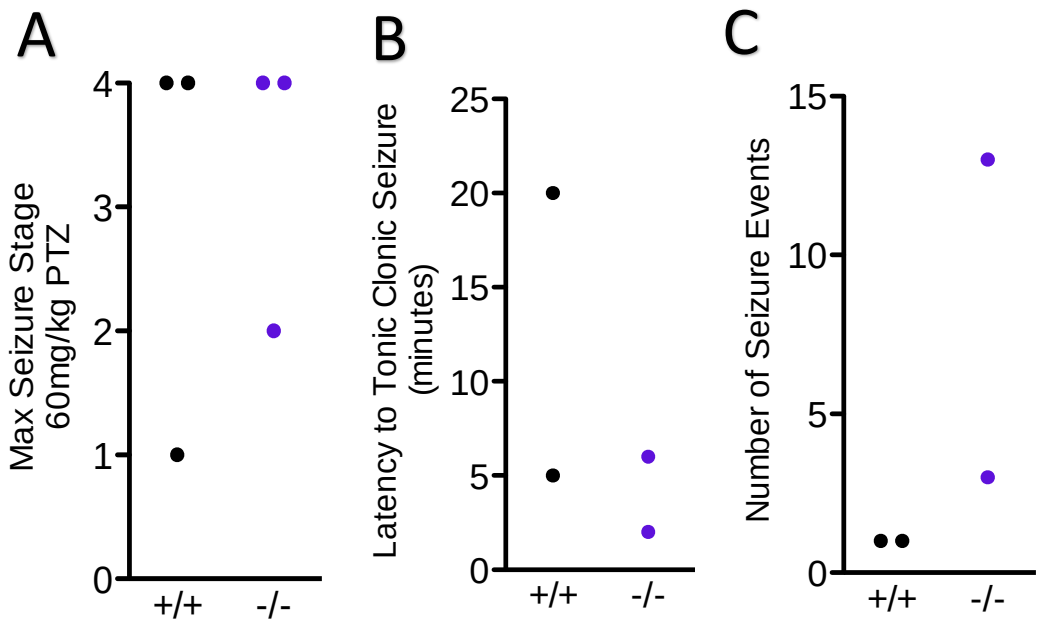

Figure 8. Maximum seizure stage after 60 mg/kg PTZ injection (left) latency to tonic-clonic seizure (middle) number of seizure events (right)

CONCLUSION

We hypothesized that if microglia play a role in regulating E/I balance, then there will be altered seizure activity in mice with defective microglia. Altogether, the data suggests that microglia do play a role in regulating E/I balance. When microglia were removed, the mice experienced a greater number of seizures and more severe seizures compared to wild type controls. Although the defective microglia did not show a difference in severity, there is a trend towards more seizure events. More trials are required and may produce a difference in severity. Overall, the hypothesis is supported because an alteration in seizure activity was observed. For future work, we turn to the question of: how do microglia regulate E/I balance? Once approach is to examine synaptic connectivity. Microglia prune synapses during development, and a lack of active microglia may lead to alterations in synapse density. This difference in wiring may have an effect on E/I balance. A second approach will examine differences in neuron-microglia communication. In the healthy brain, microglia communicate regularly with neurons. Absent or defective microglia may not communicate at the same level with neurons, influencing E/I balance.

It is critical to investigate the role of microglia in regulating E/I balance because the regulatory mechanisms are not currently well understood, yet a number of neurological disorders such as ASD, schizophrenia, and epilepsy have been associated with alterations in E/I balance (12,13). A better understanding is critical for the future design of novel therapeutics for these disorders.

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