

Toxoplasma gondii Infections Alter GABAergic Synapses and Signaling in the Central Nervous System

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ABSTRACT During infections with the protozoan parasite *Toxoplasma gondii*, gamma-aminobutyric acid (GABA) is utilized as a carbon source for parasite metabolism and also to facilitate parasite dissemination by stimulating dendritic-cell motility. The best-recognized function for GABA, however, is its role in the nervous system as an inhibitory neurotransmitter that regulates the flow and timing of excitatory neurotransmission. When this pathway is altered, seizures develop. Human toxoplasmosis patients suffer from seizures, suggesting that *Toxoplasma* interferes with GABA signaling in the brain. Here, we show that while excitatory glutamatergic presynaptic proteins appeared normal, infection with type II ME49 *Toxoplasma* tissue cysts led to global changes in the distribution of glutamic acid decarboxylase 67 (GAD67), a key enzyme that catalyzes GABA synthesis in the brain. Alterations in GAD67 staining were not due to decreased expression but rather to a change from GAD67 clustering at presynaptic termini to a more diffuse localization throughout the neuropil. Consistent with a loss of GAD67 from the synaptic terminals, *Toxoplasma*-infected mice develop spontaneous seizures and are more susceptible to drugs that induce seizures by antagonizing GABA receptors. Interestingly, GABAergic protein mislocalization and the response to seizure-inducing drugs were observed in mice infected with type II ME49 but not type III CEP strain parasites, indicating a role for a polymorphic parasite factor(s) in regulating GABAergic synapses. Taken together, these data support a model in which seizures and other neurological complications seen in *Toxoplasma*-infected individuals are due, at least in part, to changes in GABAergic signaling.

IMPORTANCE Infections of the central nervous system can cause seizures. While inflammation in the brain has been proposed to initiate the onset of the seizures, relatively little is known about how inflammation impacts the structure and function of the neurons. Here we used a parasite called *Toxoplasma gondii* that infects the brain and showed that seizures arise due to a defect in signaling of GABA, which is the neurotransmitter primarily responsible for preventing the onset of seizures.

Received 20 August 2015 Accepted 6 October 2015 Published 27 October 2015

Citation Brooks JM, Carrillo GL, Su J, Lindsay DS, Fox MA, Blader IJ. 2015. *Toxoplasma gondii* infections alter GABAergic synapses and signaling in the central nervous system. mBio 6(6):e01428-15. doi:10.1128/mBio.01428-15.

Editor Louis M. Weiss, Albert Einstein College of Medicine

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Neural activity within the brain is a balance of excitatory and inhibitory neurotransmission, and when excitatory activity becomes uncontrolled, seizures develop. A number of pathological conditions, including trauma, cancer, and inflammation, trigger seizures (1–3). Bacterial, viral, fungal, and parasitic infections of the brain also cause seizures, and several mechanisms by which seizures develop in response to infection have been proposed. These include breakdown of the blood-brain barrier, which allows an influx of either potassium, which increases neuronal firing, or autoantibodies, which target a number of proteins involved in neurotransmission (4). In addition, proinflammatory proteins, such as interleukin-1 beta (IL-1 β) and cyclooxygenase 2, can cause immune system-mediated damage to neurons and glia that leads to the onset of seizures (4, 5). Although there is a general understanding of the initial infection-induced events that trigger seizures, significantly less is understood about how they actually impact the structure and function of neurons.

Toxoplasma gondii is a protozoan parasite that infects ~30% of

the world's population (6, 7). People and animals become infected following digestion of oocysts found in feline fecal material or tissue cysts in undercooked meat. Once in the gut, the parasite infects intestinal epithelial cells and converts to the rapidly growing tachyzoite form. Inflammatory cells are recruited to combat the infection and in turn become infected and hijacked by the parasite to disseminate from the gut to target tissues, including the brain, retina, and muscle (8–11). Within these tissues, gamma interferon (IFN- γ) is key for killing most of the tachyzoites, but some evade killing and transform into the quiescent bradyzoite form (7, 12). Bradyzoites then form tissue cysts by encasing themselves within a highly glycosylated and impermeable cyst wall that renders the bradyzoites resistant to killing by either the immune response or antiparasitic drugs (13). Tissue cysts can spontaneously rupture, and the released bradyzoites develop back into tachyzoites, which continue to replicate until an immune response is mounted (7). The parasite has a clonal population structure, and the strain types display differences in virulence and clin-

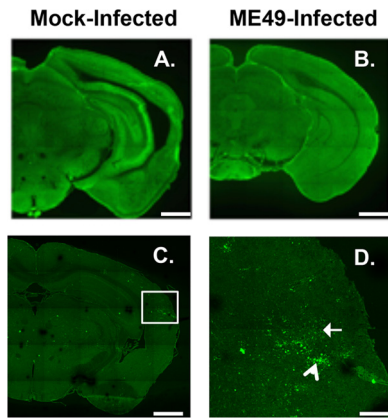


FIG 1 Decreased GABAergic synaptic protein staining in type II ME49 *Toxoplasma*-infected brains. (A and B) GAD67 immunoreactivity in mock-infected (A) or type II ME49 *Toxoplasma*-infected (B) brains. (C and D) Detection of type II ME49 *Toxoplasma* tachyzoites (white arrow in panel D) and bradyzoites (arrowhead in panel D). The image in panel D is a high-magnification image of the boxed in area in panel C. Scale bars, 1 mm (A, B, and C) and 0.25 mm (D). Lines appear in the images due to lack of overlap during image acquisition of each brain.

ical presentation. North America and Europe are dominated by four major strain types (types I, II, III, and 12 [or X]) (14, 15). In mice, type I strains are very virulent, with a 100% lethal dose (LD_{100}) of 1 parasite and death occurring within 10 days postinfection, which is a time before a chronic infection is established. Type II and III strains, with LD_{50} s of 10^3 and 10^5 , respectively, are less virulent in mice (16). Regardless, all strain types can establish long-lived chronic infections, although the numbers and sizes of cysts can differ, even between parasites belonging to the same strain type (17).

Three factors contribute to the onset and severity of toxoplasmosis. First, a host's ability to control parasite replication is key and those who are immunocompromised or were infected *in utero* are highly susceptible to developing disease (18, 19). Second, improperly regulated immune responses can cause immune system-mediated tissue damage (20). Finally, the disease type and severity are dictated by the genotypes of both the parasite and host (21–23). Symptomatic brain infection with *Toxoplasma* is known as toxoplasmic encephalitis (TE) and can clinically present with dizziness, headaches, and seizures. Despite the fact that TE has been clinically recognized for over 60 years (24), the effect that *Toxoplasma* and many other intracerebral pathogens have on neuronal structure and function is unknown. Therefore, the goal of this study was to use a murine TE model to identify the changes to neurons during TE that may underlie these neurological symptoms. Here, we report that mice with TE develop seizures due, in part, to specific changes in the synaptic localization of glutamic acid decarboxylase 67 (GAD67). Since GAD67 is the key enzyme for the neuronal biosynthesis of gamma-aminobutyric acid (GABA), which is the major inhibitory neurotransmitter in the brain, these data indicate that *Toxoplasma* directly interferes with inhibitory neurotransmission and provides a molecular basis for the development of neurological complications in TE patients.

RESULTS

***Toxoplasma* specifically alters GABAergic synapses.** Since *Toxoplasma* modulates GABAergic signaling in monocytes and uses

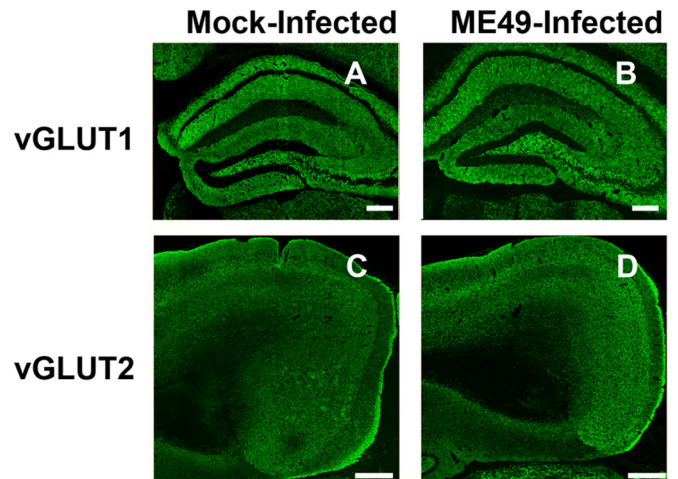


FIG 2 Unaltered glutamatergic synaptic protein staining in type II ME49 *Toxoplasma*-infected brains. (A and B) vGluT1 immunoreactivity in mock-infected or type II ME49 *Toxoplasma*-infected hippocampi. Scale bars, 0.2 mm. (C and D) vGluT2 immunoreactivity in mock-infected or type II ME49 *Toxoplasma*-infected cortices. Scale bars, 0.4 mm.

GABA as a carbon source, and since GABA is the major inhibitory neurotransmitter in the brain (25, 26), we examined the effect of type II ME49 *Toxoplasma* infection on GABAergic neurons. Thus, mice were mock infected or infected with *Toxoplasma* tissue cysts of the type II ME49 strain and, 30 days later, their brains were harvested and stained to detect GAD67, which catalyzes the conversion of glutamate to GABA and is normally clustered at presynaptic GABAergic terminals in the mature brain (27, 28). Low-magnification images of whole-hemisphere brain sections revealed an apparent loss of GAD67 immunoreactivity in the caudal areas of hippocampi from type II ME49-infected mice as well as in the cortex and thalamus (Fig. 1A and B). The effect on GAD67 immunoreactivity appeared to be more widespread than the parasites were in the brain (according to the results of an assay using an anti-*Toxoplasma* antisera that detects both tachyzoite and bradyzoite antigens) (Fig. 1C and D). The effect of type II ME49 *Toxoplasma* on inhibitory GABAergic synapses appeared to be specific, as no apparent differences were seen in staining of the presynaptic glutamatergic markers vesicular glutamate transporter 1 (vGluT1) in the hippocampus, vGluT1 or vGluT2 in the cortex, and vGluT1 in the ventral horn of the spinal cord (Fig. 2; see also Fig. S1 in the supplemental material).

***Toxoplasma* disrupts GAD67 presynaptic clustering.** Decreased GAD67 immunoreactivity could be due to either changes in GAD67 expression or its relocation from distinct puncta at synaptic terminals to a more diffuse staining within the neuron. In fact, redistribution of presynaptic machinery is known to occur during development, when transsynaptic signals induce the clustering of diffusely localized presynaptic molecules into discrete clusters specifically at synaptic sites (29, 30). To discriminate between these two possibilities, we compared brains of mock-infected and type II ME49-infected mice for mRNA levels of *gad1* (the gene that encodes GAD67) and *gphn*, which encodes gephyrin, a scaffolding protein localized to the postsynaptic specializations of GABAergic synapses. No significant differences were observed in *gad1* or *gphn* transcript levels (Fig. 3A). Similarly, no statistically significant differences in the abundances of the pro-

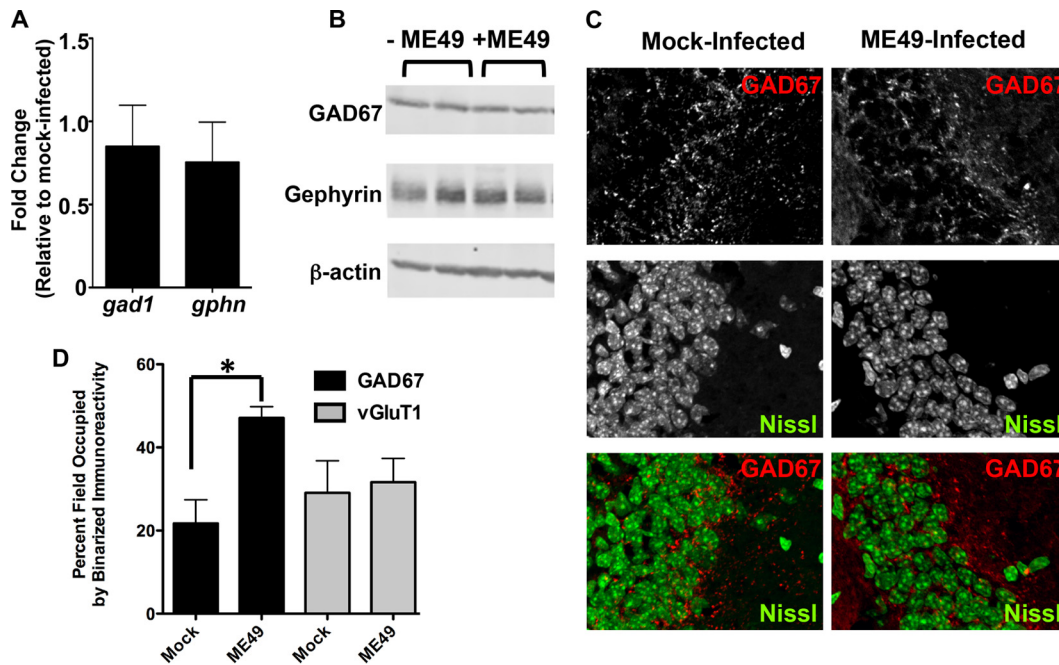


FIG 3 Type II ME49 *Toxoplasma* infections induce mislocalization of GAD67. (A) RT-PCR quantification of relative *gad1* and *gphn* transcript levels in mock-infected and type II ME49 parasite-infected brains. (B) Western blot detection of GAD67, gephyrin, and β -actin in cortical tissues harvested from mock-infected and type II ME49 parasite-infected brains. (C) High-magnification images of GAD67 staining in hippocampal CA3 regions in mock-infected and type II ME49 parasite-infected mice. Note the change from punctate staining in the mock-infected brains to a more diffuse staining in the parasite-infected brains. (D) Quantification of percent GAD67 and VGluT1 staining following binarizing of 5 images/brain ($n = 3$ mice/group). *, $P < 0.02$ (unpaired, two-tailed t test).

teins were noted in parasite-infected brains ($P > 0.7$ [two-tailed, unpaired t test]) (Fig. 3B).

Similarly to previous work (28), high-magnification analysis revealed that GAD67 localized to discrete synaptic puncta in the stratum pyramidale and other regions in the hippocampi of mock-infected mice (Fig. 3C). In contrast, GAD67 staining in these regions from the infected animals no longer appeared enriched at synaptic release sites. To quantify these differences, GAD67-stained images were binarized and the percent area of binarized immunoreactivity was calculated. Despite the minimal changes in overall GABA levels (Fig. 3A and B), these analyses revealed a statistically significant increase in the area containing binarized GAD67 immunoreactivity in the parasite-infected brains (Fig. 3D). Similarly to VGluT1 staining in Fig. 2, binary image analysis revealed no significant differences in the levels of VGluT1 coverage between mock-infected and parasite-infected brains. The impact of type II ME49 *Toxoplasma* on GAD67 localization was not restricted to the hippocampus. GAD67 localization was similarly affected in the lateral geniculate nucleus (LGN) of the thalamus and in the ventral horn of the spinal cord (see Fig. S1 and S2 in the supplemental material). Moreover, GAD67 remained restricted to neurons since it did not colocalize with either Iba1 (a macrophage marker)-expressing or glial fibrillary acidic protein (GFAP) (an astrocyte marker)-expressing cells (see Fig. S3). These data indicate that type II ME49 *Toxoplasma* globally redistributes GAD67 away from GABAergic nerve terminals.

Toxoplasma-infected mice develop spontaneous seizures. GAD67 clusters within presynaptic terminals due to its tethering to GABAergic synaptic vesicles, which is a process thought to support efficient and rapid neurotransmitter loading into the synaptic vesicles (28, 31). Thus, alterations in GAD67 localization signifi-

cantly impact GABAergic neurotransmission. We therefore assessed the neural and muscular activity of mock-infected or type II ME49 parasite-infected mice with electroencephalography (EEG) and electromyography (EMG) over a period of 5 days. EEGs were recorded from skull electrodes implanted over motor and sensory cortices of the mice, and EMGs were recorded from the nuchal musculature. EEG and EMG analyses revealed significant increases in seizure activity in the infected mice, as defined as greater than 30 s of sustained high-amplitude, high-frequency neural and muscular activity (Fig. 4A and B). Within the 5 days of recording, infected mice experienced an average of 11.3 ± 5.3 (standard error of the mean [SEM]) of these seizure events ($n = 8$) (Fig. 4D). In contrast, mock-infected mice experienced an average of 0.5 ± 0.3 (SEM) of these events during the same recording period ($n = 6$), a difference that was statistically significant ($P < 0.05$ by Tukey-Kramer honestly significant difference [HSD] test) (Fig. 4D). EMG recordings further revealed examples in type II ME49 parasite-infected mice of generalized motor seizures that persisted for >15 min (Fig. 4C). These data indicate that, similarly to human toxoplasmosis patients, type II ME49 *Toxoplasma*-infected mice develop seizures.

It has been widely speculated that interictal spikes of neural activity (rapid [<250 ms] single spikes of neural activity in EEG traces) may be predictive of seizure activity (32). For this reason, we also quantified the number of “spiking” events (defined as high-amplitude brain activity with <1 spike/s) in mock-infected and type II ME49 parasite-infected mice (33). In contrast to the significant impact of infection on seizure activity, no significant differences were seen in the numbers of “spiking” events between infected and mock-infected brains: an average of 18.5 ± 9.4 (SEM) spiking events were observed in type II ME49-infected

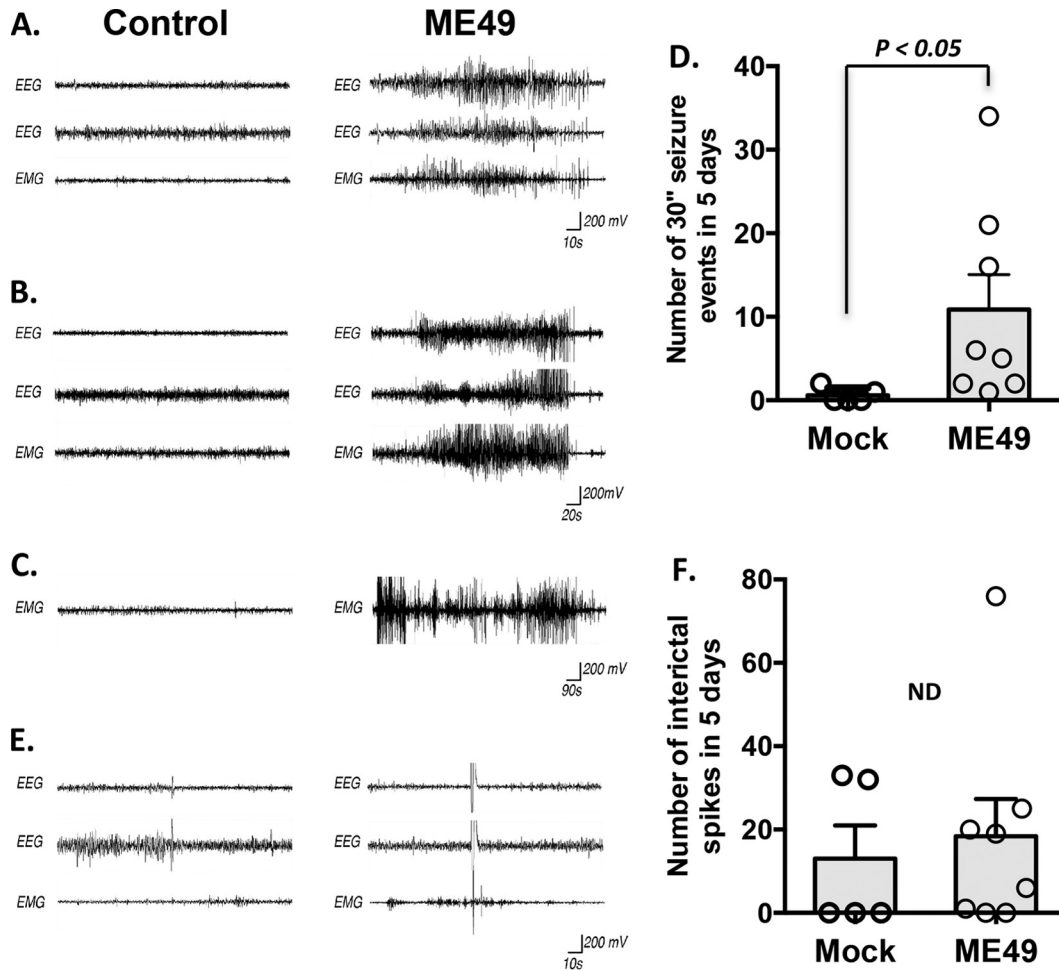


FIG 4 Type II ME49 *Toxoplasma* infection leads to spontaneous seizures in mice. (A and B) Examples of EEG and EMG recordings from control (left column) and type II ME49-infected (right column) mice. Data represent 2 EEG traces and 1 EMG trace that were obtained simultaneously from each animal and are shown in a time-locked state. The recordings represented in panel A show 60 s of neural and muscular activity in control and type II ME49-infected mice. The recordings represented in panel B show 120 s of neural and muscular activity in control and type II ME49-infected mice. (C) EMG recordings from control (left column) and type II ME49-infected (right column) mice. Note that motor seizures persisted for as long as 15 min in this type II ME49-infected mouse. (D) Quantification of the number of seizure events that exceeded 30 s in the 5-day recording period. Bar graph data represent means \pm SEM. Circles represent individual data points. $P < 0.05$ (Tukey-Kramer HSD test). (E) Individual and sparse spikes of neural and muscular activity were observed at similar frequencies in mock-infected and type II ME49-infected mice. (F) Quantification of the number of spiking events in the 5-day recording period. Bar graph data represent means \pm SEM. Circles represent individual data points. The numbers of spikes seen with mock-infected mice were not statistically significantly different from those seen with type II ME49-infected mice ($P > 0.1$ [Tukey-Kramer HSD test]).

brains during 5 days of recording versus 9.5 ± 4.6 spiking events in mock-infected controls ($P > 0.1$ [Tukey-Kramer HSD test]) (Fig. 4E and F).

***Toxoplasma*-infected mice develop more-severe seizures in response to GABA antagonists.** To assess the impact of altered GAD67 localization on epileptogenic activity in type II ME49 *Toxoplasma*-infected mice, we compared the levels of duration and intensity of seizures in mock-infected and parasite-infected mice treated with pentylentetrazol (PTZ)—a GABA receptor antagonist that induces seizures (34, 35). Thirty days after mice were either mock infected or infected with type II ME49 parasite, a single injection of PTZ was administered, EMG traces were recorded, and visual seizure scores were noted every minute for 15 min. Results demonstrated that *Toxoplasma* infection increased seizure scores and also decreased the time needed for the mouse data to reach plateau scores (Fig. 5). Thus, the onset and

magnitude of PTZ-induced seizures were increased in parasite-infected mice.

GAD67 relocation between *Toxoplasma* strains is a polymorphic phenotype. Four *Toxoplasma* haplotypes (I to III and X [or 12]) predominate in North America and Europe (15, 36–38). Of these, both type II and III strains are able to disseminate to the brain, establish chronic infections, and form tissue cysts (17, 36, 39). But genetic differences between these strains impact virulence, drug resistance, and specific host-pathogen interactions (40–45). Thus, we tested whether GAD67 localization was similarly affected by type II and III strains by infecting mice with either type II ME49 or CEP type III CEP strain parasites. We found that GAD67 staining in the hippocampi of type III CEP-infected mice closely resembled the punctate staining in uninfected animals (Fig. 6A to C). Binarized images of GAD67 immunoreactivity from mock-infected and type II ME49-infected or type III CEP-

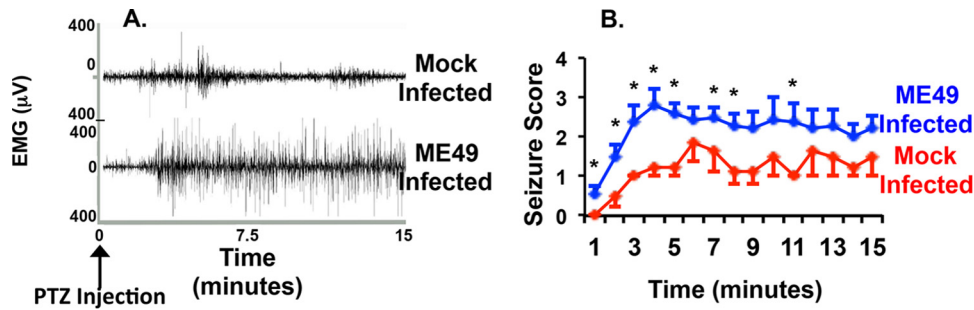


FIG 5 Increased susceptibility of type II ME49 *Toxoplasma*-infected mice to drug-induced seizures. (A) Five days after mice were analyzed for spontaneous seizures, they were injected with PTZ (40 mg/kg), and EEG/EMG data were recorded for 15 min. The data shown represent typical EMG traces from a mock-infected mouse (top) and a type II ME49 parasite-infected mouse (bottom). Notice the increased strength and intensity of muscular activity in the type II ME49 *Toxoplasma*-infected mouse. (B) Averages (\pm SEM) of seizure score data recorded every minute for 15 min after injection of PTZ. $n = 5$ mice for wild-type mice and 9 for type II ME49 mice. *, $P < 0.05$ (unpaired, two-tailed t test).

infected brains were quantified. We found that GAD67 immunoreactivity significantly increased ($P < 0.0001$ by analysis of variance [ANOVA] with Tukey's *post hoc* test) in the type II ME49-infected but not type III CEP-infected brains (Fig. 6D).

To further test our hypothesis that GAD67 mislocalization is associated with the development of seizures in *Toxoplasma*-infected mice, we compared the responses of mock-infected, type II ME49-infected, and type III CEP-infected mice to PTZ. We found that, in contrast to the severe PTZ-induced seizures in the type II ME49-infected mice, the seizures in the type III CEP-infected mice were comparable to those in the mock-infected mice (Fig. 6E). These data therefore indicate that GAD67 mislocalization and seizure susceptibility represent a polymorphic phenotype following *Toxoplasma* infections and are not a general feature of the presence of *Toxoplasma* in the brain.

DISCUSSION

A key function for GABA in the nervous system is to regulate the timing, magnitude, and flow of excitatory neurotransmission, and defects in GABAergic signaling cause seizures (46). Decreasing synaptic release from inhibitory nerve terminals can reduce GABA signaling, and GAD localization to synaptic vesicles in presynaptic terminals leads to decreased GABA release since this ensures efficient GABA synthesis at synaptic termini (28, 31). Here, we show for the first time that a microbial infection of the CNS leads to mislocalization of GAD67. These data are significant because GAD67 is the GAD isoform responsible for the majority of GABA synthesis in the mammalian brain, whereas other GAD isoforms appear to have a more auxiliary role in GABA production and inhibitory neurotransmission (47–49). The anticipated outcome of such alterations would be increased spontaneous and drug-induced epileptogenic activity (50), and we showed that both occur in type II ME49 *Toxoplasma*-infected mice. Thus, these data provide an underlying mechanistic insight into the basis for seizures in toxoplasmic encephalitis patients (51, 52). These data also add to the number of mechanisms (including blood-brain-barrier breakdown, infiltration of immune cells and antibodies, and neuronal cell death) implicated in seizure formation in individuals suffering from intracerebral infections and inflammation (4).

GAD67 localizes to synaptic vesicles through 2 distinct mechanisms. First, it dimerizes via domains within its C-terminal half with GAD65, which is a second GAD that is tethered to synaptic vesicles (31). Second, and independently of GAD65 dimerization,

GAD67 interacts with an unknown synaptic-vesicle-associated factor (28). Currently, we do not know which of these two mechanisms is affected by *Toxoplasma*. Type II ME49 *Toxoplasma* infection of cultured hippocampal neurons does not have a reproducible effect on GAD67 localization (J. M. Brooks and I. J. Blader, unpublished data), suggesting that GAD67 mislocalization is not a direct consequence of *Toxoplasma*-GABAergic neuron interactions. This conclusion is consistent with the finding that altered GAD67 mislocalization did not occur only in regions where either tachyzoites or bradyzoites are found. *Toxoplasma* can inject parasite-encoded effector proteins into neurons and other cells in the brain independently of infection (53). However, GAD67 mislocalization appears to be a more widespread phenomenon than parasite effector injection. Therefore, we hypothesize that alterations in GAD67 distribution and onset of seizures are likely controlled by a host-derived factor(s) whose expression is regulated by a polymorphic *Toxoplasma* gene product. Thus, unraveling the mechanism by which GAD67 is mislocalized will require identification of this polymorphic factor through either forward genetic quantitative trait locus (QTL) screening or reverse genetic candidate gene approaches.

Over the past decade, studies have revealed a connection between *T. gondii* and the onset of various neurological disorders (54). For example, a recent meta-analysis study demonstrated a link between chronic *Toxoplasma* infections and epilepsy (52), which is defined as an ongoing ability to spontaneously develop seizures. Other reports have also indicated that mice and humans chronically infected with *Toxoplasma* have altered behaviors such as reduced fear and inhibitions (55–57). It is tempting to speculate that the changes in GAD67 localization and GABAergic signaling reported here may underlie these long-term effects on the host's nervous system. However, note that our studies were performed in mice infected for 30 days, and although these mice had large numbers of cysts in their brains, they also contained tachyzoites and activated microglia and astrocytes (Fig. 1D; see also Fig. S3 in the supplemental material), indicating that these mice had active toxoplasmic encephalitis. Thus, longer-term studies will be needed to assess how these early effects on GABAergic signaling alter a host's behavior and neurological health.

MATERIALS AND METHODS

Animal infections. All analyses conformed to National Institutes of Health guidelines and protocols approved by the University of Buffalo

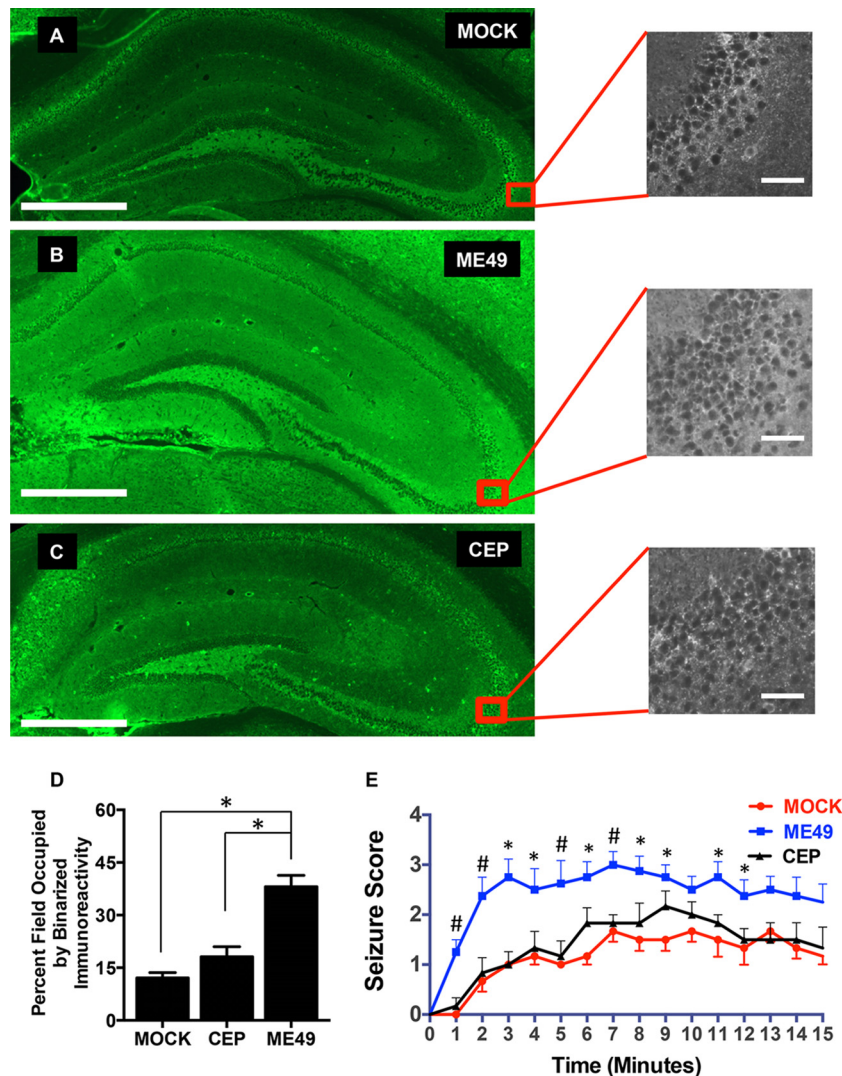


FIG 6 GAD67 mislocalization and drug-induced seizure are polymorphic responses in *Toxoplasma*-infected brains. (A to C) Representative images of coronal sections from mock-infected (A), type II ME49-infected (B), and type III CEP-infected (C) mice 30 days postinfection. Scale bars, 500 μm . Expanded images show high-magnification views of hippocampus CA3 regions. Scale bars, 50 μm . (D) Quantification of percent GAD67 staining following binarizing of images. *, $P < 0.001$ (one-way ANOVA with Tukey's *post hoc* test; $n \geq 3$ mice for each group). (E) Averages (\pm SEM) of seizure scores of mock-infected, type II ME49-infected, and type III CEP-infected mice (30 days postinfection) recorded every minute for 15 min after injection of PTZ. Each group represents score data for a minimum of 6 mice. *, statistically significant difference between type II ME49-infected and mock-infected mice only ($P < 0.05$ [one-way ANOVA with Tukey's *post hoc* test]). #, statistically significant difference between type II ME49 and both mock-infected and type III CEP-infected mice ($P < 0.05$ [one-way ANOVA with Tukey's *post hoc* test]).

(no. MIC12093Y and MIC23035Y) and Virginia Polytechnic Institute and State University (no. 12-136 and 14-090) Institutional Animal Care and Use Committees. The *Toxoplasma* type II ME49 and type III CEP strains were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum as previously described (58). Tissue cysts were passed between C57BL/6 mice by intraperitoneal injection of 10 cysts. Experimental infections were performed by injecting 10 brain cysts into 0.2 ml of DMEM. All mice were purchased from Jackson Laboratories and used at between 6 and 12 weeks of age.

Immunohistochemistry. Mice were exsanguinated and then perfused with 4% paraformaldehyde-phosphate-buffered saline (PBS). Brains were harvested, cryosectioned (16 μm), and stained as described previously (59, 60). Briefly, tissue slides were air dried for 15 min before being incubated with blocking buffer (5% normal goat serum–2.5% bovine serum albumin–0.1% Triton X-100–PBS) for 30 to 60 min. Primary anti-

bodies were diluted in fresh blocking buffer and incubated on coronal brain sections overnight at 4°C. On the following day, slides were washed in PBS and secondary antibodies (diluted 1:1,000 in blocking buffer) were applied to slides for 1 h at room temperature. After being washed in PBS, tissue slides were coverslipped with Vectashield (Vector Laboratories, Burlingame, CA) and images were acquired on either a Zeiss Examiner Z1 LSM 510 or Zeiss LSM 700 confocal microscope. For comparisons of different experimental conditions, images were acquired with and processed using identical parameters. Whole-brain images were captured with a 10 \times objective using an automated motorized stage that imaged individual tiles without overlap. Composite images of each brain were generated by stitching together the individual images using Zeiss imaging software. A minimum of three animals (per experimental condition) were compared in all imaging experiments.

The following antibodies were used: rabbit anti-vGluT1 (1:500 dilu-

tion) and rabbit anti-vGluT2 (1:500 dilution) were from Synaptic Systems, Göttingen, Germany, rabbit anti-GFAP (1:1,000 dilution) was from Dako, Carpinteria, CA, rabbit anti-Iba1 (1:1,000 dilution) was from WAKO Chemicals USA, Inc., Richmond, VA, and mouse anti-GAD67 (1:500 dilution) was from Millipore, Temecula, CA. Neurotrace 640 deep red fluorescent Nissl stain was also used according to the protocol of the manufacturer (Life Technologies, Eugene, OR). Anti-*Toxoplasma* sera (1:250 dilution) that recognize both tachyzoites and bradyzoites were kindly provided by Tajie Harris. All conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA) or Jackson ImmunoResearch (West Grove, PA).

Binary quantification. The area within an image containing immunoreactivity was determined as previously described (61). Briefly, single channels of each image were converted to binary images using ImageJ software (see Fig. S1 in the supplemental material as an example). The percent area containing positive immunoreactivity in each image was then determined in ImageJ. A Student's *t* test was used to test for statistical significance, and at least 9 images were collected from a minimum of three mock-infected and type II ME49-infected animals.

Western blotting. Brains were harvested from decapitated mock-infected or parasite-infected mice, and coronal cortical sections (500 μ m thick) were cut in ice-cold diethylpyrocarbonate (DEPC)-PBS and then flash frozen in liquid nitrogen. The tissue was then homogenized in radioimmunoprecipitation assay (RIPA) buffer supplemented with Protease Inhibitor Cocktail (Roche, Basel, Switzerland) as previously described (58). Lysates (30 μ g/sample) were separated by SDS-PAGE and subjected to Western blotting with antibodies against GAD67 and β -actin (Cell Signaling, Inc., Danvers, MA) and anti-gephyrin antibodies (Synaptic Systems) diluted in Odyssey blocking buffer (Li-COR, Lincoln, NE) containing 0.1% Tween 20. Secondary antibodies were purchased from Li-COR. Images were captured using a Li-COR Odyssey Imaging system and processed using the manufacturer's software.

RT-PCR. RNA was prepared from coronal cortical sections using a Stratagene Absolutely RNA purification kit (La Jolla, CA) and converted to cDNA with Superscript III reverse transcriptase (RT; Invitrogen). Target gene expression levels were determined by RT-PCR using the threshold cycle ($2^{-CT\Delta\Delta}$) method as previously described (62). The following primer sets were used: for the β -actin gene, 5'-GACGGCC AGGTCATCACTATTG-3' and 5'-CCACAGGATCCATACCCAAG A-3'; for the gephyrin gene, 5'-ACCCTCGCCAGAATACCAC-3' and 5'-GCTCATCAGACGGCEPCTCA-3'; and for the GAD1 gene, 5'-CTTCCAGCCAGACAAGCAGTATGA-3' and 5'-TGGGTTGGAG ATGACCATCCGGAA-3'.

Spontaneous-seizure recordings. Unless otherwise noted, all EEG/EMG hardware and software (including a 3-channel, tethered EEG/EMG system) were from Pinnacle Technologies (Lawrence, KS). Mock-infected or parasite-infected mice (30 days postinfection) were anesthetized with ketamine, the skull was exposed, and EEG/EMG head mounts were affixed to the skull with four recording electrode screws, ensuring that all 4 screws were implanted into the skull overlying the motor or sensory cortex in each cerebral hemisphere. EMG leads were implanted into nuchal muscles of the dorsal neck. The skull was then encased in a thick layer of dental acrylic (United States Dental Depot Inc., Ft. Lauderdale, FL) to prevent detachment of the head mount and EMG leads. Mice were allowed to recover for 3 to 5 days and were then tethered to the EEG/EMG recording chamber by connecting a 100 \times preamplifier (containing a 1.0-Hz high-pass EEG filter and a 10-Hz high-pass EMG filter) into the ports of the head mount. Mice remained tethered for 2 to 4 days before recording commenced, and EEG/EMG data were recorded for 5 days under normal light/dark conditions. Mice had free access to water, food pellets, and nestlets. EEG and EMG activity was recorded using *Sirenia* Acquisition software, and data acquisition was performed at a sampling rate of 10,000 Hz. Spontaneous seizures and spikes in EEG/EMG activity were identified and analyzed with *Sirenia* Seizure software. Generalized motor seizures were identified as consisting of high-amplitude, high-frequency

neural and muscular activity that persisted for more than 30 s. A total of 8 type II ME49-infected mice and 6 mock-infected C57/B6 mice were analyzed in these studies.

PTZ-induced seizures. Mice were injected with PTZ (40 mg/kg of body weight)–0.2 ml PBS and then visually monitored for 15 min while blind to their infection status. Seizures were scored manually every minute thereafter with the following scoring criteria: 0, normal activity; 1, reduced motility and prostate position; 2, partial clonus; 3, generalized clonus; 4, tonic-clonic seizure; 5, death. The PTZ dose was chosen since it reproducibly triggers high rates of mild seizures in C57/B6 mice (90% of wild-type mice develop mild seizures) but with a low (~10%) fatality rate (63).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01428-15/-/DCSupplemental>.

Figure S1, JPG file, 0.9 MB.

Figure S2, JPG file, 1.6 MB.

Figure S3, TIF file, 2.7 MB.

ACKNOWLEDGMENTS

We thank Tajie Harris for providing the anti-*Toxoplasma* sera and Emma Wilson for helpful discussions and sharing of unpublished data.

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