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What elements of the inflammatory system are necessary for epileptogenesis in vitro?

Inflammation independent epileptogenesis in vitro

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Abstract

Epileptogenesis *in vivo* can be altered by manipulation of molecules such as cytokines and complement that subserve intercellular signaling in both the inflammatory and central nervous systems. Because of the dual roles of these signaling molecules, it has been difficult to precisely define the role of systemic inflammation in epileptogenesis. Organotypic hippocampal brain slices can be maintained in culture independently of the systemic inflammatory system, and the rapid course of epileptogenesis in these cultures supports the idea that inflammation is not necessary for epilepsy. However, this preparation still retains key cellular inflammatory mediators. Here we found that rodent hippocampal organotypic slice cultures depleted of T lymphocytes and microglia developed epileptic activity at essentially the same rate and to similar degrees of severity as matched control slice cultures. These data support the idea that although the inflammatory system, neurons, and glia share key intercellular signaling molecules, neither systemic nor CNS-specific cellular elements of the immune and inflammatory systems are necessary components of epileptogenesis.

Key words: inflammatory, immune system, microglia, epileptogenesis

Significance Statement

The inflammatory and central nervous systems share many signaling molecules, compromising the utility of traditional pharmacological and knockout approaches in defining the role of inflammation in CNS disorders such as epilepsy. In an in vitro model of post-traumatic epileptogenesis, the development of epilepsy proceeded in the absence of the systemic inflammatory system, and was unaffected by removal of cellular mediators of inflammation including macrophages and T-lymphocytes. These results are not meant to disprove the idea that “inflammation causes epilepsy” but rather circumscribe the overlap between the

67 inflammatory system vs. the CNS mechanisms that are operative during post-traumatic
68 epileptogenesis.

69

70 **Introduction**

71 Local and systemic inflammation may play a role in epileptogenesis (Vezzani et al., 2012). The
72 brain is largely shielded from the systemic immune system by the blood brain barrier (Lampron
73 et al., 2013), but an active innate immune system converges to activate phagocytic final
74 effectors, the microglia. These cells comprise 10% of the cells in the brain (Benarroch, 2013),
75 where lymphocytes are also present (Ravizza et al., 2008).

76 There is robust pathological evidence for the involvement of cellular elements of the immune
77 and inflammatory systems, including T lymphocytes and microglia, in epilepsy syndromes such
78 as Rassmussen's encephalitis (Bien et al., 2002; Granata and Andermann, 2013). Other
79 epilepsy syndromes are driven by humoral elements of the immune system including antibodies
80 to N-methyl-D-aspartate (NMDA) receptors and other neuronal proteins (Davis and Dalmau,
81 2013; Gresa-Arribas et al., 2013). Inflammatory mediators including the cytokines interleukin
82 (IL)-6, IL-1β, complement cascade factor C1q, transforming growth factor (TGF)-β, and tumor
83 necrosis factor alpha (TNFα) are up-regulated in human epileptic tissue (Vezzani et al., 2012;
84 Liimatainen et al., 2013). These inflammatory mediators are increased experimentally by
85 prolonged seizures (Minami et al., 1991; Vezzani et al., 1999). Manipulation of immune and
86 inflammatory mediators alter seizures. Inhibition of leukocyte infiltration of the blood brain barrier
87 prevented experimental epilepsy (Fabene et al., 2008), and IL-1β antagonists reduced induced
88 seizures (Librizzi et al., 2012).

89 The interpretation of these intriguing findings is complicated by two issues: first, these
90 inflammatory mediators also play important roles in physiological synaptic modifications,

91 including those that underlie learning and memory. The cytokines implicated in epilepsy are also
92 produced by neurons and astrocytes in the normal, uninflamed brain (Vitkovic et al., 2000;
93 Yirmiya and Goshen, 2011; Pribiag and Stellwagen, 2014). For example, IL-1β and IL-6 are
94 increased by synaptic stimuli that induce physiological synaptic plasticity (Schneider et al.,
95 1998; Balschun et al., 2004). Cellular and cytokine elements of the immune and inflammatory
96 systems, including T lymphocytes and IL-1β, play essential roles in learning and memory
97 (Schneider et al., 1998; Yirmiya et al., 2002; Kipnis et al., 2004). Homeostatic scaling of
98 synaptic strength entails cytokines including TNFα, and possibly IL-1β (Pribiag and Stellwagen,
99 2014). Microglia participate in physiological anatomical synaptic alterations (Paolicelli et al.,
100 2011; Schafer et al., 2012). Microglial activation after epileptogenic injuries is complex and not
101 strongly correlated with neuronal loss (Papageorgiou et al. 2014). Kindling studies have not
102 found significant increases in microglia or cytokines (Khurgel et al., 1995; Tooyama et al., 2002;
103 Aalbers et al., 2014), and experimental antiepileptogenic therapies are not associated with
104 changes in microglial activation (van Vliet et al., 2012). Recent studies exploring the expression
105 of cytokines in temporal lobe epilepsy have not found evidence that inflammation is a necessary
106 for element of hippocampal sclerosis (Aalbers et al., 2014).

107 Second, inflammation and cell loss coexist (Tooyama et al., 2002). The clinical and
108 experimental situations in which the inflammatory system has been implicated in
109 epileptogenesis are also characterized by neuronal death and astrogliosis (Ravizza et al.,
110 2008). Neuronal death and astrogliosis are also epileptogenic (Dudek and Staley, 2012),
111 although it has not been demonstrated whether inflammation and cell loss act independently.

112 Studying the relation of inflammation and epilepsy by removal of elements of the systemic
113 inflammatory system *in vivo* is impractical due to ongoing monocytic immigration from the
114 bloodstream (Ransohoff and Cardona, 2010). As a proof-of-concept experiment to test the
115 necessity of the immune and inflammatory systems in epileptogenesis, we used a reduced *in*

vitro system in which the cellular effector elements of these systems can be removed and the effects on epileptogenesis observed. The hippocampal organotypic slice culture is a well-characterized *in vitro* system of epileptogenesis (McBain et al., 1989; Gutnick et al. 1989; Bausch and McNamara, 2000; Dyhrfjeld-Johnsen et al., 2010; Marchi et al., 2011; Berdichevsky et al., 2012, 2013; Ransohoff and Brown, 2012; Albus et al. 2013). Blood vessels are present but the blood brain barrier is not operative, and the systemic immune system is not present. We characterized epileptogenesis in preparations from control and T lymphocyte-deficient nude mice, with and without microglial depletion by clodronate (Kohl et al. 2003; Kumamaru et al., 2012).

Materials & Methods

Culture of organotypic hippocampal slices

All animal-use protocols were in accordance with the guidelines of the National Institutes of Health and our institution for Comparative Medicine on the use of laboratory animals, and approved by the Subcommittee on Research and Animal Care. Hippocampal slices were prepared at postnatal day 6 to 8 from C57BL/6 mice or T lymphocyte-deficient nude mice (Foxn1nu/Foxn1nu, http://jaxmice.jax.org/strain/002019.html) or Sprague-Dawley rats of either sex. 400 µm thick slices from a McIlwain tissue chopper (Mickle Laboratory Eng. Co., Surrey, United Kingdom) were mounted in clots of chicken plasma (Cocalico Biologicals, Reamstown, PA) and thrombin (Sigma-Aldrich, St. Louis, MO) on poly-L-lysine coated glass coverslips (Electron Microscopy Sciences, Hatfield, PA). Slices were incubated in roller tubes (Nunc, Roskilde, Denmark) at 37 °C within 750 µl of NeurobasalA/B27 medium supplemented with 0.5 mM GlutaMAX and 30 µg/ml gentamicin (Invitrogen). This concentration of gentamycin was less

than half the lowest concentration demonstrated to induce regenerative activity *in vitro* (Grøndahl and Langmoen 1993). Culture media were changed biweekly.

Field potential recording

Extracellular field potentials were recorded in area CA1 or CA3 of hippocampal slices in a conventional submerged chamber using tungsten coated microelectrodes (diameter, 50 μM). Oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid containing 126 mM NaCl, 3.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 11 mM glucose (pH 7.4), were continuously perfused at 33 ± 0.5 °C. Flow rate was 2.5 ml/min. Before actual recording, slices were allowed to stabilize in the recording chamber for at least 1 hour, and then extracellular field potentials were recorded for 1 hour in each slice. pCLAMP 8.2 (Axon Instruments) was used for data acquisition. Recordings were sampled at 10 KHz and filtered from 1 Hz to 1 kHz. Seizure-like activity (SLA) was defined as high amplitude (X3 baseline), high frequency (>10Hz) spikes followed by afterdischarges, with the duration of the spike and afterdischarge complex lasting more than 5 seconds. Incidence of SLA and numbers of slices and animal in the experiment are summarized in Table 1.

Chemical depletion of microglia

Clodronate is a selective macrophagic and microglial toxin without evidence of injury to other cellular components (Kohl et al. 2003; Kumamaru et al., 2012). Perivascular macrophages could be retained in slice cultures, but these cells are also depleted by clodromate at concentrations below those needed to deplete microglia (Polfliet et al. 2001). To assess whether microglia alter the course of epileptogenesis or ictogenesis, we incubated slice cultures in clodronate encapsulated liposomes (Clodrosome, purchased at www.clodrosome.com, containing 17 mM of clodronate disodium salt, 24 mM of phosphatidylcholine and 11 mM of cholesterol suspended

in phosphate buffered saline (PBS)) for 3 to 6 days as specified in Results (Fig. 4*A*). During these incubations, Clodrosome was replenished with each media change.

Immunohistochemistry of organotypic hippocampal slices

Slices were fixed overnight with 4% paraformaldehyde at room temperature. They were washed with PBS several times and stored in PBS at 4 °C until use. The hippocampi were scraped out gently from the bottom of petri dishes or coverslips and then treated with 0.5%TritonX-100 overnight. Blocking was done with 20% bovine serum albumin (BSA) for 4 hours at room temperature on a shaker. We incubated tissues overnight with the microglial specific antibody anti-ionic calcium binding adapter molecule 1 (iba-1, 1:1000, Waco Chemicals), a protein present in both perivascular macrophages and microglia (Sasaki et al. 2001), with / without the neuron-specific antibody anti-NeuN (1:50, Millipore) as primary antibodies. Goat anti-rabbit IgG Alexa 594 (1:200, invitrogen) with / without goat anti-mouse IgG Alexa 405 (1:200, Invitrogen) were used as secondary antibodies. All antibodies were dissolved in 5% BSA. 4',6-diamidino-2-phenylindole (DAPI) (300nM, Sigma Aldrich) was added for nuclear counter-staining in some slices. Imaging was done using a two-photon microscope (Olympus Optical) with 20x water immersion objective. For cell counts, the pyramidal layer of CA1 was centered on the Y axis of the microscopic field. The X coordinates of the field were determined by centering an imaginary line perpendicular to the pyramidal layer that passed through the middle of the superior blade of dentate gyrus. Ten serial XY planes images with best intensities were acquired at 2 μm vertical (Z) intervals in the CA1 subfield. Cell counting for NeuN(+) or Iba-1(+) was performed in Z-stack images from whole microscopic field using ImageJ software.

Lactate and LDH assays of clodronate effects

Organotypic hippocampal slice cultures from C57BL/6 mice were cultured in poly-D-lysine coated 6-well tissue culture plates. Three slices were treated with clodronate encapsulated

liposome (final concentration 0.2 mg/ml) and three slices were treated with an equal concentration of empty liposome from DIV0 to 6. All six slices were prepared from the same animal. Following a thorough wash-out on DIV6, culture medium with PBS was applied to both groups of slices from DIV6 to 28. Culture media were collected and changed biweekly. Images of all slices were obtained at media changes via brightfield microscopy with an Olympus CKX41 inverted microscope. Lactate was used as a biomarker of ictal activity (Berdichevsky et al. 2013) nd and LDH concentrations were used as an assay of cell death (Berdichevsky et al. 2012). measured in spent culture medium collected during the biweekly media changes.

Statistical analyses

All values are expressed as mean ± SEM. Statistical significance were evaluated with Student t-tests without or with Sidak correction for multiple comparisons where indicated, Fisher's exact test, or one-way/two-way ANOVA as indicated Alpha P value < 0.05 is considered to be significantly different.

Results

Clodronate depletes microglia in organotypic slices

The concentration and duration of clodronate treatment in organotypic slices was optimized to obtain maximal microglial depletion. An equal concentration of empty liposomes was used as the control condition in all experiments utilizing clodronate. When clodronate (final concentration of 0.2 mg/ml in the culture media) was applied for 3 days to rat slices from DIV16 to 19, the number of microglia counted in the CA1 subfield decreased by 70.4% (95.5 ± 10.9 vs. 28.25 ± 9.8 per field; $n = 4$; $p = 0.004^{a}$; Fig. 1*B*). The remaining microglia were small and rounded in shape, and had few ramified processes (Fig. 1*A*). To increase microglial depletion, the duration of treatment was extended up to 6 days (DIV16 to 22). This reduced microglia by 96.2% (91.8 ±

15.5 vs. 3.5 ± 3.2; n = 4; p = 0.001[b]; Fig. 1*B*) compared to controls. The number of Iba-1 (-) cells was not different between groups (320.3 ± 8.6 per field in clodronate group vs. 325.5 ± 10.3 per field in control group, n = 4, p = 0.77[c]). The number of CA1 microglia in control slices treated with empty liposomes was not different from slices treated with an equal volume of PBS. Following clodronate depletion, microglia were not regenerated even after 3 weeks in culture (Fig. 1*C*), indicating that clodronate also effectively eliminated microglial progenitor cells.

Microglial depletion has no anticonvulsant effects in rat slices

We compared spontaneous SLAs at DIV22 according to the presence or absence of microglia (n = 11 each group). Equal numbers of slices from each animal were allocated to clodronate or control groups. Extracellular field potentials were recorded for 1 hour in each slice. All slices showed at least one SLA in the microglia-depleted group and only 72.7% showed SLA in the control group (p = 0.07[d]. Fig. 2*C*). The frequency of SLA in microglia-depleted slices was significantly higher than control (13.3 ± 3.1 vs.3.9 ± 2.1 SLA/hr; p = 0.02[e]; Fig. 2*D*). Total duration (13.4 ± 4.1 vs. 4.3 ± 3.5 min; p = 0.11[f]; Fig. 2*E*) and mean duration of each SLA (2.1 ± 0.9 vs. 0.5 ± 0.2 min; p = 0.14[g]; Fig. 2*F*) were longer in microglia-depleted slices than control, but the differences did not reach statistical significance.

Microglial depletion has no antiepileptogenic effects in rat slices

The preceding experiments indicate that microglial depletion had no anticonvulsant effect on seizure activity in cultured slices in which epilepsy was established. To evaluate the role of microglia on epileptogenesis, we applied clodronate before the onset of seizures, that is, from the beginning of culture (DIV0) to DIV6. In the organotypic hippocampal slice model, this time period is considered as the 'latent' period, i.e. the time between brain injury and the development of spontaneous seizure activity (Berdichevsky et al., 2012). Following microglial depletion, field potentials were recorded at DIV6, 12, and 22 (Fig. 3*A*). SLAs were observed in

83.3% (vs. 50% in control, n = 6 each group, p = 0.24[h]), 83.3% (vs. 66.7% in control, n = 6 each group, p = 0.52[i]) and 37.5% (vs. 25.0% in control, n = 8 each group, p = 0.60[j]) in microglia-depleted sliceson recoding at DIV6, 12 and 22, respectively. Overall, the proportions of epileptic slices, which were defined as having more than one seizure during the observation time, were not statistically different between the microglia-depleted and control groups (Fig. 3*B*). The values for SLA frequency, duty cycle, and duration are listed in Table 2. On recording at DIV6, and 22, there were no significant differences in these parameters between microglia-depleted and control slices. These three parameters tended to be increased in microglia-depleted slices at DIV12, but the differences did not reach significance between the groups (Fig. 3*C,D,E*). Overall, there were no statistical differences between groups or ages[k].

Microglial depletion has no anticonvulsant or antiepileptogenic effects in mice

Organotypic slices from wild type C57BL/6 mice usually demonstrated more frequent SLA comparing with age-matched rat slices. To test the generalizability of the results from rat slices, we repeated the experiments described above in murine hippocampal slice cultures. Depletion of microglia by clodronate exposure from DIV0 and 6 did not alter spontaneous SLAs recorded at DIV6 (Fig. 4*A*). SLA frequency was 12.5 ± 2.3 per hour in microglia-depleted slices (n = 4) and 10.8 ± 1.9 per hour in control slices (n = 5) (p = 0.31[l]). Other parameters, total duration (5.0 ± 0.7 min vs. 4.3 ± 1.2 min; p = 0.36[m]) and mean duration (24.6 ± 1.3 sec vs. 24.3 ± 5.9 sec; p = 0.56[n]) in microglia-depleted slices were also similar to control. Next, we applied clodronate from DIV6 to DIV12 and recorded at DIV12 to assess the effect of microglial depletion on ictogenesis. The frequency was 12.0 ± 5.2 per hour for microglia-depleted (n = 4) and 9.7 ± 3.1 per hour for control (n = 6) (p = 0.99[o]). Total SLA duration and mean SLA duration were 8.0 ± 2.9 min, 37.8 ± 11.3 sec for microglia-depleted and 5.9 ± 1.9 min, 53.7 ± 23.8 sec for control, respectively and these differences were not statistically significant (p = 0.89[p] for total duration, p = 0.62[q] for mean

duration). We conclude that microglial depletion at these time periods in murine organotypic hippocampal slice cultures do not alter epileptogenesis or ictogenesis.

Epileptogenesis is not dependent on T-lymphocytes and / or microglia

T lymphocytes are a part of the adaptive immune system that play an important role in activating the innate immune, i.e. inflammatory system of the brain (Ransohoff and Brown, 2012) and have been hypothesized to contribute to epileptogenesis (Fabene et al. 2008; Marchi et al., 2011). To investigate the role of this cellular component of the immune system on epilepsy more precisely, we compared wild-type organotypic slices to organotypic slices depleted of either T lymphocytes, or both microglia and T lymphocytes by treating slices prepared from nude mice with clodronate (0.02 mg/ml). The numbers of microglia were counted in area CA1 in the three different preparations (slice cultures from wild type, nude (T lymphocyte-deficient) mice, and slices from nude mice treated with clodronate, n = 5-6 each group). Nude mice showed fewer microglia per field ($p = 0.003^{r}$ vs. rat, $p = 0.01^{s}$ vs. WT mouse; Fig. 5*A*). However, there was no correlation between the density of microglia and the incidence of epileptic slices across species (Fig. 5*A*). In mice, a lower concentration (0.02 mg/ml) of clodronate eliminated microglia as effectively as the higher concentration used in rat slices (0.2 mg/ml), so we used the lower concentration in the following experiments. Clodronate at 0.02 mg/ml did not alter neuronal populations (Fig. 5*C,D*). Epileptogenesis proceeded at a similar rate in clodronate-treated nude mouse slices as in control slices. Recordings at DIV12 demonstrated SLAs in 40.0% of double deficient slices (n = 10) vs. 62.5% in control slices prepared from nude mice (n = 8); ($p = 0.36^{t}$). Analysis of seizure parameters (Fig. 5*E*) revealed that the total duration of SLAs in clodronate-treated nude mouse slices was not different from that of control slices (20.0 ± 12.0 sec vs. 33.1 ± 18.4 sec; $p = 0.54^{u}$). SLAs were rarely observed in both groups. However, the frequency of SLAs in the double deficient preparations was decreased compared to controls (0.5 ± 0.2

SLAs/hr vs. 1.6 ± 0.5 SLAs/hr; $p = 0.051$[v]). Thus, we conclude that epileptogenesis is not dependent on T-lymphocytes and / or microglia in this model.

The time course of epileptogenesis and ictal cell death are not altered by the absence of microglia

As epileptogenesis progresses in organotypic slices, more SLAs are generated (Dyhrfjeld-Johnsen et al., 2010), and just as in human epilepsy (Lazeyras et al., 2000; Canas et al., 2010), the increased epileptiform activity is associated with increases in local lactate (Berdichevsky et al., 2013). Ictal excitotoxic neuronal injury releases the cytoplasmic lactate dehydrogenase (LDH) to the culture media (Berdichevsky et al., 2012, 2013). To be certain that we had not missed a critical compensation that normalized epileptogenesis in the slices depleted of microglia, we used the lactate and LDH assays to follow the detailed time course of epileptogenesis *in vitro*. Mouse hippocampal slices were exposed to clodronate (0.2 mg/ml) at DIV0-6 (Fig. 6*A,B*), and lactate and LDH levels were assayed at each subsequent media change in 3 depleted vs. 3 control slices from the same animal. Cumulative lactate production was slightly reduced in microglial-deficient slices (Fig. 6*C*), but this reduction was in line with the 10% reduction in total cell number due to microglial depletion (Benarroch et al., 2013), did not reach statistical significance[w] at any time point, and was much less than the lactate reduction during anticonvulsant treatment (Berdichevsky et al., 2012). Cumulative LDH release was increased early in the clodronate-treated slices (Fig. 6*D*), consistent with microglial cell death, but this difference was not sustained statistically[x].

Statistical table

	Data structure	Type of test	Power

a	Normally distributed	t-test	0.004
b	Normally distributed	t-test	0.001
c	Normally distributed	t-test	0.77
d	Categorical	Fisher's exact test	0.07
e	Normally distributed	One-way ANOVA	0.02
f	Normally distributed	One-way ANOVA	0.11
g	Normal distributed	One-way ANOVA	0.14
h	Categorical	Fisher's exact test	0.24
i	Categorical	Fisher's exact test	0.52
j	Categorical	Fisher's exact test	0.60
k	Normally distributed	Two-way ANOVA	0.39 for frequency, 0.46 for total duration and 0.30 for mean duration
l	Normally distributed	One-way ANOVA	0.31
m	Normally distributed	One-way ANOVA	0.36
n	Normally distributed	One-way ANOVA	0.56
o	Normally distributed	One-way ANOVA	0.99
p	Normally distributed	One-way ANOVA	0.89

q	Normally distributed	One-way ANOVA	0.62
r	Normally distributed	t-test	0.003
s	Normally distributed	t-test	0.01
t	Categorical	Fisher's exact test	0.36
u	Normally distributed	One-way ANOVA	0.54
v	Normally distributed	One-way ANOVA	0.051
w*	Normally distributed	t-test with Sidak correction	0.02, 0.42, 0.08, 0.07, 0.09, 0.08, 0.08, and 0.13 at each time point
x*	Normally distributed	t-test with Sidak correction	0.10, 0.02, 0.001, 0.002, 0.002, 0.002, 0.007, 0.01 at each time point

* w,x : $\alpha_{SID} = 1-(1-\alpha)^{1/m}$, $\alpha=0.05$, m=1,2,3,4,5,6,7,8. $\alpha_{SID(MAX)} = 0.06$

Discussion

In this *in vitro* study of post-traumatic epileptogenesis, electrophysiological recordings as well as lactate and LDH assays were not significantly different in slices depleted of either microglia, or both T lymphocytes and microglia. We conclude that the systemic immune system, a compromised blood brain barrier, and key cellular elements of the immune and inflammatory systems, including T lymphocytes and microglia, are not necessary for epileptogenesis, at least in this in vitro model.

Limitations

This study utilized a well-characterized *in vitro* model of epileptogenesis, the rodent hippocampal organotypic slice preparation. Our goal was to test whether cellular effectors of inflammation were necessary elements of epileptogenesis, so we did not attempt to quantify the number of T lymphoctyes present in the slices from wild-type vs. nude mice, nor did we attempt to quantify the relative effects of T lymphocyte depletion on the rate of epileptogenesis. In this *in vitro* preparation, the control slices are also separated from the systemic immune system. Thus we are not able to resolve whether epileptogenesis might proceed at a more rapid rate if systemic elements of the immune system were interacting across the blood brain barrier (Fabene et al., 2008). However, the cell loss (Berdichevsky et al., 2012), inflammatory reaction (degree of microglial activation in Fig. 1*A*), and rate of epileptogenesis (Dhyrfjeld-Johnesen et al., 2010; Berdichevsky et al., 2012) in the organotypic slice culture matches or exceeds what is observed *in vivo* (Tooyama et al., 2002; Ravizza et al., 2008; Williams et al., 2009; van Vliet et al., 2012) so there is no reason to expect that inflammation, epileptogenesis, or ictogenesis has been compromised in the control slices.

The epileptic activity that develops in organotypic slices is severe: although SLA can be temporarily suppressed with phenytoin, SLA recrudesces after three weeks in culture (Berdichevsky et al., 2012). Although this recrudescence parallels human post-traumatic epilepsy (Temkin et al., 1990; Temkin, 2009), it is possible that a less severe epilepsy model would reveal modulatory effects of immunity or inflammation more readily than the organotypic slice culture model. Thus we cannot conclude from these experiments that the immune and inflammatory systems are not involved in all epileptogeneses, but only that key cellular elements of these systems are not necessary for post-traumatic epileptogenesis, and that so far, this has only been demonstrated *in vitro*.

We did observe differences in epileptogenesis between murine slices from C57BL/6 mice (Fig. 4) vs. slice cultures from nude mice (Fig. 5). However, inbred mouse strains have variable rates of epileptogenesis (2009), wild type controls are not available for nude mice, and epileptogenesis in slices from nude mice were comparable to slices from rats (Fig. 5*A*). Thus we can deduce that epileptogenesis proceeds in the absence of T lymphocytes, but we cannot make conclusions regarding potential influences of T lymphocytes on the rate of epileptogenesis. Overall there was no correlation between microglial counts and the fraction of epileptic slices across species (Fig. 5*A*). In slices from animals other than nude mice, the increased seizure frequency after microglia depletion may reflect the neuroprotective actions of microglia (Mosser and Edwards, 2008; Smith et al., 2012; Benarroch, 2013), although the magnitude of effects were not consistent between strains and species. These minor differences in epileptogenesis do not diminish the importance of the central finding that slices without cellular mediators of inflammation exhibit robust epileptogenesis. This implies that the full inflammatory cascade cannot be a necessary component of all forms of epileptogenesis.

Because microglia were progressively eliminated from 0 to 6 days after trauma, our experiments do not preclude a necessary but very early role for microglia in epileptogenesis. However, manipulation of inflammatory mediators during this interval are ineffective *in vivo* (Noe et al., 2013), indicating that if inflammation had a necessary role in epileptogenesis, it would extend beyond the phase during which the slice cultures still contained microglia.

Although *in vitro* experiments must be extrapolated with caution, it should also be born in mind that there is no feasible way to remove the blood brain barrier, systemic immune system, and local cellular elements of the immune and inflammatory systems from current *in vivo* models.

Implications

359 Determining whether a particular system is involved in a mechanism of neural plasticity is
360 complex. This issue has been reviewed in detail for the process of long-term potentiation of
361 synaptic strength (Sanes and Lichtman, 1999). Obstacles to be overcome include the
362 heterogeneity of the process of epileptogenesis. For example, Rasmussen's encephalitis or
363 anti-NMDAR encephalitis seem unlikely to entail precisely the same molecular pathophysiology
364 as post-traumatic epilepsy. Nevertheless, inflammation has been strongly implicated in prior
365 studies of epilepsy associated with hippocampal sclerosis (Ravizza et al., 2008), which is not
366 considered to be an immune-mediated injury. However, more recent studies have not supported
367 a necessary role for inflammation in hippocampal sclerosis (Aalbers et al., 2014). Another
368 problem is distinguishing whether a system modulates or mediates, i.e. is necessary for, a
369 mechanism of plasticity. Mechanisms of plasticity that involve multiple large networks, each of
370 which is comprised of many types of cells and synapses, are particularly prone to these
371 interpretational difficulties.

372 The interpretation of experimental results is further complicated because some mediators of the
373 inflammatory response are also involved in physiological neural plasticity. Thus when these
374 inflammatory mediators are blocked, the subsequent effects on epilepsy are often interpreted to
375 be a consequence of the effects of the blockade on the inflammatory system. However it is
376 equally possible that the effects on epilepsy are a consequence of the blockade's interference
377 with normal mechanisms of synaptic plasticity. This issue is more easily appreciated when
378 considered for systems that are anatomically separated. For example, the gastrointestinal
379 system is not considered to be a mediator of neural plasticity because of the effects of
380 antagonists of vasoactive intestinal polypeptide (VIP) or cholecystokinin (CCK) on
381 epileptogenesis (Dobolyi et al., 2014). The effects of IL-1β, IL-6, TNFα antagonists on
382 epileptogenesis provide the same strength of evidence that the inflammatory system is a key
383 mediator of epileptogenesis.

One approach to these interpretational difficulties is to remove as many of the "upstream" elements of inflammation as possible and measure the effects on epileptogenesis. Based on our observations using the *in vitro* organotypic hippocampal slice culture model of post-traumatic epileptogenesis, cellular elements of the immune and inflammatory systems were not necessary for epileptogenesis. We look forward to other laboratories' dissection of this problem *in vivo* and *in vitro*, including for example focused genetic deletion experiments with wild-type littermate controls. Because microglia are considered to be the final common phagocytic pathway of the brain's inflammatory response (Schafer et al., 2013), we did not extend these experiments to consider the effects of antagonists of individual chemical mediators of the inflammatory response. Such experiments would be difficult to interpret for the reasons described above. Thus the immune and inflammatory systems may modulate, but do not mediate, at least some forms of epileptogenesis.

In the epilepsy syndromes in which the involvement of the immune and inflammatory systems appear more obvious, such as Rasmussen's encephalitis and NMDAR antibody encephalitis, we do not yet know whether the ongoing activity of the these systems is necessary, or whether they are involved in a temporally restricted number of stages of epileptogenesis, and are not subsequently necessary once those stages are complete. For example, in Rasmussen's encephalitis, anti-inflammatory therapy is ineffective (Freeman, 2005), indicating that at some point in the process of epileptogenesis, epilepsy becomes independent of the inflammatory system. On the other hand, NMDAR antibody encephalitis is often responsive to immune therapy (Titulaer et al., 2013).

In light of the current results and the effects of anti-inflammatory and immune therapies on human and experimental epilepsies, the role of inflammatory mediators in epilepsy can be interpreted in three ways: First, there are mechanisms of epileptogenesis that are independent of the immune and inflammatory systems, as demonstrated here. Second, there are epilepsy

syndromes for which the immune and / or inflammatory system may be a necessary component of at least some stages of epileptogenesis, although direct evidence for this has not yet been developed. Third, the inflammatory system may not contribute directly to epileptogenesis, but manipulation of the shared mediators of synaptic plasticity and inflammation may produce beneficial anticonvulsant or antiepileptogenic effects.

Future directions

Developing simplified *in vitro* models of epilepsy syndromes in which the immune system is suspected to play a central role, such as NMDAR antibody encephalitis, will permit further testing of the role of immunity in epilepsy. Such studies might address a question raised by the current results: if inflammation is not necessary for epileptogenesis, does it nevertheless accelerate or otherwise modulate epileptogenesis? Regardless of the role of the inflammatory system in epilepsy, it will be important to pursue the anticonvulsant and antiepileptogenic effects of the antagonists of shared mediators of inflammation and synaptic plasticity, including cyclooxygenase products, IL-1β, IL-6, and TNFα.

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575 **Figure legends**

576 **Figure 1.** Elimination of microglia using liposomal clodronate from organotypic hippocampal
577 slices of rat. ***A,*** Iba-1-positive cells (microglia and macrophages) in CA1 were depleted by using
578 liposomal clodronate (0.2 mg/ml). ***B,*** Treatment for 3 days (from DIV9 to 12) decreased the
579 number of microglia comparing liposome-control group as well as saline control group. Six-day
580 treatment from DIV16 to DIV22, eliminated more Iba-1(+) cells than 3-day treatment (96.2% vs.
581 70.4%, n = 4 per group, p = 0.07). ***C,*** Microglial depletion persisted 16 days after washout. Iba-
582 1, anti-ionic calcium binding protein-1, DAPI, 4',6-diamidino-2-phenylindole. All values are
583 expressed as mean ± SEM. *p<0.05. Scale bar, 100μm.

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596 **Figure 2.** Effect of microglial depletion on ictogenesis in cultured rat slices. ***A,*** Schematic
597 drawing of experiment protocol. ***B,*** Representative traces of field potentials recorded at DIV22
598 show that microglia-depleted slice had more frequent and longer seizure-like activities in
599 comparison with control slice. ***C,*** Bar graphs indicate the percentage of slices with more than
600 one seizure-like activity during observation period in each group. ***D-F,*** The frequency of seizure-
601 like activities was significantly greater in the microglia-depleted group (13.3 ± 3.1 vs.3.9 ± 2.1/hr,
602 n = 11 per group, p = 0.02). All values are expressed as mean ± SEM, *p<0.05.

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617 **Figure 3.** Effect of microglial depletion on epileptogenesis in cultured rat slices. ***A,*** Liposomal
618 clodronate or liposome-control was exposed to slices from DIV0 to 6 and spontaneous seizure-
619 like activities were recorded at DIV6, 12 or 22 according to the indicated protocols. ***B,*** The
620 proportions of slices demonstrating seizure-like activity during recording were not different
621 between microglia-negative group and control group (n = 6-8 per group, p = 0.24, 0.52, and
622 0.60. respectively). ***C-E,*** Seizure frequency, total recorded seizure time and mean seizure
623 duration tend to be higher in slices depleted of microglia, although none of these differences
624 were statistically different (n = 6-8 per group, p = 0.24, 0.30, and 0.28, respectively). N.S, not
625 significant. All values are expressed as mean ± SEM.

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638 **Figure 4.** Effect of microglial depletion on epileptogenesis and ictogenesis in wild-type mice
639 slices. ***A,*** Data was recorded at DIV6 after exposure of liposome clodronate from DIV0 to 6. ***B,***
640 Data was recorded at DIV12 after exposure of liposomal clodronate from DIV6 to 12. Microglial
641 depletion did not alter the frequency, total duration and mean duration of seizure-like activities
642 (*n* = 4-5 per group). ***C,*** Representative traces recorded at DIV6 from microglia-depleted and
643 control group, shows similar patterns of spontaneous seizure-like activities. All values are
644 expressed as mean ± SEM.

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Figure 5. Microglial depletion from slices of nude mouse. ***A,*** Comparison of the density of Iba1-positive cells in area CA1 in control slices from different species (*n* = 5-6 per group), vs. the percentage of slices that displayed seizure activity (*n* = 5-8 per group; different slices used for Iba-1 staining and recording). Across species, there was no significant correlation between microglial density and fraction of epileptic slices (R= -.10, *p* = 0.94). ***B,*** Examples of hippocampal cultures from nude mice at each time point. Liposomal clodronate (upper, 0.02 mg/ml) or liposome-control (lower) was applied from DIV0 to 6. ***C,D,*** Double immunostaining was performed with NeuN and Iba-1 antibodies. Quantification at CA1 reveals that liposomal clodronate did not affect the neuronal populations of nude mouse (*p* = 0.33), whereas it depleted all microglia (*n* = 5 per group). ***E,*** The proportion of epileptic slices, and total duration of seizure-like activities in microglia-depleted slices were not differ significantly from control slices, whereas the frequency of seizure-like activity was somewhat lower in microglia- depleted slices (*n* = 8-10 per group). All values are expressed as mean ± SEM. *$p<0.05$. Scale bar, 100μm.

682 **Figure 6**. Long-term assays of epileptogenesis in microglia-depleted vs. control hippocampal
683 slice cultures. ***A,*** Examples of slice culture brightfield micrographs at DIV0, prior to clodronate
684 treatment of upper slice. ***B,*** Brightfield micrographs of the same slice cultures on DIV6, at the
685 conclusion of clodronate treatment to the upper slice and empty liposome treatment of the lower
686 slice. No deleterious effects of clodronate are evident at this magnification. ***C,*** Cumulative group
687 mean lactate production, assayed in the spent culture media at 3-4 day intervals during twice
688 weekly media changes. *N* = 3 slices each group; all slices from the same animal. ***D,*** Cumulative
689 group mean LDH release, assayed in the spent culture media. Same groups slices and media
690 as for lactate assays in panel C. All values are expressed as mean ± SEM. Scale bar, 250 μm.

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703 **Table legends**

704 **Table 1.** Numbers of slices and animals used in each experiment.

705 **Table 2.** Comparison of seizure-like activities between microglia-depleted and control slices.

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722 Table 1.

Recording timing	Slice numbers in each group	Frequency of seizure (/hr)		Animal numbers used	Incidence of epilepsy (%)	
		clodronate	control		clodronate	control
Figure 2						
DIV12	11 each group	1-36	0-24	3 rats	100	72.7
Figure 3						
DIV6	6	0-2	0-2	2 rats	83.3	50
DIV12	6 each group	0-12	0-3	2 rats	83.3	66.7
DIV22	8 each group	0-3	0-2	2 rats	37.5	25.0
Figure 4						
DIV6	4-5 each group	9-19	1-16	2 mice	100	100
DIV12	4-6 each group	2-26	2-21	2 mice	100	100
Figure 5						
DIV12	4-6 each group	0-2	0-4	3 nude mice	40.0	62.5

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730 Table 2

Recording at DIV6	Microglia-depleted	Control	P value
Frequency(SLA/hr)	1.0 ± 0.3	0.5 ± 0.2	0.17
Total Duration(sec/hr)	37.7 ± 27.7	18.7 ± 9.0	0.53
Mean Duration(sec)	27.7 ± 15.3	37.3 ± 7.7	0.67
Recording at DIV12			
Frequency(SLA/hr)	3.5 ± 1.8	1.3 ± 0.5	0.24
Total Duration(sec/hr)	237.7 ± 164.2	32.8 ± 15.3	0.30
Mean Duration(sec)	59.6 ± 11.1	34.5 ± 20.9	0.28
Recording at DIV22			
Frequency(SLA/hr)	0.6 ± 0.4	0.4 ± 0.3	0.59
Total Duration(sec/hr)	88.0 ± 73.8	4.4 ± 3.1	0.28
Mean Duration(sec)	217.8 ± 191.1	11.5 ± 0.5	0.46

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