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ORIGINAL ARTICLE

Peripherally restricted viral challenge elevates extracellular glutamate and enhances synaptic transmission in the hippocampus

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Abstract

Peripheral infections increase the propensity and severity of seizures in susceptible populations. We have previously shown that intraperitoneal injection of a viral mimic, polyinosinic-polycytidylic acid (PIC), elicits hypersusceptibility of mice to kainic acid (KA)-induced seizures. This study was undertaken to determine whether this seizure hypersusceptibility entails alterations in glutamate signaling. Female C57BL/6 mice were intraperitoneally injected with PIC, and after 24 h, glutamate homeostasis in the hippocampus was monitored using the enzyme-based microelectrode arrays. PIC challenge robustly increased the level of resting extracellular glutamate. While pre-synaptic potassium-evoked glutamate release was not affected, glutamate uptake was profoundly impaired and non-vesicular glutamate release was augmented, indicating functional alterations of astrocytes. Electrophysiological examination of hippocampal slices from PIC-challenged mice revealed a several fold increase in the basal synaptic transmission as compared to control slices. PIC challenge also increased the probability of pre-synaptic glutamate release as seen from a reduction of paired-pulse facilitation and synaptic plasticity as seen from an enhancement of long-term potentiation. Altogether, our results implicate a dysregulation of astrocytic glutamate metabolism and an alteration of excitatory synaptic transmission as the underlying mechanism for the development of hippocampal hyperexcitability, and consequently seizure hypersusceptibility following peripheral PIC challenge.

Keywords: acute antiviral response, glutamate, hyperexcitability, polyinosinic-polycytidylic acid, seizures, synaptic transmission.

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Seizures represent a major neuropathological affliction and an important cause of long-term disability. Seizures result from excessive and/or synchronous neuronal activity in the brain. Cerebral inflammation following trauma, ischemia,

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[1]These co-senior authors contributed equally to the conception and experimental design of the projects as well as to the interpretation of results.

Abbreviations used: 4-AP, 4-aminopyridine; AUC, area under the curve; ACSF, artificial cerebrospinal fluid; BBB, blood–brain barrier; CA1, cornu ammonis 1; CA3, cornu ammonis 3; DG, dentate gyrus; EAAT1/2, excitatory amino acid transporters 1 and 2; EPSPs, excitatory post-synaptic potentials; fEPSPs, field excitatory post-synaptic potentials; HFS, high-frequency stimulation; i.p., intraperitoneal; IFNβ, interferon β; IL-1β, interleukin 1β; KA, kainic acid; LPS, lipopolysaccharide; LTP, long-term potentiation; MEA, microelectrode arrays; PPF, paired-pulse facilitation; PTZ, pentylenetetrazole; PIC, polyinosinic-polycytidylic acid; TBOA, dl-threo-β-benzyloxyaspartate; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNFα, tumor necrosis factor α; Xc-, cystine–glutamate antiporter.

infections, tumors, etc., has been recognized as an important pathological feature that predisposes and/or elicits seizures (Vezzani and Granata 2005; Marchi *et al.* 2009; Ravizza *et al.* 2011). The underlying mechanisms entail the activation of resident innate immune cells, chiefly microglia and astrocytes, as well as the recruitment and activation of peripheral leukocytes leading to the production of a plethora of cytokines, chemokines, prostaglandins, and other inflammatory agents. These inflammatory agents may increase excitatory inputs, decrease inhibitory inputs, or both, resulting in hyperexcitability of the neuronal networks, a hallmark of seizures.

Notably, also peripheral inflammation can increase seizure propensity in susceptible individuals (Tellez-Zenteno *et al.* 2005; Scheid and Teich 2007; Tovar *et al.* 2009). The underlying mechanisms involve relaying peripheral innate immunity signals to the brain whereby they induce a 'mirror inflammation' (Dantzer and Kelley 2007; Quan and Banks 2007; Dantzer *et al.* 2008). Several experimental studies dovetail with these clinical data. For example, the simulation of bacterial infection via intraperitoneal (i.p.) injection of a bacterial endotoxin, lipopolysaccharide (LPS) increases seizure susceptibility in mice as seen from a decrease in the threshold of clonic seizures instigated by pentylenetetrazole (PTZ) (Samland *et al.* 2003). In a rat model of inflammatory bowel diseases, intracolonical injection of 2,4,6-trinitrobenzene sulfonic acid (TNBS) increases the susceptibility to PTZ-induced seizures (Riazi *et al.* 2008). Moreover, experimental arthritis and subcutaneous granuloma decrease the onset and increase the score of PTZ-evoked seizures (Rao *et al.* 2008).

We have also shown that peripheral viral challenge robustly increases seizure susceptibility (Kirschman *et al.* 2011; Michalovicz and Konat 2014). In this experimental paradigm, i.p. injection of a viral mimetic, polyinosinic-polycytidylic acid (PIC) results in a several fold increase in the extent and duration of status epilepticus induced by kainic acid (KA) in mice (Kirschman *et al.* 2011). This seizure hypersusceptibility is protracted for 3 days after PIC challenge (Michalovicz and Konat 2014). Of note, PIC is an unstable inflammagen that is rapidly degraded in the bodily fluids (Krasowska-Zoladek *et al.* 2007), and when injected i.p. does not reach the circulation (Fil *et al.* 2011). Therefore, PIC challenge represents a bolus stimulation of the innate immune cells within the peritoneal cavity, and these peripherally generated inflammatory mediators instigate a cerebral response (Konat 2016). In particular, PIC challenge triggers a robust but transient surge of blood cytokines, that is, interferon β (IFNβ), interleukin 1β (IL-1β), IL-6, and tumor necrosis factor α (TNFα) (Cunningham *et al.* 2007; Michalovicz and Konat 2014). This cytokine surge instigates a global cerebral response as seen from the up-regulation of a myriad of inflammatory genes in all major brain regions (Cunningham *et al.* 2007; Konat *et al.* 2009; Fil *et al.* 2011). In the hippocampus, the ictal site of KA-induced seizures (Ben-Ari and Cossart 2000), PIC challenge dysregulates the expression of over 600 genes that, in addition to inflammatory and stress proteins, encode several neurotransmission-related proteins and microRNAs (Michalovicz and Konat 2014; Michalovicz *et al.* 2015). This genomic reprograming undoubtedly underlies the development of seizure hypersusceptibility, albeit specific cellular and molecular pathways have not been defined.

This study was undertaken to test the hypothesis that hyperexcitability ensuing PIC challenge features dysregulation of glutamate homeostasis. We employed the enzyme-based microelectrode technology for *in vivo* monitoring of extracellular glutamate levels in the hippocampus to identify neurotransmission-associated events affected by PIC challenge. The characterization of glutamate homeostasis was complemented with an electrophysiological study assessing synaptic transmission and plasticity in acute hippocampal slices.

Materials and methods

Animals

Eleven-week-old C57BL/6 female mice obtained from Charles River (Wilmington, MA, USA) were group-housed with free access to food and water in a temperature- and humidity-controlled colony room with a 12 : 12 light/dark cycle. Female mice were used to provide compatibility with previous studies (Cunningham *et al.* 2007; Konat *et al.* 2009; Fil *et al.* 2011; Michalovicz and Konat 2014). Acute antiviral response was induced by i.p. injection of 12 mg/kg of PIC (Invivogen, San Diego, CA, USA) in saline. Mice injected with 100 μL of saline served as vehicle controls. Mice were examined 24 h after PIC or saline injection. The West Virginia University and Auburn University Animal Care and Use Committees approved all experimental procedures.

In vivo glutamate measurement

Changes in extracellular glutamate in the hippocampus were monitored using the microelectrode array (MEA) technique (Burmeister and Gerhardt 2001) as previously described (Hunsberger *et al.* 2015a,b). Briefly, the electrodes obtained from Quanteon (Nicholasville, KY, USA) were coated with glutamate oxidase and calibrated, as exemplified in Fig. 1. A glass micropipette (Quanteon) was mounted to the arrays for intracranial drug delivery. Mice were anesthetized with isoflurane (1–4% continuous inhalation), placed in a stereotaxic device (David Kopf Instruments, Tujunga, CA, USA) and the MEA/micropipette assemblies were inserted into the hippocampal subregions, that is, dentate gyrus (DG), cornu ammonis 1 (CA1), and cornu ammonis 3 (CA3). The stereotaxic coordinates from the bregma were AP: −2.3 mm, ML: ±1.5 mm, DV: 2.1 mm for DG, AP: −2.3 mm, ML: ±2.7 mm, DV: 2.25 mm for CA3, and AP: −2.3 mm, ML: ±1.7 mm, DV: 1.4 mm for CA1. A reference electrode was implanted under the skin in a remote site. All MEA recordings were performed at 10 Hz using constant-potential amperometry. All measurements and injections were performed after a stable baseline was reached (20–

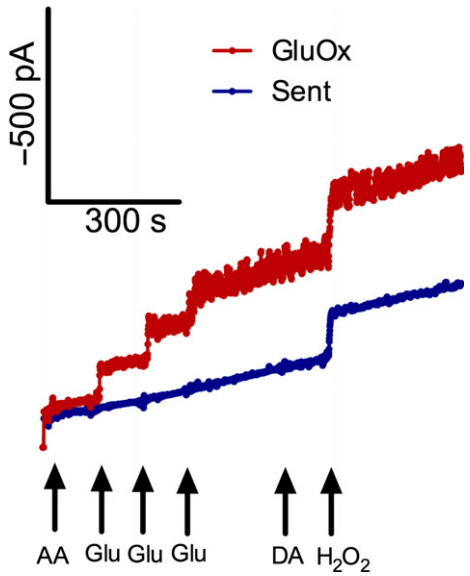

Figure 1 *In vitro* calibration of a self-referencing microelectrode measuring the change in current (pA) on a glutamate oxidase site (GluOx; red) versus a sentinel site (Sent; blue). Interferents, such as ascorbic acid (AA) and dopamine (DA), did not alter the current at either glutamate oxidase or sentinel sites. Addition of glutamate (Glu) produced a stepwise current increase on the glutamate oxidase site, but no change on the sentinel site. Hydrogen peroxide (H_2O_2) produced an increase in current on both sites. Sensitivity, slope, limit of detection, and r^2 values were calculated after calibration.

45 min). Both hemispheres were used for drug injection, and subregions within a hemisphere, were counterbalanced.

Tonic glutamate levels were calculated in all three subregions by averaging extracellular glutamate levels over 10-s periods. Evoked release was induced in a subset of animals by delivering 50–100 nL of 70 mM of potassium chloride (KCl) solution every 2–3 min. The amplitudes of 10 reproducible signals were averaged and compared. To measure glutamate uptake, a subset of animals received one to two injections at 50 nL increments within a 50–250 nL range of 200 μM glutamate (Sigma-Aldrich, St. Louis, MO, USA) delivered every 2–3 min in one hemisphere. Temporal clearance of glutamate was monitored and expressed as the net area under the curve (AUC). Glutamate release in a subset of animals was measured in the opposite hemisphere following inhibition of glutamate uptake with 50–250 nL of 500 μM dl-threo-β-benzyloxyaspartate (TBOA; Tocris, Ellisville, MO, USA). The amperometric data were analyzed using a custom Microsoft Excel software program (MatLab) as previously described (Hunsberger *et al.* 2015a,b). Data from some hippocampal regions were excluded for reasons including failure of the MEA or clogging of the micropipette. The number of mice per treatment group for glutamate measurements is indicated in Table 1.

Table 1 Number of mice used in glutamate measurement experiments.

	DG		CA3		CA1	
	PIC	Saline	PIC	Saline	PIC	Saline
Tonic	11	9	11	9	12	9
KCl	5	4	5	4	5	4
Exogenous glutamate	5	6	6	5	4	5
TBOA	6	4	6	4	6	4

CA1, cornu ammonis 1; CA3, cornu ammonis 3; DG, dentate gyrus; KCl, potassium chloride.

Hippocampal slice preparation

Animals were killed with carbon dioxide, the hippocampi were isolated and 350-μm thick transverse slices were prepared using a Leica VT1200S Vibratome (Leica Microsystems, Wetzlar, Germany). Slices were incubated at 22°C in artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 2.1 mM $CaCl_2$, 1.4 mM Na_2PO_4, 26 mM $NaHCO_3$, 20 mM dextrose, pH 7.4) saturated with 95% O_2/5% CO_2. After 1-h incubation, slices were transferred into a recording chamber for electrophysiological measurements as previously described (Wang and Zheng 2015).

Extracellular field potential recording

The slices were examined with an Olympus BX50WI microscope equipped with a high-resolution, high-sensitivity CCD camera (Dage-MTI, Michigan City, IN, USA). A bipolar stimulating electrode (100-μm separation, FHC, Bowdoinham, ME, USA) was placed in the Schaffer collateral pathway. A patch pipette drawn with the P97 Brown-Flaming Puller, (Sutter Instruments, Novato, CA, USA) and filled with ACSF (2–5 MΩ, 1.5 mm OD, 0.86 mm ID) was placed in the stratum radiatum of CA1 to record field excitatory post-synaptic potentials (fEPSPs). All parameters, including pulse duration, width, and frequency were computer controlled. Constant-current pulse intensities were controlled by a stimulus isolation unit A360 (WPI, Sarasota, FL, USA).

Basal synaptic transmission, represented by input–output responses, was determined of the slopes of fEPSP as a function of stimulus intensities. For paired-pulse facilitation (PPF), pairs of stimuli separated by varying intervals between them were delivered to the stratum radiatum at 0.05 Hz. Paired responses were averaged, and ratios of fEPSP slopes from the second stimulus (fESPS2) to fEPSP slopes from the first stimulus (fESPS1) were calculated and plotted as a function of interstimulus intervals. Long-term potentiation (LTP) was evaluated after 10 min of stable baseline period. Initial recordings were carried out with low frequency stimulation (0.05 Hz) at intensities of 0–500 μA to determine the maximal excitatory potential. For LTP experiments, the stimulus intensity was adjusted to produce 50% of the amplitude at which initial population spikes begin to appear. LTP was induced with five high-frequency stimuli (HFS; 100 pulses, 100 Hz) every 20 s. LTP was measured 55–60 min post-HFS.

The data were recorded online using the WinLTP 2.2 software (University of Bristol, Bristol, UK). Standard offline analyses of the data were conducted using Prism software (GraphPad Prism version 5.00, San Diego, CA, USA). Results are expressed as mean ± SEM.

Statistical analyses

Results were evaluated by the one-way ANOVA using JMP (SAS, Cary, NC, USA) and SPSS v.21 (SPSS Inc., Chicago, IL, USA) for glutamate and electrophysiological data, respectively. For

electrophysiological data, significant omnibus tests were followed by Student's *t*-tests. Results were presented as mean ± SEM, and differences between groups were considered statistically significant at $p \leq 0.05$.

Results

An increased glutamatergic transmission is a plausible mechanism underscoring PIC-induced hypersusceptibility to KA-induced seizures found in previous studies (Kirschman *et al.* 2011; Michalovicz and Konat 2014). Here, we assessed glutamate homeostasis in the hippocampus, the ictal site of KA-induced seizures (Ben-Ari and Cossart 2000), using the enzyme-based microelectrode technology that allows real-time monitoring of extracellular glutamate *in vivo*. We used isoflurane to avoid anesthetic-induced changes in resting glutamate levels (Mattinson *et al.* 2011), and measured glutamate in hippocampal subregions known to be rich in glutamate receptors, that is, DG, CA1, and CA3 (Pettit and Augustine 2000; Nimchinsky *et al.* 2004). All measurements were performed 24 h after i.p. injection of PIC or saline. As shown in Fig. 2a, PIC challenge induced a robust increase in tonic, resting glutamate levels in all three subregions. The highest increase of 11-fold over control was observed in DG [$F(1,18) = 41.49$, $p < 0.0001$]. CA1 [$F(1,19) = 15.58$, $p = 0.0009$] and CA3 [$F(1,18) = 18.94$, $p = 0.0004$] featured 9.8-fold and 5.8-fold increase, respectively.

Several mechanisms can be considered to account for the increase of tonic glutamate. For example, PIC challenge may alter the capacity or 'ceiling' of neuronal terminals to release glutamate (Hinzman *et al.* 2010). To test this possibility, we used the paradigm of potassium-evoked glutamate release (Day *et al.* 2006). As shown in Fig. 2b, the injection of KCl induced a transient (approximately 5 s) elevation of extracellular glutamate. No differences were observed between PIC-challenged versus control mice in any subregion (DG [$F(1,7) = 0.11$, $p = 0.75$]; CA1 [$F(1,7) = 0.002$, $p = 0.96$]; CA3 [$F(1,7) = 0.04$, $p = 0.84$]; Fig. 2c). These results indicate that PIC challenge does not increase the neurotransmitter content in pre-synaptic terminals.

A decreased glutamate clearance represents an alternative mechanism for the rise of extracellular glutamate. To test this option, we injected exogenous glutamate, and monitored its clearance by measuring net AUC. We first compared the amplitude of glutamate signals following injection of exogenous glutamate to confirm that differences in net AUC between the PIC-challenged and saline-injected mice following application of exogenous glutamate were due to alterations in the uptake and not to differences in the amount of applied glutamate (Hunsberger *et al.* 2015a,b). Prior to AUC measurement, maximal amplitudes of the glutamate signal were determined to ensure reproducibility of glutamate injection. Fig. 3a shows no significant differences in the

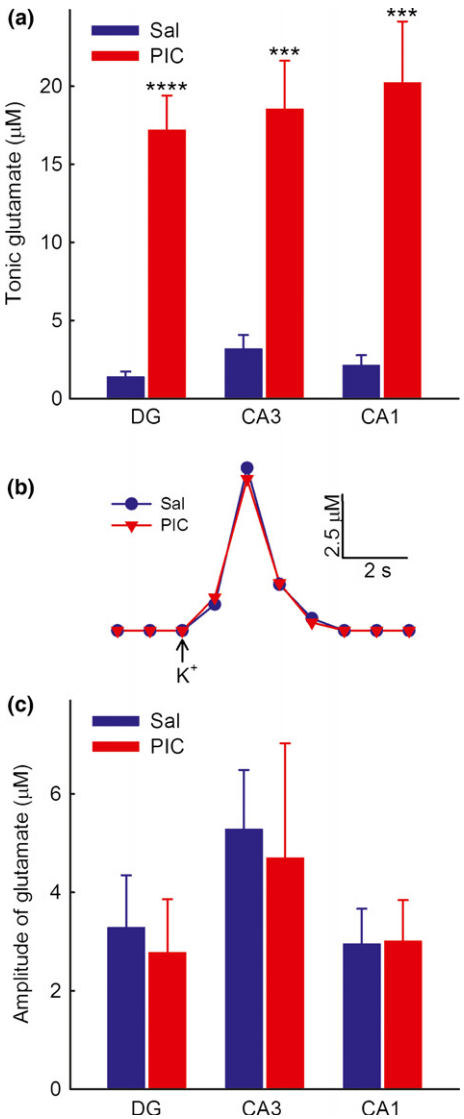

Figure 2 Tonic glutamate levels and evoked glutamate release in the hippocampus. Mice were intraperitoneally injected with PIC or saline (control). After 24 h, hippocampal glutamate was analyzed by the enzyme-based microelectrode technique in the dentate gyrus (DG), cornu ammonis 1 (CA1), and cornu ammonis 3 (CA3). (a) Extracellular tonic glutamate levels in the hippocampal subregions. (b) Baseline-matched representative traces of K^+ evoked release of glutamate in CA3. (c) The amplitudes of K^+ evoked release of glutamate in the hippocampal subregions. For details see Materials and methods. Bars represent mean ± SEM. Asterisks denote values significantly different from respective controls ***$p \leq 0.001$, ****$p \leq 0.0001$).

maximal amplitudes in any subregion in control versus PIC-challenged mice (DG [$F(1,9) = 0.06$, $p = 0.81$]; CA1 [$F(1,7) = 0.25$, $p = 0.63$]; CA3 [$F(1,9) = 0.43$, $p = 0.53$]). Also, no effect of PIC challenge on the diffusion of exogenous glutamate within the tissue expressed as the T_{rise} values, that is, the time for the signal to reach maximum amplitude (Sykova *et al.* 1998), was evident in any subregion (DG [F

(1,9) = 0.07, p = 0.80]; CA1 [F(1,7) = 0.25, p = 0.63]; CA3 [F(1,9) = 0.24, p = 0.63]; Fig. 3b), suggesting any reductions in glutamate uptake were not because of diffusion from the point source (micropipette) to the MEA. Temporal analysis of glutamate levels following its injection revealed a profoundly delayed clearance profile in PIC-challenged mice (Fig. 3c), indicative of an impairment of the neurotransmitter's uptake. The quantitation of this impairment is shown in Fig. 3d. The greatest increase in net AUC induced by PIC challenge of 8.3-fold over control was found in CA3 [F(1,9) = 11.55, p = 0.008]. The values for DG [F (1,9) = 13.77, p = 0.005] and CA1 [F(1,7) = 18.16, p = 0.004] were 6.7-fold and 3.8-fold, respectively.

The augmentation of tonic glutamate may also result from an increased release of glutamate by astrocytes. We inhibited glutamate uptake with TBOA, a competitive non-transportable excitatory amino acid transporters (EAAT) blocker (Shimamoto *et al.* 1998; Montiel *et al.* 2005; Tovar *et al.* 2009) to confirm the involvement of these receptors and to unmask the release of glutamate (Jabaudon *et al.* 1999). Local application of TBOA produced a transient increase in the extracellular glutamate concentration in both PIC-challenged and control mice (Fig. 4a), although the amplitude of this increase differed (Fig. 4b). Thus, DG [F(1,8) = 5.80, p = 0.04] and CA1 [F (1,8) = 7.06, p = 0.03] featured an 18- and 17-fold increase in PIC-challenged versus control mice. In contrast, PIC challenge had no effect on glutamate release in CA3 [F(1,8) = 0.51, p = 0.50].

The increased tonic glutamate, the impaired glutamate uptake, and the increased glutamate release implicated that PIC challenge might enhance glutamatergic neurotransmission in the hippocampus. To verify this notion, we examined basal synaptic transmission and synaptic plasticity in hippocampal slices by field recordings. Fig. 5a shows representative traces of EPSPs in hippocampi from PIC-challenged versus control mice. PIC challenge markedly increased the amplitude and slope of the EPSP. As seen from Fig. 5b, PIC challenge profoundly enhanced basal synaptic transmission denoted by input–output responses of the neuronal networks (stimulus–response curve) [F (1,41) = 30.35, p < 0.0001], at each point of measurement [ps > 0.05]. Throughout the range of stimulus intensities from 50 to 500 μA, the synaptic efficiency increased by over 2.5-fold in slices from PIC-challenged mice as compared to the slices from saline-injected mice. A change in basal synaptic transmission may result from alterations in pre-, post-, and peri-synaptic elements. To further characterize which component across the synapse actually contributed to PIC-induced increased synaptic transmission, we used the PPF protocol that reflects residual calcium levels, a pre-synaptic mechanism that plays a major role in short-term and long-term plasticity. PIC challenge decreased PPF [F (1,19) = 7.391, p = 0.014] at the short (50 ms) stimulus interval [p < 0.05], indicating an increase in pre-synaptic release probability because of alterations in either pre-synaptic compartment or astrocyte calcium signaling

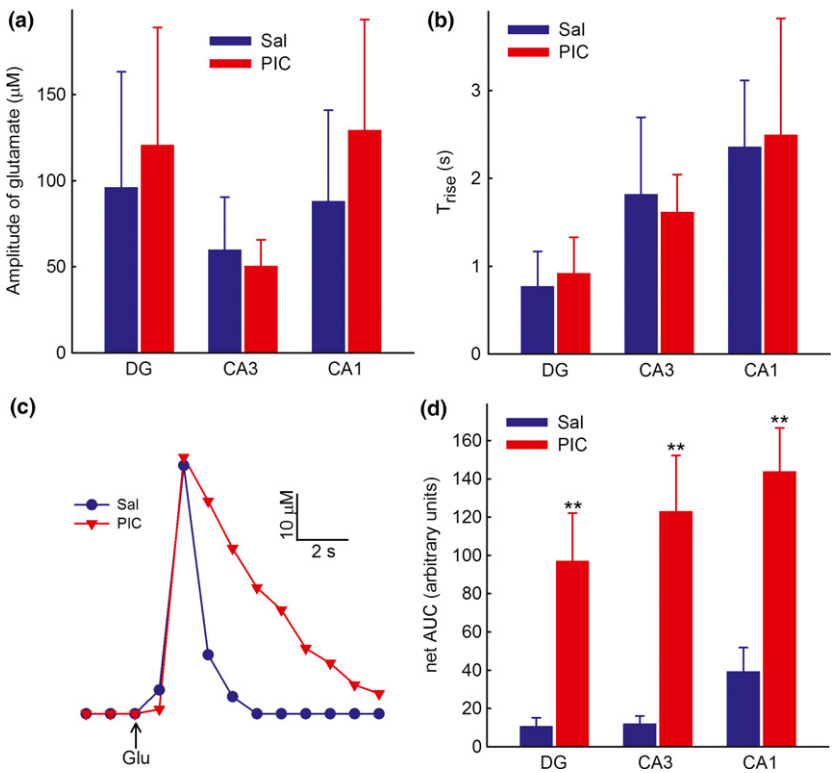

Figure 3 The uptake of exogenous glutamate in the hippocampus. Mice were intraperitoneally injected with polyinosinic-polycytidylic acid or saline (control). After 24 h, hippocampal glutamate was analyzed by the enzyme-based microelectrode technique in different hippocampal subregions as indicated. (a) The amplitude of signals following local injection of 200 μM glutamate. (b) Glutamate diffusion expressed as time to reach maximum amplitude (T_{rise}). (c) Peak-matched representative traces in the dentate gyrus. (d) Glutamate uptake expressed as the net area under the curve. For details see Materials and methods. Bars represent mean ± SEM. Asterisks denote significant differences from respective controls (**$p \leq 0.01$).

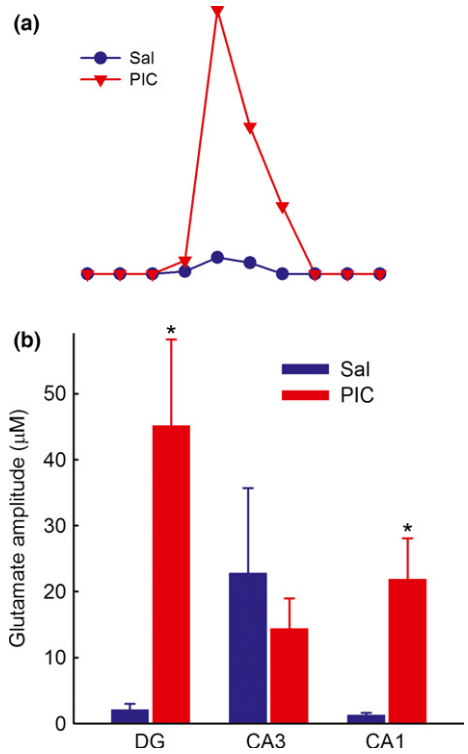

Figure 4 Spontaneous release of extracellular glutamate in the hippocampus. Mice were intraperitoneally injected with 12 mg/kg of polyinosinic-polycytidylic acid or saline (control). After 24 h, glutamate uptake was blocked by the application of 500 μM dl-threo-β-benzyloxyaspartate (TBOA) to unmask glutamate release, and the levels of extracellular glutamate were determined in different hippocampal subregions as indicated. (a) A representative trace of transient glutamate release in dentate gyrus in polyinosinic-polycytidylic acid- and saline-injected mice. (b) The amplitude of extracellular glutamate in the hippocampal subregions following TBOA application. For details see Materials and methods. Bars represent mean ± SEM. Asterisks denote significant differences from respective controls (*$p \leq 0.05$).

(Fig. 5c). Albeit, no effect was observed at longer intervals [*p*s > 0.05]. LTP, a cellular substrate of plasticity that may feature both pre- and post-synaptic expression (Padamsey and Emptage 2014), was significantly increased by PIC challenge [$F(1,68) = 2.47$, $p = 0.0007$; Fig. 5d].

Discussion

The major finding of our study is that peripheral PIC challenge disrupts cerebral glutamate homeostasis, resulting in a robust increase in the basal extracellular glutamate concentration (Fig. 2a). This increase is likely to underlie hippocampal hypersusceptibility to KA-induced seizures in PIC-challenged mice (Kirschman *et al.* 2011; Michalovicz and Konat 2014). In support of this notion, increased tonic glutamate levels have been shown to correlate positively with the severity of focal motor seizures induced by intrahippocampal injection of 4-aminopyridine (4-AP) (Stephens *et al.* 2014). Also, astrocytic release of glutamate has been shown to facilitate the initiation of seizures (Kang *et al.* 2005), while the suppression of glial glutamate release leads to decreased seizure susceptibility (De Bundel *et al.* 2011). In addition, human epileptogenic hippocampi exhibit augmented basal glutamate levels during interictal periods that may contribute to seizure generation (Cavus *et al.* 2005). Moreover, the overflow of extracellular glutamate has been recognized as a key factor in the development of neuronal hyperexcitability (Featherstone and Shippy 2008). For example, dysregulation of extracellular glutamate homeostasis has been directly linked to hyperexcitability of cortical and spinal cord neurons at diverse pathological conditions (Campbell and Hablitz 2004, 2008; Campbell *et al.* 2012, 2014; Putatunda *et al.* 2014). Glutamate-induced hyperexcitability is chiefly mediated by ionotropic glutamate receptors, in particular, NMDA receptors, but the involvement of metabotropic glutamate receptors has also been implicated (Featherstone and Shippy 2008). The mechanism entails a direct ligation of the receptors, although other more circuitous pathways may also be involved. Consequently, we posit that the amplified response of the CA1 pyramidal cells induced by the upsurge of extracellular glutamate in PIC-challenged mice contributed to the enhanced synaptic transmission (Fig. 5b), PPF (Fig. 5c), and LTP (Fig. 5d). However, a possibility that PIC challenge might also reduce inhibitory signaling can be considered. For example, a reduction in the number of inhibitory synapses was observed in the cortex of mice following repeated LPS injections (Chen *et al.* 2014).

The elevation of tonic glutamate could be because of increased glutamate release, decreased glutamate uptake, or both. Local application of potassium evoked the same amounts of glutamate in PIC and control hippocampi (Fig. 2c), indicating no alteration in the capacity or ceiling of pre-synaptic release of this neurotransmitter. However, the clearance of injected glutamate was profoundly hampered (Fig. 3d), indicating an impairment of glutamate uptake by PIC challenge. Inflammatory cytokines up-regulated in the hippocampus in response to PIC challenge might mediate this impairment. For example, IL-1β and TNFα inhibit astrocytic glutamate uptake (Ye and Sontheimer 1996; Hu *et al.* 2000), and the *Tnfa* and *Il1b* gene expression is up-regulated in the hippocampi of PIC-challenged mice as compared to controls (Michalovicz and Konat 2014).

The EAAT1 and 2 (EAAT1/2) expressed almost exclusively in astrocytes play the major role in the uptake of glutamate (Niciu *et al.* 2012). The application of TBOA, a specific competitive inhibitor of EAAT1/2 (Shimamoto *et al.* 1998), induced a transient increase of extracellular glutamate (Fig. 4), indicating the involvement of these transporters. However, the contribution of neuronal transporters cannot be ruled out. Moreover, in DG and CA1 of PIC-challenged

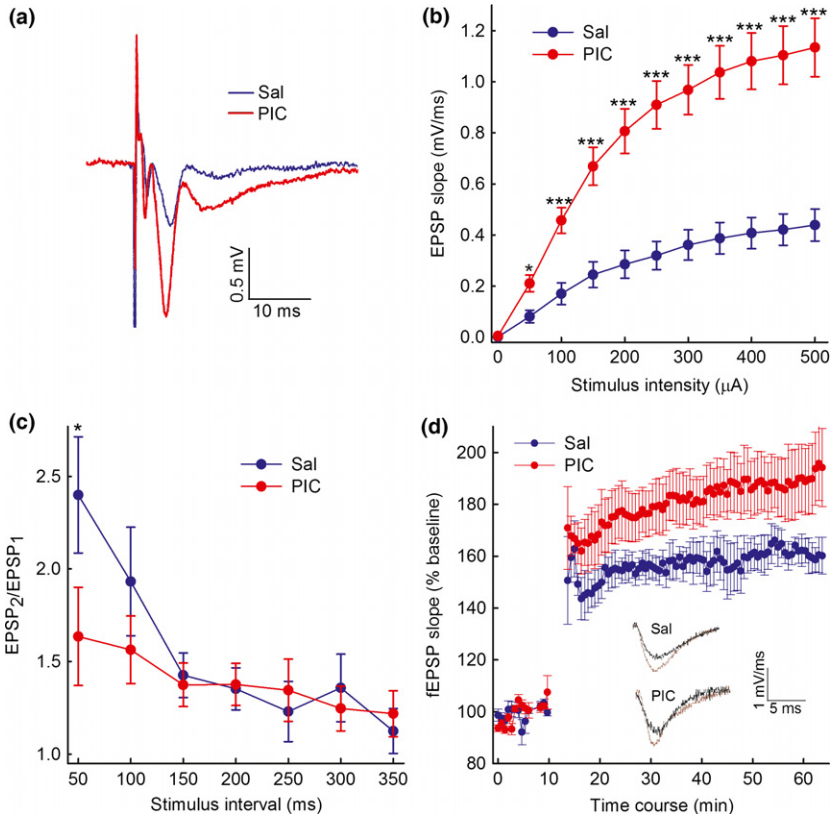

Figure 5 Synaptic transmission in hippocampal slices. Mice were intraperitoneally injected with polyinosinic-polycytidylic acid or saline (control), and after 24 h, hippocampal slices were prepared. The Schaffer collateral pathways of CA3 were stimulated, and field excitatory post-synaptic potentials (fEPSPs) evoked in the striatum radiatum of CA1 were recorded. (a) Representative traces of fEPSPs evoked at a stimulus intensity of 200 μA. (b) Basic synaptic transmission as the fEPSP slope measured at increasing stimulus intensity. (c) Paired-pulse facilitation expressed as the change of ratios of the second stimulus fEPSP slopes to the first stimulus fEPSP slopes plotted as a function of interstimulus intervals. (d) Long-term potentiation calculated as the fEPSP ratio over time. Inset: Representative traces shown include data collected from saline-injected and PIC-challenged animals during baseline recordings (black) overlayed on traces during 55–60 min LTP (brown). For details see Materials and methods. Symbols represent mean ± SEM from three mice (12 slices) per group. Asterisks denote significant differences from respective controls (Student's *t*-test; $*p \leq 0.05$, $***p \leq 0.001$).

animals, local TBOA application elicited much greater glutamate spikes than in control tissues. Because blocking of EAAT unmasks glutamate release (Jabaudon *et al.* 1999), our results suggest that PIC challenge not only impairs glutamate uptake but also augments glutamate release from astrocytes. This result is consistent with previous studies showing the enhancement of astrocytic glutamate release by inflammatory mediators, that is, IL-1β (Casamenti *et al.* 1999), TNFα, and prostaglandins (Bezzi *et al.* 2001). Altogether, our results strongly implicate astrocytes as cellular targets for inflammatory mediators generated in response to PIC challenge.

In contrast to DG and CA1, no difference between PIC-challenged and control mice in the post-TBOA glutamate amplitude was detectable in CA3 (Fig. 4), suggesting that the release of glutamate in this subregion is not affected by the inflammatory milieu instigated by PIC challenge. Because pre-synaptic glutamate release was not altered in CA3 (Fig. 2c), the astrocytic release mechanisms are likely candidates to account for this region specificity. Astrocytes release glutamate through different mechanisms, for example, Ca^{2+}-dependent exocytosis, glutamate exchange via the cystine–glutamate antiporter (Xc-), and reversal of uptake by glutamate transporters (Malarkey and Parpura 2008). It is tempting to speculate that unlike DG and CA1 astrocytes, CA3 astrocytes use mechanisms that are not susceptible to the inflammatory milieu induced by PIC challenge. Such a differential response is buttressed by a previous observation that in the presence of TBOA, tetraethylammonium chloride elicits a several fold greater glutamate release in CA3 than in CA1 or DG (Chiba *et al.* 2010). However, specific mechanisms may vary between these two paradigms.

Field recordings in hippocampal slices prepared from PIC-challenged versus control mice are congruent with the dysregulated glutamate homeostasis observed *in vivo*. Thus, the robust increase in the basal glutamatergic synaptic

transmission (Fig. 5b) likely resulted from the elevation of extracellular glutamate (Fig. 2a). The underlying mechanisms might involve the activation of extrasynaptic glutamate receptors (Petralia 2012). PPF, an index of short-term plasticity, reflects synaptic efficacy determined by the probability of pre-synaptic neurotransmitter release (Zucker and Regehr 2002). PIC challenge significantly reduced PPF (Fig. 5c), indicating that increased probability of glutamate release at the terminals of the Schaffer collaterals might contribute to the increased synaptic transmission. However, this increased pre-synaptic activity is in divergence with unchanged potassium-evoked glutamate amplitudes (Fig. 2c), another facet of the pre-synaptic neurotransmitter release. A plausible explanation is that the glutamate amplitudes measure the maximum capacity for release by depleting pre-synaptic glutamate pool with large doses of potassium, and these measurements may not be compatible with the physiological/functional release measured by PPF. Furthermore, LTP that can be expressed at post-synaptic as well as pre-synaptic loci (Padamsey and Emptage 2014) was increased by PIC challenge (Fig. 5d), indicating an enhancement of synaptic strength. Altogether, these results show that PIC challenge increases both basal synaptic transmission and synaptic plasticity.

In addition, the slice experiments provide compelling evidence for the intrinsic nature of the hippocampal alterations induced in PIC-challenged mice. For instance, peripheral inflammation might have increased the permeability of KA and/or glutamate through the blood–brain barrier, leading to hyperexcitability of hippocampal networks that would manifest as seizure hypersusceptibility. However, the robustly augmented excitatory synaptic transmission in the perfused slices from PIC-challenged as compared to control animals shows that the hyperexcitability indeed originates in the hippocampal parenchyma. This finding corroborates our previous study showing greatly increased spontaneous ictal activity elicited with 4-aminopyridine in hippocampal slices from PIC-challenged versus control mice (Konat *et al.* 2012). As discussed above, the augmentation of synaptic transmission results from the elevation of extracellular glutamate. Ergo, the slice studies also indirectly verify the intrinsic nature of glutamate dysregulation observed *in vivo*.

We have recently shown that PIC challenge profoundly up-regulates expression of the complement in the hippocampus, and that this up-regulation is commensurate with the period of seizure hypersusceptibility (Michalovicz *et al.* 2015). The complement is a major mediator of synaptic modifications (Stevens *et al.* 2007; Schafer *et al.* 2012; Stephan *et al.* 2013), and complement proteins have proconvulsive activity when injected into the hippocampus (Xiong *et al.* 2003). Therefore, it is tempting to speculate that the alteration of glutamate homeostasis and hyperexcitability might be induced by the complement proteins. The mechanisms of such alterations might entail anaphylatoxins generated through the complement activation. Anaphylatoxins can activate their cognate receptors on microglia, astrocytes, and neurons resulting in the generation of inflammatory factors that affect function of the post-synaptic terminals. For example, IL-1β (Viviani *et al.* 2003; Yang *et al.* 2005), IL-6 (Samland *et al.* 2003; Xiaoqin *et al.* 2005), TNFα (Beattie *et al.* 2002; Stellwagen *et al.* 2005), IFNβ (Hadjilambreva *et al.* 2005), CXCL10 (Ragozzino *et al.* 1998), CXCL1/2 (Giovannelli *et al.* 1998; Ragozzino *et al.* 1998), and the prostaglandin PGE2 (Chen and Bazan 2005) can enhance glutamatergic synaptic transmission. Thus, in addition to the disruption of glutamate homeostasis discussed formerly, inflammatory factors may induce hyperexcitability of the hippocampal neurons. Alternatively, complement proteins or their derivatives/complexes might bind to synaptic structures resulting in functional impairment of surface receptors that control glutamate homeostasis and/or synaptic transmission.

Recently, seizure hypersusceptibility of rats subjected to colonic inflammation (Riazi *et al.* 2008) has been linked to an increased synaptic transmission in the hippocampus (Riazi *et al.* 2015), albeit the extent of this increase was much less than the increase observed here. In contrast to our study, the colonic inflammation reduced LTP in hippocampal slices. Therefore, it seems that hippocampal hyperexcitability may be a common mechanism by which peripheral inflammation increases seizure susceptibility, but the effects on synaptic plasticity vary depending on the inflammatory paradigm.

In conclusion, our results indicate that inflammation instigated by peripheral PIC challenge enhances excitatory synaptic transmission and plasticity in the hippocampus by elevating extracellular glutamate concentration and increasing pre-synaptic activity. These putative pathways are likely responsible for the development of seizure hypersusceptibility. Our results warrant a comprehensive investigation of the underlying mechanisms at both the cellular and molecular level to provide a foundation for the development of therapeutic strategies for the management of inflammation-related seizures.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

Conflict of Interest

The authors have no conflict of interest to declare.

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