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Albumin induces excitatory synaptogenesis through astrocytic TGF-β/ALK5 signaling in a model of acquired epilepsy following blood–brain barrier dysfunction

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ABSTRACT

Post-injury epilepsy (PIE) is a common complication following brain insults, including ischemic, and traumatic brain injuries. At present, there are no means to identify the patients at risk to develop PIE or to prevent its development. Seizures can occur months or years after the insult, do not respond to anti-seizure medications in over third of the patients, and are often associated with significant neuropsychiatric morbidities. We have previously established the critical role of blood–brain barrier dysfunction in PIE, demonstrating that exposure of brain tissue to extravasated serum albumin induces activation of inflammatory transforming growth factor beta (TGF-β) signaling in astrocytes and eventually seizures. However, the link between the acute astrocytic inflammatory responses and reorganization of neural networks that underlie recurrent spontaneous seizures remains unknown. Here we demonstrate in vitro and in vivo that activation of the astrocytic ALK5/TGF-β-pathway induces excitatory, but not inhibitory, synaptogenesis that precedes the appearance of seizures. Moreover, we show that treatment with SJN2511, a specific ALK5/TGF-β inhibitor, prevents synaptogenesis and epilepsy. Our findings point to astrocyte-mediated synaptogenesis as a key epileptogenic process and highlight the manipulation of the TGF-β-pathway as a potential strategy for the prevention of PIE.

Introduction

Traumatic, ischemic, and infectious brain injuries often initiate a cascade of epileptogenic events, ultimately leading to post-injury epilepsy (PIE) after a latent period of months to years. Accumulating evidence suggests a key role of post-injury dysfunction of the blood–brain barrier (BBB) in the development of PIE: BBB dysfunction is a well-documented finding in patients following brain trauma (occurring within hours of the event and often persisting for days to weeks; for a review, see Abbott and Friedman, 2012; Cunningham et al., 2005; Rosenberg, 2012; Shlosberg et al., 2010) and is more frequent in post-traumatic patients with epilepsy (Raabe et al., 2012; Schmitz et al., 2013; Tomkins et al., 2008). In experimental animals, BBB dysfunction was associated with increased propensity for symptomatic seizures and the development of epilepsy (Friedman et al., 2009; Frigerio et al., 2012; Marchi et al., 2007; Seiffert et al., 2004; Van Vliet et al., 2007).

Previous studies have linked the brain's immune response with seizures and epilepsy, as activation of the pro-inflammatory IL-1 receptor/Toll-like receptor (IL1R/TLR) system was shown to promote seizure onset and recurrence in mice and rats (for a review, see Devinsky

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et al., 2013; Marchi et al., 2013; Vezzani et al., 2011a, 2011b). Recent findings have highlighted the involvement of a specific inflammatory pathway in PIE, identifying the epileptogenic role of transforming growth factor beta (TGF-β) signaling in animal models of BBB dysfunction. Serum albumin was shown to enter BBB-disrupted brain tissue and bind to TGF-β receptors in astrocytes (Ivens et al., 2007), inducing inflammatory signaling (Cacheaux et al., 2009) and SMAD-2/3 phosphorylation (Bar-Klein et al., 2014), thereby modifying astrocytic function (Braganza et al., 2012; David et al., 2009; Seiffert et al., 2004). TGF-β signaling was also associated with immediate changes in extracellular potassium and glutamate, and a lower threshold for neuronal activation in slices (David et al., 2009; Lapilover et al., 2012). However, the immediate and short-lived nature of these changes (Ivens et al., 2007; Lapilover et al., 2012) suggests the involvement of additional modifications, underlying permanent network changes that sustain chronic recurrent seizures. While axonal sprouting and synaptogenesis were shown to contribute to seizures and persistent network alterations (Babb et al., 1991; Bragin et al., 2000; Marco and DeFelipe, 1997), the detailed changes and mechanisms underlying network reorganization in PIE are not well understood.

Glial cells are known to play a key role in the formation and elimination of both excitatory and inhibitory synapses in developing and mature brain (Chung and Barres, 2012; Clarke and Barres, 2013; Elmariah et al., 2005; Eroglu and Barres, 2010). Specifically, astrocytes were shown to regulate synaptogenesis through secretion of thrombospondins (TSPs) (Christopherson et al., 2005; Crawford et al., 2012; Eroglu et al., 2009; Liauw et al., 2008), Hevin (Kucukdereli et al., 2011) and glypicans 4 and 6 (Allen et al., 2012). Moreover, synapse formation was also associated with activation of TGF-β signaling in Schwann cells (Feng and Ko, 2008; Packard et al., 2003), and more recently in astrocytes via induction of Smad3 (Yu et al., 2014), secretion of D-serine (Diniz et al., 2012), and CaM kinase II signaling (Diniz et al., 2014). Since the dysfunction of astroglia has been shown following brain injury, in models of acquired epilepsy (Crunelli et al., 2014; Devinsky et al., 2013; Friedman et al., 1996; Marchi et al., 2013) and in the BBB-compromised brain, here we set out to study the role of albumin-induced TGF-β pathway activation in synaptogenesis and chronic seizures. We further challenged the hypothesis that the inhibition of the TGF-β ALK5 receptor is sufficient to prevent the development of epilepsy. Through a combination of in vitro and in vivo models of inflammation post-BBB dysfunction, our study sheds light on the epileptogenic cascade following brain injury and highlights novel targets for PIE prevention in at-risk patients.

Materials and methods

All animal procedures were approved by the UC Berkeley Animal Care and Use Committee or the animal care and use ethical committees at the Ben-Gurion University of the Negev, Beer-Sheva.

Intracerebroventricular (ICV) pump implantation

Two-month-old male FVB-N mice (Harlan, Israel) were anesthetized with isoflurane (0.8–1.2%) and placed in a stereotaxic frame. A 0.7-mm-diameter hole was drilled through the skull over the somatosensory cortex (0.5 mm caudal, 1 mm lateral to bregma) and anterior to the hippocampus. Micro-osmotic pumps (ALZET, Cupertino, CA) were filled with 200 μL of either 0.4 mM bovine serum albumin (BSA; Sigma) solution or 100 ng/mL TGF-β1 (Peprotech, Rocky Hill, NJ) in artificial cerebrospinal fluid (aCSF), as previously described (Seiffert et al., 2004), and placed subcutaneously between the shoulder blades. In a subset of animals, 10% of the BSA was replaced with fluorescein isothiocyanate (FITC)-conjugated BSA (Sigma) or Alexa Fluor 488-conjugated BSA (Life Technologies) (2.68 g/L). Other animals were infused with BSA and 300 μM SJN2511 (Tocris, Bristol, UK) to block TGF-β signaling. Sham controls were implanted with pumps containing aCSF with FITC-conjugated 70 kD dextran (Sigma). Pumps delivered their contents into the right lateral cerebral ventricle via a brain infusion kit (ALZET, 0008851). Pumps were extracted under isoflurane anesthesia 7 days after implantation.

Immunohistochemistry (IHC)

Brain sections were stained according to commonly accepted protocols (Ippolito and Eroglu, 2010). Mice were cardio-perfused with phosphate-buffered saline (PBS), and brains were removed and fixed in 4% PFA in PBS overnight at 4 °C. The brains were then transferred to PBS containing 30% sucrose for 24 h and then embedded in a 2:1 mixture of 20% sucrose in PBS:Tissue-Tek OCT (VWR, Radnor, PA). Once frozen, 12 μm sections were collected using a freezing microtome. Slices were dried at 37 °C, washed three times in PBS and blocked with 10% normal goat serum in PBS for 1 h. Glial fibrillary acidic protein (GFAP) stains were performed using a monoclonal mouse anti-GFAP (Sigma). Excitatory synapses were labeled with primary antibodies for PSD-95 (Millipore, 1:200) and synapsin1 (Synaptic Systems, Goettingen, DE; 1:200). Alexa Fluor 555 and Alexa Fluor 488-conjugated secondary antibodies (Abcam, Cambridge, MA; 1:400) in 1% normal donkey serum in PBS were applied for 2 h at room temperature. The nuclear stain (ToPro3, Life Technologies; 1:1000) was added prior to mounting coverslips. Each treated slice was stained together with a matched control.

Western blot

Extracted brain tissue was immediately frozen on dry ice and homogenized in 20 mM Tris–HCl (pH 7.4), and 1 mM EDTA, 5 mM EGTA, 1 mM Na-vanadate, 2 μg/μL aprotinin, 1 μg/μL pepstatin, and 2 μg/μL leupeptin (30 mg tissue/150 μL homogenization buffer). Equal amounts of protein (30 μg) were separated by SDS–PAGE (11%) and transferred to nitrocellulose membranes (Bio-Rad Laboratories). Membranes were rinsed with PBS containing 0.1% Tween 20 and blocked with 5% nonfat milk in 1× PBS overnight at 4 °C. The membranes were incubated with mouse monoclonal antibody against GFAP (1:20000; Chemicon Int Inc.) for 1 h at room temperature. After washes in PBS–1% Tween (PBS-T), membranes were incubated with peroxidase-conjugated anti-mouse secondary antibody (1:10,000; Sigma-Aldrich) in PBS with 3% milk for 1 h. Membranes were then washed repeatedly in PBS-T and incubated with ECL. Immunoreactive bands were visualized, and series of ECL exposures were performed to ensure that non-saturated bands were used for quantification. Western blot data acquisition and analysis were accomplished by measuring the pixel density and area of Immunoreactive bands from the ECL films using ImageJ. Values were normalized to β-actin.

Telemetric electrocorticography and seizure detection

ECoG activity was monitored telemetrically (Data Science International, St. Paul, MN) for up to a month after pump implantation. During pump implantation, a subset of animals was implanted with a small transmitter (TA10EAF20), placed subcutaneously on the dorsal side. Two 0.7-mm-diameter holes were drilled through the skull over the cortex (0.5 mm anterior, 2.5 mm lateral to lambda) for screws serving as electrodes. The epicranial side of each screw was connected to a transmitter lead and secured with bone cement. Signals were transmitted to a receiver placed below the animal's cage and saved on a personal computer at a sampling rate of 1 KHz. For the unbiased detection of seizures, ECoG data were analyzed using an in-house algorithm, based on feature extraction and artificial neural network (ANN) classification (Bar-Klein et al., 2014). Seizures were defined an electrographic events lasting a minimum of 5 s.

Ex vivo GFAP and synaptic quantification

Brain slices were imaged using z-stack confocal microscopy (Olympus IX50) and a 40× (GFAP) or 63× (synaptic) objective with 1 μm z-slices. Images were analyzed automatically using Matlab (Mathworks, Natick, MA). For GFAP quantification, positive voxels were considered as those with intensities higher than the averaged intensity of control images (mean minus standard deviation). For synaptic quantification, we adapted from Eroglu et al. (2009), Ippolito and Eroglu (2010) (http://fohs.bgu.ac.il/neurophysio/). In brief, maximum intensity projections (MIPs) were generated from groups of 3 consecutive sections (1 μm each). The puncta size was set at 4–15 voxels and synapses were defined as at least 50% co-localization between pre- and post-synaptic staining.

Primary cell culture

Brains from embryonic day 19 Sprague–Dawley rats (Charles River, Hollister, CA) were removed and the temporal lobe was microdissected. Cells were dissociated with a papain solution (10 U/mL) for 20 min at 37 °C, after which they were pelleted by centrifugation, and the papain solution was replaced with HBSS (Life Technologies, Grand Island, NY). A single cell suspension was achieved by mechanical trituration and the cells were counted using a hemocytometer. The cells were plated at a density of 12.5×10^4 cells per mL in growth medium (MEM with Earle's salts, 2.5% B27 supplement (Life Technologies), 5% FBS (Life Technologies), 20 mM glucose, and 5 mM L-glutamine into glass-bottomed chamber slides (Thermo Fisher Scientific, Portsmouth, NH). After a 4-h incubation, the cell culture medium was replaced with Neurobasal medium (Life Technologies) supplemented with 2% B27 and 0.5 mM GlutaMAX (Life Technologies). The cells were maintained in 5% CO_2 at 37 °C. For pure neuronal cultures, cytosine arabinofuranoside (AraC; 10 μM; Sigma, St. Louis, MO) was added at 3 days in vitro (DIV) to the cultures to inhibit mitotically dividing cells. Cells were treated for 72 h with 0.2 mM BSA, 10 ng/mL TGF-β1, or Neurobasal media. For TGF-β blocking experiments, cells were pre-incubated for 1 h in 30 μM SJN 2511.

Immunocytochemistry (ICC)

For ICC in vitro preparations, cells were fixed with 4% PFA for 15 min at room temperature and washed in PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. Primary antibodies were incubated overnight at 4 °C in 1% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) in PBS. Excitatory synapses were labeled with primary antibodies for PSD-95 (UC Davis, Neuromab, Davis, CA; 1:800) and synapsin1 (Sigma; 1:800). Inhibitory synapses were labeled with primary antibodies for V-Gat (Millipore, Billerica, MA; 1:200) and Gephyrin (BD Bioscience, San Jose, CA; 1:100). Cy3 and Alexa Fluor 488-conjugated secondary antibodies (Jackson ImmunoResearch; 1:500) in 1% normal donkey serum in PBS were applied for 2 h at room temperature. The nuclear stain (DAPI; 1:20,000) was added prior to mounting coverslips in DABCO. Fluorescent images were obtained with an inverted fluorescence microscope using the Metamorph imaging system.

In vitro synaptic quantification

Cell culture images were collected using epifluorescence microscopy (Zeiss Axio Observer Z1) and analyzed using the Metamorph Software automatic cell scoring application. Excitatory synapses were defined as co-localization between Synapsin1 (pre-synaptic) and PSD-95 (post-synaptic) markers, while inhibitory synapses were defined as co-localization between V-Gat (pre-synaptic) and Gephyrin (post-synaptic) markers.

Real-time RT-PCR

Total RNA was isolated using the TRIzol reagent (Life Technologies) in accordance with the manufacturer's protocol. mRNA expression levels were determined by quantitative reverse transcriptase-PCR using real-time kinetic analysis with an iQ5 detection system (Bio-Rad, Hercules, CA). Real-time PCR data were analyzed using the PCR Miner program (Leonoudakis et al., 2008), and RPLP (ribosomal protein L16) RNA levels were used as internal controls for variations in sample preparation. Primer sequences were as follows: GFAP (+): AGA AAA CCG CAT CAC CAT TC; GFAP (−): TCC TTA ATG ACC TCG CCA TC. PSD-95 (+) TGC GAA GCA ACC CCA AGA GG, (−) ATG AAG CAC ATC CCC GAA GCG; VGlut1 (+) CCA CAC ACA GCA CAG TTC AGC C, (−) TCC TCG ACA CTG CCG TTT AGG

Statistics

All statistical comparisons were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Single variable comparisons between groups were performed using either one-way ANOVA with Dunnett's post hoc test or nonparametric Kruskal–Wallis test with Dunn's multiple comparisons test. Two variable comparisons were performed using two-way ANOVA with post hoc of repeated *t*-tests, corrected using the Sidak–Bonferroni method. *P*-values smaller than 0.05 were considered significant.

Results

ICV albumin induces epileptogenesis in vivo

To simulate BBB dysfunction in naïve animals and to study the role of serum albumin in epileptogenesis, mice were infused with albumin (0.4 mM in ACSF, 1 μL/h) through an ICV mini-osmotic pump for 7 days. ECoG data were acquired for up to 32 days (32 days: $n = 4$; 14 days: $n = 7$; 10 days: $n = 2$) and analyzed using an automated seizure detection algorithm (see Materials and Methods and Bar-Klein et al., 2014). Albumin treatment did not induce status epilepticus (SE) nor seizures during the first 72 h after pump implantation, in contrast to the pilocarpine model of epilepsy, in which mice develop SE shortly after drug injection (Fig. 1B). After a latent period of 4.9 ± 1.3 days, ten of the thirteen albumin-infused mice presented recurring spontaneous seizures (1.16 ± 0.16 seizures per day, lasting 47.2 ± 12.7 s). Notably, spontaneous seizures continued throughout the duration of ECoG monitoring, well after pump removal on day 7 (Fig. 1C, D). These results indicate that, similar to human PIE, exposure to albumin leads to self-sustained recurrent seizures, preceded by a latent period with no seizure activity.

ICV albumin induces activation of hippocampal astrocytes

To study the pathological changes that precede the appearance of seizures, a cohort of mice was infused with albumin for 72 h. Imaging of fluorescein-conjugated albumin revealed prominent albumin accumulation in the hippocampi and cortical regions (Fig. 2A). Western blot analysis confirmed hippocampal accumulation of albumin, comparable to the levels observed 24 h following SE in the pilocarpine model of epilepsy (ctrl, 0.58 ± 0.2 μM; 72 h-alb, 3.2 ± 0.2 μM; $n = 4$, $p < 0.05$; ctrl, 0.31 ± 0.06 ; 24 h post-SE 0.82 ± 0.2 μM; $n = 7$, $p < 0.05$). Consistent with findings following cortical exposure to albumin (Bar-Klein et al., 2014; Braganza et al., 2012; Frigerio et al., 2012; Ivens et al., 2007), fluorescent albumin was evident in astroglialelements (Fig. 2B). GFAP staining 72 h after infusion of fluorescein-conjugated albumin suggests astrocytic activation in the hippocampus, somatosensory cortex, corpus callosum, and adjacent neocortical regions. The quantification of GFAP mRNA and protein (through immunohistochemistry, ctrl = 4 alb = 5 $p < 0.0001$; real-

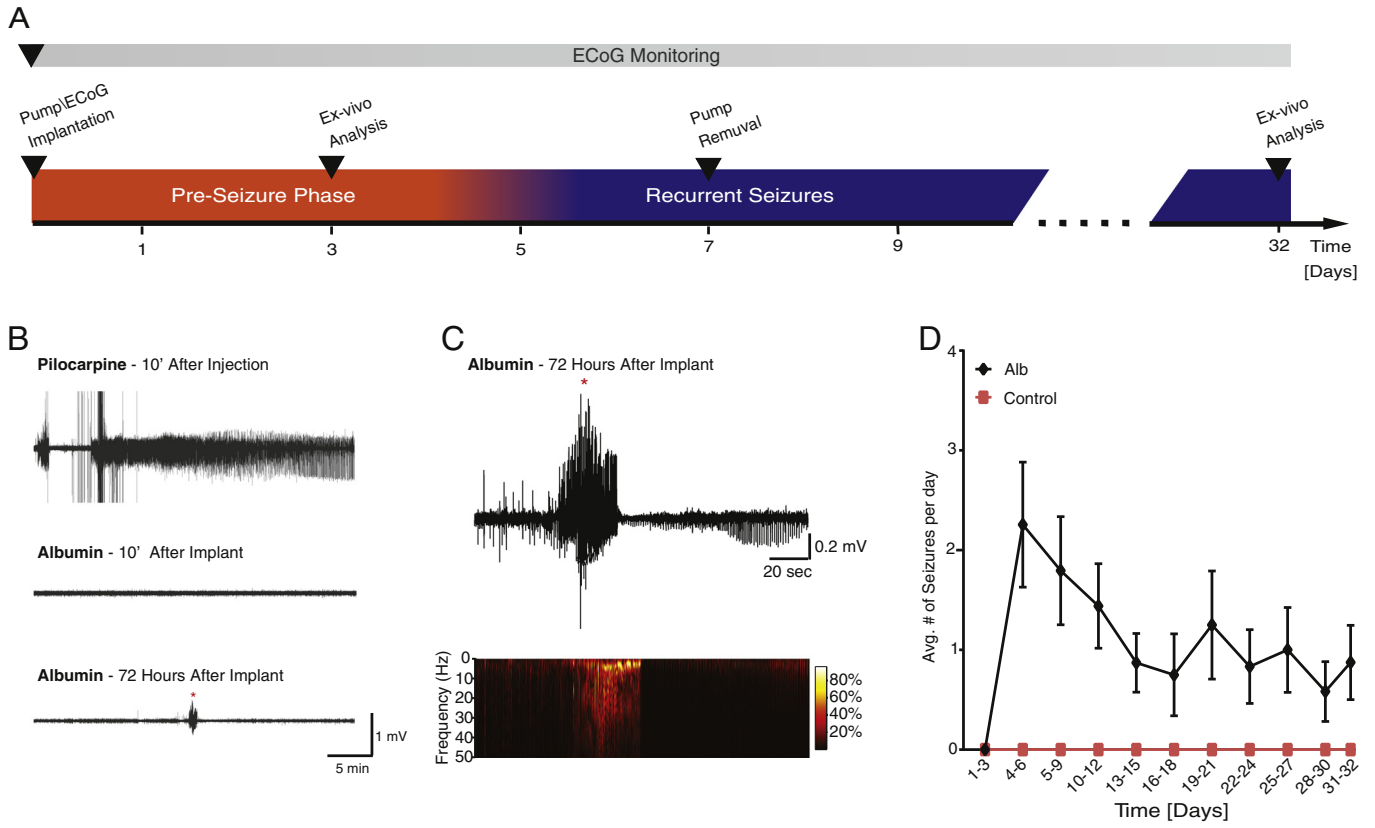

Fig. 1. ICV albumin induces latent appearance of spontaneous recurrent seizures. (A) The experimental paradigm in vivo. (B) Continuous ECoG recordings show that ICV albumin does not induce acute seizures or status epilepticus (common in other models of acquired epilepsy, e.g., pilocarpine) yet results in latent appearance of spontaneous seizures. (C) Representative seizure recorded 72 h after pump implantation. (D) Comparison of number of seizures per day (averaged over 3-day periods) shows higher incidence of seizures in the albumin-infused mice (black line, $n = 13$) compared to controls (red line, $n = 10$). Notably, seizures appeared 3 days after initiating albumin infusion, and lasted well after the extraction of the pumps on day 7. Error bars indicate SEM.

time PCR, ctrl = 3 alb = 3 $p = 0.0004$; and western blot, ctrl = 4 alb = 6 $p < 0.01$) confirmed astrocytic activation in albumin-exposed hippocampi (Fig. 2D, E). Hematoxylin and eosin staining 1 month after ICV albumin did not reveal any gross abnormalities (Fig. 2F), in contrast to SE-induced models of epilepsy.

Albumin induces excitatory synaptogenesis

Astrocytes are known to regulate the generation and elimination of both excitatory and inhibitory synapses (Chung and Barres, 2012; Clarke and Barres, 2013; Elmariah et al., 2005; Eroglu and Barres, 2010). Thus, the prominent albumin-induced astrocytic activation observed during the pre-seizure period suggested a possible role in synaptic network reorganization. To study the synaptogenic effect of albumin, we quantified excitatory and inhibitory synaptic puncta in primary mixed neuronal and glial cells of temporal origin exposed to 0.2 mM serum albumin (50% of normal serum concentration, Alb = 17; no treatment (NT) = 12) for 72 h. The quantification of excitatory pre-synaptic (synapsin1) and post-synaptic (PSD-95) markers along dendritic lengths revealed a significant increase in excitatory pre-synaptic ($p < 0.0001$), post-synaptic ($p = 0.0017$), and co-localized ($p = 0.0010$) puncta (Fig. 3A). Since inhibitory synapses occur both on dendrites and on the axon initial segment (Nusser et al., 1995), inhibitory pre-synaptic (V-Gat) and post-synaptic (Gephyrin) markers were quantified along dendritic lengths and per somatic area. No significant changes in the number of inhibitory synapses were observed (Fig. 3B, per soma: Alb = 17; NT = 15; $p = 0.2569$; and per dendrite Alb = 19; NT = 18. $p = 0.0854$), indicating that the synaptogenic effect of albumin is specific to excitatory synapses. These observations suggest a mechanism whereby vascular pathology, BBB breakdown, and albumin extravasation shift the balance of the neural network in favor of increased excitability.

Albumin-induced excitatory synaptogenesis is mediated by TGF-β/ALK5 signaling

We have previously shown that albumin initiates TGF-β signaling in astrocytes (Cacheaux et al., 2009; Ivens et al., 2007) via the ALK5 receptor (Bar-Klein et al., 2014). To test the potential involvement of TGF-β signaling in albumin-induced excitatory synaptogenesis, we treated cultures with TGF-β1 (10 ng/mL) for 72 h and repeated the synaptic quantification assay. Similar to the effect of albumin, exposure to TGF-β1 increased the number of excitatory pre-synaptic ($n = 18$, $p < 0.0001$), post-synaptic ($n = 18$, $p < 0.0001$), and co-localized ($n = 18$, $p < 0.0001$) puncta (Fig. 4A). A subset of cultures was co-incubated with the selective TGF-β type I receptor ALK5 inhibitor SJN2511 (SJN; 30 μM, applied 1 h prior to TGF-β1). SJN prevented both albumin- and TGF-β1-induced increase in excitatory pre-synaptic (Alb = 17 Alb + SJN = 21, $p < 0.0001$; TGFβ = 18 TGFβ + SJN = 20, $p < 0.0001$), post-synaptic (Alb + SJN, $p < 0.0001$; TGFβ + SJN, $p < 0.0001$), and co-localized puncta (Alb + SJN, $p = 0.00012$; TGFβ + SJN, $p < 0.0001$), to a level comparable with the untreated control group (Fig. 4A). These results demonstrate that activation of the ALK5/TGF-β pathway is critical for albumin–induced excitatory synaptogenesis. Moreover, ALK5/TGF-β1 inhibition in control cultures was sufficient *per se* to reduce synaptic counts ($p = 0.0102$, Fig. 4A), suggesting that baseline release of TGF-β1 promotes synaptogenesis in cultures.

Albumin|TGF-β-induced excitatory synaptogenesis is astrocyte dependent

We have previously demonstrated that exposure to albumin induces activation of the TGF-β pathway and secretion of TGF-β1 in astrocytes, and not in neurons (Bar-Klein et al., 2014). In order to test the role of astrocytes in albumin-induced synaptogenesis, we created neuronal enriched cultures by eliminating astrocytes with the mitotic inhibitor AraC. Notably, in the absence of a significant astrocytic population, exposure to albumin did not induce any significant changes in the number of excitatory or inhibitory synaptic puncta (NT = 15; Alb = 13; Fig. 4B). Similarly, exposure to TGF-β1 or the ALK5 blocker SJN did not induce any significant changes in the number of excitatory or inhibitory synaptic puncta in the absence of astrocytes (TGF-β1 = 14; SJN = 15; SJN&Alb = 13; SJN&TGF-β1 = 13; Fig. 4B). These results demonstrate that astrocytes are necessary for albumin- and TGF-β1-induced synaptogenesis.

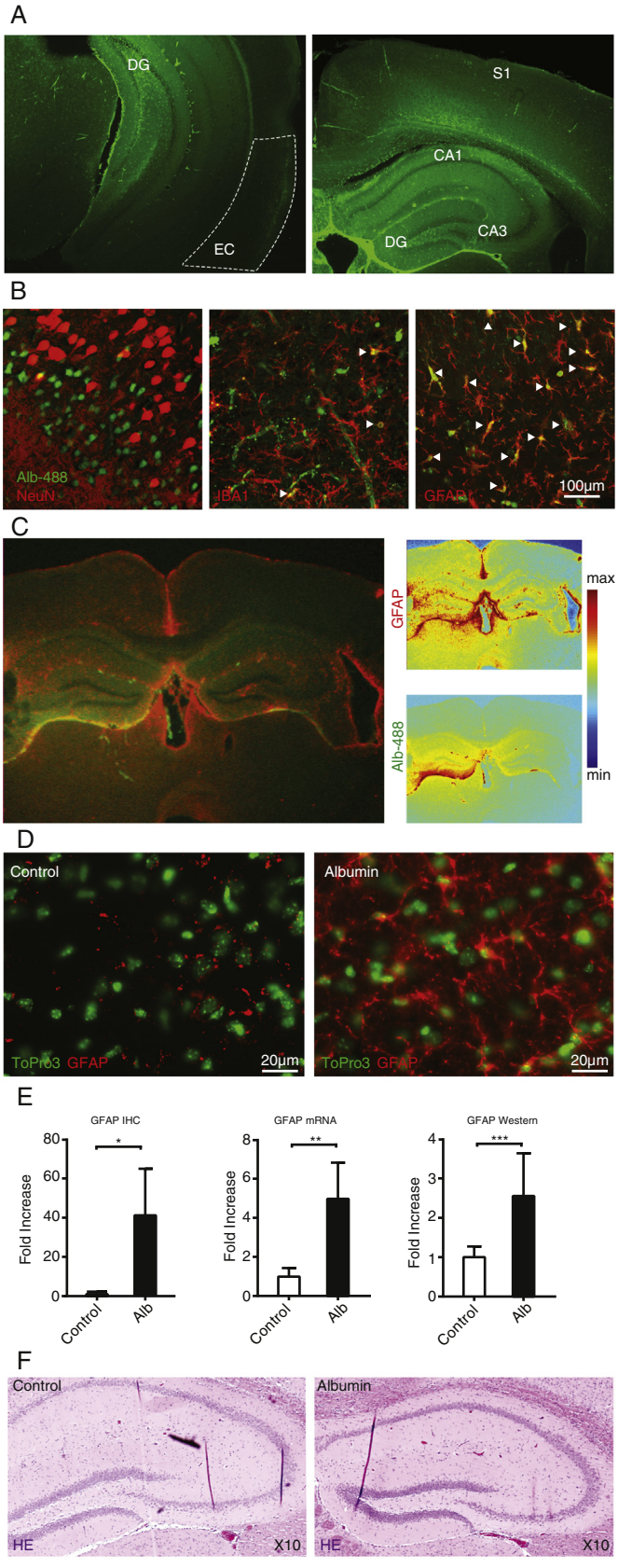

Fig. 2. ICV albumin induces activation of astrocytes. (A) 72 h infusion of fluorescein-conjugated albumin (10%; Alb-488, green) into the lateral ventricle results in florescence accumulation in the hippocampus (DG, CA1,3), the somatosensory cortex (S1), corpus callosum, entorhinal cortex (EC), and adjacent neocortical regions, as evident here in coronal slices 72 h after pump implantation (right, ~bregma −1.58 mm; left, ~bregma −2.92 mm). (B) Best representative images of albumin (Alb-488) co-localization with neurons (NeuN, somatosensory cortex), microglia (IBA1, hippocampal hilus), and astrocytes (GFAP, hippocampal hilus). White arrowheads mark the co-localization of cells with fluorescein-conjugated albumin. (C) GFAP immunohistochemical staining of a coronal slice, 72 h after infusion of fluorescein-conjugated albumin. GFAP and albumin florescent intensity maps (right panels) show high levels of fluorescence in the hippocampus, somatosensory cortex, corpus callosum, and adjacent neocortical regions. (D) Immunohistochemical (IHC) staining of cell nuclei (ToPro3, green) and GFAP-positive cells (red) demonstrates increased levels of GFAP following ICV albumin treatment in the hilus of the hippocampus. (E) Quantitative analyses of IHC staining (IHC, ctrl = 4 alb = 5) of the hippocampus reveals a significant increase in GFAP expression, further confirmed by real-time RT-PCR (mRNA, ctrl = 3 alb = 3) and western blots (western, ctrl = 4 alb = 6). (F) Hematoxylin and eosin (HE) hippocampal staining shows no gross abnormalities 1 month after albumin infusion compared to controls (ctrl = 3 alb = 3). Error bars indicate SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

ICV albumin and TGF-β induce anatomically specific excitatory synaptogenesis in vivo

To verify the in vitro evidence of albumin-induced synaptogenesis in vivo, mice were ICV infused for 72 h with aCSF (ctrl = 4), albumin (Alb = 4), TGF-β1 (TGFβ = 3), or albumin and SJN2511 (Alb + SJN = 3). Both albumin and TGF-β1 significantly increased excitatory synaptic counts in the entorhinal cortex (EC), motor cortical areas (CTX), and the CA1 area of the hippocampus ($p < 0.0001$, compared to ctrl, Fig. 5B), with no significant effect in the DG or CA3 ($p > 0.2$, compared to ctrl, Fig. 5B). Notably, co-infusion with SJN prevented synaptogenesis in all three brain areas ($p < 0.0001$, compared to albumin, Fig. 5B). In accordance with immunohistochemistry, mRNA levels revealed that exposure to albumin ($n = 8$) significantly increased excitatory synaptic proteins PSD95 ($p < 0.0001$) and VGlut ($p = 0.0025$) in the neocortex, but not in the homogenates from the entire hippocampus. Thus, similar to the in vitro findings, albumin induces excitatory synaptogenesis through TGF-β signaling, and this process can be prevented in vivo with SJN2511.

SJN2511 prevents albumin-induced seizures in vivo

Exposure to TGF-β1 was previously reported to induce increased hyperexcitability in evoked potentials recoded in vitro (Cacheaux et al., 2009). As both albumin and TGF-β1 have similar synaptogenic effects, we set out to test whether albumin-induced seizures (Fig. 1) could be replicated by infusion of TGF-β1 and prevented by ALK5 inhibition. Thus, an additional cohort of mice was implanted with epidural ECoG electrodes and ICV pumps loaded with either TGF-β1 (TGFβ = 5) or albumin and SJN2511 (Alb + SJN = 8). ECoG data were acquired over 14 days and analyzed using the automated seizure detection algorithm (see Materials and Methods and Bar-Klein et al., 2014). While 77% of albumin-treated mice developed epilepsy ($p = 0.0004$, compared to controls), TGF-β1 induced spontaneous recurring seizures in 100% of infused animals ($p = 0.0003$, compared to controls, Fig. 6A). Notably, similar to albumin, TGF-β1-induced seizures appeared after a latent period of several days (5.8 ± 1.76 days, $p = 0.8039$ compared to

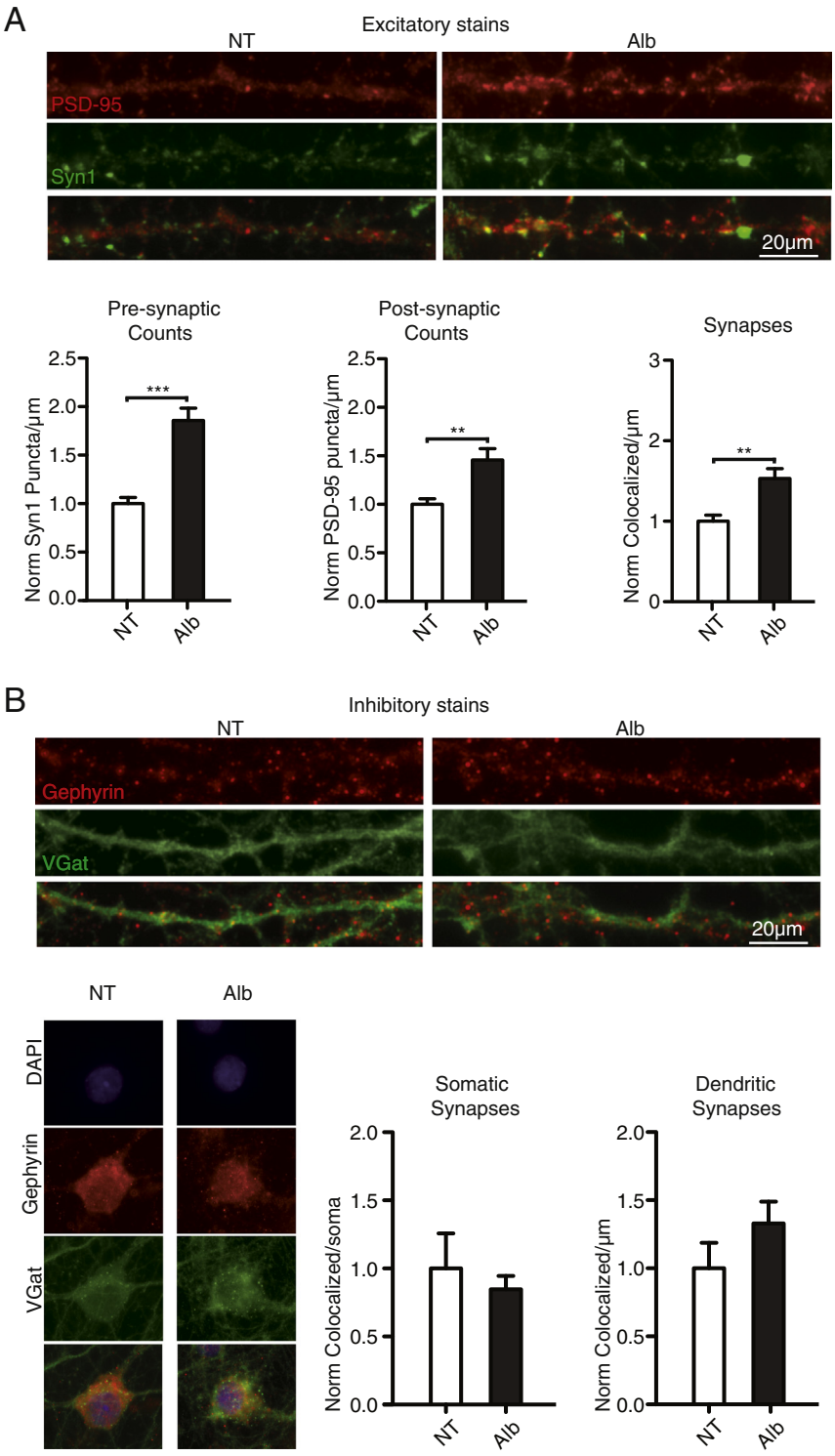

Fig. 3. Albumin induces excitatory, but not inhibitory, synaptogenesis in temporal cultures. (A) Staining for excitatory pre-synaptic (Syn1; green) and post-synaptic (PSD-95, red) protein revealed a significant albumin-induced increase in pre-synaptic, post-synaptic, and co-localized counts along dendritic lengths (Alb = 17; no treatment (NT) = 12). (B) Staining for inhibitory pre-synaptic (V-Gat, green) and post-synaptic (Gephyrin, red) proteins showed no significant albumin-induced differences in synaptic counts, quantified along dendritic lengths and the somatic area (per soma: Alb = 17; NT = 15; $p = 0.2569$; and per dendrite Alb = 19; NT = 18. $p = 0.0854$). Error bars indicate SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

albumin), having a comparable seizure duration (39.3 ± 13.4 s) and frequency (2.1857 ± 0.428 per day). Most importantly, SJN2511 prevented the development of epilepsy in all but one albumin-treated mice ($p = 0.0075$, compared to controls, Fig. 6B), demonstrating that blocking albumin activation of ALK5 signaling is sufficient to significantly reduce the incidence of epilepsy in this model.

Discussion

Here we demonstrate an albumin-induced and TGF-β-mediated synaptogenic mechanism critical in the epileptogenic process. We show both in vitro and in vivo that albumin induces excitatory synapse formation, and that this effect is astrocyte and ALK5 dependent.

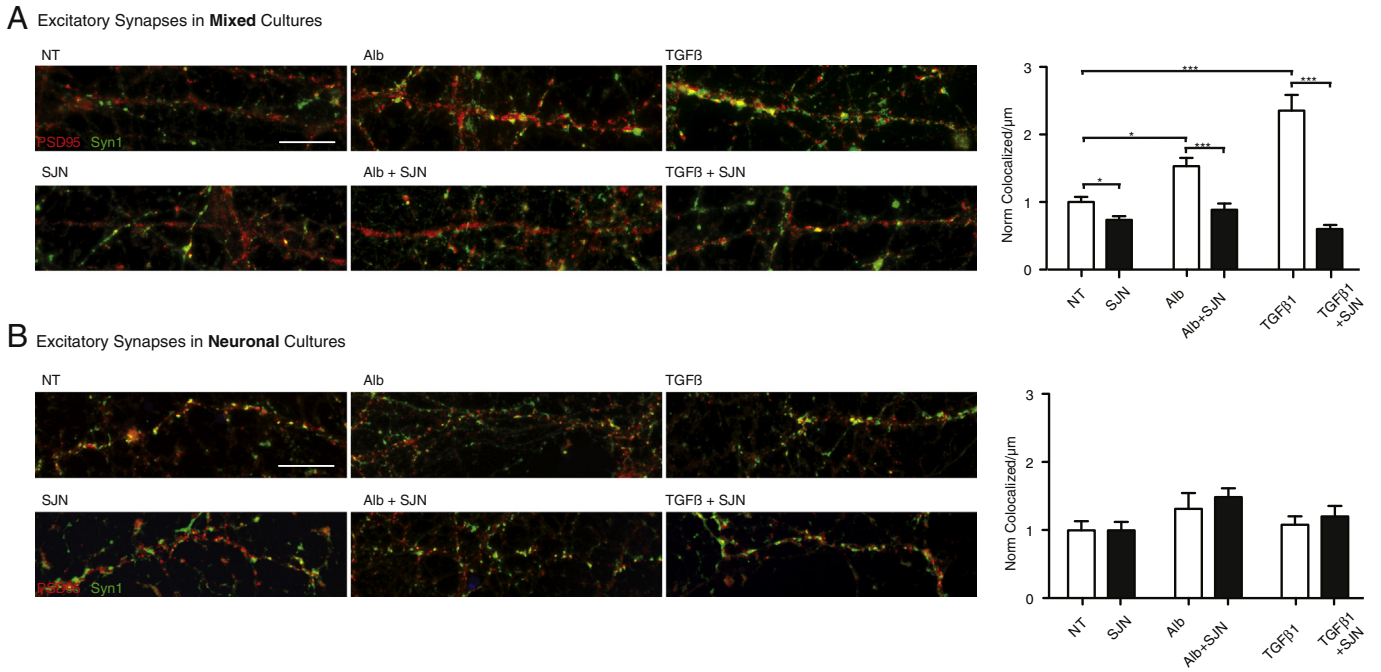

Fig. 4. Albumin-induced excitatory synaptogenesis is mediated by astrocyte-driven TGF-β\ALK5 signaling. (A) In mixed cultures of astrocytes and neurons, 72 h TGF-β1 incubation ($n = 18$) induced a synaptogenic effect similar to that of albumin ($n = 17$), with both treatments resulting in significantly higher synaptic counts compared to untreated controls (no treatment (NT) = 17). Both albumin- and TGF-β1-induced synaptogenesis was significantly blocked by the selective TGF-β type I receptor ALK5 inhibitor (SJN2511, SJN) (NT = 15;Alb + SJN = 21;TGFβ + SJN = 20). (B) No significant increase in synaptic counts was observed in enriched neuronal cultures ($p > 0.6$; Alb = 13; TGFβ = 14; NT = 15), and pre-incubation with SJN had no effect on synaptic counts in the absence of astrocytes ($p > 0.9$; SJN = 15; SJN + Alb = 13; SJN + TGFβ1 = 13). Error bars indicate SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

TGF-β signaling in astrocytes following exposure to albumin was previously shown to promote several functional changes that may be associated with increased neuronal excitability, including induction of pro-ictogenic inflammatory cytokines (Frigerio et al., 2012), down-regulation of gap junction proteins (connexin 30 and 43 (Braganza et al., 2012), and astrocytic potassium, glutamate, and water channels (Cacheaux et al., 2009; Ivens et al., 2007). This transcriptional response was then associated with a frequency-dependent increase in the concentration of extracellular potassium ($[K^+]_o$) and hyperexcitability of neighboring neurons (David et al., 2009; Lapilover et al., 2012). However, the reduced capacity of $[K^+]_o$ buffering was found to be immediate and short-lived, returning to pre-albumin levels within 4 days (Braganza et al., 2012). In contrast, the tendency of the network for seizures post-exposure to albumin was found to be delayed and long lasting (Bar-Klein et al., 2014; Seiffert et al., 2004), resembling the delayed appearance of epilepsy in patients following insults to the brain. The reorganization of the neural network has been suggested to underlie chronic seizures, in light of evidence of axonal sprouting and synapse formation in status epilepticus and traumatic injury models of epilepsy (Jin et al., 2006; Liauw et al., 2008; Scheff et al., 2005). Our findings demonstrate a mechanism linking brain injury to synaptogenesis and epilepsy, through BBB dysfunction and albumin-induced astrocytic TGF-β\ALK5-mediated selective formation of excitatory synapses.

BBB dysfunction has been well documented following epileptogenic insults and in epileptic tissue (Aronica et al., 2008; Kaya et al., 2012; Marcon et al., 2009; Raabe et al., 2012; Shlosberg et al., 2010; Tomkins et al., 2001). Animal studies indicate that BBB dysfunction promotes seizure generation (Marchi et al., 2007; Van Vliet et al., 2007) and is associated with epileptogenesis (Bar-Klein et al., 2014; Seiffert et al., 2004). Furthermore, the delayed effect of chemical BBB opening by bile salts (Ivens et al., 2007; Seiffert et al., 2004; Tomkins et al., 2007) was reproduced through direct cortical exposure to the most common serum protein, albumin, suggesting albumin as a key initiator of the epileptogenic process following BBB injury (Bar-Klein et al., 2014; Seiffert et al., 2004). Here we focused on the mechanisms underlying albumin-induced network reorganization, establishing a minimally invasive mouse model of BBB dysfunction and epilepsy, through ICV infusion of albumin.

To date, many existing models of acquired epilepsy are initiated by status epilepticus, temporal lobe sclerosis, and massive cell loss and dispersion. However, status epilepticus is a rare event in human patients, and 30–40% of patients with temporal lobe epilepsy do not have sclerosis (de Lanerolle et al., 2012). Here we present a non-status epilepticus model of chronic epilepsy, leading to delayed appearance of recurrent seizures in mice. Furthermore, although we did not rule out endfolium sclerosis or pyramidal interneuron loss, we found no apparent evidence of gross abnormalities for up to 32 days, thus allowing the study of epileptogenic mechanisms independent of sclerosis. Moreover, direct infusion of albumin mimics the pathological accumulation of albumin observed in human epileptic tissue (Raabe et al., 2012; Schmitz et al., 2013; Van Vliet et al., 2007) and in animal models of status epilepticus (Ndode-Ekane et al., 2010; Pont et al., 1995; Ruth, 1984; Saija et al., 1992; Van Vliet et al., 2007, 2014; Zucker et al., 1983). However, while albumin is sufficient to induce the development of epilepsy in a naïve brain, the interplay of BBB dysfunction and albumin-induced network alterations with other epileptogenic insults (e.g., neuronal sprouting, sclerosis, channelopathies) in the context of an epileptic brain remains to be studied (Friedman and Heinemann, 2012).

We further demonstrate that albumin and TGF-β1 have similar epileptogenic effects that are prevented by the specific inhibition of the TGF-β\ALK5 pathway. Our findings validate the role of TGF-β in PIE, supporting the epileptogenic role of the ALK5 SMAD 2,3 signaling (Bar-Klein et al., 2014). While TGF-β1 infusion induced seizures in all mice, albumin induced seizures in only 77%. Interestingly, albumin also induced a significant yet variable increase in GFAP, encouraging further studies to explore the cause of this variability and whether it may be correlated to albumin accumulation and/or seizure onset/frequency. Blocking the TGF-β pathway eliminated the occurrence of spontaneous seizures in all but one mouse, which presented a total of two seizures (Fig. 6D, E). These results suggest either an incomplete block or the involvement of additional mechanisms in albumin-induced epilepsy.

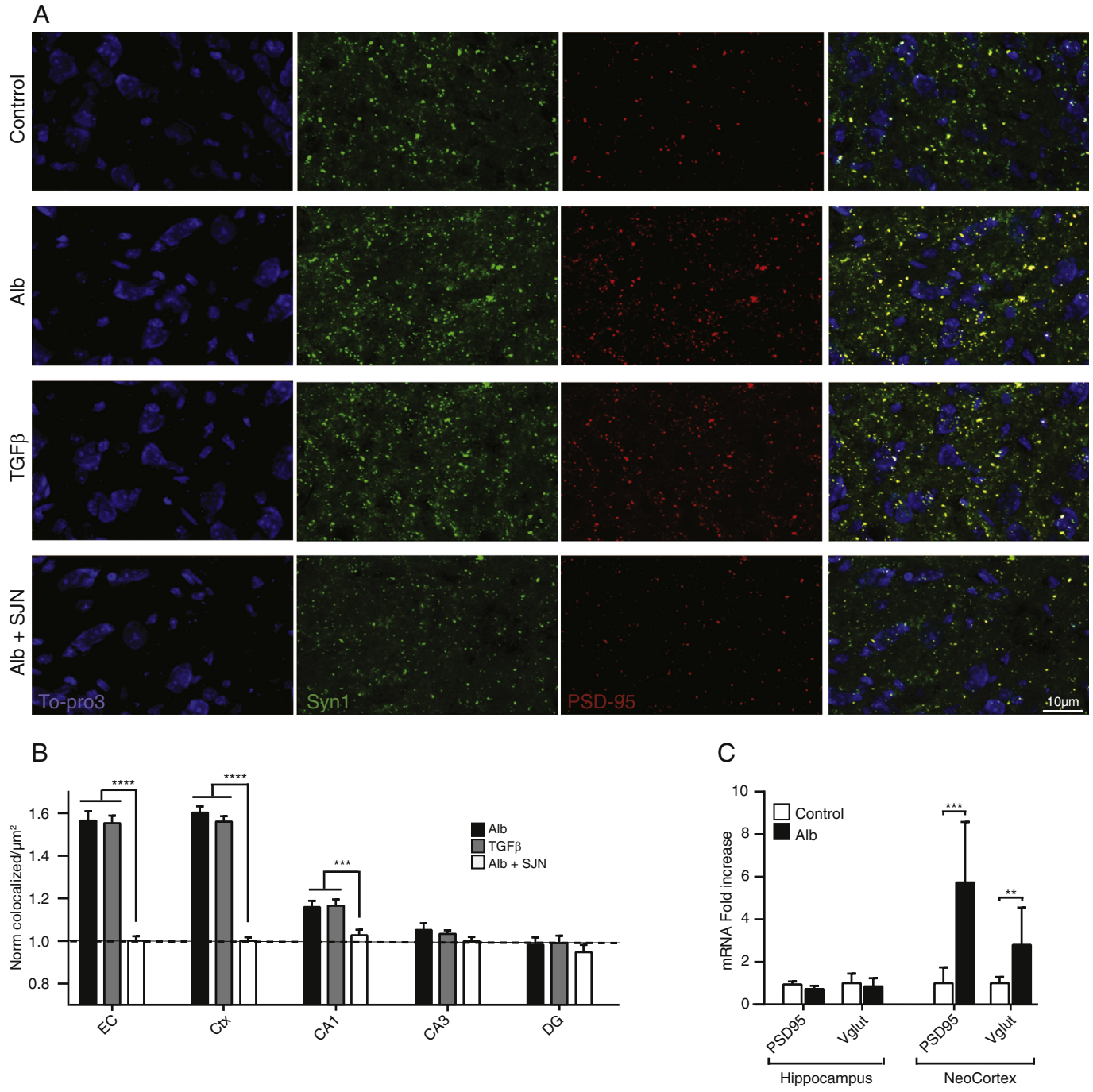

Fig. 5. Albumin exposure in vivo is associated with region-specific, TGF-β1-mediated synaptogenesis. (A) Representative z-stacked images of pre-synaptic (Syn1, green) and post-synaptic (PSD-95) markers 72 h after pump implantation. (B) Synaptic quantification demonstrates that TGF-β1 ($n = 3 \times 6$ slices) and albumin ($n = 4 \times 6$ slices) induce similar synaptogenic effects, blocked by SJN2511. (C) RT-PCR demonstrates a significant increase in cortical expression of post-synaptic (PSD95) and pre-synaptic (Vglut) protein mRNA in albumin-treated mice ($n = 8$) compared to controls ($n = 12$). Error bars indicate SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

Our in vitro results showed that both albumin and TGF-β1 induce the formation of excitatory synapses, with no significant effect on inhibitory synaptic counts in astrocyte/neuronal mixed temporal cell cultures. Moreover, the observed excitatory synaptogenesis was found to be astrocyte dependent, as it was absent in astrocyte depleted cultures. These results are consistent with the absence of TGF-β-mediated synaptogenesis in astrocyte-free cultures in retinal ganglion cells (Christopherson et al., 2005), and the plethora of reports of astrocyte-driven excitatory synapse formation, through secretion of thrombospondins (TSPs)(Cekanaviciute et al., 2014; Christopherson et al., 2005; Liauw et al., 2008), hevin (Kucukdereli et al., 2011) and glypicans 4 and 6 (Allen et al., 2012). Cell cultures are derived from temporal lobe embryonic tissue representing hippocampal and entorhinal cortex. To enable a detailed analysis of regional albumin-induced changes in synaptogenesis, we next analyzed excitatory synapses in in vivo exposure of albumin exposure and found that both albumin and TGF-β1 increase excitatory synaptic counts in the EC, CTX, and the CA1, with no significant effect in the DG or CA3. Interestingly, recent reports have associated TGF-β signaling with both excitatory and inhibitory synaptogenesis in mixed and in pure neuronal cortical cultures (Diniz et al., 2012, 2014; Yu et al., 2014). Together with these studies, our findings suggest that TGF-β may affect different

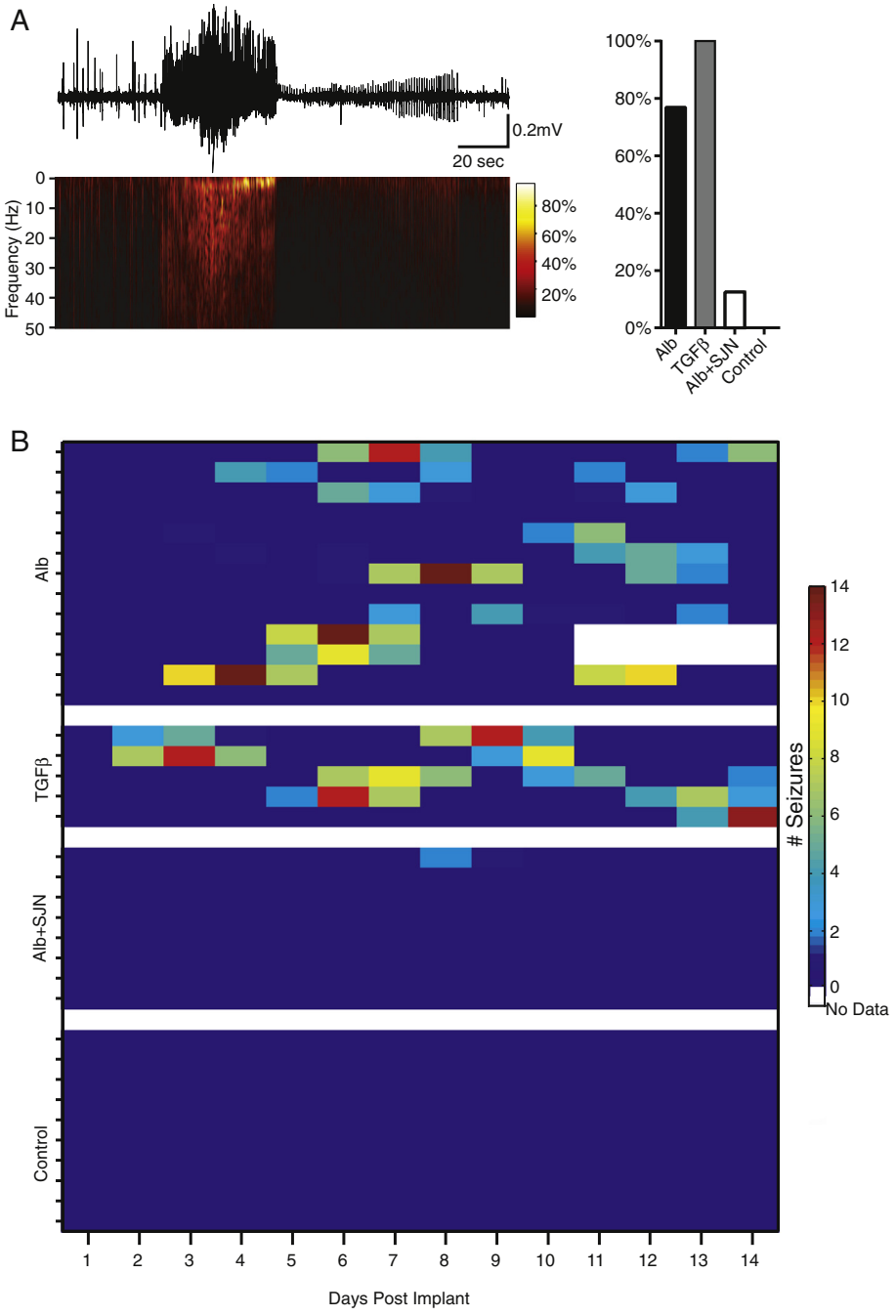

Fig. 6. Albumin-induced seizures are mediated by TGF-β and can be prevented with SJN2511. (A) ICV TGF-β induces spontaneous epileptic seizures, comparable to those observed following ICV albumin. However, while albumin induced epilepsy in 77% of mice ($n = 13$), all TGF-β-infused mice developed seizures ($n = 5$). Moreover, co-infusion with SJN2511 ($n = 8$) prevented seizures in all but one animal. (B) Albumin and TGF-β-induced seizures persisted throughout the monitoring period (and well after pump extraction on day 7). The one epileptic animal in the SJN group suffered two seizures on day 9.

cellular targets and regulate various processes in a region and\or dose-dependent manner.

Further studies are required to understand the detailed characteristics of TGF-β signaling and the mechanisms of its synaptogenic effect. NMDA post-synaptic currents are likely to be part of this mechanism since (1) TGF-β-mediated synaptogenesis is associated with secretion of the NMDA co-agonist D-serine (Diniz et al., 2012); (2) post-albumin accumulation of $[K^+]_o$ predicts the over-activation of NMDA channels (David et al., 2009), and (3) albumin-induced up-regulation of IL1β or HMGB1 in astrocytes may enhance Ca influx through NMDA receptor channels (Balosso et al., 2008; Frigerio et al., 2012; Maroso et al., 2010; Viviani et al., 2003).

Using the new model of BBB dysfunction in mice, we reveal a synaptogenic cascade leading to spontaneous recurrent seizures and demonstrate a method for preventing this outcome. While it was suggested that brain injuries elicit inflammatory signaling and synaptogenesis as part of a compensatory repair mechanism (Falo et al., 2008; Li et al., 2013; Lin et al., 2003; Ruscher et al., 2011; Scheff et al., 2005), here we suggest that this repair response may in and of itself lead to enhanced pathological connectivity and tendency for spontaneous seizures. Furthermore, we show that serum albumin can act as a potent pro-synaptogenic signaling molecule, suggesting that regulation of BBB integrity may allow controlled synaptogenesis in injured tissue. Future research is needed to understand the therapeutic role of post-injury

synaptogenesis and the regulating mechanisms required for the maintenance of a normal excitatory\inhibitory balance. While the neuronal pathways activated following albumin-mediated inflammatory response remain to be identified, they may offer an opportunity to further manipulate and refine post-injury synapse formation.

Competing interests

The authors declare no competing interests.

Acknowledgments

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References

Abbott, N.J., Friedman, A., 2012. Overview and introduction: the blood–brain barrier in health and disease. Epilepsia 53 (Suppl. 6), 1–6. http://dx.doi.org/10.1111/j.1528-1167.2012.03696.x.

Allen, N.J., Bennett, M.L., Foo, L.C., Wang, G.X., Chakraborty, C., Smith, S.J., Barres, B.A., 2012. Astrocyte glypicans 4 and 6 promote formation of excitatory synapses via GluA1 AMPA receptors. Nature 486, 410–414.

Aronica, E., Boer, K., Becker, A., Redeker, S., Spliet, W.G., van Rijen, P.C., Wittink, F., Breit, T., Wadman, W.J., Lopes da Silva, F.H., Troost, D., Gorter, J.A., 2008. Gene expression profile analysis of epilepsy-associated gangliogliomas. Neuroscience 151, 272–292. http://dx.doi.org/10.1016/j.neuroscience.2007.10.036.

Babb, T.L., Kupfer, W.R., Pretorius, J.K., Crandall, P.H., Levesque, M.F., 1991. Synaptic reorganization by mossy fibers in human epileptic fascia dentata. Neuroscience 42, 351–363.

Balosso, S., Maroso, M., Sanchez-Alavez, M., Ravizza, T., Frasca, A., Bartfai, T., Vezzani, A., 2008. A novel non-transcriptional pathway mediates the proconvulsive effects of interleukin-1beta. Brain 131, 3256–3265. http://dx.doi.org/10.1093/brain/awn271.

Bar-Klein, G., Cacheaux, L.P., Kamintsky, L., Prager, O., Weissberg, I., Schoknecht, K., Cheng, P., Kim, S.Y., Wood, L., Heinemann, U., Kaufer, D., Friedman, A., 2014. Losartan prevents acquired epilepsy via TGF-β signaling suppression. Ann. Neurol. 75. http://dx.doi.org/10.1002/ana.24147.

Braganza, O., Bedner, P., Hüttmann, K., von Staden, E., Friedman, A., Seifert, G., Steinhäuser, C., Huttmann, K., Steinhauser, C., 2012. Albumin is taken up by hippocampal NG2 cells and astrocytes and decreases gap junction coupling. Epilepsia 53, 1898–1906. http://dx.doi.org/10.1111/j.1528-1167.2012.03665.x.

Bragin, A., Wilson, C.L., Engel, J., 2000. Chronic epileptogenesis requires development of a network of pathologically interconnected neuron clusters: a hypothesis. Epilepsia 41 (Suppl. 6), S144–S152.

Cacheaux, L.P., Ivens, S., David, Y., Lakhter, A.J., Bar-Klein, G., Shapira, M., Heinemann, U., Friedman, A., Kaufer, D., 2009. Transcriptome profiling reveals TGF-beta signaling involvement in epileptogenesis. J. Neurosci. 29, 8927–8935. http://dx.doi.org/10.1523/JNEUROSCI. 0430-09.2009.

Cekanaviciute, E., Fathali, N., Doyle, K.P., Williams, A.M., Han, J., Buckwalter, M.S., 2014. Astrocytic transforming growth factor-beta signaling reduces subacute neuroinflammation after stroke in mice. Glia http://dx.doi.org/10.1002/glia.22675.

Christopherson, K.S., Ullian, E.M., Stokes, C.C.A., Mullowney, C.E., Hell, J.W., Agah, A., Lawler, J., Mosher, D.F., Bornstein, P., Barres, B.A., 2005. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120, 421–433. http://dx.doi.org/10.1016/j.cell.2004.12.020.

Chung, W.-S., Barres, B.A., 2012. The role of glial cells in synapse elimination. Curr. Opin. Neurobiol. 22, 438–445.

Clarke, L.E., Barres, B.A., 2013. Emerging roles of astrocytes in neural circuit development. Nat. Rev. Neurosci. 14, 311–321.

Crawford, D.C., Jiang, X., Taylor, A., Mennerick, S., 2012. Astrocyte-derived thrombospondins mediate the development of hippocampal presynaptic plasticity in vitro. J. Neurosci. 32, 13100–13110. http://dx.doi.org/10.1523/JNEUROSCI. 2604-12.2012.

Crunelli, V., Carmignoto, G., Steinhäuser, C., 2014. Astrocytic targets provide new avenues for the therapeutic treatment of epilepsy. Neuroscientist http://dx.doi.org/10.1177/1073858414523320 (1073858414523320–).

Cunningham, A.S., Salvador, R., Coles, J.P., Chatfield, D.A., Bradley, P.G., Johnston, A.J., Steiner, L.A., Fryer, T.D., Aigbirhio, F.I., Smielewski, P., Williams, G.B., Carpenter, T.A., Gillard, J.H., Pickard, J.D., Menon, D.K., 2005. Physiological thresholds for irreversible tissue damage in contusional regions following traumatic brain injury. Brain 128, 1931–1942. http://dx.doi.org/10.1093/brain/awh536.

David, Y., Cacheaux, L.P., Ivens, S., Lapilover, E., Heinemann, U., Kaufer, D., Friedman, A., 2009. Astrocytic dysfunction in epileptogenesis: consequence of altered potassium and glutamate homeostasis? J. Neurosci. 29, 10588–10599. http://dx.doi.org/10.1523/JNEUROSCI. 2323-09.2009.

de Lanerolle, N.C., Lee, T.S., Spencer, D.D., 2012. Histopathology of Human Epilepsy. In: Noebels, J.L., Avoli, M., Rogawski, M.A., et al. (Eds.), Jasper's Basic Mechanisms of the Epilepsies [Internet]. 4th edition. Bethesda (MD): National Center for Biotechnology Information (US) Available from: http://www.ncbi.nlm.nih.gov/books/NBK98141/.

Devinsky, O., Vezzani, A., Najjar, S., De Lanerolle, N.C., Rogawski, M.A., 2013. Glia and epilepsy: excitability and inflammation. Trends Neurosci. 36, 174–184.

Diniz, L.P., Almeida, J.C., Tortelli, V., Vargas Lopes, C., Setti-Perdigão, P., Stipursky, J., Kahn, S.A., Romão, L.F., de Miranda, J., Alves-Leon, S.V., de Souza, J.M., Castro, N.G., Panizzutti, R., Gomes, F.C.A., 2012. Astrocyte-induced synaptogenesis is mediated by transforming growth factor β signaling through modulation of D-serine levels in cerebral cortex neurons. J. Biol. Chem. 287, 41432–41445. http://dx.doi.org/10.1074/jbc.M112.380824.

Diniz, L.P., Tortelli, V., Garcia, M.N., Araújo, A.P.B., Melo, H.M., Seixas da Silva, G.S., De Felice, F.G., Alves-Leon, S.V., de Souza, J.M., Romão, L.F., Castro, N.G., Gomes, F.C.A., 2014. Astrocyte transforming growth factor beta 1 promotes inhibitory synapse formation via CaM kinase II signaling. Glia http://dx.doi.org/10.1002/glia.22713.

Elmariah, S.B., Oh, E.J., Hughes, E.G., Balice-Gordon, R.J., 2005. Astrocytes regulate inhibitory synapse formation via Trk-mediated modulation of postsynaptic GABAA receptors. J. Neurosci. 25, 3638–3650.

Eroglu, C., Barres, B.A., 2010. Regulation of synaptic connectivity by glia. Nature 468, 223–231. http://dx.doi.org/10.1038/nature09612.

Eroglu, C., Allen, N.J., Susman, M.W., O'Rourke, N.A., Park, C.Y., Ozkan, E., Chakraborty, C., Mulinyawe, S.B., Annis, D.S., Huberman, A.D., Green, E.M., Lawler, J., Dolmetsch, R., Garcia, K.C., Smith, S.J., Luo, Z.D., Rosenthal, A., Mosher, D.F., Barres, B.A., 2009. Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell 139, 380–392.

Falo, M.C., Reeves, T.M., Phillips, L.L., 2008. Agrin expression during synaptogenesis induced by traumatic brain injury. J. Neurotrauma 25, 769–783.

Feng, Z., Ko, C.-P., 2008. Schwann cells promote synaptogenesis at the neuromuscular junction via transforming growth factor-beta1. J. Neurosci. 28, 9599–9609.

Friedman, A., Heinemann, U., 2012. Role of blood–brain barrier dysfunction in epileptogenesis. In: Noebels, J.L., Avoli, M., Rogawski, M.A., Olsen, R.W., Delgado-Escueta, A.V. (Eds.), Jasper's Basic Mechanisms of the Epilepsies. Oxford University Press, Bethesda (MD).

Friedman, A., Kaufer, D., Shemer, J., Hendler, I., Soreq, H., Tur-Kaspa, I., 1996. Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. Nat. Med. 2, 1382–1385.

Friedman, A., Kaufer, D., Heinemann, U., 2009. Blood–brain barrier breakdown-inducing astrocytic transformation: novel targets for the prevention of epilepsy. Epilepsy Res. 85, 142–149. http://dx.doi.org/10.1016/j.eplepsyres.2009.03.005.

Frigerio, F., Frasca, A., Weissberg, I., Parrella, S., Friedman, A., Vezzani, A., Noé, F.M., 2012. Long-lasting pro-ictogenic effects induced in vivo by rat brain exposure to serum albumin in the absence of concomitant pathology. Epilepsia 53, 1887–1897. http://dx.doi.org/10.1111/j.1528-1167.2012.03666.x.

Ippolito, D.M., Eroglu, C., 2010. Quantifying synapses: an immunocytochemistry-based assay to quantify synapse number. J. Vis. Exp. (45), e2270 http://dx.doi.org/10.3791/2270.

Ivens, S., Kaufer, D., Flores, L.P., Bechmann, I., Zumsteg, D., Tomkins, O., Seiffert, E., Heinemann, U., Friedman, A., 2007. TGF-beta receptor-mediated albumin uptake into astrocytes is involved in neocortical epileptogenesis. Brain 130, 535–547. http://dx.doi.org/10.1093/brain/awl317.

Jin, X., Prince, D.A., Huguenard, J.R., 2006. Enhanced excitatory synaptic connectivity in layer v pyramidal neurons of chronically injured epileptogenic neocortex in rats. J. Neurosci. 26, 4891–4900.

Kaya, M., Becker, A.J., Gürses, C., 2012. Blood–brain barrier, epileptogenesis, and treatment strategies in cortical dysplasia. Epilepsia 53 (Suppl. 6), 31–36. http://dx.doi.org/10.1111/j.1528-1167.2012.03700.x.

Kucukdereli, H., Allen, N.J., Lee, A.T., Feng, A., Ozlu, M.I., Conatser, L.M., Chakraborty, C., Workman, G., Weaver, M., Sage, E.H., Barres, B.A., Eroglu, C., 2011. Control of excitatory CNS synaptogenesis by astrocyte-secreted proteins Hevin and SPARC. Proc. Natl. Acad. Sci. U. S. A. 108, E440–E449.

Lapilover, E.G., Lippmann, K., Salar, S., Maslarova, A., Dreier, J.P., Heinemann, U., Friedman, A., 2012. Peri-infarct blood–brain barrier dysfunction facilitates induction of spreading depolarization associated with epileptiform discharges. Neurobiol. Dis. 48, 495–506. http://dx.doi.org/10.1016/j.nbd.2012.06.024.

Leonoudakis, D., Zhao, P., Beattie, E.C., 2008. Rapid tumor necrosis factor alpha-induced exocytosis of glutamate receptor 2-lacking AMPA receptors to extrasynaptic plasma membrane potentiates excitotoxicity. J. Neurosci. 28, 2119–2130.

Li, L.-Y., Li, J.-L., Zhang, H.-M., Yang, W.-M., Wang, K., Fang, Y., Wang, Y., 2013. TGFβ1 treatment reduces hippocampal damage, spontaneous recurrent seizures, and learning memory deficits in pilocarpine-treated rats. J. Mol. Neurosci. 50, 109–123. http://dx.doi.org/10.1007/s12031-012-9879-1.

Liauw, J., Hoang, S., Choi, M.M., Eroglu, C., Sun, G.H., Percy, M., Wildman-Tobriner, B., Bliss, T., Guzman, R.G., Barres, B.A., Steinberg, G.K., 2008. Thrombospondins 1 and 2 are necessary for synaptic plasticity and functional recovery after stroke. J. Cereb. Blood Flow Metab. 28, 1722–1732. http://dx.doi.org/10.1038/jcbfm.2008.65.

Lin, Teng-nan, Kim, Gyoeng-Moon, Chen, Jean-Ju, Cheung, Wai-Mui, He, Yong Y., Hsu, Chung Y., 2003. Differential regulation of thrombospondin-1 and thrombospondin-2 after focal cerebral ischemia/reperfusion. Stroke 34, 177–186. http://dx.doi.org/10.1161/01.STR.0000047100.84604.BA.

Marchi, N., Angelov, L., Masaryk, T., Fazio, V., Granata, T., Hernandez, N., Hallene, K., Diglaw, T., Franic, L., Najm, I., Janigro, D., 2007. Seizure-promoting effect of blood–brain barrier disruption. Epilepsia 48, 732–742. http://dx.doi.org/10.1111/j.1528-1167.2007.00988.x.

Marchi, N., Granata, T., Janigro, D., 2013. Inflammatory pathways of seizure disorders. Trends Neurosci. 37, 55–65.

Marco, P., DeFelipe, J., 1997. Altered synaptic circuitry in the human temporal neocortex removed from epileptic patients. Exp. Brain Res. 114, 1–10.
Marcon, J., Gagliardi, B., Balosso, S., Maroso, M., Noé, F., Morin, M., Lerner-Natoli, M., Vezzani, A., Ravizza, T., 2009. Age-dependent vascular changes induced by status epilepticus in rat forebrain: implications for epileptogenesis. Neurobiol. Dis. 34, 121–132.
Maroso, M., Balosso, S., Ravizza, T., Liu, J., Aronica, E., Iyer, A.M., Rossetti, C., Molteni, M., Casalgrandi, M., Manfredi, A.A., Bianchi, M.E., Vezzani, A., 2010. Toll-like receptor 4 and high-mobility group box-1 are involved in ictogenesis and can be targeted to reduce seizures. Nat. Med. 16, 413–419. http://dx.doi.org/10.1038/nm.2127.
Ndode-Ekane, X.E., Hayward, N., Gröhn, O., Pitkänen, A., 2010. Vascular changes in epilepsy: functional consequences and association with network plasticity in pilocarpine-induced experimental epilepsy. Neuroscience 166, 312–332. http://dx.doi.org/10.1016/j.neuroscience.2009.12.002.
Nusser, Z., Roberts, J.D., Baude, A., Richards, J.G., Sieghart, W., Somogyi, P., 1995. Immunocytochemical localization of the alpha 1 and beta 2/3 subunits of the GABAA receptor in relation to specific GABAergic synapses in the dentate gyrus. Eur. J. Neurosci. 7, 630–646.
Packard, M., Mathew, D., Budnik, V., 2003. Wnts and TGF beta in synaptogenesis: old friends signalling at new places. Nat. Rev. Neurosci. 4, 113–120.
Pont, F., Collet, A., Lallement, G., 1995. Early and transient increase of rat hippocampal blood–brain barrier permeability to amino acids during kainic acid-induced seizures. Neurosci. Lett. 184, 52–54.
Raabe, A., Schmitz, A.K., Pernhorst, K., Grote, A., von der Brelie, C., Urbach, H., Friedman, A., Becker, A.J., Elger, C.E., Niehusmann, P., 2012. Cliniconeuropathologic correlations show astroglial albumin storage as a common factor in epileptogenic vascular lesions. Epilepsia 53, 539–548. http://dx.doi.org/10.1111/j.1528-1167.2012.03405.x.
Rosenberg, G.A., 2012. Neurological diseases in relation to the blood–brain barrier. J. Cereb. Blood Flow Metab. 32, 1139–1151. http://dx.doi.org/10.1038/jcbfm.2011.197.
Ruscher, K., Shamloo, M., Rickhag, M., Ladunga, I., Soriano, L., Gisselsson, L., Toresson, H., Ruslim-Litrus, L., Oksenberg, D., Urfer, R., Johansson, B.B., Nikolich, K., Wieloch, T., 2011. The sigma-1 receptor enhances brain plasticity and functional recovery after experimental stroke. Brain 134, 732–746. http://dx.doi.org/10.1093/brain/awq367.
Ruth, R.E., 1984. Increased cerebrovascular permeability to protein during systemic kainic acid seizures. Epilepsia 25, 259–268.
Saija, A., Princi, P., Pisani, A., Santoro, G., De Pasquale, R., Massi, M., Costa, G., 1992. Blood–brain barrier dysfunctions following systemic injection of kainic acid in the rat. Life Sci. 51, 467–477.
Scheff, S.W., Price, D.A., Hicks, R.R., Baldwin, S.A., Robinson, S., Brackney, C., 2005. Synaptogenesis in the hippocampal CA1 field following traumatic brain injury. J. Neurotrauma 22, 719–732.
Schmitz, A.K., Grote, A., Raabe, A., Urbach, H., Friedman, A., von Lehe, M., Becker, A.J., Niehusmann, P., 2013. Albumin storage in neoplastic astroglial elements of gangliogliomas. Seizure 22, 144–150. http://dx.doi.org/10.1016/j.seizure.2012.10.014.
Seiffert, E., Dreier, J.P., Ivens, S., Bechmann, I., Tomkins, O., Heinemann, U., Friedman, A., 2004. Lasting blood–brain barrier disruption induces epileptic focus in the rat somatosensory cortex. J. Neurosci. 24, 7829–7836. http://dx.doi.org/10.1523/JNEUROSCI.1751-04.2004.
Shlosberg, D., Benifla, M., Kaufer, D., Friedman, A., 2010. Blood–brain barrier breakdown as a therapeutic target in traumatic brain injury. Nat. Rev. Neurol. 6, 393–403. http://dx.doi.org/10.1038/nrneurol.2010.74.
Tomkins, O., Kaufer, D., Korn, A., Shelef, I., Golan, H., Reichenthal, E., Soreq, H., Friedman, A., 2001. Frequent blood–brain barrier disruption in the human cerebral cortex. Cell. Mol. Neurobiol. 21, 675–691.
Tomkins, O., Friedman, O., Ivens, S., Reiffurth, C., Major, S., Dreier, J.P., Heinemann, U., Friedman, A., 2007. Blood–brain barrier disruption results in delayed functional and structural alterations in the rat neocortex. Neurobiol. Dis. 25, 367–377. http://dx.doi.org/10.1016/j.nbd.2006.10.006.
Tomkins, O., Shelef, I., Kaizerman, I., Eliushin, A., Afawi, Z., Misk, A., Gidon, M., Cohen, A., Zumsteg, D., Friedman, A., 2008. Blood–brain barrier disruption in post-traumatic epilepsy. J. Neurol. Neurosurg. Psychiatry 79, 774–777. http://dx.doi.org/10.1136/jnnp.2007.126425.
Van Vliet, E.A., da Costa Araujo, S., Redeker, S., van Schaik, R., Aronica, E., Gorter, J.A., da Costa Araújo, S., 2007. Blood–brain barrier leakage may lead to progression of temporal lobe epilepsy. Brain 130, 521–534. http://dx.doi.org/10.1093/brain/awl318.
Van Vliet, E.A., Otte, W.M., Gorter, J.A., Dijkhuizen, R.M., Wadman, W.J., 2014. Longitudinal assessment of blood–brain barrier leakage during epileptogenesis in rats. A quantitative MRI study. Neurobiol. Dis. 63, 74–84. http://dx.doi.org/10.1016/j.nbd.2013.11.019.
Vezzani, A., French, J., Bartfai, T., Baram, T.Z., 2011a. The role of inflammation in epilepsy. Nat. Rev. Neurol. 7, 31–40. http://dx.doi.org/10.1038/nrneurol.2010.178.
Vezzani, A., Maroso, M., Balosso, S., Sanchez, M.-A., Bartfai, T., 2011b. IL-1 receptor/Toll-like receptor signaling in infection, inflammation, stress and neurodegeneration couples hyperexcitability and seizures. Brain Behav. Immun. 25, 1281–1289. http://dx.doi.org/10.1016/j.bbi.2011.03.018.
Viviani, B., Bartesaghi, S., Gardoni, F., Vezzani, A., Behrens, M.M., Bartfai, T., Binaglia, M., Corsini, E., Di Luca, M., Galli, C.L., Marinovich, M., 2003. Interleukin-1beta enhances NMDA receptor-mediated intracellular calcium increase through activation of the Src family of kinases. J. Neurosci. 23, 8692–8700.
Yu, C.-Y., Gui, W., He, H.-Y., Wang, X.-S., Zuo, J., Huang, L., Zhou, N., Wang, K., Wang, Y., 2014. Neuronal and astroglial TGFβ3-Smad3 signaling pathways differentially regulate dendrite growth and synaptogenesis. Neruomol. Med. http://dx.doi.org/10.1007/s12017-014-8293-y.
Zucker, D.K., Wooten, G.F., Lothman, E.W., 1983. Blood–brain barrier changes with kainic acid-induced limbic seizures. Exp. Neurol. 79, 422–433.