

# The GR-ANXA1 pathway is a pathological player and a candidate target in epilepsy

Emma Zub, Geoffrey Canet, Rita Garbelli, Marine Blaquière, Laura Rossini, Chiara Pastori, Madeeha Sheikh, Chris Reutelingsperger, Wendy Klement, Frédéric de Bock, et al.

▶ **To cite this version:**

Emma Zub, Geoffrey Canet, Rita Garbelli, Marine Blaquière, Laura Rossini, et al.. The GR-ANXA1 pathway is a pathological player and a candidate target in epilepsy. FASEB Journal, In press, pp.fj.201901596R. 10.1096/fj.201901596R . hal-02350122

**HAL Id: hal-02350122**
**https://hal.science/hal-02350122**

Submitted on 4 Jan 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# The GR-ANXA1 pathway is a pathological player and a candidate target in epilepsy

Emma Zub[1], Geoffrey Canet[2], Rita Garbelli[3], Marine Blaquiere[1], Laura Rossini[3], Chiara Pastori[3], Madeeha Sheikh[5], Chris Reutelingsperger[6], Wendy Klement[1], Frederic de Bock[1], Etienne Audinat[1], Laurent Givalois[2], Egle Solito[4,5] and Nicola Marchi[1]

[1]Laboratory of Cerebrovascular and Glia Research, Department of Neuroscience, Institute of Functional Genomics (UMR 5203 CNRS – U 1191 INSERM, University of Montpellier), Montpellier, France.
[2] Molecular Mechanisms in Neurodegenerative diseases, Inserm U1198, Team EiAlz, University of Montpellier, France.
[3]Epilepsy Unit, Fondazione IRCCS, Istituto Neurologico Carlo Besta, Milano, Italy,
[4]Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli "Federico II", Napoli, Italy;
[5]William Harvey Research Institute Barts and The London, Queen Mary's School of Medicine and Dentistry, UK
[6]Department of Biochemistry, Cardiovascular Research Institute, Maastricht University, Maastricht, Netherlands

**Running title:** Endogenous anti-inflammatory players and seizures

**Keywords:** AnnexinA1, epilepsy, inflammation, glucocorticoid receptor

Figures: 6
Supplemental Figure: 1
Table 1: Human data
Supplemental Tables 2: All and RT-PCR data; primers and antibodies

**Corresponding Authors:** Dr. Nicola Marchi, Cerebrovascular and Glia Research, Institute of Functional Genomics (CNRS UMR5203, INSERM U1191, and University of Montpellier) 141 rue de la Cardonille, 34094 Montpellier, Cedex 5, France. Email nicola.marchi@igf.cnrs.fr
Dr Egle Solito Charterhouse Square, London EC1M 6BQ-United Kingdom +44 (0) 207 882 2117. e.solito@qmul.ac.uk

**Acknowledgement.** This work was support by ANR-Epicyte, FRC and CURE Innovator Award to NM and by Italian Ministry of Health grant RF-2016-02362195 to RG. We thank Dr A. Maiorana for providing autoptic tissues. ES Lab is supported by Fondazione Italiana Sclerosi Multipla (FISM n. 2014/R/21) and British Heart Foundation (BHF). GC is supported by a PhD Grant from the University of Montpellier, France (CBS2 PhD program).
**Author Contributions.** Conception and design of the study (NM, EG and LG); acquisition and analysis of data (EZ, GC, RG, MB, LR, CH, MS, CR, WK, FdB). Drafting a significant portion of the manuscript or figures (NM, LG, ES, EA)

**Abstract**

*Objective.* Immune changes occur in experimental and clinical epilepsy. Here, we tested the hypothesis that during epileptogenesis and spontaneous recurrent seizures (SRS) an impairment of the endogenous anti-inflammatory pathway glucocorticoid receptor (GR) - AnnexinA1 (ANXA1) occurs. By administrating exogenous ANXA1 we studied whether pharmacological potentiation of the anti-inflammatory response modifies seizure activity and pathophysiology *in vivo*.

*Methods.* We used an *in vivo* model of temporal lobe epilepsy based on intra-hippocampal kainic acid (KA) injection. Video-EEG, molecular biology on brain and blood samples, and pharmacological investigations were performed in this model. Human epileptic cortices presenting Type II Focal Cortical Dysplasia (FCDIIa and b), hippocampi with or without hippocampal sclerosis (HS) and available controls were used to study ANXA1 expression.

*Results.* A ~~pathological~~ decrease of phosphoGR and phosphoGR/totGR~~tot~~ protein expression occurred in the hippocampus during epileptogenesis. Downstream to GR, the anti-inflammatory protein ANXA1 remained at baseline levels while inflammation installed and endured. ~~Blood m~~Monitoring of ANXA1 and corticosterone blood levels showed no significant ~~response~~ modifications throughout epileptogenesis, corroborating a lack of ANXA1 engagement in these experimental conditions. By analysing human epilepsy brain specimens we found that, where significant inflammation exists, the pattern of ANXA1 immunoreactivity was abnormal as the typical perivascular ANXA1 immunoreactivity was reduced. We next asked whether potentiation of the endogenous anti-inflammatory mechanism by ANXA1

administration modifies the disease pathophysiology. Although with varying efficacy, administration of exogenous ANXA1 reduced the time spent in seizure activity as compared to saline.

*Interpretation.* The anti-inflammatory GR-ANXA1 pathway is flawed and dysregulated during seizure progression. The prospect of potentiating this endogenous anti-inflammatory mechanism as an add-on therapeutic strategy in epilepsies~~y~~ is proposed.

## Introduction

The continuous quest for innovative anti-seizure strategies is important. This is relevant to cases where seizures cannot be controlled by using anti-epileptic drugs alone or when, regardless of drug responsiveness, unwanted side effects need to be limited. Inflammation is a pathophysiological participant to various forms of epilepsy and neutralizing specific pro-inflammatory factors was proposed as a strategy to ameliorate seizure pathophysiology with the goal of reducing seizure activity (1-3). However, inflammation in epilepsy is multi-factorial as indicated by the contribution of numerous pro-inflammatory players (4). This leads to the caveat that even when a specific pro-inflammatory factor is targeted or invalidated other pathways may take the relay to sustain inflammation.

We here focus on the flip-side of inflammation, namely endogenous anti-inflammatory mechanisms. It is today accepted that flawed resolution of inflammation partakes to disease pathophysiology and that effective resolution of inflammation requires a rapid and robust engagement of endogenous anti-inflammatory pathways neutralizing pro-inflammatory players (5). Glucocorticoids, known to activate a broad-range anti-inflammatory response, are administered in clinical cases of epileptic syndromes, encephalitis or selected instances of drug resistant epilepsy as an ad-on therapy (6-9). Nonetheless, glucocorticoid therapy bears significant side effects limiting their long-term or continuous use and a wider clinical testing (10). A novel approach to effectively and safely potentiate the endogenous anti-inflammatory pathways in epilepsies could be clinically relevant.

Building from this knowledge, we here examined the AnnexinA1 (ANXA1) - glucocorticoid receptor (GR) axis in experimental and human epilepsies. ANXA1 is a potent endogenous and broad-spectrum anti-inflammatory effector produced by peripheral and central nervous system (CNS) immune, and endothelial cells upon GR phosphorylation and nuclear translocation (11-15). After its synthesis and extracellularly, ANXA1 binds to a formyl peptide receptor mediating the potent anti-inflammatory effects of glucocorticoids. Administration of exogenous ANXA1 was proposed as an approach to bypass glucocorticoids side effects, allowing for an efficacious steroid-sparing targeting of inflammation (14) as well as a strategy to overcome GR desensitization (11, 12, 14). Initial evidence exists supporting a possible implication of ANXA1 in the epileptic pathophysiology (16, 17).

In the presented study, anti- and pro-inflammatory responses were tracked and correlated among them in a model of temporal lobe epilepsy. We studied ANXA1 expression in human temporal lobe or focal cortical dysplasia epilepsies. Finally, we tested whether administration of recombinant ANXA1 (18) is a pharmacological strategy able to modify seizure activity and inflammation *in vivo*.

## Material and Methods

### Human Subjects

We retrospectively analysed brain tissue samples previously obtained from 13 patients who underwent surgery for drug-resistant epilepsy at the Fondazione IRCCS Istituto Neurologico Carlo Besta and at the Claudio Munari Epilepsy Surgery Centre of the Niguarda Hospital (Milan, Italy). The Ethics Committees of both Institutes approved the study and informed consent was obtained from each subject ~~for the~~to use ~~of~~ brain material for research. The examined surgical specimens were obtained from i) TLE patients with or without hippocampal sclerosis, ii) patients with FCD (Type IIb and IIa). ~~*En bloc* resections were performed for strictly therapeutic reasons after the patients had given informed consent.~~ The extent of the excision was planned preoperatively to minimize risks of post-surgical deficits on the basis of the epileptogenic area location according to electro-clinical and imaging data. Seizure outcome was assessed according to Engel's classification. In addition, normal-appearing control cortices and hippocampi obtained at autopsy from four adult patients without ~~a~~ history of seizures or neurological diseases were used for comparison. All autopsies were performed within 48 hours after death and the tissue was obtained and used in a manner complaint with the Declaration of Helsinki. Table 1 summarizes relevant patients' information.

*Human t~~T~~issue preparation and immunohistochemistry*

After surgery, brain specimens were immediately immersed in fixative solution (4% paraformaldehyde) for 24-36 hours and ~~were~~ then cut in 5-mm thick slabs that were paraffin embedded. Serial 7µm thick sections were processed for hematoxylin-eosin, thionin and luxol fast blue as well as for immunohistochemistry for neuronal and glial markers. One representative paraffin block ~~per~~ each clinical case, containing the lesion ~~were~~ was used for the present study and, in the case of Type II FCD, blocks containing both lesional and perilesional area were preferred. The following primary antibodies were used: Ab against neuronal nuclei (NeuN, Chemicon, Tamecula, CA, USA, diluted 1:1000); anti-neuronal non-phosphorylated neurofilaments (SMI311R, Covance, San Diego, CA, USA, 1:250); anti-glial fibrillary acidic protein (GFAP, Chemicon, 1:1000); anti-intermediate filament protein vimentin (Dako, Carpinteria CA, USA, 1:1000); anti-ANXA1 (ANXA1, ThermoFischer Scientific, Rockford, USA, 1:1000); anti-IBA1 (Abcam, Cambridge, UK, 1:300) and anti-human leukocyte antigen (HLA-DP, DQ, DR, Dako, 1:200). Single-label immunohistochemistry was developed using avidin-biotin peroxidase method and 3,3-Diaminobenzidine (Sigma, St. Louis, USA) as chromogen, as described elsewhere (19). Sections were counterstained with hematoxylin.

*Vertebrate Animals*

Experiments were performed according to the institutional/local guidelines of laboratory animal usage (European Union Council directive 86/609EEC). Protocols were approved by the French ethical committee (Apafis#10861-2018062513068565

v1; APAFIS#3023-2016042116041644 v2; 00846.01). Adult C57BL/6j male mice were housed in a 12h light/dark cycle (food and water *ad libitum*), minimizing animal discomfort.

*Intra-hippocampal kainic acid injection and video-EEG monitoring*

The model was ~~obtained~~attained as we previously described ~~in~~ (20-22). Briefly, mice were anesthetized (chloral hydrate), positioned in a stereotaxic frame, and injected over 3 minutes in the right dorsal hippocampus (AP = −2, ML = −1.5, DV = −2 mm) with 50 nl (20 mM) of a kainic acid solution (KA; Sigma-Aldrich). Sham mice were injected with 50 nl of sterile 0.9% NaCl. From a behavioural stand point, we always confirmed that intra-hippocampal KA elicited a reproducible non convulsive status epilepticus (SE) characterized by mild asymmetric clonic movements of the forelimbs and head deviations, rotations, alternated by ~~and~~ periods of immobility. Bilateral clonic seizures associated with rearing ~~were~~ occasionally ~~observed~~occurred in ~~selected~~selected few mice, although with no impact on next epileptogenesis or development of spontaneous seizures. KA-induced SE per se did not provoke mortality. Mice undergoing long-term EEG monitoring were implanted immediately after KA injection with a bipolar electrode in the CA1 region of the dorsal hippocampi. Groups consisted of: control sham (intra-hippocampal injection of sterile saline), 24hours post-SE, 72 hours post-SE, 1 week post-SE (epileptogenesis), and >3-4 weeks post-SE (spontaneous recurrent seizures, SRS). Video-EEG recording was performed using a Pinnacle Technology system (Lawrence, KS, USA).

*Epileptogenesis and SRS pharmacology and EEG quantification*

*SRS pharmacology.* Four weeks post-SE, ~~each mouse~~ mice (total n = 18) ~~was~~ were ~~EEG~~ monitored by video-EEG ~~for~~ to establish individual baseline SRS activity. ~~in a~~Awake freely moving animals were monitored by using a Sirenia acquisition system with a sampling rate of 200 Hz and analogic low pass filter~~ing~~ (25 Hz). ~~Animals~~ Mice were placed in circular Plexiglas boxes for habituation 1 hour before recording. Baseline SRS activity was monitored ~~mMonitoring protocol consisted of~~for 6 hours/day (usually from 10am to 4pm) and for 3 consecutive days. ~~After baseline SRS~~As s next step, mice (n = 10) were ~~treated~~ injected intraperitoneally (i.p) with human recombinant ANXA1, produced and purified as previously described (~~n = 10;~~ stock solution of 2.1 µg/µl in 50 mmol/l Hepes and 140 mmol/l NaCl ph7.4; see (23)). ~~using intraperitoneal (I.P.) injection (~~We injected 1µg/100 µl (40 mM HEPES, 140 mM NaCl) in each mouse~~; see~~ (24)~~)~~ once a day and for ~~during~~ 15 days~~, once a day, during SRS~~. ~~Sham mice (n=8) received saline injections according to the same regimen. One sham animal deceased. ANXA1 was diluted in a buffer solution (40 mM HEPES, 140 mM NaCl, pH = 7).~~ Sham mice (n=8) received saline i.p. injections according to the same schedule. One sham animal was excluded due to irreparable EEG technical ~~problems~~issues. EEG recordings were re-started and acquired during the last 3 days of injections (6 hours/day). Seizure quantification was then performed using Neuroscore. Briefly, seizure activity was detected by: i) setting a threshold ~~amplitude~~ for spike detection of 2.5-3 time ~~of the~~the amplitude of the baseline; ii) minimum and maximum spike duration: 1 and 100 milliseconds respectively; iii) minimum and maximum spike interval 0.1 and 1 second respectively; iv) minimum

train duration 5 seconds; v) minimum numbers of spikes (n=5) in a seizure: 5. For each mouse data ~~are~~ were expressed as minute seizures / hour ~~(6 hours randomized)~~.

*Epileptogenesis pharmacology*. Mice were treated with ANXA1 i.p. (n = 11) or saline (n = 11) starting from SE onset at the dose of 1µg / day for 7 days and then every other day for an additional 15 days. Video-EEG r~~R~~ecordings were performed during week~~s~~ 3, when ANXA1 treatment was delivered, and at weeks 4 and 6 post-SE. The registration protocol consisted of 6h / day for each of the 22 mice (4 mice at the time)~~,~~, one day during each week. Sham and ANXA1 treated animals were always recorded as pairs (2+2). Four animals were excluded from the monitoring due to technical problems related to the EEG implant. Seizures were quantified as described for SRS. For each mouse, the data are expressed as minutes spent in seizure activity / hour ~~(2 hours randomized)~~.

*RT-qPCR*

We used available snap frozen ipsi- and contra-lateral hippocampal and parietal cortices obtained from n = 5 mice / time point (sham, 24h, 72h, 1 week and 4 week SRS). Tissues were suspended in 350 µl RLT Plus buffer (Qiagen, Venlo, The Netherlands) supplemented with 1% β-mercapto-ethanol and lysed within Lysing Matrix D tube into the FastPrep® sample preparation system (MP Biomedicals, Santa Ana, USA). Tissue lysate was homogenised on QIAshredder columns (Qiagen, Venlo, Netherlands). Total cellular RNA was extracted using the RNeasy Mini RNA isolation kit (Qiagen, Venlo, The Netherlands) and genomic DNA contamination was removed using RNase-Free DNase Set (Qiagen, Venlo, The Netherlands)~~, following~~

~~the manufacturer's instructions~~. The RNA concentration of each sample was measured with a Nanodrop 2000c spectrophotomer (Thermo Fisher Scientific, Waltham, USA). One microgram of total RNA was reverse-transcribed using random hexamers of the Transcriptor First Strand cDNA Synthesis Kit (Roche, Pleasanton, USA), following the manufacturer's instructions. RT-qPCR were ~~conducted~~ performed using ~~in a~~ 5 µL volume of 10 ng cDNA, 500 nmol/L of forward and reverse primers, and 2,5 µL of SYBR Green PCR Master Mix (Roche, Pleasanton, USA) in duplicate ~~wells~~. The program ran 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and elongation at 72°C for 15 seconds, performed in a LightCycler 480 apparatus (Roche, Pleasanton, USA). After amplification, the melting curve was assessed to ensure the presence of a single product. Each plate carried serial dilutions of a RNA calibrator to generate a standard curve of the primers efficiency during the run. The levels of cDNA were quantified by the comparative 2ΔΔCt method. Ct values of the target gene were normalized with the Ct values of the house-keeping gene GAPDH (glyceraldehyde 3-phosphatedehydrogenase). Each of the target values is expressed as *n*-fold differences relative to the experimental control. Primers are listed in Supplemental Table 1 and complete results are in Supplemental Table 2.

*Western blot analysis*

We used snap frozen ipsi- and contra-lateral hippocampi from n = 6-8 mice / time point (sham, 24h, 72h, 1 week, 4 and 8 weeks for SRS). Western blot~~B~~ were performed as previously described (25) ~~using ipsi- and contra-lateal hippocampi~~. The a~~A~~ntibodies used are listed in Supplemental Table 1. Briefly, after sacrifice, the left

and right hippocampi were independently micro-dissected, weighed, immediately snap frozen ~~on liquid nitrogen~~ and stored at -~~20~~80°C. Tissues were then sonicated (VibraCell; Sonics & Materials, Newtown, CT, USA) in a lysis buffer and centrifuged (4°C). Supernatants were collected and the protein concentration was measured using a BCA kit (ThermoFisher Scientific, Illkirch, France). Sixty µg from each sample were used ~~taken~~ for ~~WB~~ analysis. Samples were separated in SDS-polyacrylamide gel (12%) and transferred to a PVDF membrane (Merck-Millipore, Dachstein, France). The membrane was incubated overnight (4°C) with the selected primary antibody, rinsed and then incubated for 2h with the appropriate horseradish peroxidase-conjugated secondary antibody. Peroxidase activity was revealed by using enhanced-chemiluminescence (ECL) reagents (Luminata-Crescendo, Merck-Millipore). The intensity of peroxidase activity was quantified using Image-J software (NIH, Bethesda, MA, USA). β-tubulin (β-Tub) was used as a loading control for all immunoblotting experiments. Each data point in Figure 1 represents the quantificatuion of targeted protein(s) / beta-tubulin ratio, then normalized for the mean sham, the latter set as 100%.

*ANXA1, corticosterone ELISA and blood cell count*

We used ~~available~~ whole blood and serum samples collected from the right cardiac atrium using an EDTA-coated tube before perfusion (n = 5-6 mice/time point; sham, 24h, 72h, 1 week and 4 week SRS). Blood samples were centrifuged at 4°C, and samples stored at -20°C until assayed for corticosterone (25) or ANXA1 ELISA. Corticosterone concentrations were ~~assayed~~ quantified using an ~~conventional~~ ELISA kit (Enzo-Life Sciences, Farmingdale, NY, USA) ~~in~~ using 10-µl plasma sample diluted

(1:40) with the assay buffer. The assay sensitivity was 27 pg/ml. The intra- and inter-assay coefficients were 6.6 and 7.8%, respectively. Blood cell count was performed using an automated blood cell count adapted for mice (100uL samples; Horiba-ABX).

Serum ANXA1 ~~in mouse serum~~ was ~~measured~~ quantified by the ELISA as previously reported (26). ELISA-treated plates (Nunc MaxiSorp, ThermoScientific, UK) were incubated overnight with capture antibody 20 µg/ml (mouse monoclonal antibody, generated in house) in bicarbonate buffer (25 mM $NaHCO_3$, 25 mM $Na2CO_3$, pH 9.6). The plate was then washed 3 times with bicarbonate buffer and blocked ~~in blocking buffer~~using ~~(~~0.1 % BSA~~,~~ in PBS~~)~~ for 1 h at 37 °C. ~~Then~~ Next, 100 µl of each sample and standards in assay diluent (Tween-20 at 0.05 % (v/v), in PBS) where loaded and incubated for 1 h at 37 °C, then washed 5 times ~~with wash buffer~~ (0.9% (w/v) NaCl, 0.05 % (v/v) Tween-20, $dH_2O$). ~~Following this w~~Wells w~~h~~ere then incubated with 1µg/ml of detecting antibody (rabbit polyclonal anti-ANXA1; Invitrogen, UK) for 1 h at 37 °C. After 5 washes, immuno-complexes were detected by adding the goat-anti-rabbit IgG with conjugated alkaline phosphatase for 30 min. After ~~5~~ washes, the substrate~~,~~ p-Nitrophenyl phosphate (Simga Aldrich, UK) was added ~~and left~~ for 30 min for a full development of ~~colour~~color. ~~The plate was then read a~~Absorbance ~~at (~~405 nm) was acquired and corrected ~~at (~~540 nm as a reference wavelength)~~,~~ using a spectofluoro~~i~~meter (Tecan Infinite M200 Pro, Tecan, Austria).

*Immunohistochemistry*

We used fixed brains obtained from the pharmacology SRS study at end treatment. Brain sections (30 µm) were rinsed with PBS (3 times, 10 min) and placed in blocking solution (20% horse serum and triton 0.3% in PBS; 1 h at room

temperature). Slice were then incubated with primary antibodies (1:300 chicken anti-GFAP or 1:1000 rabbit anti-IBA1; PDGFRβ 1:200 rabbit; see Supplemental Table 1) in blocking solution overnight at 4°C. NG2DsRed/C57BL6 mice ~~mice were~~were also ~~used~~ included to visualize pericyte reactivity at SRS. Slices were washed with PBS and secondary antibodies (1:500) added according to the primary host: donkey anti-rabbit Alexa Fluor 488 or donkey anti-chicken AMCA. Incubation was performed at room temperature for 2h. After PBS washes, slices were mounted using Vectashield containing DAPI.

*Statistics*

Analyses were performed using GraphPad Prism 6.0. Data were analysed using parametric (Student t-test) or non-parametric (Mann-Whitney) tests depending on normality distribution (Shapiro-Wilcox). Sample sizes for biochemistry and pharmacological testing were chosen according to previously published results (18, 22). Significance: < 0.05.

## Results

*Flawed endogenous anti-inflammatory response during experimental seizure progression*

In the hippocampus, GR phosphorylation and the phosphoGR/GR protein ratio were significantly decreased during epileptogenesis and at SRS (Figure 1A-C), indicating impaired functionality of this pathway. Western blot examples are provided in Figure 1A. Downstream to GR, the anti-inflammatory protein ANXA1 remained at sham-baseline levels during epileptogenesis and at SRS, suggesting a lack of endogenous anti-inflammatory response (Figure 1D) while inflammation advances as shown by GFAP increased over time (Figure 1E). ANXA1-GR and GFAP modifications also occurred in the contralateral hippocampus where seizure activity spreads (22). GFAP astrocyte and IBA1 microglia activation were confirmed by immunohistochemistry during seizure progression (*not shown*), as we and others previously reported for this model (20, 21). RT-PCR analyses integrate these results ~~to show~~showing no significant participation of the hippocampal ANXA1-GR pathway over time, except for an early and transient increase of the ~~ANXA1~~ receptor FRP (14), the cortisone-to-cortisol converting enzyme HSD11B11, and FKBP5, a chaperone regulating GR sensitivity ((27); see Supplemental Table 2). Pro-inflammatory modifications were elicited post-SE and persisted throughout epileptogenesis and during SRS, in the hippocampal foci and contralateral, also involving the overlying cortices (Supplemental Table 2). Supplemental Figure 1A shows examples of radar plots ~~for~~ of spatial and temporal glio-vascular pro-inflammatory TMEM119, TNFα, HEXB, ICAM and VCAM as quantified by RT-PCR ~~(Table 2)~~.

We next asked whether ~~a~~ peripheral blood inflammatory ~~response~~ modifications elicit~~s~~ during seizure progression, mirroring brain changes. Blood ANXA1 remained at baseline sham levels throughout epileptogenesis (Figure 2B), except for a transient increased early post-SE. Corticosterone blood level displayed a similar pattern (Figure 2A). Complete blood cell count showed post-SE changes typical of the anti-inflammatory effects of corticosterone (Figure 2C-D), specifically a decrease of circulating T-cells and increased percentage neutrophils. The effects were transient as during epileptogenesis a peripheral pro-inflammatory state existed, sustained by increased circulating monocyte (Figure 2E). Taken together, results in Figures 1 and 2 point to a brain-peripheral deficit engagement of the ANXA1-GR anti-inflammatory mechanism during seizure progression i*n vivo*.

*Associations between anti-inflammatory ANXA1 and pro-inflammatory factors during epileptogenesis.*

We studied the correlation between anti-inflammatory ANXA1 and GFAP protein levels, the latter a sign of inflammation in epilepsy. In the epileptic foci, the progressive GFAP increase occurring at epileptogenesis and SRS was not accompanied by ANXA1 production (Figure 3A). When comparing ipsi- and contra-lateral hippocampi, divergent pattern of ANXA1/GFAP data set distribution are apparent during epileptogenesis and SRS (*light orange boxes* in Figure 3A and B), pointing to an equilibrium tilted toward inflammation prominent at, but not exclusive of, the epileptic foci.

We then linked ANXA1 to TNFα, CCL2 or CCL12 transcripts levels during disease progression (see also Table 2). Previous studies demonstrated that ANXA1 negatively regulates the expression of numerous pro-inflammatory cytokines and chemokines, while cytokines can induce ANXA1 expression as a feed-back mechanism (11, 28-31). Figure 3C-E shows that ANXA1 levels were largely unmodified despite of the surge of pro-inflammatory factors, as indicated by nearly flat r person's correlations calculated during seizure progression in the epileptic foci and as compared to sham (*red and black data sets* and line plots*)*. Figure 3C1, D1, F1 shows the changes occurring at the contra-lateral hippocampi. These results suggest modifications of the anti- and pro-inflammatory balance evolving during seizure progression.

*In human epilepsy the pattern of brain ANXA1 expression depends on inflammation*

We examined the pattern of ANXA1 expression in human brain specimens obtained from drug-resistance epileptic patients and comparable non epileptic autoptic controls (Table 1). We considered epileptic cases presenting evidence of inflammatory sign, such as Type IIb FCD and sclerotic hippocampi, in comparison to cases without inflammation (Type IIa FCD and noHS). In normal control cortex (patient ID 1-2; Table 1), ANXA1 expression was found at the perivascular level (Figure 4A-B). Similar pattern was observed in the non-malformed epileptic cortices from TLE patients (ID 8, 9; Table 1) and Type IIa FCD (ID 15-17; Table 1) not presenting evidence of activated microglial cells (Fig. 4C-D). Conversely, in Type IIb FCD cases (ID 11-14; Table 1), characterized by disrupted cortical lamination, presence of dysmorphic neurons and balloon cells (Figure 4E-F), a prominent

microglia activation was present in the core of the lesion (Figure 4G) corresponding to modification of ANXA1 expression and localization. We report a reduced ANXA1 expression at the perivascular cell compartment paralleled by immunoreactivity in balloon cells and sparse astrocytes (Figure 4H). In adjacent perilesional area, where microglia activation is reduced, ANXA1 expression was similar to control tissue (*not shown*). In the human hippocampi analysed (ID 3-10; Table 1) perivascular ANXA1 expression was observed (Figure 4I-K) together with a moderate microglia activation in 2 out of 3 cases with hippocampal sclerosis.

*Effect of ANXA1 administration on spontaneous seizure activity in vivo*

We administered recombinant ANXA1 to boost this endogenous anti-inflammatory mechanism and to investigate the impact on seizure pathophysiology *in vivo* (Figure 5A). ANXA1 was administered ~~daily~~ once a day at dosages previously shown to be beneficial in CNS or peripheral diseases (18, 24). Although outcome variability existed among the treated animals, ANXA1 administration reduced the time spent in SRS as compared to before treatment for each animal and to saline-injected sham mice, indicated as delta variation in Figure 5B. Survival Kaplan-Meier curve show fraction of animals experiencing SRS reduction after receiving ANXA1 or saline treatments (Figure 5D). ANXA1 treatment was beneficial in 7 out of 10 mice. Our results unveil cases (3 out of 7 mice) where sham-saline treatment was somehow beneficial. Figure 5E provides details of EEG measures (SRS minutes/hour) for the 5 mice that experienced a SRS reduction greater than any of the saline-treated animals. No significant side effects due to drug regimen were observed (*not shown*). The reduction of SRS was not accompanied by a reversal of the hippocampal

perivascular inflammation and pericyte-glia scarring at SRS (Supplemental Figure 1B-D) indicating that, in this experimental model, the multi-cellular modifications installed at chronic stages are permanent and do not constitute a read-out for anti-inflammatory strategies, in this case ANXA1.

We then evaluated whether ANXA1 exerts disease modifying effects when administered during epileptogenesis. After 3 weeks of ANXA1 administration 7 out of 9 ANXA1-treated mice presented with seizure activity lower than 10 min/hours, as compared to 3 out 9 in the sham group (Figure 6A-B). As ANXA1 treatment was halted, during week 4 and 6, seizure activity similarly progressed among the two experimental groups. No significant side effects were observed (*not shown*).

## Discussion

Enduring or chronic inflammation is harmful and endogenous mechanisms exist to limit and resolve the inflammatory response (13). The engagement of endogenous anti-inflammatory and pro-resolving mediators is therefore crucial to limit loss of tissue homeostasis. ANXA1 has been proved to be an endogenous anti-inflammatory mediator capable of counteracting pro-inflammatory sequel (11, 12, 15). By studying the anti- and pro-inflammatory dynamics occurring in the hippocampal foci and in blood, we unveil an inefficiency of ANXA1-GR endogenous inflammation-control mechanism during seizure progression. The progressive deterioration of the ANXA1-GR pathway occurred in hippocampal regions where capillary permeability, presence of perivascular inflammation, glia-pericyte scarring and ectopic collagens accumulation was previously reported (21). We hypothesize that loss of compartmentalized cerebrovascular ANXA1 expression during seizures could participate to the faulty anti-inflammatory protection, as previously showed for multiple sclerosis, stroke and intracerebral haemorrhage (18, 32, 33). We here report reduced capillary levels ANXA1 expression in inflammation-associated human epilepsy. We acknowledge that any attempt to localize ANXA1 by immunohistochemistry on mouse brain slices was unsuccessful and we could not find published protocols aiding us. The use of endothelial conditional knock-out mice could be instrumental to further this investigation. Thus, ANXA1 invalidation in mice was associated with increased blood-brain barrier permeability and exaggerated pro-inflammatory response (34).

We reported a rapid but transient corticosterone and ANXA1 surge in blood post-SE accompanied by leukocyte modifications. While this peripheral anti-inflammatory attempt was short-lived, monocytes remained elevated hinting to lingering blood inflammation, consistent with CCL2, CCL12, and neurovascular PDGFRβ, VCAM and ICAM increase here reported (see Table 2). This is coherent with a role for monocyte-neurovascular interplay in experimental epilepsy (35) and the participation of ANXA1 in regulating immune cell response and vascular cross-talk (15). The direct interplay between ANXA1 and soluble pro-inflammatory cytokines and chemokines was also reported (11, 14, 29-31, 34). For instance, TNFα induce ANXA1 secretion while invalidation of ANXA1 gene results in increased cytokines production due to lipopolysaccharide injections (14, 30).

*Potentiating anti-inflammatory endogenous mechanisms while reducing side effects*

Enhancing broad-spectrum endogenous anti-inflammatory mechanisms has been clinically, and sometime anecdotally, practiced by using add-on corticosteroids in cases of paediatric epilepsy (6-9). However, even when clinical improvement is reported the use of cortisol, dexamethasone or methylprednisolone may be limited due to side effects. Glucocorticoids are anti-inflammatory and the glucocorticoid-induced molecule ANXA1 mediates many of these effects. Our results are clinically relevant as, although with variable outcome, ANXA1 reduces experimental seizure activity unveiling a potential glucocorticoid-sparing, safer option to dampen inflammation and potentially applicable to specific forms of epilepsy. Moreover a progressive loss of hippocampal GR phosphorylation during seizure progression may

disclose the possibility for assessing GR desensitization in epileptic subjects that failed add-on glucocorticoid treatments (7).

Available evidence showed that hours after pilocarpine induced SE the administration of the ANXA1-derived peptide Ac 2-26 reduces inflammation and exerts neuroprotective effects (17). By administrating ANXA1 post-SE and during epileptogenesis we here report a trend anti-seizure efficacy at 3 weeks, with effects vanishing at 4 and 6 weeks when the treatment was halted. We are aware that the experimental model used in our study is associated with severe hippocampal neuronal loss and persisting glio-vascular scarring (20). Other models, such as intra-amygdala KA injections, present lesser hippocampal cell damage during epileptogenesis and SRS and are perhaps more suitable for pharmacological discovery (36). As, in this experimental, model the dosage used may be sub-optimal aAdditional investigation using higher ANXA1 dosages ~~may be~~is required. The latter is feasible as, during our experiments, ANXA1 treatment was not associated with significant side effects (*e.g.,* no weight loss, no dietary modifications, etc.). Developing this research further ~~may be~~is important as AnnexinA1 was ~~reported to be~~ efficacious in limiting inflammation in experimental multiple sclerosis and brain ischemia (18, 32, 33).

*New avenue for inflammatory-cerebrovascular targeting in epilepsies*

Our results indicate the ANXA1-GR pathway as a participant and an entry point to potentiate broad-spectrum endogenous anti-inflammatory mechanisms during seizures. Converging with our results are reports showing beneficial effects of

augmenting endogenous anti-inflammatory mechanisms, namely circulating T-regulatory cells in TLE (37) and pro-resolving cell membranes lipid mediators to target epileptogenesis (36). Moreover, ANXA1 upregulates the interleukin-1 receptor antagonist while it downregulates CCR2 (11, 12), directly implicated in seizure pathophysiology (4). ANXA1 adds to a list of novel pharmacological entry points aiming at reducing inflammation and restoring cerebrovascular integrity. The latter includes the platelet derived growth factor receptor beta PDGFRβ (20), transforming growth factor beta (38), the interleukin-1 blocker anakinra (4, 39, 40), losartan and rapamycin (41).

Our study generates a number of interrogations on: i) the cellular-specificity (endothelium vs. glia) of the pathophysiology of ANXA1 and pGR/GR modifications; ii) the pattern of ANXA1 expression across different epilepsies, including comparison to encephalitidies; iii) the cross-talk between peripheral and brain ANXA1 and the involvement of circulating leukocytes at the blood-brain barrier interface. Moreover, i~~I~~n our study, we did not investigate changes of cerebrovascular permeability in ~~SRS~~ mice post ANXA1 treatments during epileptogenesis or at SRS. Importantly, existing evidence points to brain endothelial cells as a key site for ANXA1 accumulation and anti-inflammatory, therefore vascular repairing, effects (18). We report no significant effects on the glia-pericyte inflammatory signature existing at chronic disease stages in this model (Supplemental figure 1B-D), suggesting that ANXA1, when –effective against SRS, may also act~~s~~ by interfering with the production of parenchymal pro-inflammatory soluble factors (28, 30) rather than reverting an established multi-cellular scarring. ~~(18)~~The extent of ANXA1 that reaches the brain parenchyma remains to be quantified.

In conclusion, next comprehensive and multi-model pre-clinical studies on the efficacy of ANXA1 or the modulation of the ANXA1-GR axis have the potential to disclose novel add on therapies germane to human epilepsies where inflammation represent an etiological or a pathophysiological element.

**Figure 1. Functional impairment of hippocampal ANXA1-GR during experimental seizure progression *in vivo*. A)** Western blot examples for phosphoGR, GR, ANXA1 and GFAP in the ipsi- (right) and contra-lateral (left) hippocampi. **B)** Pattern of phosphorGR protein levels from post-SE to SRS. pGR expression was significantly and consistently decreased starting from epileptogenesis, although a trending decrease occurred at earlier time points (24h and 72h post-SE). **C)** PhosphoGR/GR ratio progressively decreases during seizure progression and up to SRS, a sign of reduced receptor function. **D-E)** AnnexinA1 protein levels were unmodified during disease progression and despite of the unfolding inflammation, here shown by GFAP level increase over time. See Table 2 and Supplemental Figure 1 for a full portrait the spatio-temporal pro-inflammatory modifications. Each data point represents one hippocampal samples obtained from an individual mouse. * < 0.05, ** < 0.01, *** < 0.001 by non-parametric Mann-Whitney test.

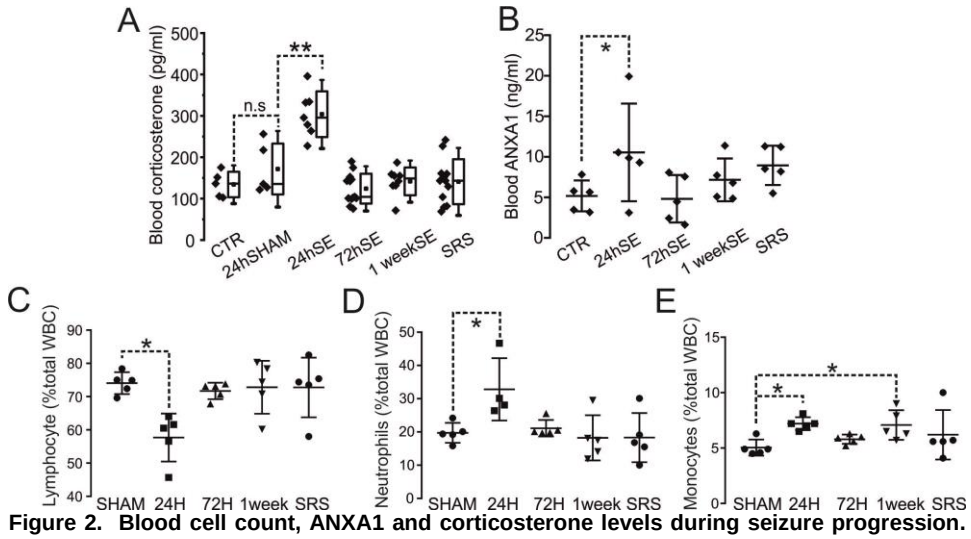



**Figure 2. Blood cell count, ANXA1 and corticosterone levels during seizure progression.** **A-B)** No significant ANXA1 and corticosterone response during seizure progression except for a transient increase 24h post-SE. Manipulation-induced stress does not significantly contribute to corticosterone levels (24h sham in A). **C-D)** Leukocyte changes occurred 24h post-SE coherently with corticosterone and ANXA1 changes. **E)** Blood monocyte inflammation lingered during epileptogenesis (1 week post-SE). Each data point represents one blood or serum samples obtained from an individual mouse. * < 0.05, ** < 0.01, *** < 0.001 by non-parametric Mann-Whitney test.

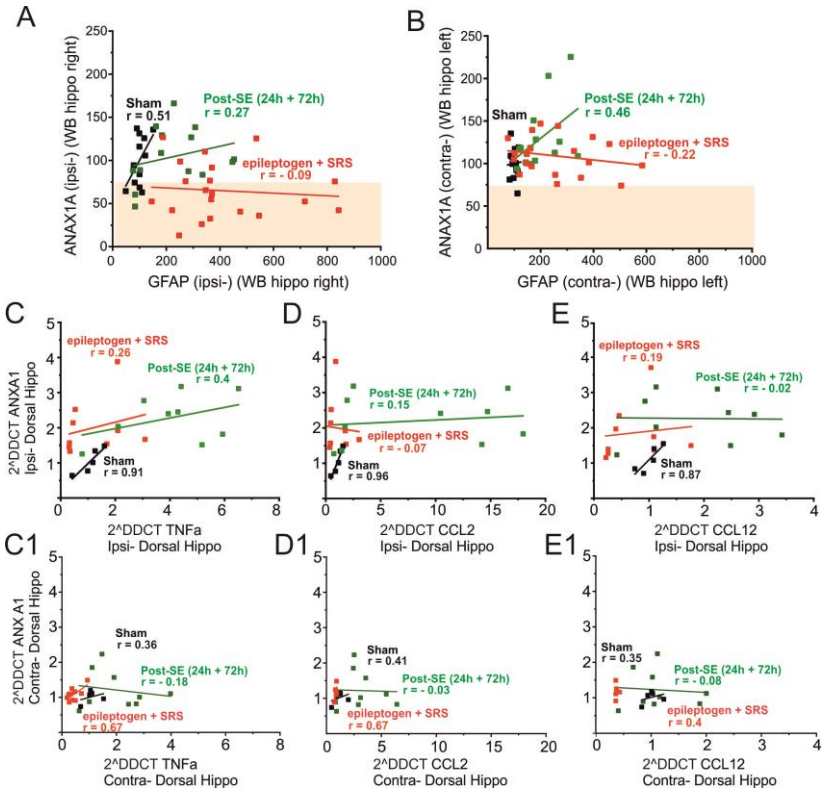



**Figure 3. The equilibrium between anti- and pro-inflammatory players is modified during seizure progression as compared to sham conditions. A-B)** Significant ANXA1/GFAP slope change during epileptogenesis and SRS as compared to sham underscore mounting GFAP inflammation at the expense of ANXA1 production (*light orange box* in B). In the contralateral hippocampi, the distribution of the epileptogenesis-SRS data set (*light orange box* in B1) suggests lesser GFAP inflammation and augmented ANXA1 levels as compared to ipsi-lateral tissue in B. **C-D-E)** At the transcript level, ANXA1 levels were unchanged and irrespective from the surge of pro-inflammatory molecules, as showed by near flat r person's correlations calculated during seizure progression. **C1, D1, F1)** Results are reported for the contra-lateral hippocampi. Each data point represents one hippocampal samples obtained from an individual mouse. See Table 2 for all RT-PCR results.

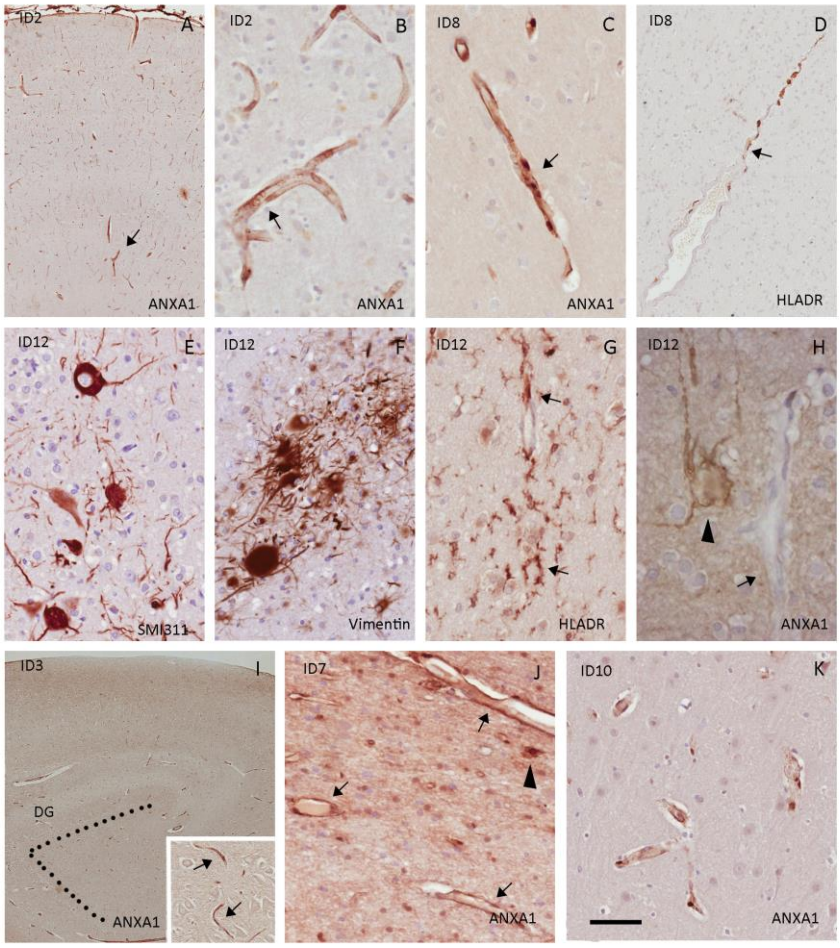



**Figure 4.** Immunohistochemistry localization of ANXA1 in human control cortex (**A**,**B**) and in non-malformed epileptic cortex from TLE patient (**C**) showing intense immunoreactivity around blood vessels (arrows) and in circulating cells; note the presence of scattered perivascular HLA-positive cells around vessels (**D**). In Type IIb FCD, characterized by dysmorphic neurons (**E**), balloon cells (**F**) and numerous activate HLA-positive microglia cells (**G**, arrows), ANXA1 immunoreactivity is lost from vessels (**H**, arrow) but is intensely present in balloon cells (arrowhead). In human hippocampi, an intense vascular ANXA1 expression is present in all considerate cases (**I-K**, arrows), moreover, in the presence of hippocampal sclerosis glial cells are also intensely labeled (arrowhead). Cal Bar: A: 480 µm; B,C,E,F,G,J,K: 50 µm; H: 55µm; D: 95 µm; I: 600 µm.

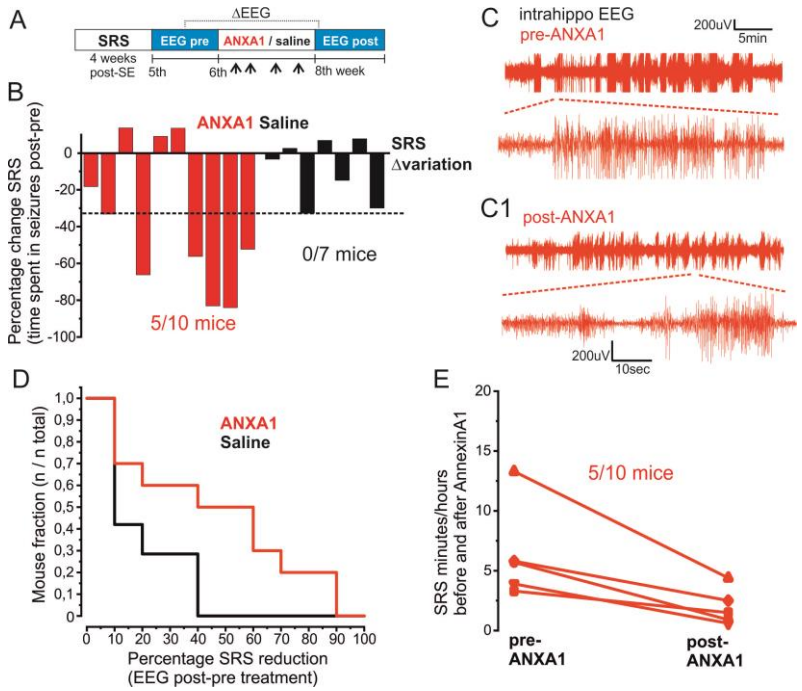



**Figure 5. ANXA1 administration reduces spontaneous recurrent seizures *in vivo*. A)** Schematic representation of experimental protocol (see Methods). Intra-hippocampal EEG recording was performed at SRS and for each mouse pre- and post- ANXA1 treatment. **B)** Percentage pre- and post-treatment EEG changes are provided. With the exception of 3 mouse, ANXA1 reduced the time spent in SRS. Saline injected SRS mice (sham) were used to control for biases related to manipulations and i.p. injections. SRS activity was decreased in 3 out of 7 mice. **C-C1)** Examples of intra-hippocampal EEG recordings pre- and post- ANXA1. **D)** Kaplan-Meier curve indicates the fraction of animals experiencing SRS reduction after receiving ANXA1 or saline regimens. **E)** Minutes seizures / hour values are provided for the 5 mice ANXA1 experiencing SRS reduction greater than any of the sham animals.

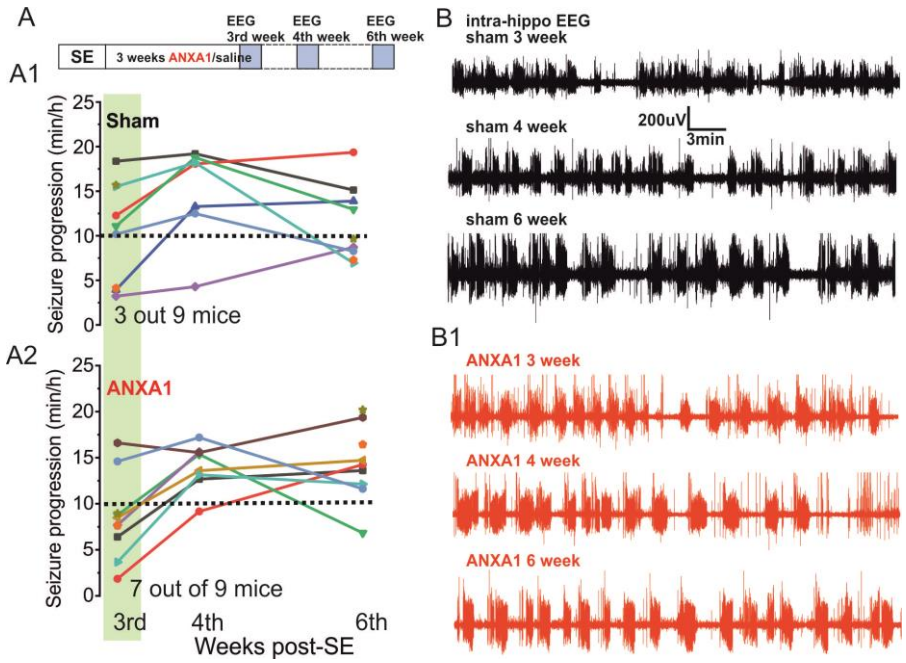
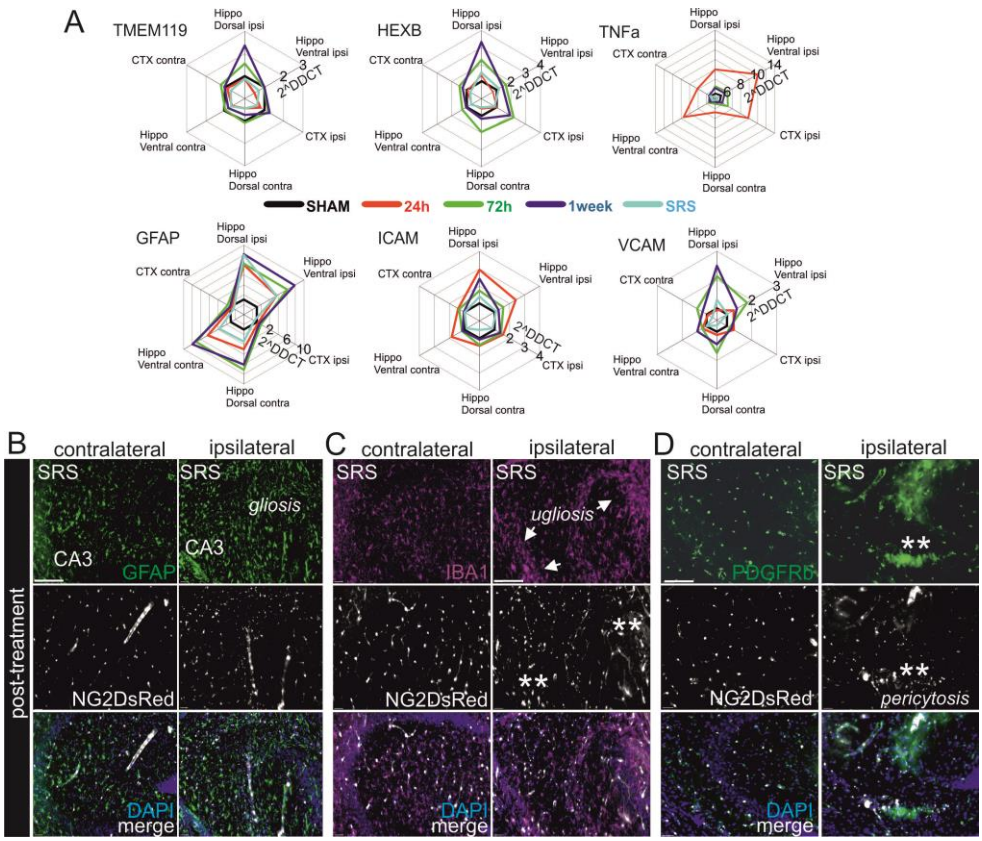



**Figure 6. ANXA1 administration during epileptogenesis and SRS outcome in vivo. A)** Schematic representation of experimental protocol (see Methods). Intra-hippocampal EEG recording was performed during epileptogenesis and for each mouse. **A1-A2)** Mouse specific seizure activity as consecutively monitored 3, 4 and 6 weeks post-SE induction in sham and ANXA1 treated animals. *Dotted line* highlight the differential distribution of seizure activity at 3 weeks between sham and ANXA1 mice receiving the treatment (*light green box*). Under this experimental condition, no effects were found on long-term SRS when ANXA1 treatment was halted. **B-B1)** Examples of intra-hippocampal EEG recordings. Each data point or line represents an individual mouse. * < 0.05 by non-parametric Mann-Whitney test.




**Supplemental Figure 1. A)** Example of radar chart displaying the spatio-temporal pro-inflammatory and cerebrovascular changes occurring during experimental seizure progression. See Table 2 for complete RT-PCR data. **B-D) ANXA1 treatment does not revert the glio-vascular hippocampal activation or scarring at SRS. B)** GFAP, **C)** IBA1 and **D)** PDGFRβ multi-cellular reactivity persisted in the ipsi-lateral hippocampi in mice where ANXA1 treatment was effective against SRS. Results are compared to contra-lateral tissue. NG2DsRed pericyte damage is consistent with capillary pathology and scarring at SRS in this model.

**Table 1. Human Brain Specimens.**

| Patient n. and diagnosis | Analyzed tissue | Site of surgery | Outcome (Engel class) |
|---|---|---|---|
| 1. control | ctx | F | na |
| 2. control | ctx | O | na |
| 3. control | hippo | T | na |
| 4. control | hippo | T | na |
| 5. TLE-HS | hippo | T | Ib |
| 6. TLE-HS | hippo | T | Id |
| 7. TLE-HS | hippo | T | Ia |
| 8. TLE-noHS | ctx-hippo | T | Ib |
| 9. TLE-noHS | ctx-hippo | T | na |
| 10. TLE-noHS | hippo | T | Ib |
| 11. FCD IIb | ctx | F | II |
| 12. FCD IIb | ctx | F | Ia |
| 13. FCD IIb | ctx | O | Ia |
| 14. FCD IIb | ctx | F | Ia |
| 15. FCD IIa | ctx | O | Ia |
| 16. FCD IIa | ctx | T | na |
| 17. FCD IIa | ctx | O | Ia |

Ctx: cortex; FCD: Focal Cortical Dysplasia; F: frontal; hippo: hippocampus; HS: hippocampal sclerosis; na: not applicable/available; O: occipital; T: temporal; TLE: temporal lobe epilepsy.

Mis en forme : Français (France)

**Table 2.** RT-PCR screening of inflammation performed on hippocampi and cortices (ipsi- and contra-) during seizure progression (sham, 24h, 72h post-SE, 1 week epileptogenesis and SRS. See separate word file. n= 4-5 mice / time point. * < 0.05 by non-parametric Mann-Whitney test.

**Supplemental Table 1. Antibodies and primers**

| Gene name | Gene code | Specie | Forward primer (5' - 3') | Reverse primer (5' - 3') | Product lenght |
|---|---|---|---|---|---|
| **Annexin A1** | Anxa1 | Mouse | TGATACAGATGCCAGGGCTTT | TCGGCAAAGAAAGCTGGAGT | 243 |
| **Formyl peptid receptor 1** | Fpr1 | Mouse | TCTCCCCCTGGACCAAAGAT | GCAAAGGACGGCTGGATTTG | 183 |
| **FK506 binding protein 5** | Fkbp5 | Mouse | GTCCAAAGCCTCAGAGTCGTTC | AGCCTTTCTCATTGGCACTGTC | 137 |
| **Hydroxysteroid 11-beta dehydrogenase 1** | Hsd11b1 | Mouse | CTCCAGAAGGTAGTGTCTCGC | CCTTGACAATAAATTGCTCCGCA | 106 |
| **Tumor necrosis factor** | Tnf / TNF-a | Mouse | GCTGAGCTCAAACCCTGGTA | CCGGACTCCGCAAAGTCTAA | 119 |
| **Interleukin 1 beta** | Il1b | Mouse | TGCCACCTTTTGACAGTGATG | AAGGTCCACGGGAAAGACAC | 220 |
| **Interleukin 6** | Il6 | Mouse | CGTGGAAATGAGAAAAGAGTTGTG | ATCTCTCTGAAGGACTCTGGCT | 250 |
| **Glial fibrillary acidic protein** | Gfap | Mouse | GGCTCGTGTGGATTTGGAGA | TGCCTCGTATTGAGTGCGAA | 190 |
| **Transmembrane protein 119** | Tmem119 | Mouse | GCATGAAGAAGGCCTGGAC | CTGGGTAGCAGCCAGAATGT | 61 |
| **Purinergic receptor P2Y, G-protein coupled 12** | P2ry12 | Mouse | CGGTACTTATCTGCTAACTAGCTTTGA | TTGGTAATTGTAGTTGCTTATGTTTTC | 94 |
| **Hexosaminidase B** | Hexb | Mouse | TTGGCAAGAAGTTTTTGATGA | TTCCACACTTCGACTACTGTGC | 63 |
| **Allograft inflammatory factor 1** | Aif1 / Iba1 | Mouse | AACCCTCTGATGTGGTCTGC | CACATCAGCTTTTGAAATCTCCTC | 170 |
| **Platelet derived growth factor receptor, beta polypeptide** | Pdgfrb | Mouse | TGTGCAGTTGCCTTACGACT | CAGGTGGGGTCCAAGATGAC | 235 |
| **Selectin, lymphocyte** | Sell | Mouse | TCATGGTCACCGCATTCTCG | TAATGTGGGAGATGCCTGCG | 206 |
| **Claudin 5** | Cldn5 | Mouse | TATGAATCTGTGCTGGCGCT | GTGCTACCCGTGCCTTAACT | 157 |
| **Tight junction protein 1** | Tjp1 /ZO-1 | Mouse | CAGAGCCCTCCGATCATTCC | GGCTGACGGGTAAATCCACA | 249 |
| **Intercellular adhesion molecule 1** | Icam1 | Mouse | AAGGTGGTTCTTCTGAGCGG | TCCAGCCGAGGACCATACAG | 185 |
| **Vascular cell adhesion molecule 1** | Vcam1 | Mouse | TGGAGGTCTACTCATTCCCTGA | GGTGGGGATGAAGGTCGTTT | 227 |
| **Integrin alpha 4** | Itga4 | Mouse | GTTTGGCTACTCGGTGGTGC | CATGTCTTCCCACAAGGCTCT | 201 |
| **Integrin beta 1 (fibronectin receptor beta)** | Itgb1 | Mouse | GCCAAATCTTGCGGAGAATGT | CCATCCCTTTGCTGCGATTG | 214 |
| **Chemokine (C-C motif) ligand 2** | Ccl2 | Mouse | CAGGTCCCTGTCATGCTTCT | TTGAGCTTGGTGACAAAAACTACAG | 209 |
| **Chemokine (C-C motif) ligand 12** | Ccl12 | Mouse | CCATCAGTCCTCAGGTATTGG | CTTCCGGACGTGAATCTTCT | 95 |

| Protein | Mol. weight | Antibody | Dilution | Ref. | Vendor |
|---|---|---|---|---|---|
| **Primary antibodies WESTERN BLOT** | | | | | |
| **ANNEXIN A1** | 35 kDA | Rabbit anti-Annexin A1 | 1/1000 | 71-3400 | ThermoFisher, France |
| **GFAP** | 55 kDa | Mouse anti-GFAP | 1/2000 | G3893 | Sigma-Aldrich, France |
| **GR** | 95 kDa | Rabbit anti-GR | 1/1000 | #3660 | Cell Signaling/Ozyme, France |
| **Iba1** | 17 kDa | Rabbit anti-Iba1 | 1/750 | 013-19741 | Wako Chem,,Osaka, Japan |
| **pGR** | 95 kDa | Rabbit anti-p[Ser211]GR | 1/1000 | #4161 | Cell Signaling/Ozyme, France |
| **β-Tub** | 50 kDa | Mouse anti-β-Tubulin | 1/7500 | T4026 | Sigma-Aldrich, France |
| **Secondary antibodies** | | | | | |
| **IgG** | Goat anti-rabbit IgG peroxidase conjugate | | 1/2000 | A61-54 | Sigma-Aldrich, France |
| **IgG** | Goat anti-mouse IgG peroxidase conjugate | | 1/2000 | A67-82 | Sigma-Aldrich, France |

| Antibodies | Host | Vendor | Reference | Dilution | Application |
|---|---|---|---|---|---|
| Anti-IBA1 | anti-rabbit | Wako Laboratory Chemicals | 019-19741 | 1/1000 | Slices |
| Anti-GFAP | anti-chicken | Abcam | Ab4674 | 1/300 | Slices |
| anti-PDGFRβ | anti-rabbit | Abcam | Ab32570 | 1/100 | OHC / Slices |
| DAPI | Vectashield : mountain medium for fluorescence with DAPI | Vector Laboratories | H-1200 | [DAPI] = 1.5µg/ml | Slice |
| **Secondary** | **Descrition** | **Vendor** | **Reference** | **Dilution** | **Application** |
| IBA1, PDGFRβ, | Donkey anti-rabbit Alexa Fluor 488 | Jackson ImmunoResearch | 711-545-152 | 1/500 | Slice / OHC |
| GFAP | Donkey anti-chicken Alexa Fluor 488 | Jackson ImmunoResearch | 703-545-155 | 1/500 | Slice |

## REFERENCE


1. Marchi, N., Granata, T., and Janigro, D. (2014) Inflammatory pathways of seizure disorders. *Trends Neurosci* **37**, 55-65
2. Vezzani, A., Balosso, S., and Ravizza, T. (2012) Inflammation and epilepsy. *Handb Clin Neurol* **107**, 163-175
3. Vezzani, A., Balosso, S., and Ravizza, T. (2019) Neuroinflammatory pathways as treatment targets and biomarkers in epilepsy. *Nat Rev Neurol* **15**, 459-472
4. Vezzani, A., French, J., Bartfai, T., and Baram, T. Z. (2011) The role of inflammation in epilepsy. *Nat Rev Neurol* **7**, 31-40
5. Fullerton, J. N., and Gilroy, D. W. (2016) Resolution of inflammation: a new therapeutic frontier. *Nat Rev Drug Discov* **15**, 551-567
6. Bakker, D. P., Catsman-Berrevoets, C. E., and Neuteboom, R. F. (2015) Effectiveness of a hybrid corticosteroid treatment regimen on refractory childhood seizures and a review of other corticosteroid treatments. *Eur J Paediatr Neurol* **19**, 553-560
7. Marchi, N., Granata, T., Freri, E., Ciusani, E., Ragona, F., Puvenna, V., Teng, Q., Alexopolous, A., and Janigro, D. (2011) Efficacy of anti-inflammatory therapy in a model of acute seizures and in a population of pediatric drug resistant epileptics. *PLoS One* **6**, e18200
8. Ramos, A. B., Cruz, R. A., Villemarette-Pittman, N. R., Olejniczak, P. W., and Mader, E. C., Jr. (2019) Dexamethasone as Abortive Treatment for Refractory Seizures or Status Epilepticus in the Inpatient Setting. *J Investig Med High Impact Case Rep* **7**, 2324709619848816
9. Verhelst, H., Boon, P., Buyse, G., Ceulemans, B., D'Hooghe, M., Meirleir, L. D., Hasaerts, D., Jansen, A., Lagae, L., Meurs, A., Coster, R. V., and Vonck, K. (2005) Steroids in intractable childhood epilepsy: clinical experience and review of the literature. *Seizure* **14**, 412-421
10. Schacke, H., Docke, W. D., and Asadullah, K. (2002) Mechanisms involved in the side effects of glucocorticoids. *Pharmacol Ther* **96**, 23-43
11. Perretti, M., and D'Acquisto, F. (2009) Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat Rev Immunol* **9**, 62-70
12. Perretti, M., Leroy, X., Bland, E. J., and Montero-Melendez, T. (2015) Resolution Pharmacology: Opportunities for Therapeutic Innovation in Inflammation. *Trends Pharmacol Sci* **36**, 737-755
13. Sugimoto, M. A., Vago, J. P., Teixeira, M. M., and Sousa, L. P. (2016) Annexin A1 and the Resolution of Inflammation: Modulation of Neutrophil Recruitment, Apoptosis, and Clearance. *J Immunol Res* **2016**, 8239258
14. Yang, Y. H., Morand, E., and Leech, M. (2013) Annexin A1: potential for glucocorticoid sparing in RA. *Nat Rev Rheumatol* **9**, 595-603
15. Purvis, G. S. D., Solito, E., and Thiemermann, C. (2019) Annexin-A1: Therapeutic Potential in Microvascular Disease. *Frontiers in immunology* **10**, 938
16. Yao, B. Z., Yu, S. Q., Yuan, H., Zhang, H. J., Niu, P., and Ye, J. P. (2015) The Role and Effects of ANXA1 in Temporal Lobe Epilepsy: A Protection Mechanism? *Med Sci Monit Basic Res* **21**, 241-246
17. Gimenes, A. D., Andrade, B. F. D., Pinotti, J. V. P., Oliani, S. M., Galvis-Alonso, O. Y., and Gil, C. D. (2019) Annexin A1-derived peptide Ac2-26 in a pilocarpine-induced status epilepticus model: anti-inflammatory and neuroprotective effects. *J Neuroinflammation* **16**, 32
18. Cristante, E., McArthur, S., Mauro, C., Maggioli, E., Romero, I. A., Wylezinska-Arridge, M., Couraud, P. O., Lopez-Tremoleda, J., Christian, H. C., Weksler, B. B., Malaspina, A., and Solito, E. (2013) Identification of an essential endogenous regulator of blood-brain barrier integrity, and its pathological and therapeutic implications. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 832-841


Mis en forme : Italien (Italie)

19. Garbelli, R., Munari, C., De Biasi, S., Vitellaro-Zuccarello, L., Galli, C., Bramerio, M., Mai, R., Battaglia, G., and Spreafico, R. (1999) Taylor's cortical dysplasia: a confocal and ultrastructural immunohistochemical study. *Brain pathology* **9**, 445-461
20. Klement, W., Blaquiere, M., Zub, E., deBock, F., Boux, F., Barbier, E., Audinat, E., Lerner-Natoli, M., and Marchi, N. (2019) A pericyte-glia scarring develops at the leaky capillaries in the hippocampus during seizure activity. *Epilepsia*
21. Klement, W., Garbelli, R., Zub, E., Rossini, L., Tassi, L., Girard, B., Blaquiere, M., Bertaso, F., Perroy, J., de Bock, F., and Marchi, N. (2018) Seizure progression and inflammatory mediators promote pericytosis and pericyte-microglia clustering at the cerebrovasculature. *Neurobiol Dis* **113**, 70-81
22. Runtz, L., Girard, B., Toussenot, M., Espallergues, J., Fayd'Herbe De Maudave, A., Milman, A., deBock, F., Ghosh, C., Guerineau, N. C., Pascussi, J. M., Bertaso, F., and Marchi, N. (2018) Hepatic and hippocampal cytochrome P450 enzyme overexpression during spontaneous recurrent seizures. *Epilepsia* **59**, 123-134
23. Kusters, D. H., Chatrou, M. L., Willems, B. A., De Saint-Hubert, M., Bauwens, M., van der Vorst, E., Bena, S., Biessen, E. A., Perretti, M., Schurgers, L. J., and Reutelingsperger, C. P. (2015) Pharmacological Treatment with Annexin A1 Reduces Atherosclerotic Plaque Burden in LDLR-/- Mice on Western Type Diet. *PLoS One* **10**, e0130484
24. Purvis, G. S. D., Chiazza, F., Chen, J., Azevedo-Loiola, R., Martin, L., Kusters, D. H. M., Reutelingsperger, C., Fountoulakis, N., Gnudi, L., Yaqoob, M. M., Collino, M., Thiemermann, C., and Solito, E. (2018) Annexin A1 attenuates microvascular complications through restoration of Akt signalling in a murine model of type 1 diabetes. *Diabetologia* **61**, 482-495
25. Pineau, F., Canet, G., Desrumaux, C., Hunt, H., Chevallier, N., Ollivier, M., Belanoff, J. K., and Givalois, L. (2016) New selective glucocorticoid receptor modulators reverse amyloid-beta peptide-induced hippocampus toxicity. *Neurobiol Aging* **45**, 109-122
26. Yang, Y. H., Morand, E. F., Getting, S. J., Paul-Clark, M., Liu, D. L., Yona, S., Hannon, R., Buckingham, J. C., Perretti, M., and Flower, R. J. (2004) Modulation of inflammation and response to dexamethasone by Annexin 1 in antigen-induced arthritis. *Arthritis and rheumatism* **50**, 976-984
27. Binder, E. B. (2009) The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. *Psychoneuroendocrinology* **34 Suppl 1**, S186-195
28. Yang, Y. H., Toh, M. L., Clyne, C. D., Leech, M., Aeberli, D., Xue, J., Dacumos, A., Sharma, L., and Morand, E. F. (2006) Annexin 1 negatively regulates IL-6 expression via effects on p38 MAPK and MAPK phosphatase-1. *J Immunol* **177**, 8148-8153
29. Trentin, P. G., Ferreira, T. P., Arantes, A. C., Ciambarella, B. T., Cordeiro, R. S., Flower, R. J., Perretti, M., Martins, M. A., and Silva, P. M. (2015) Annexin A1 mimetic peptide controls the inflammatory and fibrotic effects of silica particles in mice. *Br J Pharmacol* **172**, 3058-3071
30. Yang, Y. H., Aeberli, D., Dacumos, A., Xue, J. R., and Morand, E. F. (2009) Annexin-1 regulates macrophage IL-6 and TNF via glucocorticoid-induced leucine zipper. *J Immunol* **183**, 1435-1445
31. Peshavariya, H. M., Taylor, C. J., Goh, C., Liu, G. S., Jiang, F., Chan, E. C., and Dusting, G. J. (2013) Annexin peptide Ac2-26 suppresses TNFalpha-induced inflammatory responses via inhibition of Rac1-dependent NADPH oxidase in human endothelial cells. *PLoS One* **8**, e60790
32. Wang, Z., Chen, Z., Yang, J., Yang, Z., Yin, J., Zuo, G., Duan, X., Shen, H., Li, H., and Chen, G. (2017) Identification of two phosphorylation sites essential for annexin A1 in blood-brain barrier protection after experimental intracerebral hemorrhage in rats. *J Cereb Blood Flow Metab* **37**, 2509-2525
33. Gussenhoven, R., Klein, L., Ophelders, D., Habets, D. H. J., Giebel, B., Kramer, B. W., Schurgers, L. J., Reutelingsperger, C. P. M., and Wolfs, T. (2019) Annexin A1 as

Mis en forme : Italien (Italie)

Neuroprotective Determinant for Blood-Brain Barrier Integrity in Neonatal Hypoxic-Ischemic Encephalopathy. *J Clin Med* **8**

34. Roviezzo, F., Getting, S. J., Paul-Clark, M. J., Yona, S., Gavins, F. N., Perretti, M., Hannon, R., Croxtall, J. D., Buckingham, J. C., and Flower, R. J. (2002) The annexin-1 knockout mouse: what it tells us about the inflammatory response. *J Physiol Pharmacol* **53**, 541-553
35. Varvel, N. H., Neher, J. J., Bosch, A., Wang, W., Ransohoff, R. M., Miller, R. J., and Dingledine, R. (2016) Infiltrating monocytes promote brain inflammation and exacerbate neuronal damage after status epilepticus. *Proceedings of the National Academy of Sciences of the United States of America* **113**, E5665-5674
36. Frigerio, F., Pasqualini, G., Craparotta, I., Marchini, S., van Vliet, E. A., Foerch, P., Vandenplas, C., Leclercq, K., Aronica, E., Porcu, L., Pistorius, K., Colas, R. A., Hansen, T. V., Perretti, M., Kaminski, R. M., Dalli, J., and Vezzani, A. (2018) n-3 Docosapentaenoic acid-derived protectin D1 promotes resolution of neuroinflammation and arrests epileptogenesis. *Brain* **141**, 3130-3143
37. Xu, D., Robinson, A. P., Ishii, T., Duncan, D. S., Alden, T. D., Goings, G. E., Ifergan, I., Podojil, J. R., Penaloza-MacMaster, P., Kearney, J. A., Swanson, G. T., Miller, S. D., and Koh, S. (2018) Peripherally derived T regulatory and gammadelta T cells have opposing roles in the pathogenesis of intractable pediatric epilepsy. *J Exp Med* **215**, 1169-1186
38. 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., and Friedman, A. (2014) Losartan prevents acquired epilepsy via TGF-beta signaling suppression. *Ann Neurol* **75**, 864-875
39. DeSena, A. D., Do, T., and Schulert, G. S. (2018) Systemic autoinflammation with intractable epilepsy managed with interleukin-1 blockade. *J Neuroinflammation* **15**, 38
40. Clarkson, B. D. S., LaFrance-Corey, R. G., Kahoud, R. J., Farias-Moeller, R., Payne, E. T., and Howe, C. L. (2019) Functional deficiency in endogenous interleukin-1 receptor antagonist in patients with febrile infection-related epilepsy syndrome. *Ann Neurol*
41. van Vliet, E. A., Forte, G., Holtman, L., den Burger, J. C., Sinjewel, A., de Vries, H. E., Aronica, E., and Gorter, J. A. (2012) Inhibition of mammalian target of rapamycin reduces epileptogenesis and blood-brain barrier leakage but not microglia activation. *Epilepsia* **53**, 1254-1263