Aberrant expression of PAR bZIP transcription factors is associated with epileptogenesis, focus on hepatic leukemia factor

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Epilepsy is a widespread neurological disease characterized by abnormal neuronal activity resulting in recurrent seizures. There is mounting evidence that a circadian system disruption, involving clock genes and their downstream transcriptional regulators, is associated with epilepsy. In this study, we characterized the hippocampal expression of clock genes and PAR bZIP transcription factors (TFs) in a mouse model of temporal lobe epilepsy induced by intrahippocampal injection of kainic acid (KA). The expression of PAR bZIP TFs was significantly altered following KA injection as well as in other rodent models of acquired epilepsy. Although the PAR bZIP TFs are regulated by proinflammatory cytokines in peripheral tissues, we discovered that the regulation of their expression is inflammation-independent in hippocampal tissue and rather mediated by clock genes and hyperexcitability. Furthermore, we report that hepatic leukemia factor (Hlf), a member of PAR bZIP TFs family, is invariably downregulated in animal models of acquired epilepsy, regulates neuronal activity in vitro and its overexpression in dentate gyrus neurons in vivo leads to altered expression of genes associated with seizures and epilepsy. Overall, our study provides further evidence of PAR bZIP TFs involvement in epileptogenesis and points to Hlf as the key player.
seizures. Additionally, a significant reduction in seizure threshold was reported in Bmal1 knockout mouse and decreased levels of CLOCK, CRY1, PER1 and DBP protein were found in epileptic tissue obtained from surgical resection.

Proinflammatory cytokines, which may be produced following seizures and contribute to their generation and susceptibility, have been shown to be key regulators of clock gene expression. Decreased expression of clock genes as well as PAR bZIP TFs was observed in NIH-3T3 cells or human synovial fibroblasts exposed to tumor necrosis factor α (TNF-α) or interleukin-1β (IL-1β), and in the liver of mice injected with TNF-α or with CD40 agonistic antibodies. The functional significance of cytokine-mediated suppression of clock genes remains elusive.

In this study, our objective was to characterize the hippocampal expression of clock genes and PAR bZIP TFs in a kainic acid (KA) model of TLE and to dissect the interaction between them and neuroinflammation. The intrahippocampal KA injection in adult mice induces an acute status epilepticus, followed by 14 days of a latent period without ictal activity. Afterwards, animals develop spontaneous recurrent seizures. In this model, seizures are accompanied by progressive neurodegeneration and neuroinflammation in hippocampus.

We found significant changes in PAR bZIP TFs expression characterized by downregulation of Hlf, Dbp, Tef and upregulation of E4bp4. Surprisingly, we were not able to mimic these changes by simulating only neuroinflammation. This suggests a tissue-specific and inflammation-independent pathway. The changes in Clock, Bmal1, Npas2 and Period genes expression at an early stage of KA-induced epilepsy might be driving the subsequent changes in PAR bZIP TFs expression. Additionally, we demonstrated that Hlf over-expression in primary hippocampal neurons leads to altered neuronal excitability in vitro and differential expression of genes involved in neuronal excitability, seizures and epilepsy in vivo.

Methods

Animals. All methods were carried out in accordance with guidelines and regulations of University of Zurich, Switzerland and University of Los Andes, Santiago, Chile. All experimental protocols were approved by the Cantonal Veterinary Office of Zurich, Switzerland and the Bioethical Committee of University of Los Andes, Santiago, Chile. Adult C57Bl6/J male mice (12–14 weeks old, from Charles River) and Sprague Dawley female rats (from the breeding colony of Universidad de los Andes, Chile) were used in the study. Animals were housed at standard conditions (20–24 °C, minimum 40% relative humidity) under a 12-hour light/dark cycle (lights on at 7 a.m., lights off at 7 p.m.), with access to food and water ad libitum.

Stereotaxic injections into hippocampus. Mice were anaesthetized with isoflurane and placed into a motorized stereotaxic robot (Neurostar) equipped with the drill and microinjection Nanoinject II (Drummond). The concentration of isoflurane was adjusted until all pain reactions disappeared. After fixation, a small opening was drilled in the skull to allow for the injection needle to access the hippocampal formation. Afterwards, sterile solutions of PBS, KA (5 nM, 70 nL, Sigma Aldrich), N-methyl-D-aspartic-acid (NMDA, 70 nM, 140 nL, Sigma Aldrich), (-)-Bicuculline methiodide (1 μM, 0.5 μL, Tocris), mouse recombinant cytokines TNF-α (15 pmol, 0.5 μL, Peprotech) and IL-1β (5 pmol, 0.5 μL, Peprotech), lipopolysaccharide (LPS, 10 μg/1 μL, 1 μL, Sigma Aldrich) or AAV vectors (described below) were stereotaxically injected into the right hippocampus (~1.8 AP, +1.6 ML, +1.9 DV). The injection capillary was left in the brain for additional 5 min after the injection. Control animals received a matching volume of sterile PBS or control AAV vector. All injections were performed between 7 a.m. and noon. The concentration and volume injected are summarized in Table 1. Additionally, before and immediately after surgery, mice received an injection of Temgesic (buprenorphine, 0.06 mg/kg, i.p.).

In KA model experiments (n = 125, 4–7 mice per group), animals were sacrificed at either 3 hours, 1-day post injection (dpi), 6 dpi, 14 dpi or 28 dpi. Additionally, at 1 and 28 dpi, mice were sacrificed at four different Zeitgeber time-points (ZT = Zeitgeber time, ZT0 lights on, ZT12 lights off): ZT0, ZT6, ZT12 and ZT18. At 6 and 14 dpi, mice were sacrificed at two ZT: ZT0 and ZT12. In experiments investigating acute effect of recombinant murine cytokines TNF-α and IL-1β (n = 15, 5 mice per group), animals were sacrificed at 6 hours after their injection. In experiments with LPS (n = 10, 5 mice per group), NMDA and (-)-Bicuculline methiodide (n = 15, 5 mice per group), animals were sacrificed at 24 h after the injection to resemble the 1 dpi time-point in KA experiment.

AAV-vectors. Self-complementary (sc) adenovirus-associated virus (AAV) vector TNF (AAV-TNF) ssAAV-2/8-hCMV-chl-flexedm1NFalpha-SV40p(A) and its corresponding control AAV vector (AAV-control) ssAAV-2/8-hCMV-chl-loxp-SV40p(A) were kindly provided by the laboratory of Christopher Pryce (University of Zurich, Switzerland). The AAV vectors were characterized in their recent publication. AAV-control and AAV-TNF were injected as described above at a final titer of 1.1 × 1011 vg/ml (280 μl, n = 10, 5 mice per group). Animals were sacrificed 14 days after AAV injection and their brain processed for the gene expression analysis. Single-stranded (ss) AAV vector HLF (AAV-HLF) ssAAV-2/8-hSyn1-chl-EGFP_2A_mHLF-WPRE-SV40p(A) and its corresponding control AAV vector (AAV-EGFP) ssAAV-2/8-hSyn1-chl-EGFP-WPRE-SV40p(A) were produced by the Viral Vector Facility (VVF, University of Zurich) as described before. AAV-HLF was constructed by inserting the PCR-amplified mHLF ORF (Origene, MR204073, NM_172563) downstream and in-frame of an EGFP-2A sequence. AAV-EGFP and AAV-HLF vectors were injected at a final titer of 1.2 × 1012 vg/ml (280 μl, n = 26, 7 mice per group for transcriptorics; 12 mice were used in the pilot study to determine the transgene expression levels, localization and subcellular specificity in the hippocampus, Fig. 1a–c). After 14 days of expression, animals were sacrificed, and their brain processed for the gene expression and fluorescent microscopy analyses. Histology and immunohistochemistry were performed as described in our previous work.

Brain sections were stained with the following antibodies: GFAP (DAKO Schweiz, Z334, 1:20000), Iba-1 (Wako, 019–19741, 1:3000) and NeuN (Chemicon, MAB377, 1:1000).
package25. performed to evaluate statistical significance in the gene expression using the function “t-test” on R statistical

1-rat 1–6, Coulter-control-rat 1–6, Dingledine-day 1-rat 1–6, Dingledine-control-rat 1–6; n

Nadler-day 1-rat 1–6, Nadler-control-rat 1–6; n = ing samples were included in the analysis: kainic acid (Wadman-day 1-rat 1–6, Wadman-control-rat 1–6, = AAV-HLF or AAV-EGFP (MOI 1013 vg/ml). Medium was changed every 3 days. At ~12–16 DIV , ×

Animal Care and Use Committee. Cells were cultured on cover slips and transduced with at 2 DIV with

EGFP . Whole-cell pipettes were pulled from thin wall glass capillaries (1.5 O.D. an inverted NIKON TE-2000U microscope to visualize transduced primary hippocampal neurons expressing

10 HEPES, 4 MgATP , 0.3 NaGTP , (pH 7.3, 290 mOsM/Kg). Patch clamp experiments were performed under

intracellular solution consisted of (in mM) 146 KGluconate (KGluc), 1 NaCl, 1 MgSO4, 0.2 CaCl2, 2 EGTA,

CaCl2, 2 MgSO4, 1 NaH2PO4, 25 NaHCO3, and 20 glucose (pH 7.4) for electrophysiology experiments. The

the coverslip was transferred to a 35 mm plate containing ACSF which contained (in mM) 120 NaCl, 3 KCl, 2.5

Sprague Dawley rats as previously described 28. This protocol was approved by the Universidad de los Andes

RNA isolation and gene expression analysis. In all experiments, the dorsal parts of ipsilateral and contralateral hippocampi were dissected on ice and were immediately frozen in RNAPure PeqGOLD solution (VWR). At the day of RNA isolation, brain tissue samples were homogenized (Qiagen TissueLyser) and whole cell RNA extracted using NucleoSpin-RNA II kit (Machery-Nagel). 500 μg RNA was subsequently reverse-transcribed using random hexamer primers (Thermo Scientific) and M-MuLV reverse transcriptase (Life Technologies). 20 ng of cDNA was amplified in a CFX384 detection system (Biorad) using PrecisionPLUS qPCR Mastermix (Primersdesign). The relative levels of each RNA sample were calculated by the 2− △△ CT method24 using qbase software (Biogazelle). Hprt1 and eEF1a1 were used as housekeeping genes. Each CT value used for these calculations was the mean of triplicates of the same reaction. All primers used were synthesized by Eurofins genomics. Sequences of all used primers are listed in Table 1. The t-test with multiple-testing correction was performed to evaluate statistical significance in the gene expression using the function “t-test” on R statistical package25.

Analysis of GSE47752 dataset. The Affymetrix microarray data described by Dingledine et al.26 was obtained from the Gene expression omnibus (GEO), under accession number GSE47752. This data is composed of 172 samples and 31099 features and was analyzed using the R statistical software 25. The follow-

ing samples were included in the analysis: kainic acid (Wadman-day 1-rat 1–6, Wadman-control-rat 1–6, Nadler-day 1-rat 1–6, Nadler-control-rat 1–6; n = 12 control, n = 12 kainic acid), pilocarpine (Coulter-day 1-rat 1–6, Coulter-control-rat 1–6, Dingledine-day 1-rat 1–6, Dingledine-control-rat 1–6; n = 12 control, n = 12 pilocarpine), kindling (McNamara-stage 1-rat 1–6, McNamara-control-rat 1–6, Rogawski-stage 1-rat 1–6, Rogawski-control-rat 1–6; n = 12 control, n = 12 kindling) and self-sustained status epilepticus (Wasterlain-day 1-rat 1–6, Wasterlain-control-rat 1–5; n = 5 control, n = 6 self-sustained status epilepticus).

Normalization was performed using the RMA normalization using all probes regardless of Affymetrix ‘Present’ (P) or ‘Absent’ (A) calls. Only probes with the “at” suffix were retained to ensure that only probes specific to a single gene were carried forward for further analysis. Differential expression of the genes Hlf, Tef, Dhp and E4bp4 was performed using the Limma (Linear models for microarray) package27.

Primary neuronal cultures & patch clamp. Primary hippocampal cultures were prepared from E18 Sprague Dawley rats as previously described 28. This protocol was approved by the Universidad de los Andes Animal Care and Use Committee. Cells were cultured on cover slips and transduced with at 2 DIV with AAV-HLF or AAV-EGFP (MOI = 50000, 1.2 × 1013 vg/ml). Medium was changed every 3 days. At ~12–16 DIV, the coverslip was transferred to a 35 mm plate containing ACSF which contained (in mM) 120 NaCl, 3 KCl, 2.5 CaCl2, 2 MgSO4, 1 NaH2PO4, 25 NaHCO3, and 20 glucose (pH 7.4) for electrophysiology experiments. The intracellular solution consisted of (in mM) 146 KGluc, 1 NaCl, 1 MgSO4, 0.2 CaCl2, 2 EGTA, 10 HEPES, 4 MgATP, 0.3 NaGTP, (pH 7.3, 290 mOsM/Kg). Patch clamp experiments were performed under an inverted NIKON TE-2000U microscope to visualize transduced primary hippocampal neurons expressing EGFP. Whole-cell pipettes were pulled from thin wall glass capillaries (1.5 O.D. × 1.17 I.D., Harvard Apparatus, Massachusetts, USA) using a Sutter puller9 (model P-97). Electrode resistances in the bath were 5–7 MΩ. Series resistance was monitored by a 5 mV step, and cells were discarded if this changed significantly (~20%). Cells were clamped at a holding potential of ~70 mV with an axopatch 200B (Molecular Devices, Pennsylvania, USA), filtered a 2 KHz and digitized at 5KHz using a Digidata 1550 (Molecular Devices) and Clampex 10.0 (Molecular

| Gene Name | Forward primer | Reverse primer |
|-----------|----------------|----------------|
| Hlf       | TGCTTTGCTGTTGCTTCTC | CAAGAGGAAATGGAGAAGATGGAAC |
| Tef       | TCCCCAGTCCCCGCTC | CTCCAAGAAACAGCAGACAGAT |
| Dhp       | TTAGAAGGACGCTTCTAGT | GCAACCTCCAGATATCAGAGGAAC |
| E4bp4     | ATGGGAGCTCTTCTCACCAC | TACCCCGAGTTCCATGTTTTC |
| Npm2      | TGCGAGCAGCTCTAACGAAGC | GTATTCTGTTTGCTGAGAGAT |
| Clock     | TTCCCTCCCTTAAAGGACGACT | CTTAATGCTACCTTGAGAGATAG |
| Bmal1     | AAATCAGACCCACATTTCTCAG | TTCCCTCGGTGATACCTTCAC |
| Per1      | CAGGCTAACCAGAGATTACACAGC | CAGCCACAGAGAAGTTGACCTTGG |
| Per2      | CTACAGCCTCCTCCTATATAATGT | ACGAGCAGCACGAGACAGAG |
| Per3      | TCAGAAGAAGCCAAGCCAAATC | GGTGTTCTGCTTGGCTGATTG |
| CRY1      | GATCCACCATTAGAGAGACAC | ACAGCCACATCAACTTCCCA |
| CRY2      | CAAAGCATTGGAGACGGAAG | GAAGAGGGCGAGGAGAG |
| Rev-erb-a | GTCTCCCTCGTGCTGCTTCTG | CCAAGTGACATGGGCGCTT |
| Rev           | ACTACGGGAGTATCACTGCTGAG | GTGCAAGAGTGAAGCAGCCTACATC |
| Hprt      | TCTCTCTCAGACCGCTTCTTT | AGGTATACAAACAAACTCTAGGTCAT |
| Ef1a1    | AAAGGCGATGTTGTTGAGAGG | CTTCCAGCGCTTCCTGGT |
| TNF-α    | GAAAGTGCAGTCTCCTGTGTT | CGCTCAGGTCATGATGTTAA |
| B-13     | AAACAGATGAAGTGTAGG | TGGAGAAGACACATTTGGT |
| B-10     | CAGGGATCTTAGCTAAGGGAAAGA | GCTCAGTGAAATAATGAGAAGG |

Table 1. Primers used in the RT-qPCR.
Figure 1. Identification of genes associated with Hlf over-expression in dentate gyrus neurons of adult male mice. (a) The representative expression pattern of EGFP reporter expression after AAV-HLF injection in the ipsilateral hippocampus. Scalebar = 400 μm (b) Relative mean expression (±SD) of PAR bZIP TFs and inflammatory cytokines after AAV-HLF injection. The t-test was performed to evaluate significance. ***p < 0.001. (c) Co-localization of EGFP reporter expression with immunohistological staining specific for neurons (NeuN), astrocytes (GFAP) and microglia (Iba1). White arrows show co-localization of HLF-EGFP reporter with neuronal NeuN staining. Scalebar = 50 μm. (d) Heatmap shows the z-score from the transformed counts of each individual sample replicate, for all differentially expressed genes. (e) Summary of enrichment for GO molecular function and (f) Biological process. Figures were created using Adobe Illustrator version 23.1.1. www.adobe.com/Illustrator, R version 3.4.3.; https://www.R-project.org and GraphPad Prism version 8.3.1 for macOS, www.graphpad.com.
Devices. Stimulation of neurons was achieved by adding bicuculline (20 μM) to the recording bath, while delivering glutamate (2.5 mM) with a patch pipette with a resistance of ~2 MΩ placed near the recorded cell (~50 μm away). Glutamate concentration in the pipette was 2.5 mM, and delivery of glutamate was obtained by a 50 μl Hamilton syringe mounted to a SP101i syringe pump (flow rate of 1 μl/min, World Precision Instruments, FL, USA) and connected to the pipette by a tight silicon tubing. Action potentials were obtained by applying a series of current steps (50 pA each) under current clamp mode. Traces were recorded with pClamp 10 and analysis of intrinsic properties and sPSCs were performed with MiniAnalysis (Synapsoft). Average traces were obtained by selecting events in MiniAnalysis, aligning the peak of the events and averaging those traces under the “group analysis” function. Average traces from each cell were averaged to obtain one trace per group. Decay fit was calculated in MiniAnalysis by using a 2 exponential function approach (Amplitude1 exp(−time/τ1) + Amplitude2 exp(−time/τ2).

Two-way ANOVAs and two-tailed t-tests were performed with GraphPad Prism version 8 (GraphPad Software). The amplitude and interevent interval of PSCs were analyzed according to the highest level of activity shown, in one-minute long traces. To make the KS tests more stringent we applied a bootstrap estimation of the p-value by extracting random samples of size 100 (either currents or amplitudes) using the function “sample” on R statistical package25. After obtaining a p-value from these samples the procedure was repeated 1000 times. The percentage of times the random samples gave a p-value < 5% was used to establish if cumulative distributions are different. We considered distributions to be significantly different when more than 50% of random samples had a p-value < 5% This ensures that the KS test is not as sensitive to small differences between the associated functions.

Density plot of sPSCs amplitudes and interevent intervals were used to visually confirm our statistical results.

Transcriptomics analysis. Next-generation sequencing was performed by Functional Genomics Center Zurich, University of Zurich, Switzerland. Consequent data analysis was than performed by the Swiss Institute of Bioinformatics, Switzerland. Total RNA was processed using the TruSeq RNA stranded protocol (Illumina) in order to produce paired-end sequencing libraries. Libraries were then sequenced on the Illumina HiSeq 4000, single-end, 125 cycles. Reads were aligned against Mus Musculus.GRCm38.86 genome using STAR (v.2.5.2b)26. The number of read counts per gene locus was summarized with htseq-count (v.0.6.1)27 using Mus Musculus.GRCm38.86 gene annotations. Quality of the RNA-seq data alignment was assessed using RSeQC (v. 2.3.7)31. Reads were also aligned to the Mus Musculus.GRCm38.86 transcriptome using STAR (v. 2.5.2b)29 and the estimation of the isoforms abundance was computed using RSEM (v. 1.2.31)32. To assess differential expression, we used the R Bioconductor package DESeq. 2 (version 1.14.1)33. Differentially expressed Genes were identified at the Benjamini-Hochberg (BH) adjusted P < 0.05 level, using the Wald test. For gene set enrichment analysis, no direction criterion on fold change was applied. Enriched GO (Gene Ontology) categories were identified using the enrichment analysis package in R/Bioconductor, clusterProfiler34 with significant terms at BH P adjusted <0.05 reported.

Results

Status epilepticus induces changes in the expression of PAR bZIP transcription factors and does not affect expression of clock core genes. We evaluated the hippocampal expression of core clock genes and PAR bZIP TFs at different stages during epileptogenesis by injecting KA into the right dorsal hippocampus, a well-established model of TLE35. The pathogenesis of this model follows a stereotypic pattern23,35, characterized by an initial status epilepticus lasting up to 1 dpi, followed by a silent latent period and the occurrence of spontaneous recurrent seizures in the chronic period starting at about 14 dpi. The expression of clock genes and PAR bZIP TFs was evaluated at two (6 and 14 dpi) or four ZT time-points (1 and 28 dpi) over a day to exclude possible phase shift in clock gene expression amplitude. We found significant alteration in the expression of PAR bZIP TFs (Fig. 2). The transcriptional activators Hlf, Dhp and Tef were significantly down-regulated while the transcriptional repressor E4bp4 was up-regulated. Changes were most evident during the acute and epileptogenesis phases (1 and 6 dpi, respectively) of the status-epilepticus and tend to normalize thereafter. The expression of PAR bZIP factors in these models at 24 h after induction of status epilepticus. Our analysis revealed significant downregulation of Hlf in all four models (See Fig. 5a). In addition, Tef was significantly downregulated and the transcriptional repressor E4bp4 was significantly up-regulated in all models except for SSSE. Dhp was significantly downregulated in the pilocarpine model. Taken together the microarray data of four rat models of epilepsy support our finding of altered expression of PAR bZIP TFs in KA induced epilepsy.
Excitotoxicity and seizure activity induce the downregulation of Hlf. Next, we investigated whether acute hyperexcitability in general is responsible for the altered behavior of PAR bZIP TFs. To explore this possibility, we injected either N-methyl-D-aspartic acid (NMDA) or bicuculline into the right dorsal hippocampus. Hippocampal injection of NMDA stimulates ionotropic NMDA receptors, induces acute excitotoxicity and status epilepticus; however, unlike KA-induced status epilepticus, does not cause an early loss of hippocampal interneurons. Bicuculline is a competitive antagonist of GABAA receptors and it is known to produce seizures in the absence of neurodegenerative events. In both models, Hlf was significantly downregulated 24 h after status epilepticus (Fig. 5b), whereas Dbp was upregulated only after NMDA injection. These effects were mirrored also in the contralateral hippocampus (Fig. 5b).

Changes in the expression of PAR bZIP transcription factors are independent of inflammation. Both clinical and experimental evidence suggest that inflammatory processes are involved in the pathogenesis of TLE. Hence, we characterized the expression of proinflammatory cytokines TNF-α and IL-1β, as well as the anti-inflammatory cytokine interleukin 10 (IL-10) in the intrahippocampal KA model of TLE (Fig. 6). TNF-α expression was significantly upregulated 1, 6 and 14 days after the KA infusion (15-fold, 10-fold and 5-fold respectively). IL-1β remained upregulated at all timepoints studied, the effect being about 5-fold. The expression of IL-10 was increased at later time points, at 14 and 28 dpi. These cytokines were also significantly upregulated in the contralateral hemisphere, albeit the increase being less pronounced (Fig. 6).

As outlined above, inflammation regulates the expression of PAR bZIP TFs. Thus, we hypothesized that the KA induced inflammatory response drives the observed changes of PAR bZIP TFs expression. To test this hypothesis, we infused either mouse cytokine TNF-α or IL-1β into the hippocampus. Whereas TNF-α mRNA expression did not increase upon TNF-α or IL-1β injection, the expression of IL-1β mRNA was augmented about 5- and 10-fold respectively (Fig. 7a). However, we observed no changes in the expression of PAR bZIP factors (Fig. 7a). As cytokine levels reached by this treatment may have been insufficient to inhibit PAR bZIP TFs, we decided to...
simulate sub-chronic TNF-α exposure by infusion of an adeno-virus-associated viral TNF vector (AAV-TNF). The AAV-TNF induced robust TNF-α expression (100-fold) in the hippocampus; however, it did not affect the expression of PAR bZIP TFs (Fig. 7b). We corroborated this result by inducing neuroinflammation by infusion of lipopolysaccharide (LPS). LPS activates microglia interacting with their Toll-like receptor-4 and induces production of inflammatory cytokines. The intrahippocampal infusion of LPS induced significant overexpression of TNF-α (50-fold) and IL-1β (100-fold); however, the expression of PAR bZIP TFs remained unchanged (Fig. 7c).

Surprisingly, the hippocampal expression of PAR bZIP TFs was not affected by a local increase in the expression of pro-inflammatory cytokines such as TNF-α and IL-1β, as has been described for other murine tissues15. This evidence is in line with our previous finding that the expression of PAR bZIP TFs is unchanged in brains of mice with experimental autoimmune encephalomyelitis, a model of multiple sclerosis (unpublished data). Hence, this evidence suggests that early changes in hippocampal expression of PAR bZIP TFs in rodent models of TLE are not a direct response to inflammatory processes that occur during the initial stages of epileptogenesis. Thus, PAR bZIP TFs in the hippocampal tissues are regulated in a different way than in the periphery.

KA induces early response of clock genes. To further explore the mechanism responsible for the down-regulation of PAR bZIP TFs during epileptogenesis, we decided to characterize the hippocampal expression of PAR bZIP TFs and core clock genes, at 3 hours after the induction of status epilepticus by intrahippocampal KA (Fig. 8). At this early time point, we observed significant reduction of Clock, Bmal1, Npas2 and Per3 expression. Additionally, the expression of Per1 and Per2 was significantly upregulated. The expression of PAR bZIP TFs Hlf and Dbp did not show statistically significant decrease, while the expression of Tef was significantly downregulated. This expression pattern suggests that early changes in core clock genes might be involved in the initiation of changes in PAR bZIP TFs levels. The positive regulators of their expression (Clock, Bmal1, Npas2) were down-regulated and their negative regulators (Per1 and Per2) were upregulated. Additionally, the expression of TNF-α
and IL-1β was not altered yet at this early time point. These results might explain the changes in PAR bZIP TFs expression at 1 dpi after the KA lesion.

Neurons over-expressing Hlf showed a significant decrease in the frequency and increase in the amplitude of spontaneous events in the presence of bicuculline and glutamate. As we observed consistent downregulation of Hlf expression in various models of epilepsy, likely as a result of acute hyperexcitability, we examined the effects of Hlf expression in neurons under basal and hyperexcitable conditions. Hence, we overexpressed Hlf by transducing primary hippocampal neurons at 2 DIV with either a control vector (AAV-EGFP) or a vector overexpressing Hlf (AAV-HLF) and recorded spontaneous currents at ~12–16 DIV by whole-cell voltage clamp. Under basal activity levels (Fig. 9a,b,c), overexpression of Hlf resulted in a significant reduction in the amplitude of sPSCs, with no differences in the frequency or decay kinetics (Fig. 9d, AAV-EGFP: n = 10, 8.72 ± 0.89 msec; AAV-HLF: n = 11, 8.01 ± 1.06 msec, p = 0.6204), of events compared to neurons transduced with an AAV-EGFP. In the presence of bicuculline and pressure-ejected glutamate (Fig. 9e,f,g), neurons overexpressing Hlf showed a significant decrease in the frequency and an increase in the amplitude of sEPSCs compared to neurons carrying the EV control. We observed no differences between groups in the number of action potentials [AAV-EGFP: n = 10, 5.1 ± 0.86, AAV-HLF: n = 11, 4.64 ± 0.92, AAV-EGFP (BIC + GLU): n = 12, 5 ± 0.75, AAV-HLF/(BIC + GLU): n = 6, 5.67 ± 0.56. ANOVA: F (3, 35) = 0.2016, P = 0.8946] or amplitude [AAV-EGFP: n = 10, 71.49 ± 4.45 mV, AAV-HLF: n = 11, 64.57 ± 4.92 mV, AAV-EGFP/(BIC + GLU): n = 12, 61.83 ± 3.51 mV, AAV-HLF/(BIC+GLU): n = 6, 57.08 ± 5.03 mV ANOVA: F (3, 35) = 1.545, P = 0.2200]; Fig. 9i,j].
Figure 5. The expression of PAR bZIP TFs in other models of acquired epilepsy. (a) Hippocampal expression of PAR bZIP transcription factors (Hlf, Dbp, Tef and E4bp4) in four different rat models of status epilepticus: systemic KA (n = 12 in each group) and pilocarpine administration (n = 12 in each group), self-sustained status epilepticus (SSSE, n = 5 in control group, n = 6 in SSSE group) and amygdala kindling (n = 12 in each group)26. All rats involved in the study were male. White and grey boxes represent control and epilepsy model groups, respectively. Differential expression of the genes Hlf, Tef, Dbp and E4bp4 was performed using the Linear models for microarray (Limma) (b) Relative expression of PAR bZIP TFs after intrahippocampal injection of NMDA or bicuculline in adult male mice (n = 5 in each group). The t-test was performed to evaluate significance. *p < 0.05, **p < 0.005, ***p < 0.001. Figures were created using R version 3.4.3, https://www.R-project.org and GraphPad Prism version 8.3.1 for macOS, www.graphpad.com.
Gene expression analysis. To determine which genes are regulated by hepatic leukemia factor, we performed transcriptome analysis of the dorsal hippocampal tissue from mice over-expressing mouse Hlf restricted to the dentate gyrus (Fig. 1a). It has been shown that mouse endogenous Hlf is expressed mostly in the dentate gyrus, while in CA1 and CA3 subregions of hippocampus the expression is limited43, (Experiment: 565 and 566, Probe Name: Rp_Baylor_103366)44. These samples were compared with samples from mice injected with a control vector (AAV-EGFP). Hlf was over-expressed 7-fold in the dorsal hippocampal tissue while there were no changes in the expression of Tef and Dbp (Fig. 1b). In addition, the AAV-HLF injection did not induce
local neuroinflammation detected as expression of TNF-α and IL-1β (Fig. 1b). The expression of AAV-HLF vector was restricted to neurons and was detected in neither astrocytes nor microglia (Fig. 1c). Only samples with confirmed overexpression of Hlf using qPCR were sequenced (AAV-HLF n = 7, AAV-EGFP n = 7). In total, the expression of 65 genes was significantly altered (Fig. 1d). The list of all differentially expressed genes is included in the Supplementary Table S1. Raw data are available at NCBI GEO database under accession number GSE140046. Additionally, the gene ontology classification for molecular function and biological process were performed using all 65 significantly differentially expressed genes (Fig. 1e,f).

We identified genes whose expression is influenced by neuronal Hlf. We found changes in expression of genes coding for ion channels such as Trpa1, P2rx5, Grin3a, Kcng1 and Gabrd. Next, the expression of Synpr, Gfra2, Lcn2, Slc30a3, Rasd2, Igfbp5, Fxyd7, Cdkn1a and Slc6a8 was altered. Synaptoporin, the protein coded by Synpr gene, is a marker of mossy fiber sprouting, a phenomenon associated with epileptogenesis after KA injection. Gfra2 was reported to modulate threshold of kindling evoked seizures, Lcn2 was identified as a chemokine inducer in KA model and Slc30a3 modulates transport of zinc into synaptic vesicles that is co-released with glutamate and regulates excitability and its loss is associated with febrile seizures. Rasd2 and Igfbp5 were identified by a recent study as candidate genes associated with epileptogenicity in KA model of TLE and Fxyd7 to be altered after seizure preconditioning using pilocarpine model. Human studies reported Cdkn1a to be upregulated in patients with epilepsy and deficiency in Slc6a8 was reported to result in intractable epilepsy and cognitive impairment. Although, with the given dataset, we are not able to deduce any direct involvement in seizure modulation, many of those genes were previously described to be associated with neuronal excitability, seizures or epilepsy.

**Discussion**

Even though the occurrence of epileptic seizures in circadian patterns has been documented in animal models as well as humans the relationship between epileptic seizures and the circadian system at the molecular level is still unclear. A recent study reported decreased Clock expression in human epileptogenic tissue and decreased seizure...
threshold in mice with Clock deletion in excitatory cortical neurons suggesting that alterations in Clock expression are epileptogenic. Furthermore, mice deficient for Bmal1 exhibited reduced seizure thresholds.

In this study, we utilized the KA model of TLE to study the expression of core clock genes and their downstream transcription factors, the PAR bZIP TFs family. We showed that the expression of Hlf, Dbp, Tef is suppressed while the expression of E4bp4 is significantly upregulated during epileptogenesis. This effect was most evident during the acute phase (1 dpi); however, it was significant also during epileptogenesis (6 dpi) and at the beginning of the chronic phase (14 dpi). Additionally, our analysis of existing microarray data set (Dingledine R. 2013. NCBI Gene Expression Omnibus. GSE47752) revealed identical trends in the expression of PAR bZIP TFs in rat models of acquired epilepsy. Surprisingly, the expression of core clock-genes was not altered at 1, 6, 14 or 28 dpi after KA injection. These data are in accordance with another study performing gene profiling in dorsal hippocampus after 6 hours, 15 days and 6 months post KA injection. They did not find significant changes in core-clock gene expression while they identified Hlf to be an important transcription factor associated with changes in gene expression at 15 dpi. The functional importance of PAR bZIP TFs in regulating neuronal excitability was proven by a study showing that triple-knockout mice deficient for Hlf, Dbp and Tef develop audiogenic and spontaneous seizures. The authors pointed at Tef as the gene associated with seizures by regulating expression of pyridoxal kinase, an enzyme involved in the metabolism of neurotransmitters. In another study, the deletion of Hlf in mouse exacerbated seizures and reduced survival in the Snc2a Q54 mouse model of epilepsy. Likewise, DBP was found downregulated together with CLOCK, CRY1 and PER1 at protein levels in patients with focal cortical dysplasia. Furthermore, neuroprotective effects were described for E4bp4, which we found here to be upregulated after induction of status epilepticus. Besides modulating circadian rhythms, E4bp4 serves as a survival factor in motoneurons during their development. By limiting neuronal injury, the upregulation of E4bp4 in experimental epilepsy may be a part of the response to neurotoxic insults.

Interestingly, KA induced changes in the expression of PAR bZIP TFs as well as neuroinflammation were mirrored in the contralateral (non-injected) hippocampus. Hlf remained downregulated there even at the late chronic phase (28dpi). At the acute phase, this effect is most likely attributed to hyperactivity spreading from the
Figure 9. (a–c) Hlf overexpression decreases the amplitude but does not affect the frequency of spontaneous currents under basal conditions. (a) Representative traces (sPSCs). (b) (left) Cumulative amplitude distributions for sPSCs. 64.3% of simulated samples had a p-value < 5%. Inset: Mean spontaneous currents. (right) Cumulative interevent interval distributions for sPSCs. 20.7% of simulated samples had a p-value < 0.05. (c) Density plot of sPSCs (left) amplitudes and (right) interevent intervals. (d) Decay kinetics of sPSCs. (e,f) Hlf overexpression reduces the frequency of spontaneous excitatory currents in the presence of bicuculline and glutamate (BIC + GLU). (e) Representative sPSCs traces. (f) (left) Cumulative amplitude distributions for sPSCs. 67.6% of simulated samples had a p-value < 0.05. (right) Cumulative interevent interval distributions for sPSCs. 72.6% of the simulated samples had a p-value < 0.05. Inset: Mean spontaneous currents. (g) Density plot of sPSCs (left) amplitudes and (right) interevent intervals. (h) No significant differences were observed in decay kinetics between groups. (i–j) There are no differences in action potential number between neurons transduced with AAV-EGFP or a AAV-HLF in basal conditions or in the presence of bicuculline and glutamate. (i)
injecte hippocampus via commissural projections\textsuperscript{57}. However, during the chronic phase the epileptic activity does not spread into the contralateral hippocampus\textsuperscript{58}. Additionally, it has been shown that contralateral hippocampus can provide important regulatory input and modulate ongoing seizures\textsuperscript{59}. Thus, it is not clear whether the downregulation of \textit{Hlf} in contralateral hippocampus during the chronic phase is caused by ongoing changes in the ipsilateral hippocampus or it is entrained by intrinsic changes in the contralateral hippocampus induced during the status epilepticus.

What induces the changes in PAR bZIP TFS expression? We have shown in our previous research that the expression of PAR bZIP TFS is suppressed by the proinflammatory cytokines TNF-\textgreek{\alpha} and IL-1\textgreek{\beta} by interfering with their E-boxes in mouse liver and fibroblasts\textsuperscript{15}. Besides suppressing \textit{Hlf}, \textit{Dbp} and \textit{Tef}, TNF-\textgreek{\alpha} was found to enhance the expression of \textit{Ebp4} in synovial fibroblasts\textsuperscript{65}. This picture is identical to the one detected here in our KA induced model of epilepsy. Since we observed an elevated expression of TNF-\textgreek{\alpha} and IL-1\textgreek{\beta} in the KA model, we thought that the cytokine induced neuroinflammatory effect might be responsible for the modulation of PAR bZIP TFS. However, we were not able to suppress the expression of PAR bZIP TFS in hippocampus by simulating the proinflammatory environment. This suggests that their expression in the hippocampus is regulated by mechanisms than differ from those in periphery. Distinct circadian oscillations of PAR bZIP TFS in different tissues supports this hypothesis. In liver and kidney, PAR bZIP TFS oscillate diurnally with a high amplitude\textsuperscript{60,61} while in brain tissue they oscillate with a low amplitude or not at all\textsuperscript{11,12}.

Early changes in clock core-genes expression might be driving the initial downregulation of PAR bZIP TFS. At an early time-point (3h) after KA injection, when the inflammatory cytokines are not yet elevated, the expression of \textit{Clock}, \textit{Npas2} and \textit{Bmal1} were significantly downregulated while \textit{Per} genes 1 and 2 were upregulated. The expression of \textit{Dbp} and presumably also \textit{Hlf} and \textit{Tef} is directly activated by CLOCK-BMAL\textgreek{\alpha}\textgreek{\beta}\textsuperscript{62,63} and inhibited by \textit{Per} and CRY\textgreek{\beta}\textsuperscript{64}, thus their deficient expression results in altered expression of PAR bZIP TFS. In addition, it has been shown that \textit{Hlf} and \textit{Tef} expression in neurons is reduced in mouse with neuronal \textit{Clock} deletion\textsuperscript{7}. However, it is unclear whether these early changes in core clock gene expression contribute to alterations in PAR bZIP TFS at later stages of KA model, since their expression is already normalized at this stage. Alternatively, the neuroinflammation, excitotoxicity or seizure activity itself might play a role in sustaining the altered levels of PAR bZIP TFS.

To further investigate the role of PAR bZIP TFS in the regulation of neuronal excitability, we induced \textit{Hlf} over-expression in primary neurons under hyperexcitable conditions induced by glutamate and bicuculline exposure. It resulted in decrease in the frequency and an increase in the amplitude of sEPSCs. In another study, \textit{Hlf} deletion has been shown to increase the frequency of spontaneous seizures in two mouse models involving mutations on voltage-gated sodium channels; the gain-of-function \textit{Scn2a}\textsuperscript{54} mouse model and the heterozygous loss-of-function \textit{Scn1a} mouse model\textsuperscript{66}. Interestingly, these models seem to induce seizures from different mechanisms; a reduction in GABAergic inhibition in the \textit{Scn1a} model and an increase in excitability in CA1 glutamatergic neurons in the \textit{Q54} mouse model\textsuperscript{66}. This posits the possibility that \textit{Hlf} is involved in regulation of both pathways, which may explain the lower excitability we observed when exposing hippocampal primary neurons to hyperexcitable conditions. This decrease in excitability does not appear to involve changes in intrinsic properties of neurons nor in dendritic filtering, as we did not observe changes in the number or amplitude of action potentials, nor in the decay time of spontaneous currents. This result suggests that \textit{Hlf}, as a member of PAR bZIP TFS, is an intrinsic regulatory element modulating synaptic activity by a yet unknown mechanism.

Taken together, \textit{Hlf} seems to be a promising transcription factor associated with epileptogenesis. We found consistent deficiency of \textit{Hlf} in animal models of acquired epilepsy, demonstrated that \textit{Hlf} regulates neuronal activity and its overexpression in neurons leads to altered expression of genes associated with epilepsy. Upon resolving the molecular mechanism and causality between PAR bZIP TFS alterations and seizures, these findings should initiate further studies into PAR bZIP TFS as a target to prevent epileptogenesis or to modulate seizure activity.

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Author contributions
L.R., T.G., A.F. and J.F. designed the study. L.R., T.G. and C.L. performed the experiments. J.P. created the AAV vectors. L.D. performed bioinformatics analyses. L.R., T.G. and C.L. wrote the manuscript. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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