

1 **Title:** Large-scale changes to mRNA polyadenylation in temporal lobe epilepsy

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3 **Abbreviated title:** mRNA polyadenylation and epilepsy

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33

34 **Abstract**

35 The molecular mechanisms that shape the gene expression landscape during the development
36 and maintenance of chronic states of brain hyperexcitability are incompletely understood.
37 Here we show that cytoplasmic mRNA polyadenylation, a posttranscriptional mechanism for
38 regulating gene expression, undergoes widespread reorganisation in temporal lobe epilepsy.
39 Specifically, over 25% of the hippocampal transcriptome displayed changes in their poly(A)
40 tail in mouse models of epilepsy, particular evident in the chronic phase. The expression of
41 cytoplasmic polyadenylation binding proteins (CPEB1-4) was found to be altered in the
42 hippocampus in mouse models of epilepsy and temporal lobe epilepsy patients and CPEB4
43 target transcripts were over-represented among those showing poly(A) tail changes.
44 Supporting an adaptive function, CPEB4-deficiency leads to an increase in seizure severity
45 and neurodegeneration in mouse models of epilepsy. Together, these findings reveal an
46 additional layer of gene expression control during epilepsy and point to novel targets for
47 seizure control and disease-modification in epilepsy.

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59 **Introduction**

60 Epilepsy is one of the most common chronic neurological disorders, affecting approximately
61 70 million people worldwide^{1,2}. Temporal lobe epilepsy (TLE) is the most common
62 refractory form of epilepsy in adults and typically results from an earlier precipitating insult
63 that causes structural and functional reorganisation of neuronal-glia networks within the
64 hippocampus resulting in chronic hyperexcitability³. These network changes, which include
65 selective neuronal loss, gliosis and synaptic remodelling, are driven in part by large-scale
66 changes in gene expression⁴⁻⁷. The gene expression landscape continues to be dysregulated
67 once epilepsy is established⁸.

68 Recent studies have uncovered important roles for post-transcriptional mechanisms during
69 the development of epilepsy. These include the actions of small noncoding RNA, such as
70 microRNA and post-translational control of protein turnover via the proteasome contributing
71 to altered levels of ion channels, changes in neuronal micro- and macro-structure and glial
72 responses within seizure-generating neuronal circuits⁹⁻¹³. The molecular mechanisms
73 underlying the transcriptional and translational landscape in epilepsy remain, however,
74 incompletely understood.

75 In the cell nucleus, the majority of mRNAs acquire a non-templated poly(A) tail. Although
76 the addition of a poly(A) tail seems to occur by default, the subsequent control of poly(A) tail
77 length is highly regulated both in the nucleus and cytoplasm¹⁴. Cytoplasmic mRNA
78 polyadenylation contributes to the regulation of the stability, transport and translation of
79 mature transcripts, and is therefore an essential post-transcriptional mechanism for regulating
80 spatio-temporal gene expression¹⁴.

81 The cytoplasmic polyadenylation element binding proteins (CPEBs) are sequence-specific
82 RNA-binding proteins (RNABPs) and key regulators of mRNA translation via the
83 modulation of poly(A) tail length¹⁵. The CPEB family is composed of four members in

84 vertebrates (CPEB1-4) with CPEB2-4 sharing more homology than with CPEB¹⁶. To
85 function, CPEBs bind to cytoplasmic polyadenylation element (CPE) sequences, located at
86 the 3' untranslated region (3'UTR) of the target mRNAs. They nucleate a complex of factors
87 that regulate poly(A) tail length, thereby controlling both translational repression and
88 activation¹⁵. In the brain, CPEBs mediate numerous cellular processes including long-term
89 potentiation, synaptic plasticity and expression of neurotransmitter receptors¹⁷⁻¹⁹. Functional
90 roles for CPEBs in brain diseases include CPEB4 a critical regulator of risk genes associated
91 with autism²¹ and as protective against ischemic insults *in vivo*²⁰. Notably, evoked seizures
92 result in increased CPEB4 expression²² and mice lacking *Cpeb1* and the *Fmr1* gene display
93 decreased susceptibility to acoustic stimulation-induced seizures²³. Here, we explored
94 changes in mRNA polyadenylation in experimental mouse models of epilepsy, revealing
95 large-scale alterations as a major feature of the gene expression landscape in epilepsy.

96

97 **Results**

98 **Genome-wide mRNA polyadenylation profiling reveals deadenylation of epilepsy-** 99 **related genes**

100 To investigate whether seizures and epilepsy impact on mRNA polyadenylation, we used the
101 well characterized intraamygdala kainic acid (KA)-induced status epilepticus mouse model²⁴.
102 In this model of acquired epilepsy, an intraamygdala microinjection of KA leads to status
103 epilepticus and wide-spread neurodegeneration involving the ipsilateral cortex and CA3
104 subfield of the hippocampus. All mice treated with intraamygdala KA develop epilepsy
105 following a short latency period of 3-5 days, experiencing 2-5 seizures per day²⁴.
106 We first used mRNA microarrays to map both, changes in the rate of gene transcription and
107 potential genome-wide alterations in poly(A) tail length in the ipsilateral hippocampus at two
108 time-points; 8 hours (h) post-status epilepticus (acute injury) and at 14 days post-status

109 epilepticus (epilepsy) (Fig. 1a). Changes in poly(A) tail length were analyzed via poly(U)
110 chromatography. Here, by using a differential elution with either 25% or 90% formamide,
111 total hippocampal mRNA extracts were separated into two fractions, one enriched in mRNAs
112 with a short and one enriched in mRNAs with a long poly(A) tail, respectively²¹ (Fig. 1b).
113 This approach revealed that large-scale increases in mRNA levels accompany the early phase
114 after status epilepticus (4991 genes) when compared to established epilepsy (968 genes) (Fig.
115 1b). Therefore, regulation of mRNA levels seems to dominate the altered gene expression
116 landscape immediately following status epilepticus which is later reduced in epilepsy. In
117 sharp contrast to alterations in transcript levels, changes in poly(A) tail length were much
118 more pronounced in samples from mice with established epilepsy, affecting 28.6% of the
119 total mRNA pool (6177 genes) when compared to status epilepticus affecting 9.6% of the
120 genome (2088 genes) (Fig. 1b).

121 In order to identify functional groups of genes and pathways affected by changes in
122 polyadenylation, transcripts with poly(A) tail alterations were analysed by Gene Ontology
123 (GO) terms using the bioinformatic tool DAVID²⁵. Notably, pathways related to epilepsy
124 were particularly abundant among genes undergoing a shortening in their poly(A) tail
125 following status epilepticus and during epilepsy (Extended Data Fig. 1a). Shortening of
126 selected deadenylated transcripts was validated by high-resolution poly(A) tail (Hire-PAT)
127 assay (Extended Data Fig. 1b). To explore whether changes in mRNA polyadenylation
128 disproportionately affect genes implicated in the pathogenesis of epilepsy, we compiled a set
129 of epilepsy-related genes from three recent independent studies. These studies included: a)
130 genes where mutations cause epilepsy²⁶, b) genes with ultra-rare deleterious variations in
131 familial genetic generalized epilepsies (GGE) and non-acquired focal epilepsies (NAFE)²⁷
132 and c) genes localized in loci associated with epilepsy²⁸ (Supplementary Table 2).
133 Remarkably, transcripts displaying poly(A) tail shortening showed an enrichment in genes

134 implicated in epilepsy at both time-points, post status epilepticus and during epilepsy (Fig.
135 1b). This enrichment persisted after comparing our dataset with genes specifically expressed
136 in the brain, thus proving this enrichment to be specific for epilepsy-related genes (Extended
137 Data Fig. 1c). Genes identified in our array to undergo mRNA deadenylation following status
138 epilepticus, also showed a reduction in their protein levels, demonstrating that mRNA
139 deadenylation leads to reduced protein expression. This included the Glutamate ionotropic
140 receptor NMDA type subunit 2B (GRIN2B), previously shown to play a role during
141 epilepsy²⁶, Syntaxin 6 (STX6) and N6-adenosine-methyltransferase (METTL3), genes not
142 associated with epilepsy before (Fig. 1c). No significant changes in transcript levels of our
143 selected genes was observed when analysed post-status epilepticus, further suggesting this
144 decrease in expression is due to mRNA deadenylation (Fig. 1d). Previous *in vivo* studies
145 showed that deadenylation, by resulting in decreased protein output, is more disruptive than
146 poly(A) tail elongation in the brain²¹. Our results therefore indicate that deadenylation may
147 play a role during the development of epilepsy through diminishing the translation of specific
148 target genes.

149 Taken together, our results demonstrate changes in mRNA polyadenylation following
150 seizures and epilepsy affecting over 25% of the total transcriptome with deadenylation
151 disproportionately affecting transcripts encoding proteins related to epilepsy. Our results,
152 therefore, reveal mRNA polyadenylation as a previously unrecognized layer of gene
153 expression control in epilepsy.

154

155 **Increased expression of CPEBs in the hippocampus of TLE patients**

156 To identify possible candidate RNA Binding Proteins (RNABPs) responsible for driving
157 seizure-induced alterations in poly(A) tail length, we first compiled a list of genes whose
158 transcripts are known to bind to the main RNABPs involved in cytoplasmic polyadenylation²⁹

159 including human antigen R (HUR), which prevent deadenylation³⁰; PUMILIO, which
160 promotes deadenylation³¹ and CPEBs²¹ (Fig. 2a and Supplementary Table 3). This analysis
161 revealed that CPEB binders were highly enriched among genes that showed poly(A) tail
162 alterations following status epilepticus and during epilepsy (Fig. 2b). Interestingly, binders of
163 CPEB1 and of CPEB4 (the latter representing the CPEB2-4 subfamily) have been identified
164 in the brain²¹; while targets of both CPEBs were increased among genes with poly(A) tail
165 changes, the percentage of CPEB4 binders was significantly higher when compared to
166 CPEB1 binders, particularly during epilepsy (Extended Data Fig. 2a).

167 Next, to investigate the expression profile of the CPEB protein family during epilepsy, we
168 analyzed resected hippocampal tissue obtained from drug-refractory TLE patients. Here, we
169 found an increased expression of all CPEB family members, in particular of CPEB2 and
170 CPEB4 (Fig. 2c).

171 Together, the enrichment of CPEB-binders among altered genes and the increased levels of
172 CPEBs in TLE, indicate that members of the CPEB protein family, particularly the CPEB2-4
173 subfamily, are likely to be the main drivers of poly(A) tail changes during epilepsy.

174

175 **Increased expression of CPEB4 in experimental models of status epilepticus**

176 Next, to provide further evidence of CPEB driving poly(A) tail changes during epilepsy
177 development, we also analyzed the expression profile of the CPEB protein family in our
178 mouse models of status epilepticus. Whereas hippocampal *Cpeb3* and *Cpeb4* transcript levels
179 were increased at the early time points following status epilepticus in the intraamygdala KA
180 mouse model, no changes were found in *Cpeb1* and *Cpeb2* transcription levels (Fig. 3a). At
181 the protein level, only CPEB4 was significantly increased at short time-points, starting at 4 h
182 post-status epilepticus and returning to baseline control levels at 24 h (Fig. 3b), the time-point
183 at which a slight increase in CPEB1 and slight decrease in CPEB2 protein levels was also

184 observed. Interestingly, increased transcription of *Cpeb4* was most evident in the dentate
185 gyrus (Extended Data Fig. 3a), a subfield of the hippocampus largely resistant to seizure-
186 induced neuronal death in the model²⁴. CPEB4 protein was also upregulated in the
187 hippocampus following status epilepticus induced by the cholinergic mimetic pilocarpine
188 (Extended Data Fig. 3b), another widely used epilepsy model³². To provide additional proof
189 of a role for CPEBs during epilepsy, we analyzed epilepsy-related genes within targets of
190 CPEB1 and CPEB4 (Supplementary Table 2). Notably, only CPEB4 binders were enriched
191 within epilepsy-related genes, further suggesting that CPEB4 is an important regulatory
192 protein during epilepsy (Fig. 3c).

193 In summary, our data show altered expression of CPEBs in the hippocampus following status
194 epilepticus, in particular CPEB4, with CPEB4 binders being enriched among epilepsy-related
195 genes.

196

197 **Altered CPEB4 function may explain changes in poly(A) tail length in epilepsy**

198 Next, to obtain functional evidence that CPEB4 is responsible for changes in mRNA
199 polyadenylation occurring during epilepsy, we compared our identified global poly(A) profile
200 following status epilepticus and during epilepsy to the poly(A) profile present in CPEB4
201 knock-out (KO) mice²¹. Notably, analysis of the global transcript polyadenylation status in
202 CPEB4^{KO/KO} mice revealed an opposing poly(A) tail length pattern to that observed in our
203 epilepsy mouse model (Fig. 4a). Moreover, in contrast to the observed polyadenylation
204 signature during epilepsy, in CPEB4^{KO/KO} mice the enrichment in epilepsy-related genes was
205 found in the set of genes showing poly(A) tail lengthening (Fig. 4b). Together, these results
206 further corroborate poly(A)-tail alterations observed in our intraamygdala KA mouse model
207 to be attributable to altered CPEB4 function.

208

209 **CPEB4 deficiency increases seizure susceptibility and seizure-induced brain damage**

210 To determine whether mRNA polyadenylation and CPEB4 contribute to brain excitability or
211 the pathophysiology of status epilepticus, we characterized seizures and their
212 neuropathological sequelae in CPEB4 heterozygous (CPEB4^{KO/+}) and homozygous
213 (CPEB4^{KO/KO}) knockout mice³³. Immunoblot and transcript analysis confirmed a partial and
214 full reduction of CPEB4 protein in the hippocampus of heterozygous and homozygous mice,
215 respectively (Extended Data Fig. 4a). CPEB4-deficient mice also showed normal levels of
216 different cell-type markers and kainate receptor levels in the hippocampus (Extended Data
217 Fig. 4b). Furthermore, hippocampal mRNA levels of the neuronal activity-regulated gene *c-*
218 *Fos* and baseline EEG recordings were similar between wildtype (WT), CPEB4^{KO/+} and
219 CPEB4^{KO/KO} mice suggesting loss of CPEB4 does not noticeably alter normal brain function
220 (Extended Data Fig. 4c, d).

221 We then investigated the impact of CPEB4-deficiency on status epilepticus triggered by an
222 intraamygdala microinjection of KA. Both CPEB4^{KO/+} and CPEB4^{KO/KO} mice showed a
223 shorter latency to the first seizure burst compared to WT mice (Fig. 5a). CPEB4^{KO/+} and
224 CPEB4^{KO/KO} mice also experienced more severe seizures, as evidenced by higher total power
225 (Fig. 5b, c) and amplitude (Extended data Fig. 4e) during the time of KA injection until the
226 administration of the anticonvulsant lorazepam 40 min later. This increase in seizure severity
227 persisted for an additional 60 min recording period in CPEB4^{KO/KO} mice (Fig. 5b, c).
228 Analysis of high frequency high amplitude (HFHA) paroxysmal discharges which correlate
229 with seizure-induced brain pathology³⁴, revealed that both CPEB4^{KO/+} and CPEB4^{KO/KO} mice
230 showed longer durations of HFHA spiking (Fig. 5d). Behavioral seizures were also more
231 severe in CPEB4^{KO/+} and CPEB4^{KO/KO} mice during status epilepticus (Fig. 5e). Next, we
232 analysed brain sections to determine whether loss of CPEB4 affects neuropathological
233 outcomes. Status epilepticus in the intraamygdala model produces a characteristic lesion

234 within the ipsilateral CA3 subfield comprising select neuron loss and gliosis. Both
235 CPEB4^{KO/+} and CPEB4^{KO/KO} mice displayed increased neuronal death following status
236 epilepticus as evidenced by more Fluorojade (FjB)-positive cells in all subfields of the
237 hippocampus and in the cortex (Fig. 5f, g).

238 We also investigated neuropathological outcomes in a second model. CPEB4^{KO/+} mice
239 subjected to status epilepticus induced by intraperitoneal pilocarpine showed increased
240 mortality (Extended data Fig. 5a) and underwent more severe seizures (Extended data Fig.
241 5b, c), including longer durations of HFHA spiking (Extended data Fig. 5d). Analysis of
242 brain sections from these mice also identified more neurodegeneration in the hippocampus
243 and cortex of CPEB4^{KO/+} mice compared to WT controls (Extended data Fig. 5e).

244 Together, our results demonstrate that loss of CPEB4 increases vulnerability to seizures and
245 hippocampal damage indicating that CPEB4 is an important regulator of brain excitability
246 and seizure-induced neurodegeneration.

247

248 **Discussion**

249 In the present study, we are the first to describe changes in the global mRNA polyadenylation
250 profile following status epilepticus and during epilepsy. This represents an important post-
251 transcriptional level of regulation of gene expression in which CPEBs play a key role. This is
252 evidenced by bioinformatics analysis of various RNABPs and of epilepsy-related genes,
253 together with the alteration of CPEBs in samples from human TLE and KA mouse models.
254 We further identify CPEB4 as a key neuroprotective regulator of mRNA polyadenylation
255 during epilepsy, and this is corroborated in CPEB4-deficient mice.

256 Despite the availability of over 25 anti-epileptic drugs (AEDs), pharmacological
257 interventions remain ineffective in 30% of patients and, even when seizure-suppressive,
258 current AEDs are merely symptomatic and have no beneficial effect on the development of

259 epilepsy³⁵. While both acute status epilepticus and chronic epilepsy are characterized by
260 altered gene transcription, other post-transcriptional and post-translational mechanisms have
261 been shown to be crucial during the development of epilepsy³⁶. We are, however, still far
262 from a complete picture of the pathological molecular changes occurring during
263 epileptogenesis and epilepsy, a critical requirement for the development of much needed new
264 therapeutic strategies. Adding to the complexity of gene regulation during epileptogenesis,
265 we have now identified a novel regulatory gene expression mechanism, cytoplasmic mRNA
266 polyadenylation, potentially contributing to how protein expression is controlled during
267 epilepsy and thereby contributing to the generation of hyperexcitable networks.

268 Besides the long-known role of CPEB-dependent cytoplasmic mRNA polyadenylation during
269 early development, a role has more recently been recognized in the adult brain¹⁸, where it is
270 associated with synaptic plasticity, long-term potentiation, learning and memory³⁷. Long
271 forms of synaptic plasticity involving prion-like tag-mechanisms are particularly reliant on
272 CPEB-dependent polyadenylation³⁸. Epilepsy is a long lasting persistence of the
273 hyperexcitability observed in status epilepticus and, thus, amenable to be regulated by CPEB-
274 and poly(A)-mediated post-transcriptional regulation. In fact, while transcriptional changes
275 are profuse during status epilepticus, they decline in epilepsy, while the percentage of genes
276 showing altered poly(A) tail length increases from status epilepticus to epilepsy, with almost
277 30% of the genome affected in chronic epilepsy. These results suggest that transcriptional
278 changes may predominate in the early phases of hyperexcitability while poly(A)-dependent
279 regulation plays a larger role in the maintenance of epilepsy.

280 Despite multiple lines of evidence pointing to a key role of CPEBs in poly(A) changes in
281 epilepsy reported here, we cannot however exclude a contribution of other RNABPs such as
282 HUR or PUMILIO, which have previously been associated with epilepsy^{39,40}. Similarly,
283 among the various CPEBs, we show here that CPEB4 is a key player in epilepsy related

284 poly(A)-dependent gene regulation, but we cannot discard that other CPEBs also play a role.
285 The strongest evidence suggesting a role for CPEB4 include its early increase in expression
286 following acute seizures in two mouse models of status epilepticus and that a polyadenylation
287 profile opposite to the one observed during epilepsy is observed in CPEB4 knock-out mice.
288 Our data indicate that CPEB4 induction during epileptogenesis is neuroprotective and that the
289 high levels of expression found in human TLE patient tissue most likely represent an
290 endogenous antiepileptogenic adaptive mechanism to protect the brain once pathological
291 processes are initiated. Accordingly, CPEB4 deficiency in mice, despite having no apparent
292 impact on normal brain physiology⁴¹, lowers the seizure threshold upon challenge with
293 proconvulsants and leads to an increase in seizure severity and resulting brain damage.
294 Interestingly, further evidence of the neuroprotective role of CPEB4 was also previously
295 obtained in an *in vitro* model of ischaemia²⁰.
296 In conclusion, our results uncover cytoplasmic mRNA polyadenylation as an important layer
297 of gene expression regulation in epilepsy, which must be considered when analyzing
298 molecular patho-mechanisms during epileptogenesis and open the door for the development
299 of novel therapies targeting mRNA polyadenylation for the treatment of drug-refractory
300 epilepsy.

301

302 **Methods**

303

304 **Human brain tissue**

305 This study was approved by the Ethics (Medical Research) Committee of Beaumont Hospital,
306 Dublin (05/18), and written informed consent was obtained from all patients. Briefly, patients
307 (n = 6) were referred for surgical resection of the temporal lobe for the treatment of
308 intractable TLE. After temporal lobe resection, hippocampi were obtained and frozen in
309 liquid nitrogen and stored at -70°C until use. A pathologist (Dr. Michael Farrell) assessed
310 hippocampal tissue and confirmed the absence of significant neuronal loss. Control (autopsy)
311 temporal hippocampus (n = 6) was obtained from individuals from the Brain and Tissue Bank
312 for Developmental Disorders at the University of Maryland, Baltimore, MD, U.S.A. Samples
313 were processed for Western blot analysis. Brain sample and donor metadata are available in
314 Supplementary Table 4.

315

316 **Animal models of status epilepticus and epilepsy**

317 Animal experiments were carried out in accordance with the principles of the European
318 Communities Council Directive (2010/63/EU). Procedures were reviewed and approved by
319 the Research Ethics Committee of the Royal College of Surgeons in Ireland (REC 1322) and
320 the Irish Health Products Regulatory Authority (HPRA) (AE19127/P038). All efforts were
321 maximized to reduce the number of animals used in this study. Mice used in our experiments
322 were 8-12 weeks old male and female C57Bl/6, obtained from Harlan Laboratories (Bicester,
323 UK) and from the Biomedical Research Facility (BRF), Royal College of Surgeons in Ireland
324 (Dublin, Ireland). CPEB4-deficient mice (CPEB4^{KO/+}, CPEB4^{KO/KO}) harbour a heterozygous
325 or homozygous deletion of constitutive exon 2, respectively, resulting in a premature stop
326 codon and thereby a partial or full suppression of CPEB4 protein expression³³. Animals were

327 housed in a controlled biomedical facility on a 12 h light/dark cycle at 22 ± 1 °C and humidity
328 of 40-60% with food and water provided *ad libitum*. Status epilepticus was induced as
329 described previously either via an intraamygdala injection of KA or an intraperitoneal
330 injection of pilocarpine⁴². Before implantation of cannulas (intraamygdala KA injection) and
331 electrodes (EEG recordings), mice were anesthetized using isoflurane (5% induction, 1-2%
332 maintenance) and maintained normothermic by means of a feedback-controlled heat blanket
333 (Harved Apparatus Ltd, Kent, UK). Next, mice were placed in a stereotaxic frame and a
334 midline scalp incision was performed to expose the skull. A guide cannula (coordinates from
335 Bregma; AP = -0.94 mm, L = -2.85 mm) and three electrodes (Bilaney Consultants Ltd,
336 Sevenoaks, UK), two above each hippocampus and one above the frontal cortex as reference,
337 were fixed in place with dental cement. Intraamygdala KA (0.3 µg KA in 0.2 µl phosphate-
338 buffered saline (PBS)) (Sigma-Aldrich, Dublin, Ireland) was administered into the basolateral
339 amygdala nucleus. Vehicle-injected control animals received 0.2 µl of PBS. To reduce
340 morbidity and mortality, mice were treated with an i.p. injection of the anticonvulsant
341 lorazepam (6 mg/kg) 40 min post-KA or PBS injection (Wyetch, Taplow, UK). As described
342 previously, all mice develop epilepsy after a short latency period of 3-5 days²⁴. Status
343 epilepticus was also induced by an i.p. injection of pilocarpine (340 mg/kg body weight)
344 (Sigma-Aldrich, Dublin, Ireland) 20 min following the injection of methyl-scopolamine (1
345 mg/kg) (Sigma-Aldrich, Dublin, Ireland)⁴². Mice were treated with i.p. lorazepam (6 mg/kg)
346 90 min following i.p. pilocarpine. EEG was recorded using the Xltek recording system
347 (Optima Medical Ltd, Guildford, UK) starting 20 min before administration of pro-
348 convulsant (KA or pilocarpine) to record baseline and during status epilepticus (40 min for
349 intraamygdala KA-treated mice and 90 min for mice treated with i.p. pilocarpine). EEG was
350 continued for 1 h post-administration of lorazepam. Mice were euthanized at different time-
351 points following status epilepticus (1 h, 4 h, 8 h, 24 h) and during chronic epilepsy (14 days)

352 and brains flash-frozen whole in 2-methylbutane at -30°C for FjB staining, perfused with
353 PBS and paraformaldehyde (PFA) 4% for immunofluorescence or microdissected and frozen
354 for protein or RNA extraction.

355

356 **Poly(U) chromatography**

357 C57Bl/6 WT mice were sacrificed 8 h following status epilepticus (acute pathology) (KA and
358 PBS, $n = 9$ per group) or 14 days post-status epilepticus (time-point at which all mice suffer
359 from chronic epilepsy²⁴) (KA and PBS, $n = 9$ per group). Ipsilateral hippocampi were quickly
360 dissected, pooled into 3 groups ($n = 3$ per pooled sample) and stored at -80°C until use. RNA
361 was extracted using the Maxwell® 16 LEV simplyrna Tissue Kit (Promega, AS1280).
362 Poly(A) RNA fraction was purified by poly(U) chromatography as before⁴³. Poly(U)-agarose
363 (Sigma, p8563) was suspended in swelling buffer (0.05 M Tris-HCl, pH 7.5, 1 M NaCl) 35
364 ml/g, incubated overnight at room temperature and loaded into a chromatography column. An
365 aliquot of total RNA was stored at -80°C (“Input”) and the remaining sample incubated with
366 sample buffer (0.01 M Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS) for 5 min at 65°C and
367 chilled on ice. Binding buffer was added (0.05 M Tris-HCl, pH 7.5, 0.7 M NaCl, 10 mM
368 EDTA, 25% [v/v] formamide) which was followed by loading of samples into the poly(U)-
369 agarose chromatography column (Mobitec, M1002s). Samples were then incubated for 30
370 min at room temperature (25°C) with agitation. Next, columns containing samples were
371 washed three times at 25°C and six times at 55°C with washing buffer (0.05 M Tris-HCl, pH
372 7.5, 0.1 M NaCl, 10 mM EDTA, 25% [v/v] formamide). The 55°C washes were collected and
373 stored at -80°C (“Short poly(A)-tail fraction”). The remaining poly(A) RNA (“Long poly(A)-
374 tail fraction”) was eluted with elution buffer (0.05 M HEPES, pH 7, 10 mM EDTA, 90%
375 [v/v] formamide) at 55°C and stored at -80°C . RNA of the two poly(A) fractions was
376 precipitated by adding 1 volume of isopropanol, 1/10th volume of sodium acetate 3 M pH 5.2

377 and 20 µg of glycogen (Sigma, G1767). Samples were incubated at -20°C for 20 min and
378 centrifuged for 15 min at 14000 g at 4°C. Supernatant was removed and pellet was washed
379 with 750 µl of ethanol and centrifuged at 14000 g at 4°C for 5 min. Then supernatant was
380 removed again and pellet was air-dried for 5 min. Next, RNAs were resuspended in 300 µl of
381 nuclease-free water and 300 µl of acid Phenol:Chloroform (5:1) were added. Then, samples
382 were vortexed and centrifuged for 10 min at 14000 g at 4°C. The aqueous phase was
383 recovered, mixed with 1 volume of chloroform, vortexed and centrifuged again. The aqueous
384 phase was recovered and precipitated again using the isopropanol precipitation. To confirm
385 the average length in each fragment, when setting up the method, we performed digestion of
386 the non-poly(A) mRNA regions followed by end-labelling of the poly(A) tail for each eluted
387 fraction and Urea-PAGE. Ensuring proper functioning of our technique, poly(A)-tail changes
388 were assessed by HIRE-PAT assays of control genes in Input, Washed and Eluted fractions.
389 RNA quantification was performed by Qubit Fluorimeter using Qubit RNA Hs Assay kit
390 (Thermo-Fisher Scientific, Q32852). RNA integrity QC was performed with Agilent
391 Bioanalyzer 2100, using RNA Nano Assay (Agilent Technologies 5067-1511) and RNA Pico
392 Assay (Agilent Technologies 5067-1513).

393

394 **GeneAtlas MG-430 PM microarray analysis**

395 cDNA library preparation and amplification were performed according to the manufacturer's
396 instructions (Sigma-Aldrich) using the WTA2 kit with 25 ng starting material. cDNA was
397 amplified for 17 cycles and purified using PureLink Quick PCR Purification Kit (Invitrogen,
398 K310001). Quantification of amplified cDNA was carried out on a Nanodrop ND-1000
399 spectrophotometer (Thermo-Fisher Scientific, Waltham, MA, USA). 8.5 µg of the cDNA
400 from each sample were fragmented and labelled with GeneChip Mapping 250K Nsp assay kit
401 (Affymetrix, 900753) following the manufacturer's instructions. Hybridization was

402 performed using the GeneAtlas Hyb, Wash and Stain Kit for 3' IVT arrays. Samples ready to
403 hybridize were denatured at 96°C for 10 min prior to incubation with mouse MG-430 PM
404 Array Strip (Affymetrix, 901570). Hybridization was performed for 16 h at 45°C in the
405 GeneAtlas Hybridization Oven (Affymetrix, 00-0331). Washing and staining steps after
406 hybridization were performed in the GeneAtlas Fluidics Station (Affymetrix, 00-0079),
407 following the specific script for Mouse MG-430 PM Arrays. Finally, arrays were scanned
408 with GeneAtlas Scanner (Affymetrix) using default parameters, and the generation of CEL
409 files for bioinformatics analysis was performed using GeneAtlas software (Affymetrix).
410 Processing of microarray samples was performed using R⁴⁴ and Bioconductor⁴⁵. Raw CEL
411 files were normalized using RMA background correction and summarization⁴⁶. Standard
412 quality controls were performed in order to identify abnormal samples⁴⁷ regarding: a) spatial
413 artefacts in the hybridization process (scan images and pseudo-images from probe level
414 models); b) intensity dependences of differences between chips (MvA plots); c) RNA quality
415 (RNA digest plot); and d) global intensity levels (boxplot of perfect match log-intensity
416 distributions before and after normalization and RLE plots). Probeset annotation was
417 performed using the information available on the Affymetrix web page
418 (<https://www.affymetrix.com/analysis/index.affx>) using version na35. Expression values
419 were adjusted for technical biases as described⁴⁸ using a linear model and implemented with
420 the R package "limma"⁴⁹. For each biological replicate the log₂ fold change was computed
421 between "WASHED (Short)" and "ELUTED (Long)" samples and used to find significant
422 differences between intraamygdala KA or vehicle-treated control mice sacrificed at two
423 different time-points (8 h (acute) and 14 days (chronic epilepsy) following status epilepticus).
424 Differential expression was performed using a linear model with fluidics and amplification
425 batch as covariates. *P*-values were adjusted with the Benjamini and Hochberg correction. We
426 considered one transcript as shortened when *P*-value was < 0.05 and FC was negative and

427 lengthened when P -value was < 0.05 and FC was positive, in at least one probe. If the same
428 transcript showed opposite results for different probes, the poly(A) tail was considered as not
429 changed.

430

431 **Gene Ontology analysis**

432 Genes with changes in poly(A) tail length between intraamygdala KA or vehicle control mice
433 sacrificed at two different time-points (8 h and 14 days following status epilepticus) were
434 analysed by GO terms with the bioinformatic tool DAVID Bioinformatics Resources 6.7²⁵.

435

436 **High-Resolution poly(A) tail (HIRE-PAT) assay**

437 To measure poly(A) tail length of mRNAs, USB® Poly(A) Tail-Length Assay Kit
438 (Affymetrix, 76455) based on the HIRE-PAT method was performed. We used total RNA
439 obtained of Washed and Eluted fractions (enriched in mRNA short and long poly(A) tail
440 respectively), from the ipsilateral hippocampi of mice sacrificed 8 h following status
441 epilepticus. G/I tailing (1 μ g of total RNA) and reverse transcription were performed
442 according to the manufacturer's instructions. Poly(A) tail size was determined by subtracting
443 the PCR amplicon size obtained with the Universal primer and forward specific primers. To
444 verify that the measured poly(A) tail corresponds to a specific gene, at least two different
445 forward specific primers were tested (Supplementary Table 5). PCR products were resolved
446 on a 2.5% sybr green agarose gel (Biotium, 41004) run at 120 V for 1.5 h.

447

448 **Western blot**

449 Western blot was carried out as before⁴². Samples (mouse and human) were prepared by
450 homogenizing extracted brain tissue in ice-cold extraction buffer (20 mM HEPES pH 7.4,
451 100 mM NaCl, 20 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 1 μ M okadaic

452 acid, 5 mM sodium pyrophosphate, 30 mM β -glycerophosphate, 5 mM EDTA, protease
453 inhibitors (Complete, Roche, Cat. No 11697498001)). Protein concentration was determined
454 by Quick Start Bradford kit assay (Bio-Rad, 500-0203) or BCA (Thermo Scientific, Ref
455 23235) following the manufacturer's instructions. Between 15 - 40 μ g of total protein were
456 electrophoresed on 8 - 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred to
457 a nitrocellulose blotting membrane (Amersham Protran 0.45 μ m, GE Healthcare Life
458 Sciences, 10600002) and blocked in TBS-T (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.1%
459 Tween 20) supplemented with 5% non-fat dry milk. Membranes were incubated overnight at
460 4°C with the primary antibody in TBS-T supplemented with 5% non-fat dry milk. On the next
461 day, following washing with TBS-T, membranes were incubated with secondary HRP-
462 conjugated anti-mouse or anti-rabbit IgG (1:1000, Jackson Immuno 266 Research, Plymouth,
463 PA, U.S.A), or anti-goat IgG-Fc fragment (1:5000, Bethyl, A50-104P) and protein bands
464 visualized using chemiluminescence Merck Millipore, Billerica, MA, U.S.A (Pierce
465 Biotechnology, Rockford, IL, USA). Gel bands were captured using a Fujifilm LAS-4000
466 (Fujifilm, Tokyo, Japan), analysed using Alpha-EaseFC4.0 software and quantified using
467 ImageJ software⁵⁰. Protein quantity was normalized to the loading control (ACTB or
468 GAPDH). The following primary antibodies were used: rabbit GRIN2B (1:1000, Abcam,
469 ab65783); rabbit METTL3 (1:1000, Abcam, ab195352); mouse STX6 (1:1000, BD
470 transduction Laboratories, Cat 610635); rabbit CPEB1 (1:350, Santacruz, sc-33193, for
471 human samples and 1:1000, proteintech, 13274-1-AP for mouse samples); rabbit CPEB2
472 (1:1000, Abcam, ab51069); rabbit CPEB3 (1:1000, Abcam, ab10883); rabbit CPEB4
473 (1:1000, Abcam, ab83009); rabbit NEUN (1:1000, Millipore, ABN78); mouse GFAP
474 (1:1000, Sigma, G3893); rabbit GLUR6/7 (1:1000, Millipore, 1497226); mouse ACTB
475 (1:5000, Sigma, A2228); mouse GAPDH (1:5000, Cell Signaling Technology, 14C10);
476

477 **RNA extraction and quantitative polymerase chain reaction (qPCR)**

478 RNA extraction was performed using the Trizol method, as described before⁴². Quantity and
479 quality of RNA was measured using a Nanodrop Spectrophotometer (Thermo Scientific,
480 Rockford, IL, U.S.A). Samples with a 260/280 ratio between 1.8 – 2.2 were considered
481 acceptable. 500 ng of total mRNA was used to produce complementary DNA (cDNA) by
482 reverse transcription using SuperScript III reverse transcriptase enzyme (Invitrogen, CA,
483 U.S.A) primed with 50 pmol of random hexamers (Sigma, Dublin, Ireland). qPCR was
484 performed using the QuantiTech SYBR Green kit (Qiagen Ltd, Hilden, Germany) and the
485 LightCycler 1.5 (Roche Diagnostics, GmbH, Mannheim, Germany). Each reaction tube
486 contained 2 µl cDNA sample, 10 µl SyBR green Quantitect Reagent (Quiagen Ltd, Hilden,
487 Germany), 1.25 µM primer pair (Sigma, Dublin, Ireland) and RNase free water (Invitrogen,
488 CA, U.S.A) to a final volume of 20 µl. Using LightCycler 1.5 software, data were analysed
489 and normalized to the expression of *Actb*. Primers used are detailed in Supplementary Table 5.

490

491 **Enrichment analysis**

492 To evaluate whether a gene set is enriched over the background, enrichment analysis studies
493 were carried out using one-sided Fisher's exact test. For our analysis, we used curated gene
494 lists of epilepsy-related genes generated from three independent studies. This included genes
495 with mutation that cause epilepsy (n = 84); mutations in these genes cause pure or relatively
496 pure epilepsies, or syndromes with epilepsy as the core symptom²⁶. Genes with ultra-rare
497 deleterious variation in familial genetic generalized epilepsy (GGE) and non-acquired focal
498 epilepsy (NAFE) (n = 18); we chose the most significant genes per group (top 15)²⁷. Genes
499 localized in loci associated with epilepsy (n = 21); the 21 most likely epilepsy genes at these
500 loci, with the majority in genetic generalized epilepsies, were selected²⁸. The complete set of
501 epilepsy-related genes used in our study is shown in Supplementary Table 2.

502 **Identification of mRNA targets of RNA binding proteins**

503 CPEB1 and CPEB4 binders were determined previously by RNA immunoprecipitation²¹;
504 PUM1 and PUM2 targets were identified by individual nucleotide resolution CLIP (iCLIP)³¹;
505 genes that interact with HUR were identified by photoactivatable ribonucleoside-enhanced
506 crosslinking and immunoprecipitation (PAR-CLIP)³⁰. The complete list of RNABP targets is
507 shown in Supplementary Table 3. The list of brain-specific genes was obtained from the
508 human protein atlas (<http://proteatlas.org/humanproteome/brain>).

509

510 **Electroencephalogram (EEG) analysis**

511 To analyse EEG frequency, amplitude signal (power spectral density and EEG spectrogram
512 of the data) and seizures onset, EEG data was uploaded into Labchart7 software (AD
513 instruments Ltd, Oxford, UK). EEG total power (μV^2) is a function of EEG amplitude over
514 time and was analysed by integrating frequency bands from 0 to 100 Hz. Power spectral
515 density heat maps were generated using LabChart (spectral view), with the frequency domain
516 filtered from 0 to 40 Hz and the amplitude domain filtered from 0 to 50 mV. The duration of
517 high-frequency (>5 Hz) and high-amplitude (>2 times baseline) (HFHA) polyspike
518 discharges of ≥ 5 s duration, synonymous with injury-causing electrographic activity³⁴, were
519 counted manually by a reviewer who was blinded to treatment. Seizure onset was calculated
520 as first seizure burst from time of intraamygdala KA injection.

521

522 **Behaviour assessment of seizure severity**

523 Changes in seizure-induced behaviour were scored according to a modified Racine Scale as
524 reported previously⁵¹. Score 1, immobility and freezing; Score 2, forelimb and or tail
525 extension, rigid posture; Score 3, repetitive movements, head bobbing; Score 4, rearing and
526 falling; Score 5, continuous rearing and falling; Score 6, severe tonic-clonic seizures. Mice

527 were scored by an observer blinded to treatment every 5 min for 40 min after KA injection.
528 The highest score attained during each 5 min period was recorded.

529

530 **Fluoro-Jade B staining**

531 To assess status epilepticus-induced neurodegeneration, FjB staining was carried out as
532 before⁵². Briefly, 12 µm coronal sections at the medial level of the hippocampus (Bregma AP
533 = -1.94 mm) were cut on a cryostat. Tissue was fixed in formalin, rehydrated in ethanol, and
534 then transferred to a 0.006% potassium permanganate solution followed by incubation with
535 0.001% FjB (Chemicon Europe Ltd, Chandlers Ford, UK). Sections were mounted in DPX
536 mounting solution. Then, using an epifluorescence microscope, cells including all
537 hippocampal subfields (dentate gyrus (DG), CA1 and CA3 regions) and cortex were counted
538 by a person unaware of treatment under a 40x lens in two adjacent sections and the average
539 determined for each animal.

540

541 **Data analysis**

542 Statistical analysis was performed using SPSS 21.0 (SPSS® Statistic IBM®). Data are
543 represented as mean ± S.E.M. (Standard Error of the Mean) with 95% confidence interval.
544 The normality of the data was analysed by Shapiro-Wilk test ($n < 50$) or Kolmogorov-
545 Smirnov ($n > 50$). Homogeneity of variance was analysed by Levene test. For comparison of
546 two independent groups, two-tail unpaired t-Student's test (data with normal distribution),
547 Mann-Whitney-Wilcoxon or Kolmogorov-Smirnov tests (with non-normal distribution) was
548 performed. To compare dependent measurements, we used a paired t-test (normal
549 distribution) or Wilcoxon signed-rank tests (non-normal). For multiple comparisons, data
550 with a normal distribution were analysed by one way-ANOVA test followed by a Tukey's or
551 a Games-Howell's post-hoc test. Statistical significance of non-parametric data for multiple

552 comparisons was determined by Kruskal-Wallis one-way ANOVA test. Enrichment tests
553 were carried out by using one-sided Fisher's exact test. A critical value for significance of $P <$
554 0.05 was used throughout the study.

555

556 **Data availability.** The data that support the findings of this study are available from the
557 corresponding authors upon reasonable request. All records have been approved and assigned
558 GEO accession numbers, however, until the acceptance of the manuscript, these will be kept
559 private. GSE132523 - Identification of the mRNA polyadenylation profile in the
560 hippocampus following intraamygdala kainic acid-induced of status epilepticus in mice

561

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572

573 **Author contributions**

574 A.P. performed bioinformatics and statistical analysis, L.D.-G. performed and was involved
575 in most of experiments, and A.P. and L.D.-G contributed to study design and were involved
576 in all assays and data collection. M.A. performed Western-Blot, qRT-PCR and EEG and data

577 analysis. E.B. performed mouse modelling and behavioural studies. G.C. contributed to the
578 microarray analysis. J.M. analysed data. I.O. performed semiquantitative qRT-PCR. Y.H.-S
579 carried out Western-Blot and qRT-PCR analysis. N.D., M.A.F. and D.F.O. provided human
580 tissue. D.C.H. and R.M. made intellectual contributions to experimental design and
581 discussion. J.J.L. and T.E. directed and conceived the study. T.E. wrote the paper with input
582 from all authors.

583

584 **Conflict of Interest**

585 The authors declare no competing interests. We confirm that we have read the Journal's
586 position on issues involved in ethical publication and affirm that this report is consistent with
587 those guidelines.

588

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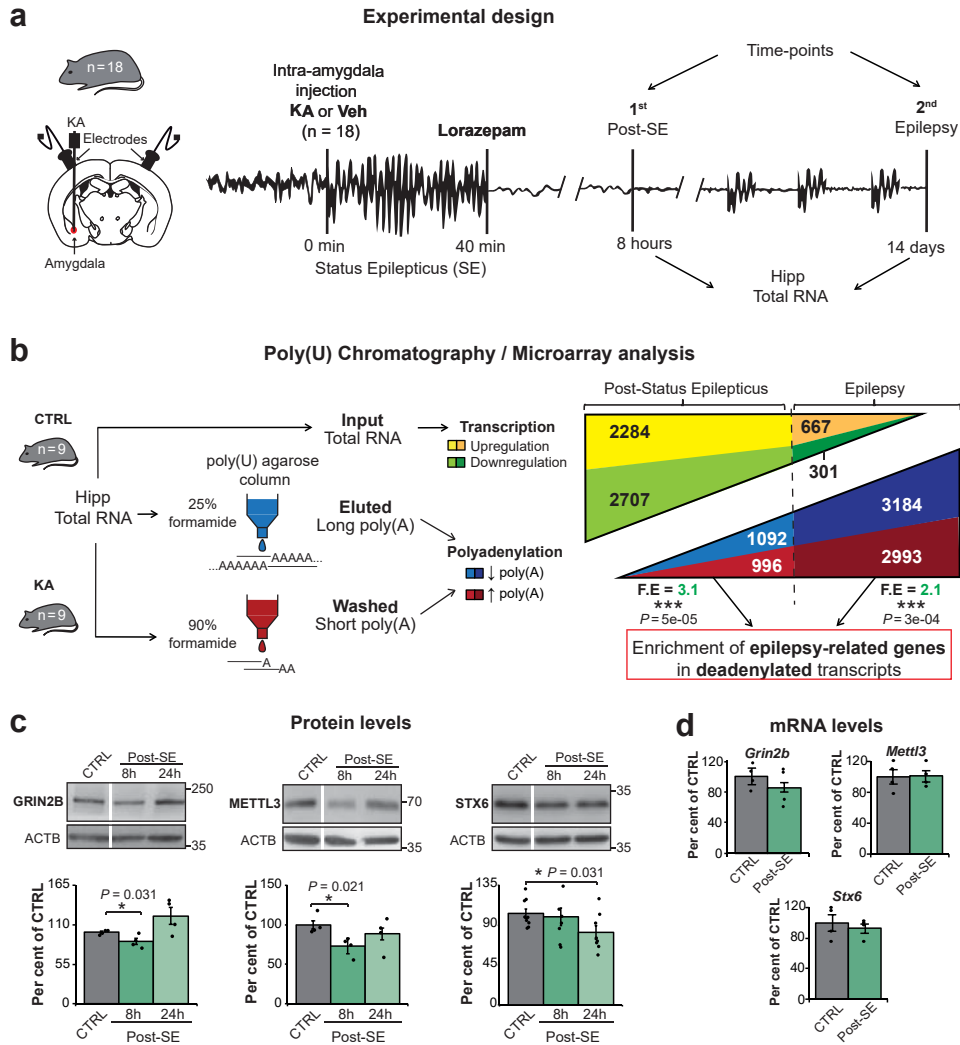


Fig. 1 | Genome-wide mRNA polyadenylation changes following status epilepticus and during epilepsy.

a, Schematic showing the experimental design using the intraamygdala KA mouse model of acquired epilepsy. Hippocampi were collected at two time-points: 8 hours post-status epilepticus (acute injury, $n = 9$) and 14 days post-status epilepticus (epilepsy, $n = 9$). KA, kainic acid; SE, status epilepticus. **b**, Experimental design of poly(U) chromatography and microarray analysis (left) and comparison between genes with dysregulated transcription vs. mRNA polyadenylation changes (right) (Total amount of analyzed genes = 21566). F.E, fold enrichment. **c**, Protein levels in the ipsilateral hippocampus of WT mice injected with vehicle (Control) vs. KA (status epilepticus) at 8 h and 24 h post-status epilepticus of GRIN2B ($n = 4$), METTL3 ($n = 4$) and STX6 ($n = 8$). CTRL, control; WT, wildtype; SE, status epilepticus. **d**, mRNA levels in the ipsilateral hippocampus at 8 h post-status epilepticus, vehicle vs. KA-treated mice ($n = 4$). Data were analyzed and normalized to the expression of ACTB. **b**, One-sided Fisher's exact test, P values of deadenylated epilepsy-related genes versus total deadenylated transcripts. **c**, **d**, Two-sided unpaired t-test. Data are mean \pm S.E.M. 95% CIs. * $P < 0.05$.

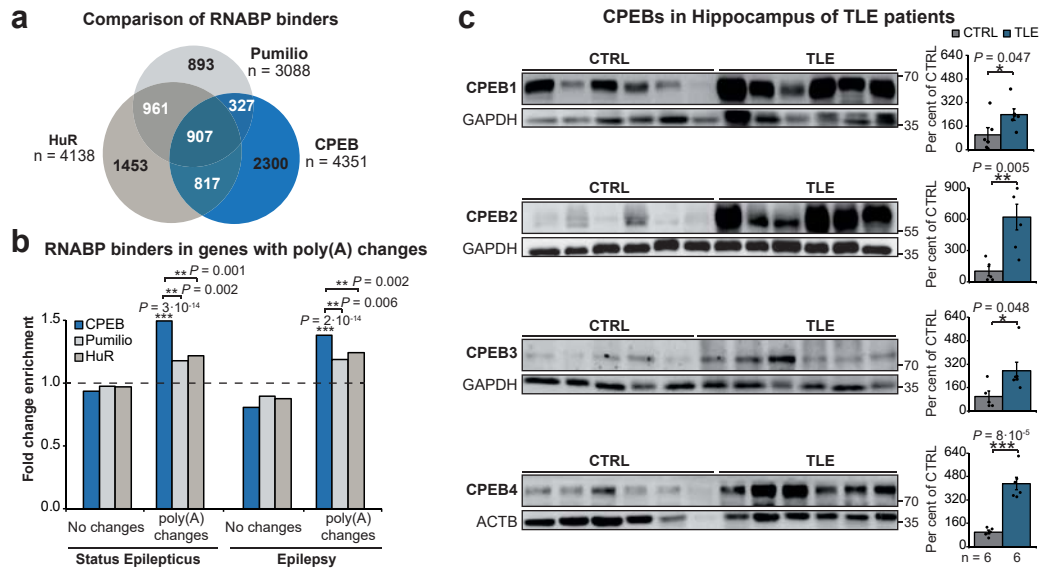


Fig. 2 | Poly(A) changes in RNABP binders and CPEBs expression in TLE.

a, Venn diagram of identified targets of main RNABP families implicated in polyadenylation: PUMILIO, HUR and CPEBs. RNABP, RNA Binding Protein. **b**, Enrichment of RNABP binders of genes undergoing poly(A) tail changes post-status epilepticus and during epilepsy. **c**, CPEB protein levels in the hippocampus of control and patients with TLE (n = 6 per group). Protein quantity was normalized to the loading control (ACTB or GAPDH). TLE, Temporal Lobe Epilepsy; **b**, One-sided Fisher's exact test. **c**, Two-sided unpaired t-test. Data are mean \pm S.E.M. 95% CIs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

CPEB hippocampal expression in intraamygdala KA mouse model

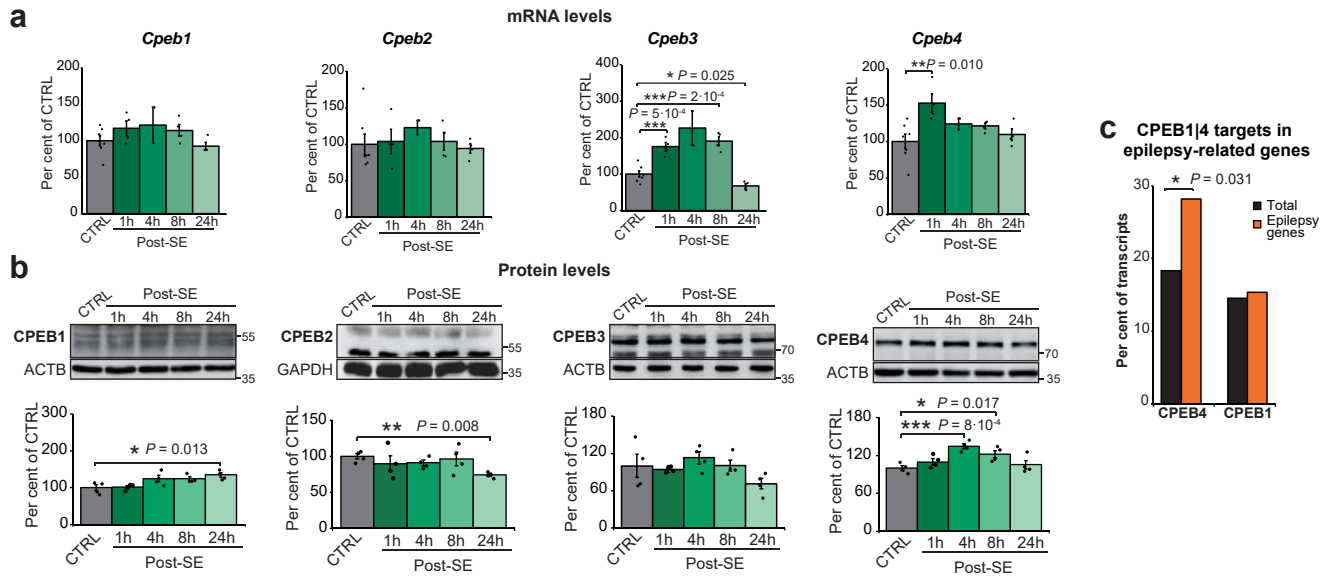


Fig. 3 | Expression of CPEBs in intraamygdala KA mouse model

a, mRNA levels of *Cpebs* in the ipsilateral hippocampus of WT mice injected with vehicle (CTRL) vs. KA at 1 h, 4 h, 8 h and 24 h post-status epilepticus (post-SE) ($n = 4$). Data were analyzed and normalized to the expression of *Actb*. **b**, Protein levels of CPEB family members at same time-points (vehicle $n = 7$, KA $n = 4$). Protein quantity was normalized to the loading control (ACTB or GAPDH). **c**, Percentage of transcripts bound by CPEB4 and CPEB1 in whole transcriptome and in epilepsy-related genes. **a**, **b**, Two-sided unpaired t-test, **c**, One-sided Fisher's exact test. Data are mean \pm S.E.M. 95% CIs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

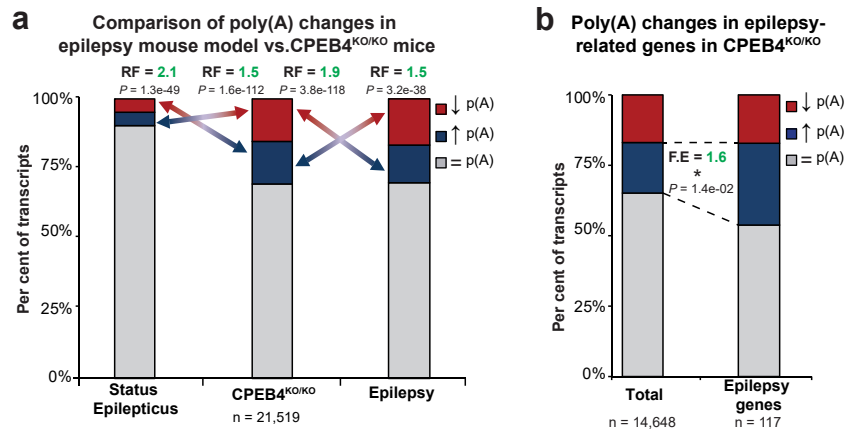


Fig. 4 | CPEB4 as main driver of poly(A) changes in epilepsy

a, Comparison of genes with poly(A)-tail changes in CPEB4^{KO/KO} and genes with changes in poly(A) tail length post-status epilepticus and during epilepsy; RF, representation factor; FE, fold enrichment. **b**, Percentage of epilepsy-related genes with poly(A) tail changes in CPEB4^{KO/KO}. **a**, Hypergeometric test. **b**, One-sided Fisher's exact test, *P* values of epilepsy-related transcripts lengthened vs. total.

Intraamygdala KA mouse model

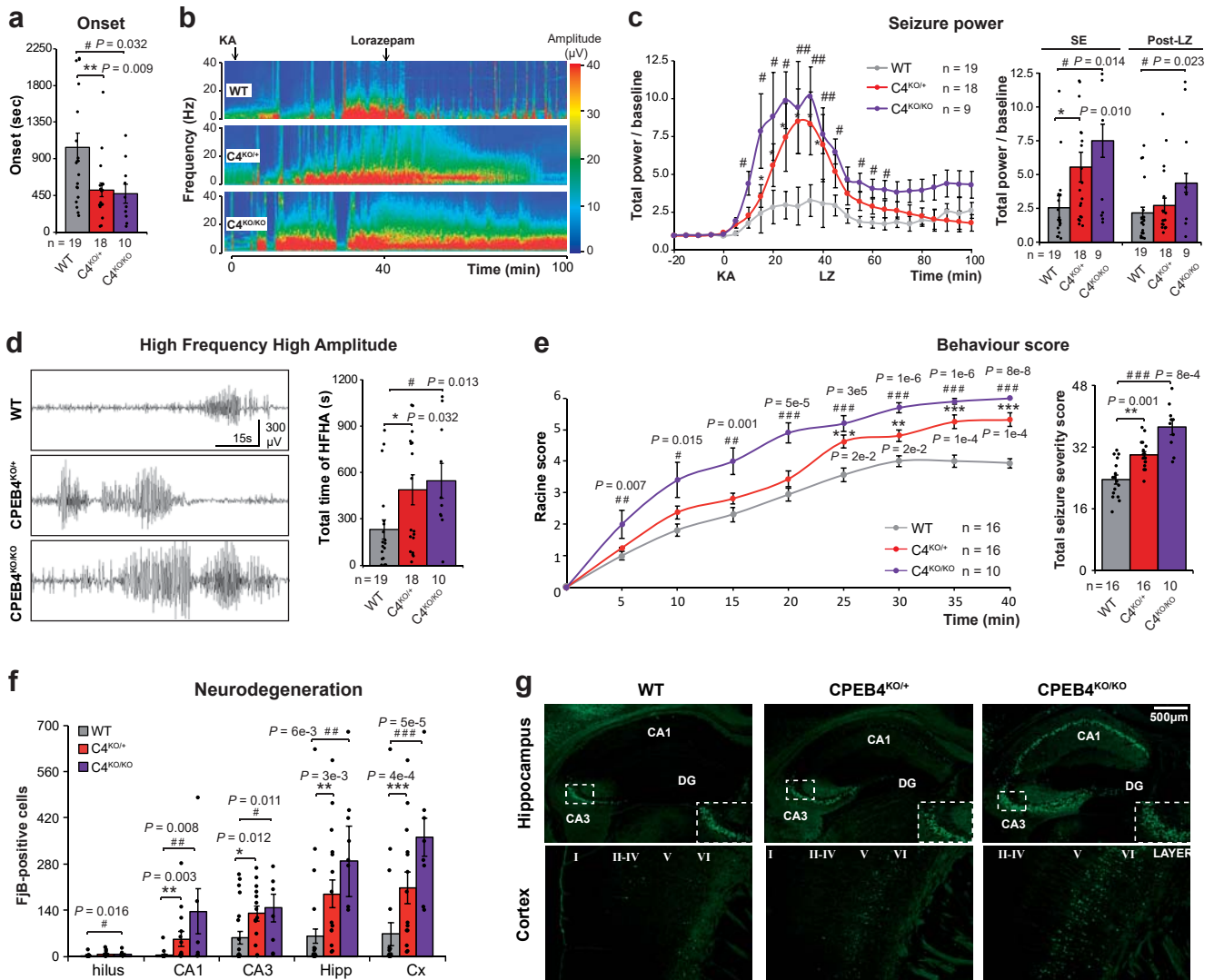


Fig. 5 | CPEB4-deficiency increases seizure susceptibility during status epilepticus and seizure-induced brain damage.

a, Later seizure onset in CPEB4-deficient mice. **b**, Representative heatmap showing increased total seizure power during a 100 min recording period starting from intra-amygdala KA injection ($t = 0$). **c**, Total power normalized to baseline post-KA injection represented in 5 min segments during status epilepticus (0 – 40 min) and post-LZ administration (40 – 100 min). LZ, lorazepam. **d**, Representative electroencephalogram (EEG) traces during status epilepticus and total time of high-frequency high-amplitude (HFHA) spiking. **e**, Behavioral severity of seizures (mean Racine score) scored each 5 min and total score. **f**, Quantitative analysis of Fluoro-Jade-B (FJB) positive cells and **g**, representative sections of neurodegeneration in ipsilateral cortex and hippocampus 72 h post-status epilepticus. Data are mean \pm S.E.M. 95% CIs. WT vs CPEB4^{KO/+} * $P < 0.05$, ** $P < 0.01$. WT vs CPEB4^{KO/KO} # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.