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1 **Title**

2

3 Epilepsy gene therapy using non-integrating lentiviral delivery of an engineered potassium channel

4 gene

5

6

7 **Running title**

8

9 Engineered K channel therapy for epilepsy

10

11

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13

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25 **Abstract**

26

27 Refractory focal neocortical epilepsy is a devastating disease for which there is frequently no
28 effective treatment. Gene therapy represents a promising alternative, but treating epilepsy in this
29 way involves irreversible changes to brain tissue, so vector design must be carefully optimized to
30 guarantee safety without compromising efficacy. We set out to develop an epilepsy gene therapy
31 vector optimized for clinical translation. The gene encoding the voltage-gated potassium channel
32 Kv1.1, *KCNA1*, was codon-optimized for human expression and mutated to accelerate the channels'
33 recovery from inactivation. For improved safety, this engineered potassium channel (EKC) gene was
34 packaged into a non-integrating lentiviral vector under the control of a cell type-specific *CAMK2A*
35 promoter. In a blinded, randomized, placebo-controlled pre-clinical trial, the EKC lentivector robustly
36 reduced seizure frequency in a rat model of focal neocortical epilepsy characterized by discrete
37 spontaneous seizures. This demonstration of efficacy in a clinically relevant setting, combined with
38 the improved safety conferred by cell type-specific expression and integration-deficient delivery,
39 identify EKC gene therapy as ready for clinical translation in the treatment of refractory focal
40 epilepsy.

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41 **Keywords**

42

43 Epilepsy / gene therapy / lentivirus / non-integrating / potassium channel

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44 **Introduction**

45

46 Epilepsy affects over 60 million people worldwide (Ngugi et al., 2010). Even with optimal treatment
47 approximately 30% remain resistant to pharmacotherapy (Kwan et al., 2011; Picot et al., 2008). The
48 development of new anti-epileptic drugs in the last 20 years has had little impact on refractory
49 epilepsy; people with inadequately controlled seizures continue to experience major co-morbidities,
50 social exclusion, and an annual rate of sudden unexpected death in epilepsy (SUDEP) of 0.5-1%
51 (Devinsky, 2011; Hoppe and Elger, 2011). Although surgical resection of the epileptogenic zone can
52 result in seizure freedom, it is unsuitable for over 90% of refractory epilepsy patients (Lhatoo et al.,
53 2003). Surgical intervention in focal neocortical epilepsy (FNE) is further complicated by the high risk
54 of damage to eloquent regions of the cortex involved in functions such as memory, language, vision
55 or fine motor control (Schuele and Lüders, 2008). People with FNE are therefore often left with very
56 few, usually palliative, treatment options, and there is an urgent need to develop alternative
57 therapies.

58

59 Gene therapy is one promising option (Kullmann et al., 2014), but major hurdles remain in achieving
60 stable, predictable and safe transgene expression with viral vectors. Because focal seizures often
61 arise from brain areas very close to eloquent cortex, lentiviral vectors, which generally lead to rapid,
62 stable and, most importantly, spatially-restricted transgene expression (Lundberg et al., 2008), are
63 an attractive delivery tool. In addition, the large packaging capacity of lentivectors allows a wide
64 choice of promoter-transgene combinations (Kantor et al., 2014), which can further increase the
65 specificity of expression. Hitherto, clinical trials with lentivectors for CNS disorders have been mainly
66 restricted to *ex-vivo* treatment of hematopoietic stem cells (Biffi et al., 2013, 2013; Cartier et al.,
67 2009). However, a recent trial using a lentivector injected directly into the striatum has
68 demonstrated safety and tolerability in Parkinson's disease, with evidence of decreased L-DOPA
69 requirement (Palfi et al., 2014).

70

71 Early studies of gene therapy for epilepsy focused on acutely precipitated seizures, which often
72 translate poorly (Galanopoulou et al., 2012). More recent strategies, mainly involving delivery of
73 adeno-associated viral (AAV) vectors to models of temporal lobe epilepsy, have shown that the
74 development of seizures after an epileptogenic insult (epileptogenesis) can be attenuated
75 (Bovolenta et al., 2010; Haberman et al., 2003; Kanter-Schlifke et al., 2007; Lin et al., 2006; McCown,
76 2006; Nikitidou et al., 2014; Noè et al., 2008; Richichi et al., 2004; Woldbye et al., 2010). We have
77 recently reported several approaches to gene therapy in a model of *epilepsia partialis continua* (EPC)
78 induced by tetanus neurotoxin (TeNT) injection into the rat motor cortex (Kätzel et al., 2014; Wykes
79 et al., 2012). In this model pathological high-frequency electrocorticographic (ECoG) activity is

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80 prominent, but discrete seizures lasting over 20 seconds are rare. Lentiviral overexpression of the
81 human potassium channel Kv1.1, encoded by *KCNA1,* was highly effective at reducing pathological
82 high frequency activity (Wykes et al., 2012). *In vitro* studies showed that Kv1.1 overexpression
83 reduced both intrinsic neuronal excitability and glutamate release from transduced pyramidal
84 neurons (Heeroma et al., 2009; Wykes et al., 2012). Importantly, both effects were graded, with
85 neither neuronal excitability nor neurotransmitter release completely abolished. However, it
86 remains unclear whether these graded effects on excitability and transmitter release, and the
87 reduction of pathological ECoG activity in the motor cortex, can translate to the therapeutic
88 suppression of intermittent discrete seizures.
89
90 Gene therapy based on overexpression of Kv1.1, or other proteins that reduce neuronal activity,
91 requires effective targeting of transgene expression to excitatory neurons. Our previous work relied
92 on driving *KCNA1* overexpression with a strong viral promoter, CMV. Although this promoter may
93 bias expression to excitatory neurons in rat, recent data suggests it is not capable of doing so in non-
94 human primates (Lerchner et al., 2014; Yaguchi et al., 2013). Restricting transgene expression to
95 particular neuronal subtypes can however be achieved with the use of cell type specific promoters,
96 but it is not yet known if these can support a level of expression sufficient to dampen neuronal
97 activity. Finally, current clinical guidance seeks to reduce the risk of insertional mutagenesis
98 associated with viral integration into the genome (Baum et al., 2004; Hacein-Bey-Abina et al., 2003),
99 but there are few data indicating whether non-integrating lentiviral constructs can yield strong,
100 stable transgene expression within the CNS.
101
102 To bring potassium channel gene therapy closer to the clinic, we have developed an optimized
103 lentiviral vector designed to boost Kv1.1 expression and reduce its inactivation with an engineered
104 *KCNA1* gene (EKC), and improve safety with a cell type specific (*CAMK2A*) promoter and non-
105 integrating delivery vector (Rahim et al., 2009; Yáñez-Muñoz et al., 2006). The lentivector was tested
106 for efficacy in a rat model of FNE characterised by long-lasting, discrete occipital cortex seizures
107 (Chang et al., 2018). In a blinded, randomized, placebo-controlled pre-clinical trial, EKC gene therapy
108 rapidly and persistently suppressed spontaneous seizures relative to a control lentivector without
109 EKC.
110

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111 **Results**

112

113 **A pilot study shows that *KCNA1* gene therapy suppresses spontaneous seizures in a visual cortex**
114 **epilepsy model**

115

116 We first asked whether the CMV-driven *KCNA1* lentivector (CMV-*KCNA1*) used previously in a model
117 of EPC (Wykes et al., 2012) was also effective in an epilepsy model characterized by discrete
118 seizures. Epilepsy (Fig. 1A; Supp. Fig. 1) was induced in adult rats with a single injection of TeNT into
119 the primary visual cortex. Seizures in this model typically last between 50 and 200 s, are
120 accompanied by unilateral, bilateral or generalized convulsions, and evolve over several weeks
121 before fading (Chang et al., 2018). To monitor local electrographic activity, a wireless ECoG
122 transmitter was implanted with a subdural intracranial recording electrode positioned above the
123 injection site. Two weeks after TeNT administration, following the establishment of epilepsy, animals
124 were randomized into two groups and injected via a pre-implanted cannula with either the CMV-
125 *KCNA1* lentivector or a control vector expressing only green fluorescent protein (GFP). Injections
126 were delivered directly into the seizure focus and followed by a further 4 weeks of ECoG recording
127 (Fig. 1B).

128

129 The CMV-*KCNA1* lentivector transduced neurons within a narrow column of the cortex (Fig. 1C). As is
130 typical of this model (Chang et al., 2018), the total number of seizures experienced by each animal
131 over the 6 weeks of recording was highly variable (Fig. 1D). Consequently, to compare seizure
132 frequency between the two treatment groups the numbers of seizures experienced each week were
133 normalized to the number experienced in the week preceding treatment (week −1, or baseline (Bl)
134 week). Despite the small sample size (6 treated vs. 5 controls), the CMV-*KCNA1* lentivector
135 significantly reduced normalized seizure frequency compared to controls in the weeks following
136 treatment (generalized log-linear mixed model on weeks 0 – 3, treatment*week interaction effect:
137 $F(1,40) = 4.851$, $p = 0.033$; Fig. 1E). The therapeutic effect emerged rapidly; plots of normalized
138 cumulative daily seizure frequency for the two groups diverged within 3 days of lentivector injection,
139 consistent with rapid transgene expression, as seen previously in the motor cortex model (Fig. 1F).

140

141 This pilot study strongly suggests that *KCNA1* gene therapy can suppress spontaneous discrete
142 seizures. However, the CMV-*KCNA1* lentivector tested is poorly suited for clinical translation. We
143 therefore set out to develop an optimized vector with improved safety and efficacy.

144

145 **Design and characterization of an EKC gene therapy optimized for clinical translation**

146

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147 The transfer plasmid used to synthesize the optimized lentivector differed from the original CMV-
148 *KCNA1* construct in several ways (Fig. 2A). The non-cell type specific CMV promoter was replaced
149 with a 1.3 kb human *CAMK2A* promoter to bias expression to excitatory neurons (Dittgen et al.,
150 2004; Yaguchi et al., 2013). The *KCNA1* gene was codon-optimized for expression in human cells, and
151 mutated to introduce an I400V amino acid substitution normally generated by RNA editing. This
152 substitution elicits a 20-fold increase in the rate at which Kv1.1 channels recover from inactivation
153 (Bhalla et al., 2004). For pre-clinical evaluation, the coding sequence of a short-lived dscGFP reporter
154 was linked to the EKC gene by a T2A element, which permits dual peptide expression from a single
155 promoter. To ensure that the EKC construct could produce functional Kv1.1 channels, we performed
156 whole-cell patch clamp recordings in transfected Neuro-2a cells, a line selected for its high *Camk2a*
157 promoter activity. Robust non-inactivating Kv1.1 currents were recorded in cells transfected with the
158 EKC plasmid (Fig. 2B).
159
160 The EKC transfer plasmid was packaged into a non-integrating lentiviral vector (Yáñez-Muñoz et al.,
161 2006). When injected into the rat visual cortex, this lentivector drove strong, localized expression of
162 the dscGFP reporter (Fig. 2C). Imaging of sequential brain slices yielded an estimated transduction
163 volume of approximately 0.074 mm^3 (Supp. Fig. 2). Immunohistochemistry revealed no visible
164 overlap between dscGFP expression and glial fibrillary acidic protein (GFAP) staining (0/512 dscGFP+
165 cells stained for GFAP, n = 3 animals; Fig. 2Di). In contrast, all dscGFP+ cells stained positively for the
166 neuronal marker NeuN (714/714, n = 3 animals; Fig. 2Dii). These data indicate that transgene
167 expression from the EKC lentivector is restricted to neurons. There was minimal overlap between
168 dscGFP expression and staining for glutamic acid decarboxylase 67 (GAD67), an enzymatic marker
169 for GABAergic neurons (3/603 dscGFP+ cells stained for GAD67, n = 3 animals; Fig. 2Diii). This
170 suggests that EKC transgene expression is largely restricted to excitatory neurons.
171
172 **EKC gene therapy reduces seizure frequency in a blinded, randomized pre-clinical trial**
173
174 To test the therapeutic efficacy of the EKC lentivector, we designed a blinded, randomized, placebo-
175 controlled pre-clinical trial, and selected normalized seizure frequency as the primary outcome
176 measure. Eleven days after injection of TeNT into the visual cortex, 26 rats were randomized into
177 two groups and injected via a pre-implanted cannula with either the EKC lentivector or its dscGFP-
178 only control. ECoG recordings were continued for a further 4 weeks. The timeline was altered from
179 that of the pilot study to treat after 11 days in order to capture the period when seizure activity is at
180 its highest (2 – 4 weeks following TeNT injection) (Fig. 3A).
181

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182 To minimize the confounding influence of animals that displayed a very low seizure frequency prior
183 to treatment, subjects were excluded if they exhibited fewer than five seizures in the week
184 preceding lentiviral delivery (the baseline week). This criterion, applied before unblinding, led to the
185 exclusion of eight animals (6 EKC, 2 control). Of the remaining 18, all but one survived for the
186 duration of recording. This rat (from the EKC group) was culled in the final week due to detachment
187 of its headpiece. However, because the subject had already passed through the period of peak
188 seizure activity, and in order to maximise the amount of data obtained from the study, this
189 incomplete dataset was included in the overall analysis. Again, this decision was made before
190 unblinding.
191
192 There was no significant difference between the treatment groups in the number of seizures
193 experienced in the week preceding virus injection (control median = 11 (IQR 10 – 26), EKC median =
194 10 (IQR 7.5 – 12); Mann Whitney U test, $p = 0.185$). Analysis of the primary outcome measure
195 indicated that EKC therapy robustly decreased normalized seizure frequency compared to controls in
196 the weeks following treatment (generalized log-linear mixed model on weeks 0 – 3, treatment*week
197 interaction effect: $F(1,67) = 29.704$, $p < 0.001$; Fig. 3B). The size of the effect was larger than that
198 observed in the pilot study, suggesting that the EKC gene is more effective than its wild-type *KCNA1*
199 counterpart at suppressing neuronal hyperexcitability. As in the pilot study, the reduction in seizure
200 frequency lasted for the duration of recording, and the absolute effect size only decreased as
201 seizures abated in the control group. Again the therapeutic effect emerged rapidly, with plots of
202 normalized cumulative daily seizure frequency for the two groups diverging 2 days after treatment
203 (Fig. 3C).

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204 **Discussion**

205

206 EKC gene therapy represents an effective new treatment for focal neocortical seizures in a format
207 adapted to improve safety and suitability for human use. The results presented here provide strong
208 justification for the further clinical development of EKC therapy.

209

210 We have previously shown that overexpression of Kv1.1 can reduce the frequency of brief (< 1 s),
211 high-frequency epileptiform discharges in a motor cortex TeNT model of EPC (Wykes et al., 2012).
212 However, this study did not investigate whether Kv1.1 overexpression could inhibit discrete seizures
213 lasting 1 – 2 minutes, more typical of common forms of focal epilepsy. We show here, in two
214 independent trials, that Kv1.1 overexpression is indeed sufficient to reduce the frequency of
215 seizures, although interestingly seizure duration was not altered. This is most simply explained by
216 proposing that seizure initiation is rapidly accompanied by propagation to other brain areas, beyond
217 the lentivector-treated region, consistent with the convulsions seen in the majority of seizures in the
218 visual cortex TeNT model (Chang et al., 2018).

219

220 Injection of TeNT into the occipital cortex induced seizures that lasted markedly longer (50 – 150 s)
221 than the epileptiform bursts evoked by TeNT injection in motor cortex (< 1 s) (Wykes et al., 2012).
222 The difference, which parallels that seen with occipital lobe seizures and EPC in human patients, may
223 be a consequence of different connectivity in the occipital and motor cortices. Further studies will be
224 needed to determine how cortical architecture impacts the type of epileptiform activity induced by
225 TeNT insult.

226

227 Lentiviral gene therapy approaches are becoming more common in CNS disorders, and have shown
228 good safety and tolerability even in extended trials (Palfi et al., 2014). However, a potential safety
229 concern with retroviral vectors is the inherent risk of insertional mutagenesis (Baum et al., 2004;
230 Hacein-Bey-Abina et al., 2003). This risk can be minimized by rendering vectors integration-deficient.
231 The popularity of non-integrating lentiviruses for therapeutic gene transfer is growing, and the
232 vectors have already demonstrated pre-clinical efficacy in the treatment of degenerative retinal
233 disease and haemophilia B (Suwanmanee et al., 2014; Yáñez-Muñoz et al., 2006). The non-
234 integrating EKC lentivirus described here drove strong, localized transgene expression after direct
235 injection into the rat neocortex, and rapidly and persistently suppressed focal seizure activity. This
236 supports the use of integration-deficient vectors as safe, effective delivery tools for gene therapy of
237 neurological disease.

238

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239 In the case of epilepsy, an additional safety concern is the possibility of potassium channel
240 overexpression in interneurons, which could aggravate seizure activity by exacerbating rather than
241 attenuating local excitability. To mitigate this risk we have used a human *CAMK2A* promoter that in
242 rats led to very little expression in GABAergic cells. Promoter specificity can differ between species
243 (Lerchner et al., 2014; Yaguchi et al., 2013), and the specificity of the human *CAMK2A* promoter for
244 excitatory glutamatergic neurons will ultimately need to be validated in the human brain. Evidently,
245 if EKC gene therapy is to progress to the clinic, such validation will need to be performed in the
246 absence of a fluorescent reporter.
247
248 Because the role of potassium channels, including Kv1.1, in regulating neuronal excitability is
249 conserved across a broad range of neurons, potassium channel overexpression may hold therapeutic
250 promise in the treatment of other diseases characterized by neuronal hyperexcitability. There is
251 currently an unmet clinical need for new treatments of chronic pain, and a variety of gene therapy
252 approaches aimed at reducing the excitability of dorsal root ganglion neurons have already
253 demonstrated pre-clinical efficacy (Snowball and Schorge, 2015). Other disorders such as Parkinson's
254 disease are associated with excessive activity in specific groups of neurons (Lobb, 2014), and could
255 be candidates for treatment with an appropriate combination of potassium channel subtype and cell
256 type specific promoter.

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257 **Materials and methods**

258

259 *Molecular biology*

260 Lentiviral transfer plasmids were constructed using standard subcloning techniques. *KCNA1* was
261 codon optimized for human expression using GeneOptimizer® software, and synthesized using
262 GeneArt® (Thermo Fisher Scientific). All plasmids were fully sequenced before use. Sequences are
263 available on request.

264

265 *Voltage clamp recordings*

266 Neuro-2a cells were grown in Gibco® Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX™
267 (Thermo Fisher Scientific) supplemented with 10% heat-inactivated foetal bovine serum (Thermo
268 Fisher Scientific), 1% penicillin/streptomycin (Thermo Fisher Scientific) and 1% non-essential amino
269 acids (Sigma). Cultures were maintained in logarithmic growth phase in a humidified 5% CO_2
270 atmosphere at 37 °C. Transfections were performed according to the manufacturer's instructions
271 using TurboFect™ transfection reagent (Thermo Fisher Scientific). Transfected cells were plated onto
272 13 mm borosilicate glass coverslips (VWR). Coverslips were placed into the chamber of a BX51WI
273 fixed-stage upright microscope equipped with UMPLFLN 10× and LUMPLFLN 40× water-immersion
274 objectives (Olympus). Coverslips were submerged in a static bath of extracellular solution with the
275 following composition (in mM): 140 NaCl, 4 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 10 HEPES (pH 7.35, osmolarity
276 ~301 mOsm/L). Filamented borosilicate glass micropipettes (GC150-F; Warner Instruments) were
277 pulled to tip resistances between 2.0 and 3.0 MΩ using a P-97 Flaming/Brown micropipette puller
278 (Sutter Instrument Company). Micropipettes were filled with an intracellular solution of the
279 following composition (in mM): 140 KCl, 10 HEPES, 10 EGTA (pH 7.35, osmolarity ~291 mOsm/L).
280 Macroscopic currents were recorded under voltage clamp using the whole-cell patch clamp
281 configuration. The voltage step protocol used was as follows: cells were held at a resting potential of
282 −80 mV and currents evoked by 200 ms depolarising steps delivered in 10 mV increments up to +20
283 mV. A 40 ms hyperpolarising step to −100 mV was included before returning to baseline. Data were
284 filtered at 3 kHz and acquired at 10 kHz using WinWCP software (J. Dempster, University of
285 Strathclyde) and an Axon Multiclamp 700B amplifier (Molecular Devices). Series resistance
286 compensation was employed throughout, with prediction and correction components adjusted to
287 80% and the bandwidth set to 1.2 kHz. Cells with series resistance greater than 10 MΩ were
288 excluded from the analysis. All recordings were made at room temperature (23 – 26 °C). The liquid
289 junction potential, calculated to be +4.1 mV, was left uncorrected. Leak currents were minimal and
290 left unsubtracted.

291

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292 For analysis, evoked currents were taken as the steady-state current in the last 40 ms of each
293 voltage step. Baseline holding currents were subtracted before division by cell capacitance to
294 generate current density values. To calculate normalized conductance, the current density at each
295 voltage step was divided by the step potential minus the potassium reversal potential (−91.34 mV).
296 This generates raw conductance values that are corrected for the variation in K^+ driving force which
297 accompanies stepwise changes in membrane potential. Plots of raw conductance against voltage for
298 each EKC-transfected cell were fit with individual Boltzmann functions given by the equation:
299

$$G = A_2 + \frac{A_1 - A_2}{1 + e^{\frac{V - V_{0.5}}{k}}}$$

300
301 where G is the conductance, V the voltage, A_1 the initial (minimum) conductance, A_2 the final
302 (maximum) conductance, $V_{0.5}$ the voltage of half-maximal conductance, and k the slope factor. Raw
303 conductance values were normalized to A_1 and A_2 of their own Boltzmann functions. Normalized
304 conductance was then plotted against voltage for all EKC-transfected cells and mean values fit with a
305 single Boltzmann function (Fig. 2Biii).
306
307 *Lentiviral synthesis*
308 The CMV-*KCNA1* lentivector was identical to that used in Wykes *et al.*, 2012 (Wykes et al., 2012). For
309 the EKC lentivector and its dscGFP-only control, HEK293T producer cells were grown in Gibco®
310 DMEM + GlutaMAX™ supplemented with 10% heat-inactivated foetal bovine serum and 1%
311 penicillin/streptomycin. Cultures were maintained in logarithmic growth phase in a humidified 5%
312 CO_2 atmosphere at 37 °C. Cells were split every 3 – 4 days using 0.05% Trypsin-EDTA (Thermo Fisher
313 Scientific) and never grown for more than 15 passages. Cells were co-transfected with pMDG-VSV.G,
314 pCMVdR8.74^{D64V} and either the EKC transfer plasmid or its dscGFP-only control. The mass ratio of
315 envelope to packaging to transfer plasmids was 1 : 2.5 : 1.5. Transfections were performed according
316 to the manufacturer's instructions using Lipofectamine® 2000 (Thermo Fisher Scientific). The
317 transfection medium was replaced after 18 hours. Two media harvests were collected, at 40 hours
318 and 60 hours after transfection. Harvested media were pre-cleaned by centrifugation at 1000 rpm
319 for 3 minutes at 4 °C and filtered through 0.45 μm micropores. Media were overlaid on a sucrose
320 solution with the following composition (in mM): 50 Tris-HCl, 100 NaCl, 0.5 EDTA (pH 7.4, 10% w/v
321 sucrose), and centrifuged at 20,000 rpm for 2 hours at 4 °C. Lentiviral pellets were resuspended in
322 sterile PBS, aliquoted, snap-frozen and stored at −80 °C. Viral titre was approximated using the Lenti-
323 X™ p24 rapid titer kit (Clontech). Each titration was performed in triplicate with 3 separate aliquots.
324 Estimated titres were 2.42×10^9 IU/ml (EKC lentivector) and 4.26×10^9 IU/ml (dscGFP-only control).
325

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326 *Surgical procedures*

327 All experiments were performed in accordance with the United Kingdom Animals (Scientific
328 Procedures) Act 1986. Adult male rats (Sprague Dawley; 300-400g) were anesthetized and placed
329 into a stereotaxic frame (Kopf). 15 ng of TeNT was injected into layer 5 of the right visual cortex in a
330 final volume of 1.0 µl at a rate of 100 nl/min (coordinates: 3 mm lateral, 7 mm posterior of bregma;
331 1.0 mm deep from the pia). An ECoG transmitter (A3028E; Open Source Instruments, MA, USA) was
332 implanted subcutaneously with a subdural intracranial recording electrode positioned above the
333 injection site. A reference electrode was implanted in the contralateral hemisphere. A cannula
334 (Plastics One) was positioned above the injection site for delivery of lentiviral vectors 11 or 14 days
335 later. Each rat received a maximum of 2.0 µl of lentivirus injected directly into the seizure focus.
336 Animals injected with TeNT were housed separately in Faraday cages for the duration of the study.

337

338 *ECoG acquisition and analysis*

339 ECoG was recorded continuously for up to 6 weeks after surgery. Data were acquired using A3028E
340 implantable transmitters (0.3 – 160 Hz, 512 samples/s) and ancillary receivers and software (Open
341 Source Instruments, Inc.). The method of seizure detection differed for the pilot study and the final
342 pre-clinical trial. For the pilot study, ECoG traces were first divided into 1 s epochs. Four metrics
343 (power, coastline, intermittency and coherence) were then quantified for each epoch, and their
344 values compared to those from a user-curated library of epochs validated by video as representing
345 seizure activity (Wykes et al., 2012). Matched values were fed into a consolidation script that
346 returned all instances of 5 or more sequential epochs identified as containing seizure activity. All
347 seizures in the consolidation output were verified by an experimenter. For the final pre-clinical trial,
348 6 metrics were quantified for each epoch (power, coastline, intermittency, coherence, asymmetry
349 and rhythm) and all matched values were manually checked for seizure activity without the use of a
350 consolidation script. Seizure counts in this trial were performed by an experimenter blinded to the
351 treatment. For all datasets the minimum duration for a seizure was set at 10 s.

352

353 *Immunohistochemistry*

354 One week after lentivirus injection rats were terminally anesthetized with sodium pentobarbital
355 (Euthatal; Merial) and transcardially perfused with cold (4 °C) heparinized PBS (80 mg/L heparin
356 sodium salt; Sigma) followed by 4% paraformaldehyde (PFA) in PBS (Santa Cruz Biotechnology).
357 Brains were removed and post-fixed in 4% PFA at 4 °C for a further 24 hours. After washing in PBS
358 brains were sliced into 70 µm coronal sections using a vibrating microtome (Leica) and stored free-
359 floating at 4 °C in PBS plus 0.02% sodium azide (Sigma). For antibody staining, slices were
360 permeabilized for 20 minutes in PBS plus 0.3% Triton X-100 (Sigma) before blocking for 1 hour in PBS
361 plus 0.3% Triton X-100, 1% bovine serum albumin (Sigma) and 4% goat serum (Sigma). Slices were

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362 incubated overnight at 4 °C in PBS plus 0.3% Triton X-100 and a rabbit anti-NeuN (diluted 1:750;
363 ab177487; Abcam), mouse anti-GFAP (diluted 1:500; MAB3402; Merck Millipore) or mouse anti-
364 GAD67 (diluted 1:500; MAB5406; Merck Millipore) primary antibody. After three 10 minute washes
365 in PBS, slices were incubated at room temperature for 3 hours in PBS plus the relevant Alexa Fluor®
366 594-conjugated secondary antibody (goat anti-rabbit (A-11037; Thermo Fisher Scientific) or goat
367 anti-mouse (A-11005; Thermo Fisher Scientific); both diluted 1:750). After a further three 10 minute
368 washes in PBS, slices were mounted onto plain glass microscope slides (Thermo Fisher Scientific)
369 using Vectashield® HardSet™ mounting medium (Vector Laboratories) and borosilicate glass
370 coverslips (VWR). Bright-field and fluorescence images were acquired using one of two microscopes:
371 an Axio Imager A1 fluorescence microscope (Axiovision LE software) equipped with 2.5×, 10× and
372 40× EC Plan-Neofluar non-immersion objectives, or an inverted LSM 710 confocal laser scanning
373 microscope (ZEN 2009 software) equipped with 40× and 63× EC Plan-Neofluar oil-immersion
374 objectives (all Zeiss). For the confocal microscope, dscGFP and Alexa Fluor® 594 were excited with
375 the 488 nm and 561 nm lines of an argon or diode pumped solid state (DPSS) laser, respectively. All
376 image processing was performed using ImageJ software. Composite images were assembled using
377 the MosaicJ ImageJ plugin.
378
379 *Statistics*
380 Data from the pilot study were used to determine sample sizes for the final pre-clinical trial. We
381 estimated that the maximal weekly seizure frequency would double from baseline, and we wished to
382 detect with 80% power a 40% reduction from this maximum at $p < 0.05$. Given a mean baseline
383 weekly seizure frequency of 5 or above, a modification of Lehr's formula (Lehr, 1992) for the Poisson
384 distribution suggested 7 – 8 animals per group would be sufficient to detect a reduction in seizure
385 frequency from 10 to 6 per week. Our modified Lehr's formula is given by the following equation:
386

$$n = \frac{4}{\left(\sqrt{\lambda_1} - \sqrt{\lambda_2}\right)^2}$$

387
388 where n is the size of each sample (treatment group), λ_1 the mean weekly seizure frequency before
389 treatment, and λ_2 the mean weekly seizure frequency after treatment.
390
391 Efficacy of treatment data (Fig. 1E, 3B) were analysed using a generalized log-linear mixed model
392 with random effect of animal (autoregressive covariance) and fixed effects of treatment group,
393 week, and the interaction between treatment group and week. Seizure counts in the week preceding
394 treatment were compared using a Mann Whitney U test. Current densities at +20 mV (Fig. 2Bii) were
395 compared using a Welch's one-way ANOVA followed by Games-Howell post-hoc tests.

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396 **Acknowledgments**

397

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405

406

407 **Author contributions**

408

409 AS designed, synthesized and characterized the EKC lentivector, and analyzed and interpreted data
410 from the final pre-clinical trial. EC performed the final pre-clinical trial and analyzed and interpreted
411 data from it. RCW performed the pilot study and analyzed and interpreted data from it. AL provided
412 technical assistance for video/ECoG recordings. KSH designed and built the ECoG recording system,
413 and analyzed and interpreted data from the pilot study. SS, DMK and MCW designed the study,
414 supervised the experiments and interpreted the data. AS, SS, DMK and MCW wrote the manuscript
415 with input from all co-authors.

416

417

418 **Conflicts of interest**

419

420 The authors have intellectual property on the use of engineered potassium channels. KSH is the
421 majority share-holder of Open Source Instruments, Inc.

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422 **The Paper Explained**

423

424 Problem:

425 Focal neocortical epilepsy is a serious and common disease that is frequently resistant to anti-
426 epileptic drugs. Gene therapy is a promising treatment alternative to surgery to remove the seizure
427 focus. In previous work, overexpression of *KCNA1*, encoding the voltage-gated potassium channel
428 Kv1.1, suppressed pathological, high-frequency brain activity evoked by injecting tetanus toxin into
429 the rat motor cortex. However, several features of the lentiviral vector used to deliver the *KCNA1*
430 gene made it unsafe for human administration, and it was unclear if the reduction of pathological
431 high-frequency activity would extend to a suppression of discrete seizures. We therefore developed
432 a lentiviral vector optimized for clinical translation, and tested its effectiveness in a model of
433 epilepsy characterized by discrete seizures.

434

435 Results:

436 To boost the efficacy of the gene therapy, *KCNA1* was codon-optimized for human expression and
437 mutated to accelerate the recovery of Kv1.1 channels from inactivation. To improve safety, this
438 engineered potassium channel gene was placed under the transcriptional control of a *CAMK2A*
439 promoter to restrict expression to excitatory neurons, and packaged into a non-integrating lentiviral
440 vector to reduce the risk of insertional mutagenesis. In a blinded, randomized, placebo-controlled
441 pre-clinical trial, the EKC lentivector robustly reduced seizure frequency in a rat model of focal
442 neocortical epilepsy characterized by discrete seizures.

443

444 Impact:

445 This demonstration of the anti-epileptic efficacy of *KCNA1* gene therapy in a clinically relevant
446 setting, combined with the improved safety conferred by cell type specific expression and
447 integration-deficient delivery, suggests EKC gene therapy is well placed for clinical translation in the
448 treatment of drug-resistant focal epilepsy. To our knowledge this study represents the first
449 successful use of a non-integrating lentiviral vector to treat an experimental model of a neurological
450 disease, and addresses a major treatment gap for over 5 million people suffering from refractory
451 focal epilepsy worldwide.

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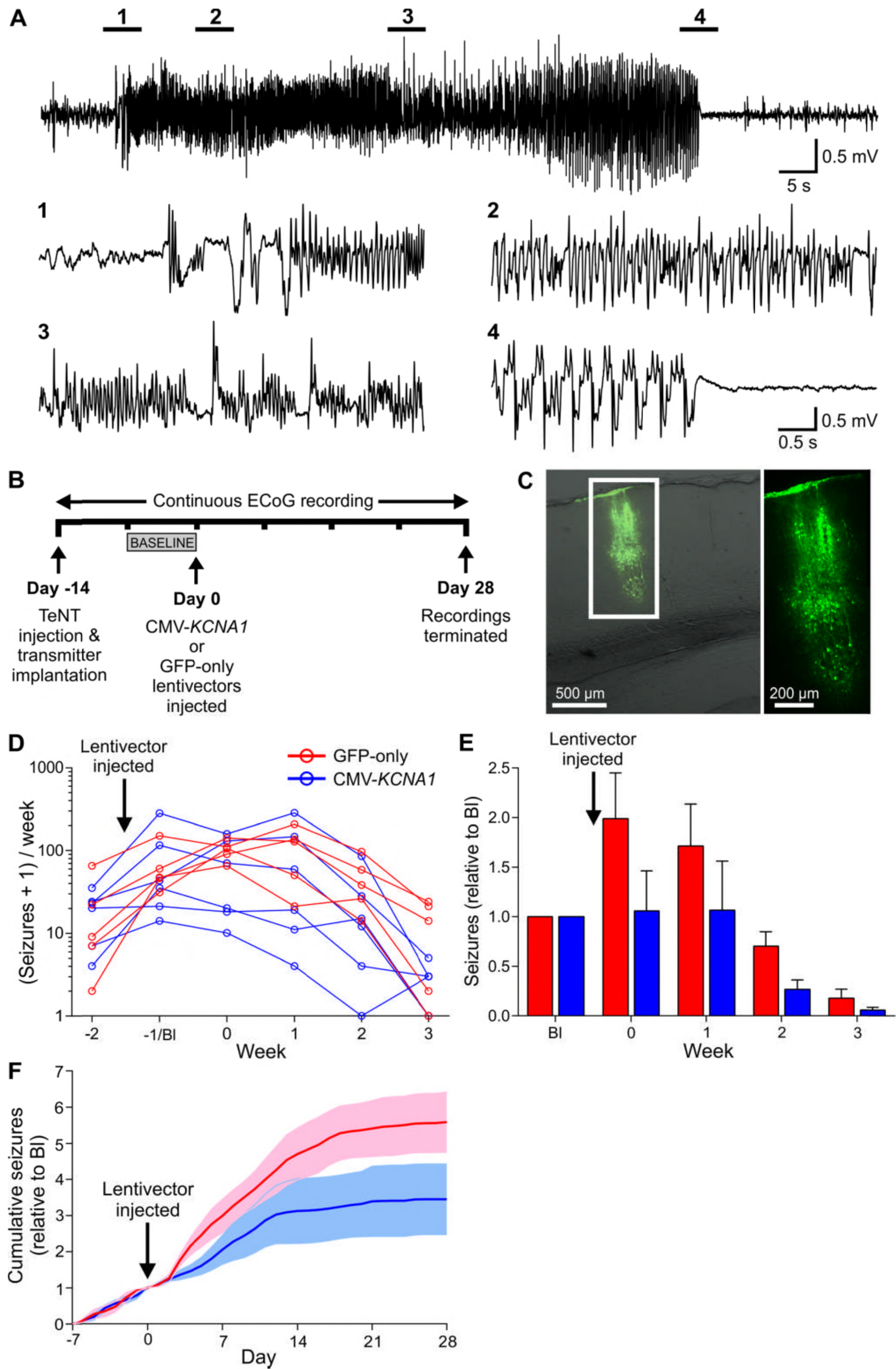
564 **Figures and legends**

565



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567 **Figure 1: A pilot study suggests *KCNA1* gene therapy can suppress genuine discrete seizures in the**
568 **visual cortex TeNT model of FNE.**
569 A. Representative occipital cortex seizure experienced by an adult rat 2 weeks after injection of TeNT
570 into the primary visual cortex. Expanded sections are taken at the times indicated. Video footage of
571 the behaviours associated with this electrographic seizure is provided in Fig EV1.
572 B. Timeline highlighting key experimental milestones.
573 C. Neuronal transduction with the CMV-*KCNA1* lentivector was restricted to a narrow column of
574 cortex surrounding the site of injection additional images in Fig EV2.
575 D. Number of seizures (per week) experienced by animals injected with the CMV-*KCNA1* lentivector
576 (blue; n = 6) or its GFP-only control (red; n = 5). Data are plotted on a logarithmic scale after
577 incrementing each seizure count by 1 to avoid zero values.
578 E. Normalized seizure frequency (per week) for the two groups. The numbers of seizures
579 experienced each week were normalized to the number experienced by each animal in the week
580 preceding treatment (week Bl).
581 F. Normalized cumulative seizure frequency (per day). Cumulative seizure counts were also
582 normalized to the total number experienced in week Bl.
583 Data in panels E and F are presented as mean ± the standard error of the mean (SEM).
584

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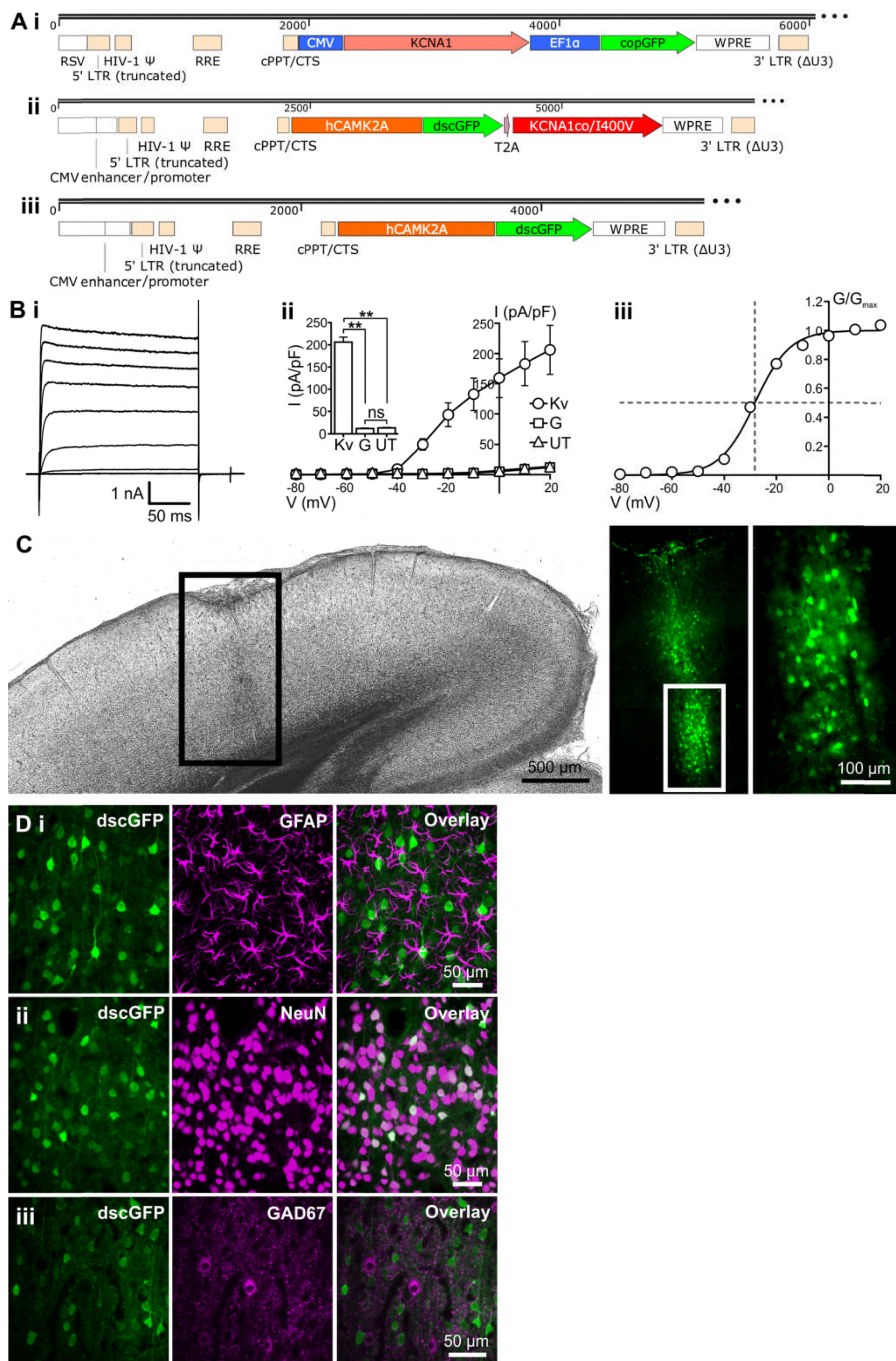

585

586

587 **Figure 2: Design and characterization of an EKC gene therapy optimized for clinical translation.**

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588 A. Lentiviral transfer plasmid maps for the CMV-*KCNA1* pilot vector (i), the optimized EKC vector (ii)
589 and its dscGFP-only control (iii). Abbreviations: RSV – Rous sarcoma virus promoter; LTR – long
590 terminal repeat; HIV-1 Ψ – HIV-1 packaging signal; RRE – Rev response element; cPPT/CTS – central
591 polypurine tract and central termination sequence; EF1α – elongation factor 1 α promoter; WPRE –
592 woodchuck hepatitis virus post-transcriptional regulatory element.
593 B. Heterologous expression of functional Kv1.1 channels from the optimized EKC transfer plasmid.
594 (i): Representative current-time trace from a Neuro-2a cell transfected with the EKC transfer
595 plasmid. (ii): Plot of mean current density against voltage for cells transfected with the EKC transfer
596 plasmid (Kv; n=13), cells transfected with the dscGFP-only control plasmid (G; n=8), and
597 untransfected controls (UT; n=10). Inset: histogram showing differences in current density between
598 the three groups during the voltage step to +20 mV (Kv vs. UT: p=0.0013; Kv vs. G: p=0.0012; UT vs.
599 G: p=0.82; ns = not significant; Welch's one-way ANOVA with Games-Howell post-hoc tests). (iii):
600 Plot of mean normalized conductance against voltage for cells transfected with the EKC transfer
601 plasmid. Data are fit with a single Boltzmann function. The $V_{0.5}$ (voltage of half-maximal
602 conductance) of −28.2 mV is similar to values obtained from human embryonic kidney 293 (HEK293)
603 cells transfected with CMV-driven, wild-type *KCNA1* (−32.8 ± 0.9 mV)(Tomlinson et al., 2013). All
604 error bars represent SEM.
605 C. Bright-field and fluorescence images of a brain slice from a rat injected in the left visual cortex
606 with 1.25 µl (~3.0 x 10^6 infectious units (IU)) of the EKC lentivector. The pattern of transduction is
607 similar to that observed with the CMV-*KCNA1* vector.
608 D. Immunohistochemical assessment of the cell type specificity of EKC expression. (i): There was no
609 overlap between transduced neurons expressing dscGFP and astrocytes stained for GFAP. (ii): There
610 was 100% overlap between dscGFP+ cells and neurons stained for NeuN. (iii): Minimal overlap was
611 observed between dscGFP+ cells and inhibitory interneurons stained for GAD67.
612

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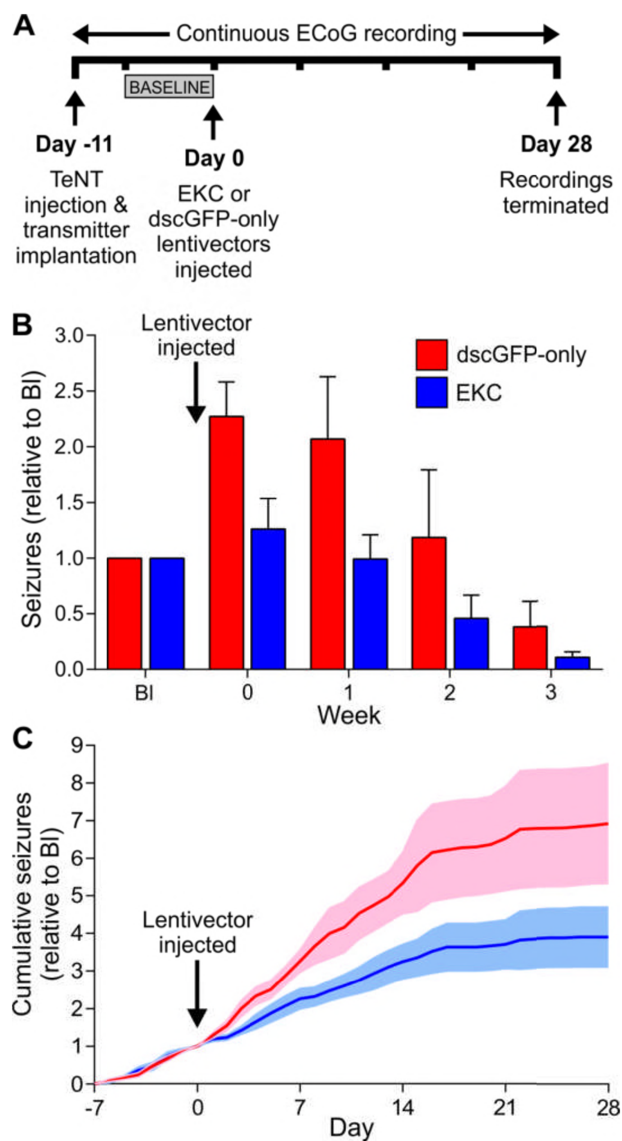

613

614

615 **Figure 3: EKC gene therapy robustly reduces seizure frequency in a blinded, randomized, placebo-**

616 **controlled pre-clinical trial.**

617 A. Timeline highlighting key experimental milestones. Note the injection of lentiviral vectors 11 days

618 after TeNT delivery.

619 B. Normalized seizure frequency (per week) for animals treated with the EKC lentivector (blue; n =

620 7/6) or its dscGFP-only control (red; n = 11).

621 C. Normalized cumulative seizure frequency (per day).

622 Data in panels B and C are presented as mean ± SEM.

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623 **Expanded View figures and legends**

624

625 See separate .mp4 file for Expanded View Figure 1.

626

627 **Fig EV1: Time-locked video-ECoG recording of a spontaneous discrete seizure in the visual cortex**
628 **TeNT model of FNE.**
629 As previously described (Chang et al., 2018) electrographic seizures in this model were accompanied
630 by overt behaviours including sudden increases in arousal, repetitive eye blinking, rearing, bilateral
631 limb twitching and wet dog shakes. Displayed is a representative video-ECoG recording of a rat
632 experiencing a focal to bilateral tonic-clonic seizure.

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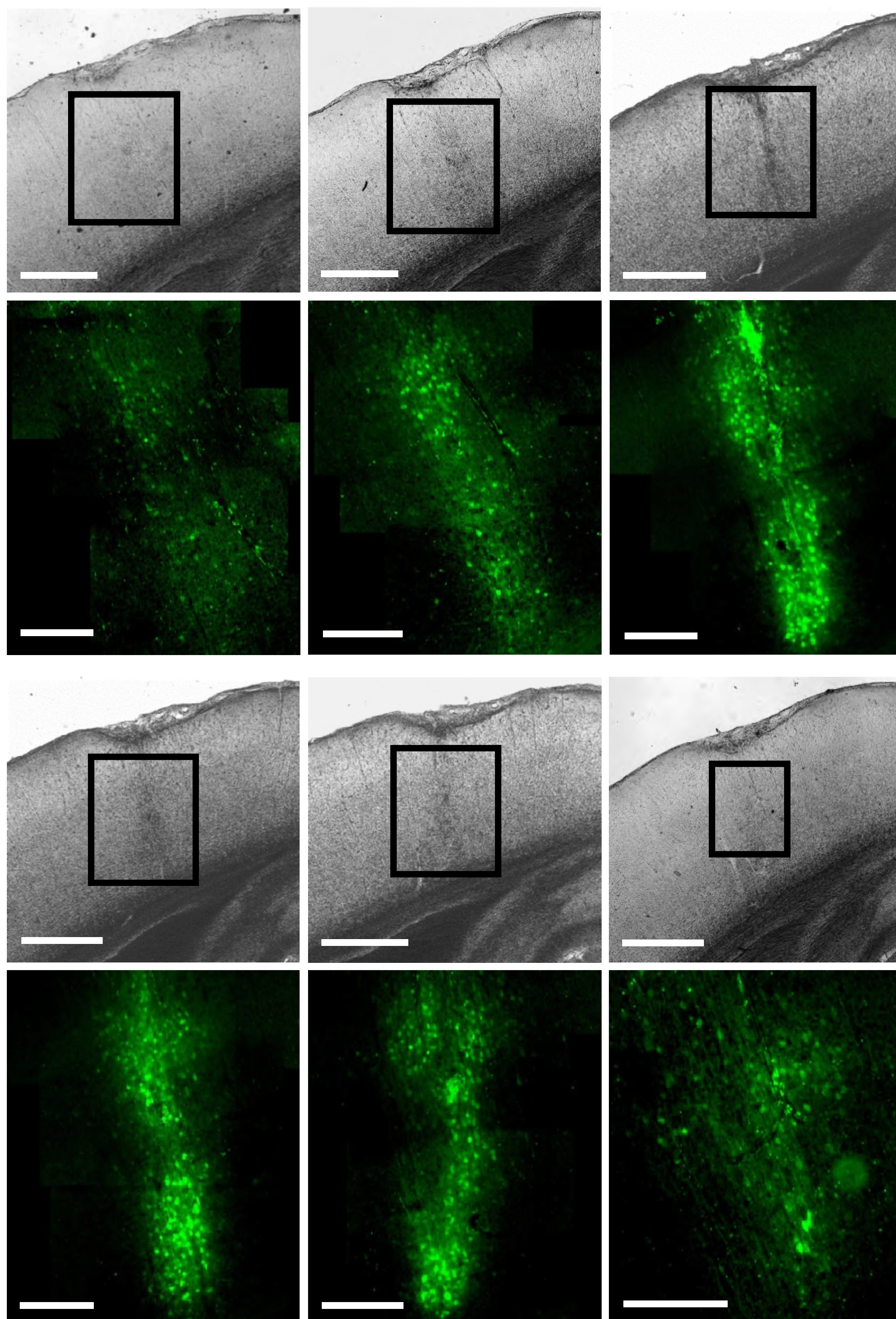

633

634 **Fig EV2: Spread of transduction with the EKC lentivector**.

635 Bright-field and fluorescence images of 6 sequential left-hemisphere visual cortex slices (70 µm

636 thick) from a rat brain injected with 1.25 µl (~3.0 x 10^6 IU) of the EKC lentivector. Slices are ordered

637 from top left (rostral) to bottom right (caudal). Scale bars represent 600 µm and 200 µm for bright-

638 field and fluorescence images, respectively.