eNeuro

http://eneuro.msubmit.net

eN-NWR-0005-14

Cerebellar directed optogenetic intervention inhibits spontaneous hippocampal seizures in a mouse model of temporal lobe epilepsy

Title:

Cerebellar directed optogenetic intervention inhibits spontaneous hippocampal seizures in a mouse model of temporal lobe epilepsy

Abbreviated Title:

Cerebellar control of temporal lobe epilepsy

Abstract

Temporal lobe epilepsy is often medically refractory and new targets for intervention are needed. We used a mouse model of temporal lobe epilepsy, on-line seizure detection, and responsive optogenetic intervention to investigate the potential for cerebellar control of spontaneous temporal lobe seizures. Cerebellar targeted intervention inhibited spontaneous temporal lobe seizures during the chronic phase of the disorder. We further report that the direction of modulation as well as the location of intervention within the cerebellum can affect the outcome of intervention. Specifically, on-demand optogenetic excitation or inhibition of parvalbumin-expressing neurons, including Purkinje cells, in the lateral or midline cerebellum results in a decrease in seizure duration. In contrast, a consistent reduction in spontaneous seizure frequency occurs uniquely with on-demand optogenetic excitation of the midline cerebellum, and was not seen with intervention directly targeting the hippocampal formation. These findings demonstrate that the cerebellum is a powerful modulator of temporal lobe epilepsy, and that intervention targeting the cerebellum as a potential therapy for epilepsy should be revisited.

Significance Statement

Epilepsy is a condition of spontaneous recurrent seizures. Current treatment options for epilepsy can have major negative side effects and for many patients fail to control seizures. We detected seizures on-line and tested a new selective intervention using a mouse model of temporal lobe epilepsy. In this form of epilepsy, seizures typically arise in the hippocampus. We report that targeting intervention to a distinct brain region, the cerebellum, significantly inhibits hippocampal seizures. This identifies the cerebellum as a potential future therapeutic target for temporal lobe epilepsy.

Introduction

Temporal lobe epilepsy (TLE) is the most common form of epilepsy in adults. Many TLE patients that are unresponsive to anti-epileptic drugs are also not good candidates for surgical resection, leaving a large population of patients with a great and currently unaddressed need for seizure control. On-demand optogenetics (Armstrong et al., 2013) provides a powerful tool to study the potential influence of networks outside the hippocampus on temporal lobe seizures and thereby identify new targets for intervention and improve understanding of network interactions in epilepsy.

Although the cerebellum is not typically considered to play a role in seizure activity in distant cortical regions such as the hippocampus, a recent study indicated that the cerebellum may exert surprisingly potent effects on normal hippocampal information processing (Rochefort et al., 2011). Given the need for new intervention targets, we asked if on-demand optogenetic intervention targeting the cerebellum could provide seizure control. Using the unilateral intrahippocampal kainate mouse model of temporal lobe epilepsy and on-line seizure detection (Armstrong et al., 2013), we found that cerebellar targeted intervention can inhibit spontaneous temporal lobe seizures during the chronic phase of the disorder, and that the direction of modulation as well as the location of intervention within the cerebellum can affect the outcome of intervention. While on-demand optogenetic excitation or inhibition of cerebellar parvalbumin (PV) expressing neurons, including Purkinje cells, results in a decrease in seizure duration, a consistent reduction in spontaneous seizure frequency occurs uniquely with on-demand optogenetic excitation of the midline cerebellum. This inhibition of seizure initiation outlasts the duration of intervention, and was not seen with intervention directly targeting the hippocampal formation.

Materials and Methods

Animals

All animal procedures were performed according to the regulation of the Animal Care Committee at the institution were the research was performed. Mice with restricted opsin expression were generated by crossing mice expressing Cre selectively in parvalbumin-expressing neurons (PV-Cre; B6;129P2-Pvalbtm1(Cre)Arbr/J; stock 008069 from Jackson labs (Hippenmeyer et al., 2005)) with either floxed-STOP channelrhodopsin (ChR2) mice (Ai32; Rosa-CAG-LSLChR2H134R-EYFP-deltaNeo generated by Hongkui Zeng, obtained from the Allen Institute and maintained by crossing with C57BL/6J mice (Jackson labs stock number 000664); Ai32D is available from Jackson labs, stock 012569 (Madisen et al., 2012)) or floxed-STOP halorhodopsin (HR) mice (Ai39; B6;129S-Gt(ROSA)26Sortm39(CAGHOP/EYFP)Hze/J; generated by Hongkui Zeng, available from Jackson labs, stock 014539 (Madisen et al., 2012), maintained by crossing with C57BL/6J mice). These crosses generated mice expressing ChR2 or HR in PV-containing cells, including cerebellar Purkinje neurons, and are referred to as PV-ChR2 or PV-HR, respectively, in the text. Obtaining mice with opsin expression restricted to Purkinje neurons was attempted by crossing B6.129-Tg(Pcp2-cre)2Mpin/J (Jackson labs stock number 004146, a mouse line with Cre inserted into the *Pcp2* (Purkinje cell protein 2) gene) with the above Cre-dependent opsin lines. However, as discussed in the Results section, we found this line to have considerable non-specific expression. The goal of mice with truly restricted expression was instead achieved by crossing a second line (B6.Cg-Tg(Pcp2-cre)3555Jdhu/J, Jackson labs stock number 010536, referred to in the text simply as "Pcp-Cre") with the above Cre-dependent opsin lines.

Male and female offspring were used for experiments. Opsin-negative littermates were used as non-expressing epileptic controls. For visualization of Cre expression, PV-Cre and Pcp-Cre mice were crossed with a tdTomato reporter strain (B6;129S6-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J; stock 007905 from Jackson labs). For some slices, a DAPI (4',6-diamidino-2-phenylindole) mounting media (Vectashield) was used.

Epilepsy induction and monitoring

To obtain chronically epileptic mice which reproduce key features of human unilateral hippocampal sclerosis (Cavalheiro et al., 1982, Bragin et al., 1999, Staba et al., 2012) we used the intrahippocampal kainate mouse model of temporal lobe epilepsy, in which kainate is focally injected unilaterally into the hippocampus of adult mice. In this model, after a period of weeks, spontaneous recurrent seizures emerge, which typically originate near the sclerotic region in the hippocampal formation (Bragin et al., 1999, Haussler et al., 2012). On or after postnatal day 46, kainic acid (50-100nL, 20mM in saline, Tocris Bioscience) was stereotaxically injected into the left dorsal hippocampus (2.0mm posterior, 1.25mm left, and 1.6mm ventral to bregma) under isoflurane anesthesia. At least two weeks later, animals were stereotaxically implanted with bipolar, twisted wire, depth electrodes (PlasticsOne) and optical fibers (0.37NA, 200μm or 400μm diameter, ThorLabs) terminated in 1.25mm ceramic ferrules (Kientec Systems, Inc.). Hippocampal optrodes (electrode+optical fiber) were implanted ipsilateral to kainate injection (posterior 2.6mm, left 1.75mm, ventral 1.4mm with respect to bregma). The hippocampal electroencephalogram (EEG) signal was derived from this single twisted wire electrode (i.e., local hippocampus reference). Cerebellar optical fibers were targeted to the cerebellar surface 5.7mm posterior to bregma, at the midline (0mm lateral), 2.3mm left, and 2.3 right, or to the

medial DCN at posterior 6.5mm, lateral 0.6mm, and ventral 2mm. A subset of animals additionally had a cerebellar bipolar electrode to record simultaneously the cerebellar EEG signal. As with hippocampal EEG recordings, the cerebellar EEG signal was derived from this single twisted wire electrode (i.e., local cerebellar reference). Implants were fixed to the skull using screws (McMaster-Carr) and dental cement (Teets Cold Curing), and the animals were allowed to recover for several days before beginning 24 hour video and EEG (vEEG) monitoring for seizures and subsequent closed-loop seizure detection and light delivery (described below). On average, animals were implanted 18.6±1.8 weeks after KA injection (range: 2 to 66 weeks) and the effect of light on seizures was examined starting 23±2.6 weeks after KA injection. There was no correlation between seizure duration reduction and time since kainate ($p=0.99$, Spearman test).

Closed-loop seizure detection and light delivery

For vEEG monitoring and on-demand optogenetic intervention, animals were connected via optical patch cords (Thorlabs, Doric Lenses; terminated in a 1.25mm ferrule which was connected to the implanted optical fiber with a ceramic split sleeve (Precision Fiber Products, Inc.)) connected through an optical commutator (Doric lenses) to a fiber-coupled diode laser (Shanghai Laser & Optics Century Co., Ltd) of an appropriate wavelength to activate the opsin expressed (ChR2: blue = 473nm; HR: amber = 589nm), and through an electrical commutator (PlasticsOne) to an analog Brownlee 410 amplifier (Armstrong et al., 2013, Krook-Magnuson et al., 2013). EEG signals were digitized by an NI USB-6221-BNC digitizer (National Instruments) sampled at 500-1000Hz, and analyzed in real-time by a PC running custom MATLAB seizure detection software.

On-line seizure detection was achieved with this custom MATLAB software, a version of which is available for download (Armstrong et al., 2013). Briefly, an experimenter identifies features of the early ictal electrographic signal to be used in triggering the real-time closed-loop seizure detection software and tunes the detector to achieve appropriate seizure detection. The experimenter selects from a number of different inclusion or exclusion criteria and adjusts thresholds to optimally detect the seizures with maximum sensitivity and specificity. The following features can be used alone or in combination to tune the seizure detection algorithm 1) signal power properties (magnitude, rate of change), 2) spike features (amplitude, width, rate, regularity), 3) signal coastline, and 4) frequency properties (changes in energy within specific frequency bands). Note that a simple spike detection algorithm would be insufficient in this model of epilepsy. As previously described, this real-time seizure detection method, once properly tuned, is able to achieve low false positive and false negative rates (Armstrong et al., 2013, Krook-Magnuson et al., 2013).

Once tuned, the custom closed-loop MATLAB software detects seizures in real-time, and triggers the delivery of laser light for 50% of events in a random fashion, allowing each animal to serve as its own internal control (in addition to opsin-negative littermate controls). Intervention consisted of 3s of pulsed light delivery. Both short light pulses (50ms on, 100ms off) and longer pulses (1000s on, 50ms off) were investigated.

Juxtacellular recordings

Craniotomies (~1mm in diameter) were placed above the left hippocampus (ipsilateral to previous kainate injection; posterior 2.6mm, lateral 1.75mm), the left cerebellum (posterior 5.7-6mm, lateral 2.3mm), and the vermis (posterior 5.7-6mm, midline) while the mouse was under

isoflurane anesthesia. Mice were then transferred to an 8-inch Styrofoam ball levitated by pressurized air and used as a spherical treadmill (Varga et al., 2012). Mice were allowed to recover for a minimum of one hour before electrophysiological recordings began. During recordings, mice were freely running or resting on the treadmill. During periods of rest, the mice adjusted their posture, had their eyes open, and occasionally groomed.

Borosilicate glass long-tapered electrodes (Sutter Instruments) were pulled using a micropipette puller (Sutter P1000). One electrode was filled with 0.5M NaCl and lowered into the hippocampus (1100-1400μm below the dura) to record the hippocampal field potential (referred to in the text as the hippocampal EEG). A second electrode (filled with 0.5M Na-acetate, 12-20 MΩ) was lowered into either the lateral cerebellum or vermis and was used to record juxtacellularly from cerebellar Purkinje neurons (identified by the presence of complex spikes). Recordings were performed using an ELC-03XS universal amplifier (NPI Electronics). Both recorded channels were low-pass filtered at 5kHz and digitized at 20kHz using NIDAQ data acquisition cards (National Instruments) and the data was recorded using custom made recording software written in MATLAB (Varga et al., 2012). The animal's movement was continuously video recorded, and video recordings were synchronized with electrophysiological recordings.

Slice electrophysiology

Whole-cell patch-clamp recordings were made from 300μm thick cerebellar slices prepared in ice-cold sucrose solution contained the following (in mM): 85 NaCl, 75 Sucrose, 2.5 KCl, 25 Glucose, 1.25 NaH_2PO_4, 4 MgCl2, 0.5 $CaCl_2$, and 24 $NaHCO_3$. Recordings were done at 36°C using artificial cerebrospinal fluid containing (in mM) 2.5 KCl, 10 Glucose, 126 NaCl,

1.25 NaH_2PO_4, 2 $MgCl_2$, 2 CaCl, 26 $NaHCO_3$; with intracellular solution containing (in mM) 126 potassium gluconate, 4 KCl, 10 HEPES, 4 Mg-ATP, 0.3 Na_2-GTP, 10 phosphocreatine, 8 biocytin; pH 7.2; 270–290 mOsm; pipette resistance: 3–4.5 MΩ. Slices were visualized with an upright microscope (Eclipse FN-1; Nikon) with infrared (750 nm) Nomarski differential interference contrast optics (Nikon 40× NIR (near infrared) Apo N2 NA0.8W WD3.5 objective with 1.5× magnification lens). Light was delivered through the epifluorscence port of the Nikon Eclipse FN-1, using a Lambda DG-4 with smart shutter and LAMDA SC controller (Shutter Instruments), and TTL input from a Digidata 1322A (Axon Instruments). After the recordings, slices were fixed in 0.1 M phosphate buffer containing 4% paraformaldehyde and 0.1% picric acid at 4°C. Streptavidin conjugated to Alexa-594 was used to detect biocytin and visualize recorded cells. Large cells in the Purkinje cell layer were targeted for recordings, and all cells included in the analysis were recovered for post-hoc analysis and showed Purkinje cell morphology.

Statistical analysis

Seizure duration after the time of the trigger and time to next seizure were analyzed off-line by reviewers blinded to the light condition and genotype of the animal. On average, 100±1 events were analyzed per animal per condition. Comparisons between light and no light conditions for post-detection seizure duration and time to next seizure were compared in each animal using two-sample Kolmogorov-Smirnov and two-tailed Mann-Whitney tests. Effects on post-detection seizure duration and time to next seizure (light versus no light) were examined at the group level using Wilcoxon Signed Ranks Test. Comparison between opsin-expressing animals and opsin-negative littermates for percent post-detection seizure duration reduction with

light, and for percent increase in median time to next seizure with light, was done with a two-tailed Mann-Whitney test. Comparison of percent duration reduction and comparisons of increases in time to next seizure between conditions (Factor A: light location; Factor B: opsin expression) was made using a two-way ANOVA. Data recorded from head-fixed animals was analyzed using custom-written routines in MATLAB using the Signal Processing Toolbox. The juxtacellular recorded signal was high-pass filtered at 400Hz, and single unit spikes were detected after crossing a threshold manually set by the experimenter (typically between 200 and 400 μV). Superimposing all detected spikes revealed single units in all cases. The firing rate was determined in 100ms long windows throughout the recording, and firing rates during ictal and interictal periods were compared using Mann-Whitney tests.

Values presented are mean ± standard error (s.e.m.). A p value <0.05 was considered significant. Statistical analysis was done using Microsoft Excel 2007, OriginPro 8 and 9, MATLAB, and Google documents. To estimate the power (**Statistical Table**) of the Mann-Whitney and the Kolmogorov-Smirnov tests for effects on seizure duration at the individual animal level, we used a sample size of 100 events (50% receiving intervention, similar to our actual data sets), a desired detectable difference of a 20% reduction in mean post-detection seizure duration, and assumed gamma distributions for the underlying data. Estimates of the parameters for the gamma distributions for seizure durations of events receiving light and not receiving light were based on a data set showing approximately a 20% reduction in duration with intervention. Shape (k) was estimated as the mean squared divided by the variance, and spread (θ) was estimated as variance divided by the mean. The power was then estimated by simulation, using 10,000 randomly generated data sets with the desired sample size and distribution. The power for Mann-Whitney and Kolmogorov-Smirnov tests for time to next

seizure at the individual animal level, as well as changes in firing for juxtacellular recordings, was estimated in a similar manner. Power values for two-way ANOVAs are post-hoc values, using the actual data sets tested, and should be interpreted as such. For those with a low power (e.g., due to little or no underlying difference), the power based on a sample size of 200 animals is also included in the **Statistical Table** for reference. Power for group level statistics for Mann-Whitney and Wilcoxon tests were estimated via simulation, using 10,000 randomly generated data sets using Gaussian distributions, a desired detectable difference of 30%, our typical group sample size of nine, and a typical standard deviation for these data sets (20%).

Results

Lateral cerebellar intervention in PV-ChR2 animals

In order to gain optogenetic control over cerebellar neurons, animals expressing Cre in parvalbumin-expressing neurons ("PV-Cre"), including cerebellar Purkinje cells (**Fig. 1a**), were crossed with mice expressing the excitatory opsin channelrhodopsin (ChR2) (Boyden et al., 2005, Madisen et al., 2012) in a Cre-dependent fashion (Madisen et al., 2012), as detailed in the **Methods** section above. Whole-cell patch clamp recordings from cerebellar slices from PV-ChR2 mice revealed strong light-induced excitatory currents (1,580 ±140pA, n=14 Purkinje cells), which were capable of driving Purkinje cells to fire action potentials (**Fig. 1b**). No currents or depolarization were induced in cells recorded from opsin-negative animals (**Fig. 1b**).

To examine the effect of cerebellar-directed on-demand optogenetic intervention on temporal lobe seizures, the unilateral intrahippocampal kainate mouse model of temporal lobe epilepsy was used. During the chronic phase of the disorder, spontaneous seizures were recorded from the hippocampus, and custom-designed software provided on-line seizure detection and closed-loop optogenetic intervention (Armstrong et al., 2013, Krook-Magnuson et al., 2013) (**Fig. 1c,d**).

Delivery of 3s of short pulses of light (473nm; 50ms on, 100ms off) to the lateral cerebellar cortex (lobulus simplex) ipsilateral or contralateral to the site of prior intrahippocampal kainate injection produced a significant reduction in seizure duration (ipsilateral: 33±9% seizure duration reduction, n=12 animals; **Fig. 1e & f**; contralateral: 30±15% reduction; n=6 animals; **Fig. 1g**; ipsilateral versus contralateral: $p=0.12$; opsin-expressing versus opsin-negative littermate controls: $p<0.05$; interaction: $p=0.21$; two-way ANOVA [a], subscript

refers to line in the Statistical Table; **Fig. 1i**). There was no significant change in time to next seizure with light application for either light location (ipsilateral: 18±10% increase in median time to next seizure; contralateral: 24±13% increase; opsin expressing versus opsin negative: p=0.36; ipsilateral versus contralateral: p=0.21; interaction: p=0.45; two-way ANOVA [b]), indicating the absence of a rebound effect, or a long-lasting inhibitory effect on seizures with lateral cerebellar light delivery.

We next examined whether the frequency of light delivery had an effect on seizure control by using 3s of pulsed light with longer pulses of light ("long": 1000ms on, 50ms off, rather than the "short" generally used: 50ms on, 100ms off). This intervention also inhibited seizures (ipsilateral: 32±10% duration reduction; contralateral: 35±10% duration reduction; ipsilateral versus contralateral: p=0.14; opsin-expressing versus opsin-negative littermate controls: $p<0.05$; interaction: p=0.27; two-way ANOVA [c]; **Fig. 1i**). Indeed, long pulses were as efficacious at inhibiting seizure duration as short pulses (32±10% versus 33±9% and 35±10% versus 30±15%, as discussed above; location: p=0.98; pulse length: p=0.87; interaction: p=0.78; two-way ANOVA [d]), indicating that the frequency of light pulses is not a critical factor for inhibiting seizure duration.

Taken together, these data indicate that inhibition of seizure duration is achievable by activating PV-expressing neurons, including Purkinje cells, of either lateral cerebellar hemisphere.

Hippocampal intervention in PV-ChR2 animals

It has been reported that on-demand activation of hippocampal PV-expressing interneurons can inhibit seizure duration (Krook-Magnuson et al., 2013). We therefore also examined hippocampal directed intervention in order to directly compare cerebellar to hippocampal intervention. Similar levels of reduction of seizure duration were achieved with these two intervention strategies (3s of short pulses, hippocampal intervention: 35±5% duration reduction, $p<0.001$, Wilcoxon test [i], n=14 animals; versus opsin-negative controls $p<0.001$, Mann-Whitney [j]; hippocampal (35±5%) vs lateral cerebellar (32±7%) intervention: $p=0.75$, Mann-Whitney [j]). Intervention targeting the hippocampus with 3s of long light pulses also reduced seizure duration (50±9% duration reduction, $p<0.05$ Wilcoxon test [i]). Hippocampal directed intervention did not affect time to next seizure (short pulses: $p=0.19$, Wilcoxon [i]; long pulses: $p=0.19$, Wilcoxon test [i]), matching previous reports (Krook-Magnuson et al., 2013).

Lateral cerebellar intervention in PV-HR animals

We next asked if on-demand cerebellar intervention required activation of Purkinje cells per se. That is, can disruption of cerebellar activity in either direction inhibit temporal lobe seizures, or is excitation required? Could inhibition of Purkinje cells actually worsen seizures? To address this, we crossed PV-Cre animals with mice expressing the inhibitory opsin halorhodopsin (HR) (Gradinaru et al., 2010, Madisen et al., 2012) in a Cre-dependent manner (Madisen et al., 2012). Light application to cerebellar slices from PV-HR animals produced robust inhibitory currents in recorded Purkinje cells (1090±100pA, n=8 Purkinje cells). This inhibition was sufficient to inhibit action potential firing induced by depolarizing current

injection (**Fig 2a**). No current or change in membrane potential was induced by light delivery to slices from opsin-negative animals (n=9 Purkinje cells).

We then tested the effect of on-demand light delivery *in vivo* on spontaneous seizures, as previously done with PV-ChR2 animals. Surprisingly, 3s of on-demand cerebellar light (589nm) delivery in PV-HR animals also reduced seizure duration. This was true for both short light pulses (ipsilateral cerebellum: 32±13% duration reduction; contralateral cerebellum: 33±11% duration reduction; opsin expressing versus opsin negative: $p<0.05$; location: $p=0.90$; interaction: $p=0.87$, two-way ANOVA [k]) and long light pulses (ipsilateral cerebellum: 37±12% duration reduction; contralateral cerebellum: 44±12% duration reduction; opsin expressing versus opsin negative: $p<0.01$; location: $p=0.45$; interaction: $p=0.77$, two-way ANOVA [l]). We next asked if there was an effect on time to next seizure with lateral cerebellar targeted intervention in PV-HR animals. Similar to intervention in PV-ChR2 animals, short pulses of light targeting the lateral cerebellum had no effect on time to next seizure (ipsilateral: $p=1$, Wilcoxon [i]; contralateral: $p=0.64$, Wilcoxon [i]). These data indicate that inhibition of seizure duration is achievable with long or short pulses targeting either lateral cerebellar hemisphere in PV-HR animals. Therefore, for cerebellar directed intervention, the direction of modulation is not a critical factor for inhibition of seizure duration.

Hippocampal intervention in PV-HR animals

Given these findings with lateral cerebellar intervention, we next asked if the same would hold for hippocampal directed intervention. While PV-expressing neurons in the cerebellum include the large projection neurons of the cerebellar cortex (i.e., Purkinje cells), in the

hippocampus PV is not expressed in principal cells. Therefore, inhibition of PV expressing neurons in the two locations have different effects on the respective networks, and may have different effects on seizure duration. Indeed, we found that on-demand optogenetic inhibition of PV neurons in the hippocampus did not affect seizure duration, regardless of pulse duration (opsin-expressing versus opsin negative, short pulses: p=0.73, Mann-Whitney [j]; long pulses: p=0.70, Mann-Whitney [j]; **Fig. 2f**). There was also no effect on time to next seizure (light versus no light, short pulses: p=0.50, Wilcoxon [i]; light versus no light, long pulses: p=1; Wilcoxon [i]). Note that intervention was of limited duration (3s of pulsed light). This indicates that whether the direction of modulation (i.e., excitation or inhibition) is a critical factor in inhibiting seizure duration depends on the underlying circuitry (e.g., alteration of local inhibitory neurons versus altering activity of inhibitory output neurons at a distant site), with inhibition of seizure duration in PV-HR animals occurring with cerebellar, but not hippocampal, directed intervention.

Intervention directed to the midline cerebellum

While interventions in which light was delivered to the hippocampus or lateral cerebellum did not consistently affect the frequency of seizures, when light (3s, short pulses) was instead delivered to the midline cerebellum (targeting the vermis) in PV-ChR2 animals, in addition to a reduction in seizure duration (39±14% duration reduction p<0.05, Wilcoxon [i]; n=7 animals; **Fig. 3a**), there was a significant and consistent prolongation of the interseizure interval (175±64% increase in time to next seizure; p<0.05, Wilcoxon [i]; at the individual animal level, significant in 6 of 7 animals, p<0.05, Mann-Whitney [e]), a therapeutically desirable outcome. This inhibition of seizure generation outlasted the duration of the applied light (**Fig. 3b**), and for

short pulses was unique to vermal intervention (**Fig. 3c**). Vermal directed intervention with long pulses also increased time to next seizure (363±92% increase; $p<0.05$ Wilcoxon [i]; at the individual animal level, significant in 6 of 7 animals, $p<0.05$, Mann-Whitney [e]).

We next asked whether the effect on seizure frequency required excitation of PV cells per se, or whether disruption of vermal cerebellar activity alone could alter seizure frequency, similar to effects on seizure duration. We therefore examined the effect of midline cerebellar light delivery in PV-HR animals. Light delivery (589nm) produced no significant effect on time to next seizure, regardless of pulse duration (percent change time to next seizure, short pulses: -10±7%, $p=0.15$, Wilcoxon [i], n=8 animals; **Fig. 3c;** long pulses: -2±15%; $p=0.64$, Wilcoxon [i]). These findings indicate that a disruption of cerebellar activity alone is not sufficient to cause a decrease in seizure frequency. Rather, the direction of modulation (excitation), as well as the location of light delivery (vermis), are critical to achieve a consistent reduction in seizure frequency (**Fig. 3c**).

Purkinje neurons project to the deep cerebellar nuclei (DCN). In separate experiments, we therefore targeted light delivery (3s, short pulses) to the axon terminals of Purkinje neurons in the medial DCN (fastigial nucleus, which receives input from the medial cerebellar cortex). Targeting light delivery to the DCN in PV-ChR2 animals decreased seizure duration (27±16.1% reduction) and, importantly, also increased time to next seizure (361±110% increase; $p<0.01$ in 5 of 5 animals; Mann-Whitney [e]; versus midline cerebellar cortex: $p=0.19$, Mann-Whitney [j]).

Selective excitation of Purkinje cells

Purkinje cells not only express PV (and thus the opsins in PV-ChR2 and PV-HR animals) but also are the sole output of the cerebellar cortex. Therefore, any intervention targeting the cerebellar cortex will ultimately have its effect through an alteration of Purkinje cell firing. Still, other neurons in the cerebellum expresses PV, and we therefore sought to replicate our key findings in a mouse line expressing opsins selectively in Purkinje cells. Therefore, we tested the effect of cerebellar intervention using B6.129-Tg(Pcp2-cre)2Mpin/J –ChR2 animals. While we replicated an effect on seizure duration (percent duration reduction ipsilateral: 91%, $p<0.0000001$, n=1 animal, Kolmogorov-Smirnov [h] & Mann-Whitney [g]; contralateral: 82%, $p<0.0000001$, n=1 animal, Kolmogorov-Smirnov [h] & Mann-Whitney [g]) and time to next seizure with vermal intervention (percent increase time to next vermis: 66%, $p<0.001$, n=1 animal, Mann-Whitney [e]; ipsilateral: $p=0.37$, Mann-Whitney [e]; contralateral: $p=0.34$, Mann-Whitney [e]), we found that expression was not selective. Crossing B6.129-Tg(Pcp2-cre)2Mpin/J with a tdTomato reporter strain revealed Cre expression in Purkinje cells, as well as in cerebellar molecular layer interneurons, deep cerebellar neurons, scattered cerebral cortex neurons, and even some hippocampal granule cells (data not shown).

We therefore obtained a second line (B6.Cg-Tg(Pcp2-cre)3555Jdhu/J – referred to here simply as "Pcp-Cre"), which we found to be highly specific (**Fig 4a**). We performed whole-cell patch clamp recordings from Purkinje neurons from cerebellar slices from Pcp-ChR2 animals. Light delivery produced strong excitatory currents (2,040±140pA, n=6 Purkinje cells), capable of inducing Purkinje cell firing (**Fig. 4b**). We then tested *in vivo* on-demand optogenetic intervention in Pcp-ChR2 animals. As in PV-ChR2 animals, cerebellar light delivery significantly reduced seizure duration (percent duration reduction ipsilateral: 46±15%, n=4 animals, $p<0.05$ in 4 of 4, Kolmogorov-Smirnov [h] & 3 of 4, Mann-Whitney [g]; contralateral:

49±9%, n=4 animals, $p<0.05$ in 4 of 4, Kolmogorov-Smirnov [h] & Mann-Whitney [g]; vermis: 42±9%, n=6 animals; $p<0.05$ in 6 of 6, Kolmogorov-Smirnov [h] & Mann-Whitney [g] **Fig. 4c,e**). As opsins are not expressed in the hippocampus in Pcp-ChR2 animals, we predicted no change in seizure duration with hippocampal light delivery. Indeed, in Pcp-ChR2 animals hippocampal light delivery produced no significant change in seizure duration ($p=0.15$, Wilcoxon [i]; **Fig. 4d**; cerebellar versus hippocampal intervention, $p<0.05$, Mann-Whitney [j]). These data indicate that direct optogenetic modulation of cerebellar Purkinje cells alone is sufficient to reduce spontaneous temporal lobe seizure duration.

We next examined the effect on seizure frequency of midline cerebellar light delivery in animals with Purkinje cell selective ChR2 expression. Intervention targeting the vermis in Pcp-ChR2 animals was able to significantly increase the time to next seizure (long pulses: $p<0.05$ in 4 of 5 animals, Kolmogorov-Smirnov [f] & Mann-Whitney [e]; **Fig. 4f**; short pulses: $p<0.05$ in 2 of 6 animals, Kolmogorov-Smirnov [f] & Mann-Whitney [e]). These data therefore support that selective optogenetic excitation of cerebellar Purkinje neurons is capable of not only reducing seizure duration, but also of inhibiting seizure induction (i.e., increasing time to next seizure).

Bidirectional functional connectivity

The above data demonstrate that cerebellar activity can modulate hippocampal activity, in that cerebellar directed intervention can inhibit hippocampally recorded temporal lobe seizures. We additionally find that temporal lobe seizures can modulate cerebellar activity: in 17 of 24 animals, temporal lobe seizures produced a change in cerebellar EEG (**Fig. 5a, b**). In a subset of these animals, we recorded from Purkinje neurons (identified by the presence of complex spikes)

using the juxtacellular method from awake, head-fixed animals (Varga et al., 2012). Temporal lobe seizures significantly modulated ($p<0.01$, Mann-Whitney ^{m}) the firing of 7 of 21 recorded Purkinje cells (**Fig. 5c, d**). These findings indicate that not only can the cerebellum affect temporal lobe seizures, but that temporal lobe seizures can also affect cerebellar activity – that is, there is a bidirectional functional connectivity.

Discussion

We demonstrate that cerebellar directed intervention can inhibit temporal lobe seizures, that temporal lobe seizures can modulate cerebellar activity, and that excitation of midline cerebellar Purkinje neurons produces a decrease in seizure frequency (increase in time to next seizure). As this inhibition of seizure frequency was seen with only certain interventions, it reveals a fundamentally novel dissociation of an inhibition of on-going seizure events and an inhibition of seizure generation. Additionally, our findings are critically important from a clinical perspective, as they demonstrate that interventions targeting the cerebellum as a potential therapy for medically intractable epilepsies should be revisited.

While the cerebellum is presently often considered to be outside the realm of seizures and epilepsy, cerebellar tumors can result in seizures (Norden and Blumenfeld, 2002) and there are changes in cerebellar blood flow and unit activity during seizures (Gartside, 1979, Miller, 1992, Kandel and Buzsaki, 1993, Blumenfeld et al., 2009). Indeed, there was considerable interest in the cerebellum in seizures in the 70s, but results of studies examining electrical stimulation of the cerebellum on seizures produced mixed results, and interest waned (Miller, 1992, Fountas et al., 2010). Results of these early studies may have been mixed as they relied on electrical stimulation, which is wrought with lack of specificity -- a hurdle which can be overcome with optogenetics.

We report that on-demand optogenetic intervention targeting the cerebellum – a region outside of the temporal lobe -- inhibited spontaneous temporal lobe seizures, and critically, that the site and direction of cerebellar intervention mattered, with consistent inhibition of seizure frequency only occurring with midline cerebellar excitation. We report in this study that there

was no increase in time to next seizure with optogenetic intervention targeting PV-expressing neurons in the hippocampus, and it has been previously reported that 30s of direct on-demand optogenetic inhibition of excitatory cells in the hippocampus also fails to increase time to next seizure (Krook-Magnuson et al., 2013). This highlights the unique and powerful role of the cerebellum in inhibiting temporal lobe seizures.

Although these findings could not be predicted from earlier on-demand optogenetic studies (Krook-Magnuson et al., 2013, Paz et al., 2013), they are generally in line with studies indicating that the cerebellum has powerful effects on upper brain centers beyond classical motor control in both health and disease (Ivry and Baldo, 1992, Buckner, 2013), including recent work demonstrating a role in hippocampal spatial navigation (Rochefort et al., 2011) and disturbances in cognitive functions related to autism (Tsai et al., 2012). As in these recent studies, the precise circuits mediating the cerebellar effects on temporal lobe seizures are not yet understood. Although plausible pathways for the robust, cerebellar-hippocampal seizure regulatory effects described here may involve the ventral lateral thalamus (Haroian et al., 1981, Angaut et al., 1985) (in the case of the lateral cerebellar modulation of seizure duration) and the broader modulatory actions associated with the reticular formation (Elisevich et al., 1985, Verveer et al., 1997) (in the case of prolongation of the time to next seizure following intervention targeting the vermis), it should be noted that the cerebellum has connections to numerous other brain areas that could also be involved, including the hypothalamus (Zhu et al., 2006, Soussi et al., 2014), superior colliculus (Gale et al., 1993, Katoh and Benedek, 2003), and septum (Harper and Heath, 1973, Paul et al., 1973).

Given the prevalence of TLE, the major negative side effects of current treatment options, and the high percentage of patients with uncontrolled seizures, the need for new

1 therapeutic approaches is clear, and our data provide strong support for reevaluation of cerebellar
2 targeted intervention to control temporal lobe seizures.

References

Angaut P, Cicirata F, Serapide F (1985) Topographic organization of the cerebellothalamic projections in the rat. An autoradiographic study. Neuroscience 15:389-401.

Armstrong C, Krook-Magnuson E, Oijala M, Soltesz I (2013) Closed-loop optogenetic intervention in mice. Nature protocols 8:1475-1493.

Blumenfeld H, Varghese GI, Purcaro MJ, Motelow JE, Enev M, McNally KA, Levin AR, Hirsch LJ, Tikofsky R, Zubal IG, Paige AL, Spencer SS (2009) Cortical and subcortical networks in human secondarily generalized tonic-clonic seizures. Brain 132:999-1012.

Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K (2005) Millisecond-timescale, genetically targeted optical control of neural activity. Nature neuroscience 8:1263-1268.

Bragin A, Engel J, Jr., Wilson CL, Vizentin E, Mathern GW (1999) Electrophysiologic analysis of a chronic seizure model after unilateral hippocampal KA injection. Epilepsia 40:1210-1221.

Buckner RL (2013) The cerebellum and cognitive function: 25 years of insight from anatomy and neuroimaging. Neuron 80:807-815.

Cavalheiro EA, Riche DA, Le Gal La Salle G (1982) Long-term effects of intrahippocampal kainic acid injection in rats: a method for inducing spontaneous recurrent seizures. Electroencephalogr Clin Neurophysiol 53:581-589.

Elisevich KV, Hrycyshyn AW, Flumerfelt BA (1985) Cerebellar, medullary and spinal afferent connections of the paramedian reticular nucleus in the cat. Brain Res 332:267-282.

Fountas KN, Kapsalaki E, Hadjigeorgiou G (2010) Cerebellar stimulation in the management of medically intractable epilepsy: a systematic and critical review. Neurosurg Focus 29:E8.

Gale K, Pazos A, Maggio R, Japikse K, Pritchard P (1993) Blockade of GABA receptors in superior colliculus protects against focally evoked limbic motor seizures. Brain Res 603:279-283.

Gartside IB (1979) The activity of cerebellar neurones during epileptiform activity induced by penicillin in the cerebral cortex of the rat. Electroencephalogr Clin Neurophysiol 46:189-196.

Gradinaru V, Zhang F, Ramakrishnan C, Mattis J, Prakash R, Diester I, Goshen I, Thompson KR, Deisseroth K (2010) Molecular and cellular approaches for diversifying and extending optogenetics. Cell 141:154-165.

Haroian AJ, Massopust LC, Young PA (1981) Cerebellothalamic projections in the rat: an autoradiographic and degeneration study. J Comp Neurol 197:217-236.

Harper JW, Heath RG (1973) Anatomic connections of the fastigial nucleus to the rostral forebrain in the cat. Exp Neurol 39:285-292.

Haussler U, Bielefeld L, Froriep UP, Wolfart J, Haas CA (2012) Septotemporal position in the hippocampal formation determines epileptic and neurogenic activity in temporal lobe epilepsy. Cereb Cortex 22:26-36.

Hippenmeyer S, Vrieseling E, Sigrist M, Portmann T, Laengle C, Ladle DR, Arber S (2005) A developmental switch in the response of DRG neurons to ETS transcription factor signaling. PLoS Biol 3:e159.

Ivry RB, Baldo JV (1992) Is the cerebellum involved in learning and cognition? Curr Opin Neurobiol 2:212-216.

Kandel A, Buzsaki G (1993) Cerebellar neuronal activity correlates with spike and wave EEG patterns in the rat. Epilepsy Res 16:1-9.

Katoh YY, Benedek G (2003) Cerebellar fastigial neurons send bifurcating axons to both the left and right superior colliculus in cats. Brain Res 970:246-249.

Krook-Magnuson E, Armstrong C, Oijala M, Soltesz I (2013) On-demand optogenetic control of spontaneous seizures in temporal lobe epilepsy. Nature communications 4:1376.

Madisen L, Mao T, Koch H, Zhuo JM, Berenyi A, Fujisawa S, Hsu YW, Garcia AJ, 3rd, Gu X, Zanella S, Kidney J, Gu H, Mao Y, Hooks BM, Boyden ES, Buzsaki G, Ramirez JM, Jones AR, Svoboda K, Han X, Turner EE, Zeng H (2012) A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. Nature neuroscience 15:793-802.

Miller JW (1992) The role of mesencephalic and thalamic arousal systems in experimental seizures. Prog Neurobiol 39:155-178.

Norden AD, Blumenfeld H (2002) The role of subcortical structures in human epilepsy. Epilepsy Behav 3:219-231.

Paul SM, Heath RG, Ellison JP (1973) Histochemical demonstration of a direct pathway from the fastigial nucleus to the septal region. Exp Neurol 40:798-805.

Paz JT, Davidson TJ, Frechette ES, Delord B, Parada I, Peng K, Deisseroth K, Huguenard JR (2013) Closed-loop optogenetic control of thalamus as a tool for interrupting seizures after cortical injury. Nature neuroscience 16:64-70.

Rochefort C, Arabo A, Andre M, Poucet B, Save E, Rondi-Reig L (2011) Cerebellum shapes hippocampal spatial code. Science 334:385-389.

Soussi R, Boulland JL, Bassot E, Bras H, Coulon P, Chaudhry FA, Storm-Mathisen J, Ferhat L, Esclapez M (2014) Reorganization of supramammillary-hippocampal pathways in the rat

pilocarpine model of temporal lobe epilepsy: evidence for axon terminal sprouting. Brain structure & function.

Staba RJ, Ekstrom AD, Suthana NA, Burggren A, Fried I, Engel J, Jr., Bookheimer SY (2012) Gray matter loss correlates with mesial temporal lobe neuronal hyperexcitability inside the human seizure-onset zone. Epilepsia 53:25-34.

Tsai PT, Hull C, Chu Y, Greene-Colozzi E, Sadowski AR, Leech JM, Steinberg J, Crawley JN, Regehr WG, Sahin M (2012) Autistic-like behaviour and cerebellar dysfunction in Purkinje cell Tsc1 mutant mice. Nature 488:647-651.

Varga C, Golshani P, Soltesz I (2012) Frequency-invariant temporal ordering of interneuronal discharges during hippocampal oscillations in awake mice. Proc Natl Acad Sci U S A 109:E2726-2734.

Verveer C, Hawkins RK, Ruigrok TJ, De Zeeuw CI (1997) Ultrastructural study of the GABAergic and cerebellar input to the nucleus reticularis tegmenti pontis. Brain Res 766:289-296.

Zhu JN, Yung WH, Kwok-Chong Chow B, Chan YS, Wang JJ (2006) The cerebellar-hypothalamic circuits: potential pathways underlying cerebellar involvement in somatic-visceral integration. Brain Res Rev 52:93-106.

Figure Legends

Figure 1. Lateral cerebellar intervention in PV-ChR2 mice.

(**a**) In PV-Cre animals, Cre is expressed in cerebellar neurons, including Purkinje neurons. Layers indicated: M: molecular, P: Purkinje, G: granular. (**b**) Blue light produces action potentials in Purkinje neurons in PV-ChR2 animals (top) but not in opsin negative animals (bottom trace). (**c**) On-line detection of spontaneous temporal lobe seizures allows on-demand light delivery during the chronic phase of the disorder. (**d**) The EEG is recorded from the hippocampus ipsilateral to previous kainate injection (KA), and light is delivered to the cerebellum. (**e**) Example detected seizures not receiving light (top trace) and receiving cerebellar directed light-intervention (bottom). Gray bars denote seizure detection. Blue box denotes 3s of pulsed light delivery. In opsin-expressing PV-ChR2 animals, light delivered to the ipsilateral (**f**) or contralateral (**g**) cerebellum produces a significant reduction in seizure duration, but there is no effect of light in an opsin-negative animal (**h**). 3s of pulsed light delivery produces a significant reduction in seizure duration in opsin expressing animals with both short pulses (50ms on, 100ms off) or long pulses (1000ms on, 50ms off). Asterisks denote $p<0.05$. Scale bars: (a) 200μm; (b) 50ms, 20mV; (e) 5s, 0.05mV.

Figure 2. On-demand optogenetic intervention in PV-HR animals.

(**a**) Crossing PV-Cre mice with mice expressing in a Cre-dependent manner the inhibitory opsin halorhodopsin (HR) produces mice expressing HR in PV-expressing neurons, including cerebellar Purkinje cells. Light delivery to cerebellar slices from PV-HR mice produces strong inhibition, capable of inhibiting Purkinje cell firing induced by direct current injected. *In vivo*

on-demand intervention in PV-HR animals directed to the cerebellar cortex ipsilateral (**b**) or contralateral (**c**) to previous hippocampal kainate injection reduced post-detection seizure duration in opsin-expressing, but not opsin negative (**d**) animals. (**e**) Both short and long light pulses were effective at stopping seizures. (**f**) In contrast, light delivery to the hippocampus in PV-HR animals did not affect seizure duration. "dur red": duration reduction. Scale bars: (a) 500ms, 20mV.

Figure 3. Optogenetic intervention targeting the midline cerebellum.

On-demand optogenetic intervention with 3s of pulsed light delivered to the midline cerebellum (vermis) in a PV-ChR2 mouse reduces seizure duration (**a**) and increases time to next seizure (**b**). Blue bars and traces: events receiving light. Gray: events not receiving light. Inset: Box and whisker plot indicating median, 25^{th} & 75^{th} percentiles, and 25^{th} & 75^{th} percentile ± 1.5 times the interquartile range. (**c**) With three seconds of short light pulses (50ms on, 100ms off), there is an increase in time to next seizure with vermal directed intervention in PV-ChR2 animals. CB: cerebellum; hipp: hippocampus; ipsilat or ipsi: ipsilateral to KA; contrlat: contralateral.

Figure 4. On-demand optogenetic intervention with Purkinje cell specific expression.

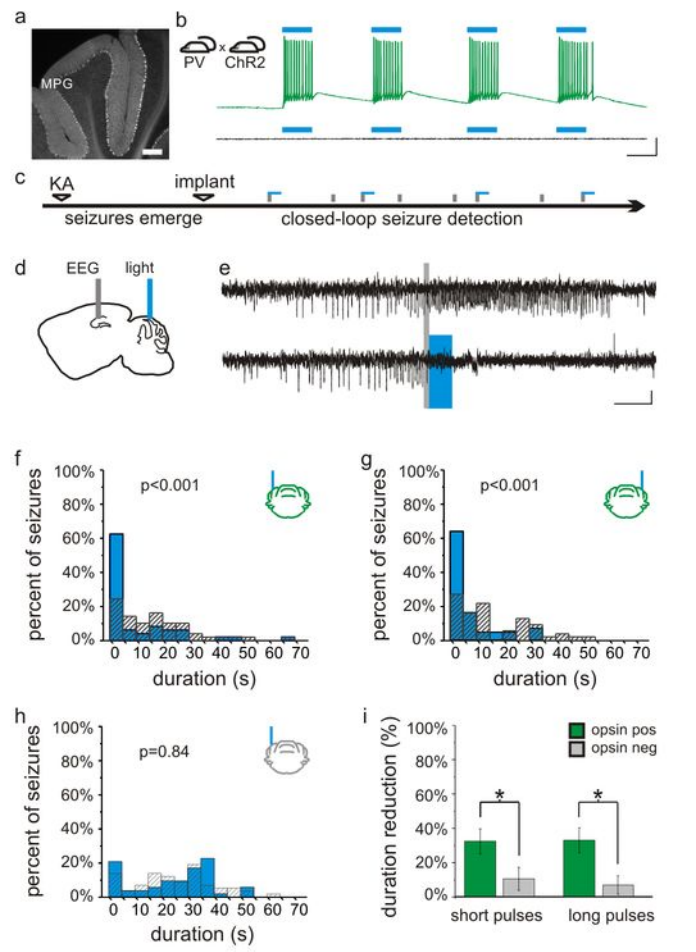
(**a**) In Pcp-Cre mice, cerebellar Cre is selectively expressed in Purkinje neurons. Cre expression (white) was visualized by crossing with a reporter strain. Bottom panel, blue: DAPI fluorescent stain. (**b**) Crossing Pcp-Cre mice with mice expressing ChR2 in a Cre-dependent manner produces mice with ChR2 expressed selectively in Purkinje neurons. Light delivery to cerebellar

slices from Pcp-ChR2 animals induces firing in Purkinje neurons. (**c**) *In vivo* on-demand optogenetic intervention targeting the lateral cerebellum produces an inhibition of seizure duration. (**d**) Light delivery to the hippocampus (which does not express opsin in Pcp-ChR2 animals) does not inhibit seizures. Light delivery to the midline cerebellum is capable of reducing seizure duration (**e**) and increasing time to next seizure (**f**). Scale bars: (a, top) 500μm; (a, bottom) 50μm; (b) 100ms, 20mV.

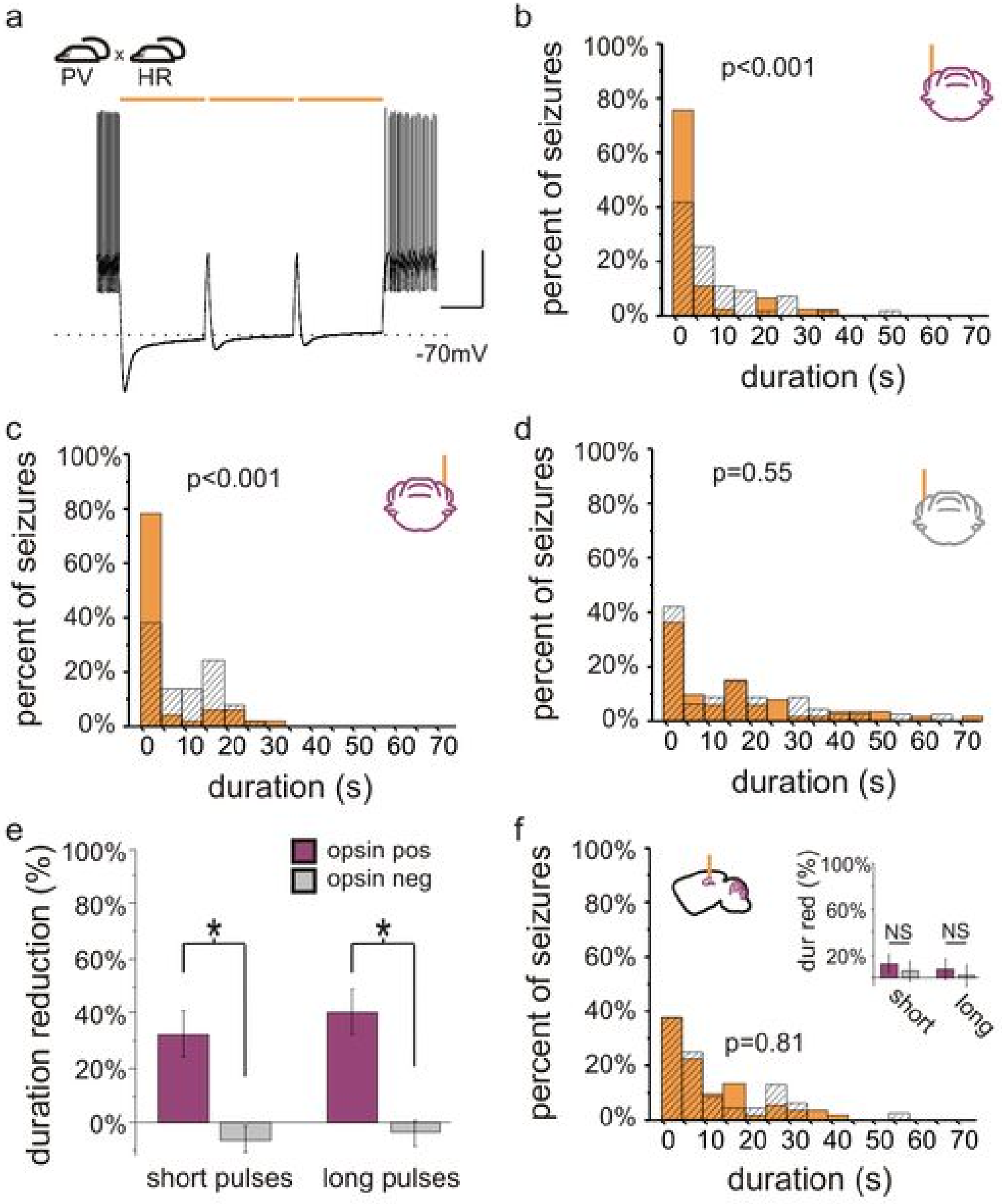
Figure 5. Temporal lobe seizures modulate cerebellar activity.

An example temporal lobe seizure recorded in the hippocampus (**a**) which produced changes in the cerebellar EEG (**b**) and in the firing rate of a juxtacellularly recorded Purkinje cell over the course of the seizure (**c**) as well as on a shorter time scale (**d**). Green trace denotes detected Purkinje cell spikes. Scale bars: 1mV (a) or 0.5mV (b), 10s (a-c); 0.5mV or 0.1kHz change in firing rate, 0.1s (d).

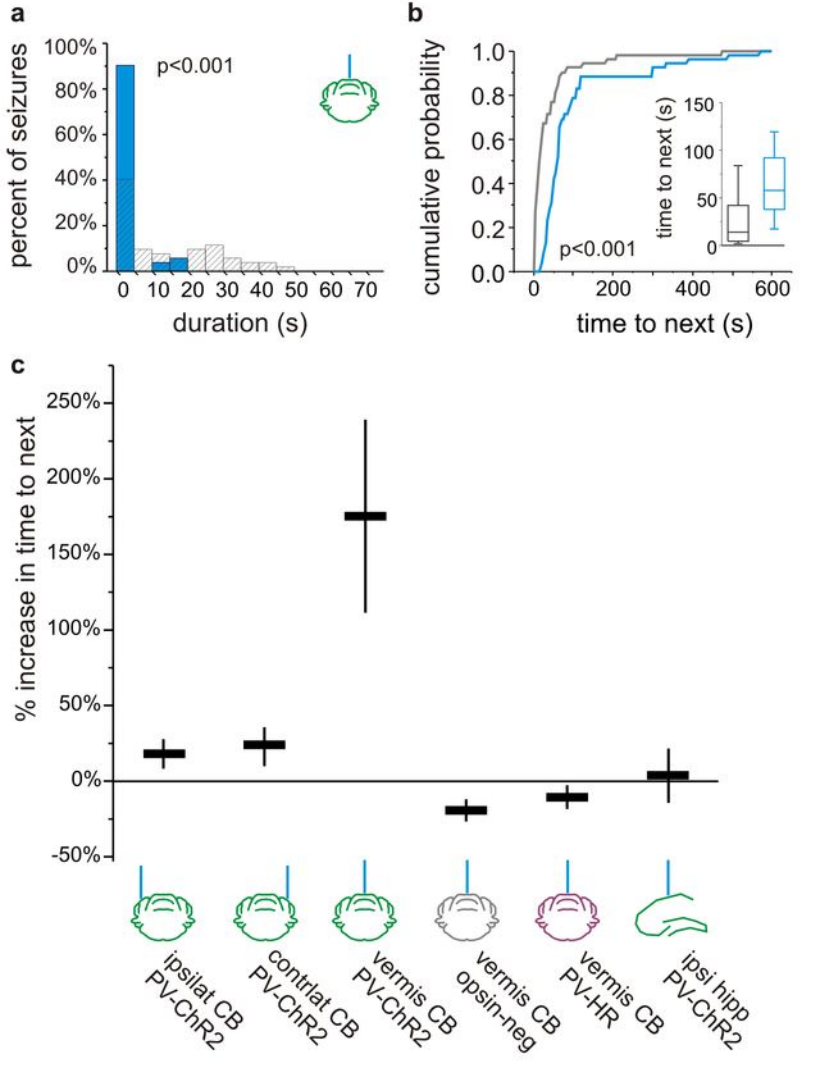
Krook-Magnuson et al Figure 1



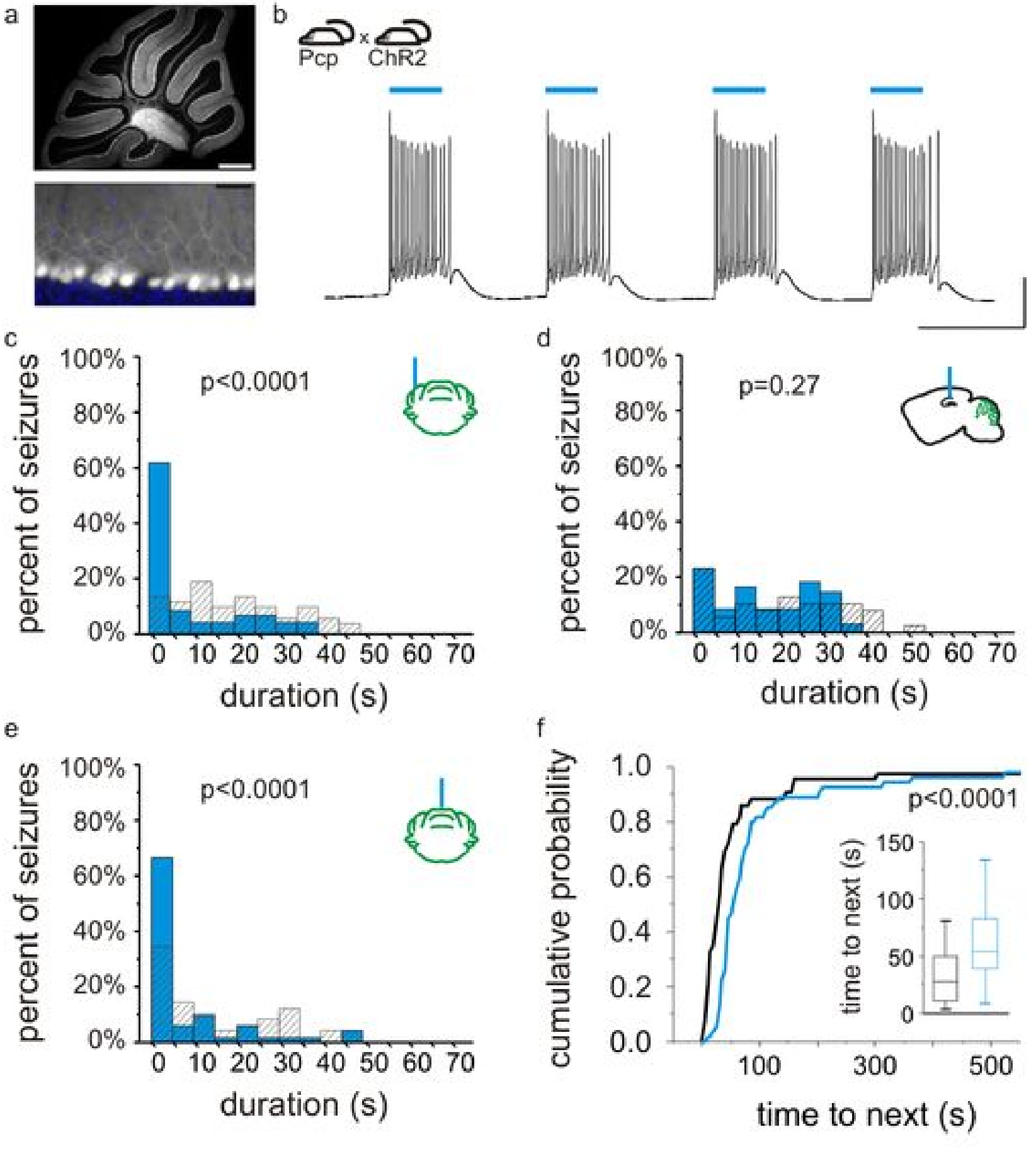
Krook-Magnuson et al Figure 2



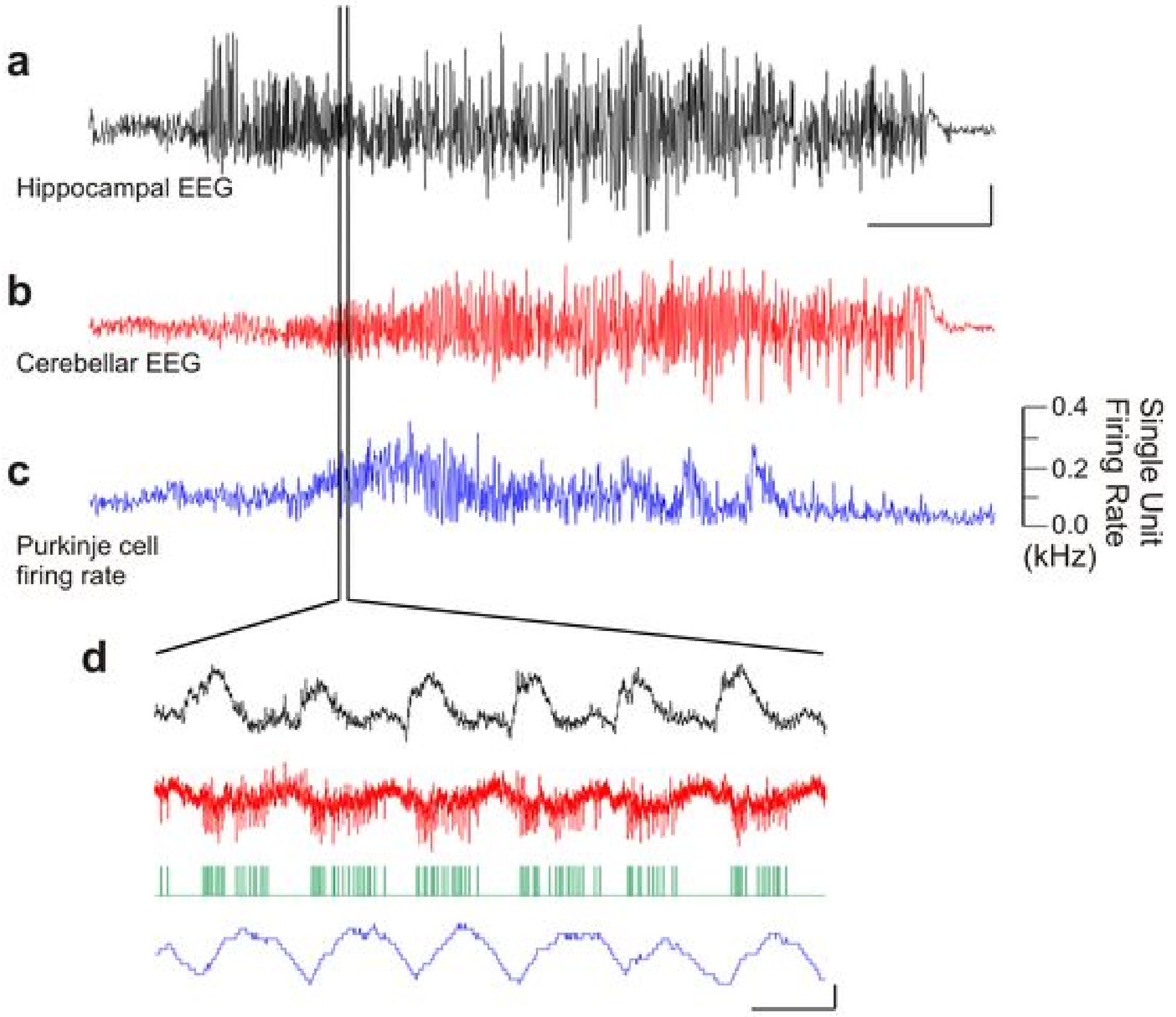
Krook-Magnuson et al. Figure 3



Krook-Magnuson et al Figure 4



Krook-Magnuson et al Figure 5



Statistical Table.

	Data Structure	**Type of Test**	Power		
a	multivariate	Two-way ANOVA	genotype	0.71	
			location	0.33	
			interaction	0.43	
b	multivariate	Two-way ANOVA	genotype	0.15	
			location	0.23	
			interaction	0.18	
c	multivariate	Two-way ANOVA	genotype	0.64	
			location	0.31	
			interaction	0.52	
d	multivariate	Two-way ANOVA			Power with n=200
			location	0.05	0.05
			pulse type	0.05	0.06
			interaction	0.07	0.10
e	normality not assumed	Mann-Whitney	0.50		
f	normality not assumed	Kolmogorov-Smirnov	0.42		
g	normality not assumed	Mann-Whitney	0.82		
h	normality not assumed	Kolmogorov-Smirnov	0.89		
i	normality not assumed	Wilcoxon	0.96		
j	normality not assumed	Mann-Whitney	0.795		
k	multivariate	Two-way ANOVA			Power with n=200
			genotype	0.72	
			location	0.05	0.07
			interaction	0.06	0.08
l	multivariate	Two-way ANOVA	genotype	0.86	
			location	0.11	
			interaction	0.07	
m	normality not assumed	Mann-Whitney	.99		