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Excitation, but not inhibition of the fastigial nucleus provides powerful control over temporal lobe seizures

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Abstract

Temporal lobe epilepsy (TLE) is the most common form of epilepsy in adults, but current treatment options provide limited efficacy, leaving as many as one third of patients with uncontrolled seizures. Recently, attention has shifted towards more closed-loop therapies for seizure control, and on-demand optogenetic modulation of the cerebellar cortex was shown to be highly effective at attenuating hippocampal seizures. Intriguingly, both optogenetic excitation and inhibition of cerebellar cortical output neurons, Purkinje cells, attenuated seizures. The mechanisms by which the cerebellum impacts seizures, however, are unknown. In the present study, we targeted the immediate downstream projection of vermal Purkinje cells -- the fastigial nucleus -- in order to determine whether increases and/or decreases in fastigial output can underlie seizure cessation. Though Purkinje cell input to fastigial neurons is inhibitory, direct optogenetic inhibition of the fastigial nucleus had no effect on seizure duration. Conversely, however, fastigial excitation robustly attenuated hippocampal seizures. Seizure cessation was achieved at multiple stimulation frequencies, regardless of laterality relative to seizure focus, and even with single light pulses. Seizure inhibition was greater when selectively targeting glutamatergic fastigial neurons than when an approach that lacked cell-type specificity was used. Together, these results suggest that stimulating excitatory neurons in the fastigial nucleus may be a promising approach for therapeutic intervention in TLE.

INTRODUCTION

Temporal lobe epilepsy (TLE), a disorder characterized by spontaneous, recurrent seizures typically emerging from the hippocampus, is the most common form of epilepsy in adults, with 150,000 new diagnoses per year in the US alone (England et al., 2012). Total healthcare costs associated with epilepsy in the US overall exceed $2.7 billion (Vivas et al., 2012). Despite this high prevalence, current treatment options have limited efficacy and carry the potential for problematic side effects, leaving 30-40% of epilepsy patients with uncontrolled

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Author contributions

MLS and EKM conceived of the work, MLS and EKM designed the experiments. MLS collected and analyzed the data, with input from EKM. MLS and EKM interpreted the data for the work. MLS and EKM wrote the manuscript.

Competing interests

The authors declare no competing financial interests

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seizures (England et al., 2012). Clearly, there is an immense need to improve both current treatment options and our understanding of the seizure network in temporal lobe epilepsy.

Recent attention has shifted towards more targeted interventions such as deep brain and on-demand stimulation, which has been shown to reduce seizure duration in some patients (Fisher et al., 2010; Valentin et al., 2013; Bergey et al., 2015; Pakdaman et al., 2016). Targeting areas outside the seizure focus has shown some promise (Fisher et al., 2010; Salanova et al., 2015; Elder et al., 2019), and recent optogenetic work has renewed interest in the cerebellum as a potential therapeutic target (Krook-Magnuson et al., 2014; Kros et al., 2015a; Miterko et al., 2019). For example, on-demand optogenetic modulation of the midline cerebellar cortex, the vermis, was recently found to robustly terminate hippocampal seizures (Krook-Magnuson et al., 2014). The mechanisms by which cerebellar stimulation inhibits seizures, however, is unknown, and complications associated with targeting the cerebellar cortex may limit translational opportunities.

Vermal Purkinje cells project to and inhibit neurons in the fastigial nucleus (It , 1984). Glutamatergic fastigial neurons in turn project to further downstream targets, including the superior colliculus, thalamus, and reticular formation (Angaut & Bowsher, 1970). An immediate and important question is whether fastigial neurons can be leveraged, similar to Purkinje cells, to attenuate hippocampal seizures. Intriguingly, both on-demand excitation and inhibition of Purkinje cells was highly effective in reducing seizure duration (Krook-Magnuson et al., 2014). As both excitation or inhibition of Purkinje cells can attenuate seizures, it is possible that simply disrupting on-going activity in the cerebellum is sufficient to stop seizures. If true, then excitation or inhibition of the fastigial nucleus would also inhibit seizures. However, alternative explanations are possible. During optogenetic excitation, Purkinje cell firing increases overall during light stimulation, including robust increases during each pulse of light (Tsubota et al., 2011; Chaumont et al., 2013; Witter et al., 2013; Krook-Magnuson et al., 2014; Kruse et al., 2014; Lee et al., 2015). However, after each individual pulse of light there is a decrease in Purking cell firing (Krook-Magnuson et al., 2014; Lee et al., 2015; El-Shamayleh et al., 2017; Brown & Raman, 2018). Similarly, with optogenetic inhibition, Purkinje cell firing decreases overall during the period of pulsed light delivery, but there are brief increases in firing between pulses (Krook-Magnuson et al., 2014; Lee et al., 2015). Previous work has shown that even brief pauses in Purkinje cell firing results in increased firing of downstream deep cerebellar nuclear neurons (Gauck & Jaeger, 2000; Lee et al., 2015; Brown & Raman, 2018). Therefore, interpreting previous optogenetic targeting of the cerebellar cortex, in the context of the cerebellar nuclei, is not straightforward. Additionally, Purkinje cells project to multiple types of neurons in the nuclei, including both excitatory and inhibitory neurons (Bagnall et al., 2009; Uusisaari & Knopfel, 2012).

A major unanswered question then is what ultimately can lead to the cessation of seizures, such that we could mimic the effects through direct manipulation of the fastigial nucleus. Is seizure suppression mediated through i) merely the disruption of ongoing cerebellar activity, such that either excitation or inhibition of fastigial neurons would be sufficient to terminate seizures, ii) inhibiton of fasitigial neurons, or iii) excitatation of fastigial neurons? To test

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these possibilities, we implemented direct inhibition and excitation of the fastigial nucleus during hippocampal seizures in a mouse model of TLE.

We find that on-demand excitation of the fastigial nucleus is required for hippocampal seizure attenuation. Direct on-demand inhibition of the fastigial nucleus, tested across multiple different stimulation frequencies, has no effect on the duration of hippocampal seizures. In contrast, robust attenuation of hippocampal seizures is observed with on-demand excitation of the fastigial nucleus. Stimulation is highly effective regardless of the side targeted relative to the seizure focus or the stimulation frequency tested. Even a single pulse of light is able to truncate seizures. We additionally find that excitation of glutamatergic nuclear neurons selectively provides greater seizure inhibition than non-cell-type-selective excitation of the fastigial nucleus. While additional circuit elements may be able to also provide some degree of seizure control, these results clearly indicate that increases in excitatory fastigial output, rather than decreases, provide strong inhibition of seizures. These results further suggest that the fastigial nucleus is a potential target for therapeutic neuromodulation in TLE.

MATERIALS AND METHODS

Ethical approval

The present study conforms to the ethical principles and regulations of the *Journal of Physiology*. All experimental protocols were approved by the University of Minnesota's Institutional Animal Care and Use Committee (IACUC protocol 1801-35497A).

Animals

For all experiments, mice were bred in-house and had *ad libitum* access to food and water in all housing conditions. Mice with opsin expression were generated by crossing mice expressing Cre selectively in VGluT2-expressing neurons (Vong et al., 2011)(B6J. 129S6(FVB)-Slc17a6$^{tm2(cre)Lowl}$/MwarJ; Jackson Laboratory stock 028863), referred to as VGluT2-cre in the text, with either floxed-STOP channelrhodopsin (ChR2) mice (Madisen et al., 2012)(B6;129S-Gt(ROSA)26Sor$^{tm32.1(CAG-COP4*H134R/EYFP)Hze}$/J; Jackson Laboratory stock 012569) or floxed-STOP halorhodopsin (HR) mice (Madisen et al., 2012) (B6;129S-Gt(ROSA)26Sor$^{tm39(CAG-HOP/EYFP)Hze}$/J; Jackson Laboratory stock 014539). These crosses generated mice with opsin expression restricted to VGluT2 expressing neurons, referred to as VGluT2-ChR and VGluT2-HR in the text. For experiments with virally induced opsin expression, Black-6 (C57BL/6J; Jackson Laboratory stock 000664) mice were used for cre-independent viruses and VGluT2-cre mice for cre-dependent viruses.

Opsin-negative littermates were used as light only controls for experiments with VGluT2-ChR and -HR animals. For virally induced opsin expression, mice injected with virus inducing expression of fluorescent protein only were used as controls.

Both male and female mice were used for experiments. Until optical fiber and electrode implantation, animals were housed in standard housing conditions in the animal facility at the University of Minnesota. Following implantation, animals were singly housed in

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investigator managed housing. In all conditions, animals were allowed ad libitum access to food and water, and were on a 12 hour light; 12 hour dark (/low red light) cycle.

Stereotactic surgeries

Epilepsy induction—The mouse unilateral intra-hippocampal kainic acid model of epilepsy was utilized (Bouilleret et al., 1999; Bragin et al., 1999), as described previously with minor modifications (Armstrong et al., 2013). Briefly, adult mice (postnatal day 45 or later) under isoflurane anesthesia were injected with 100nL of kainic acid (KA) unilaterally into the right hippocampus (2.0 mm posterior, 1.25 mm right, 1.6 mm ventral from bregma). Animals were subsequently removed from isoflurane less than five minutes post injection. In this model, spontaneous recurrent electrographic seizures emerge after a period of weeks (Bouilleret et al., 1999).

Electrode and fiber implantation—A minimum of 1 week post kainic acid injection, mice were implanted with a twisted wire bipolar (local reference, differential) electrode (PlasticsOne) ipsilateral to the site of kainate (2.6 mm posterior, 1.75 mm right, 1.6 mm ventral from bregma). Optical fibers (ThorLabs) targeting the left and right fastigial nucleus were implanted 6.48 mm posterior, 0.75 left/right, and 3.5 mm ventral from bregma. Implants were secured to the skull using surgical screws and dental cement, as described previously (Armstrong et al., 2013). Animals were allowed to recover a minimum of five days prior to video and local field potential (LFP) monitoring for seizures and closed-loop interventions.

Viral targeting—For all viral experiments, AAV serotype 9 was selected from other options due to its optimal expression in the fastigial nucleus with no apparent retrograde expression. Mice were injected with 120nL of virus via a Hamilton Neuros syringe into the left cerebellar fastigial nucleus (6.48 posterior, 0.75 left, 3.7 mm ventral from bregma) under isoflurane anesthesia. Black-6 mice were injected with virus encoding Channelrhodopsin fused to enhanced yellow fluorescent protein (AAV9-CAG-ChR2-GFP, titer of $2.1\times10e^{12}$, UNC vector core lot #AV5406D, provided by Edward Boyden), or AAV9-CAG-GFP (titer 2×10^{12}, UNC vector core lot #AV5221, provided by Edward Boyden). VGluT2-cre mice were injected with virus encoding Channelrhodopsin in a cre-dependent manner (pAAV-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA, titer of 2.2×10^{13} Addgene viral prep #202198-AAV9, lot #V22125, gift from Karl Deisseroth)(Gradinaru et al., 2007), virus encoding Halorhodopsin in a cre-dependent manner (pAAV-Ef1a-DIO eNpHR 3.0-EYFP, titer of 2.2×10^{13}, Addgene viral prep #26966-AAV9, lot #V44904, gift from Karl Deisseroth)(Gradinaru et al., 2010), or GFP alone in a cre-dependent manner (AAV9-CAG-Flex-GFP, titer 3.7×10^{12}, UNC vector core lot #AV5220C, provided by Edward Boyden). After injection, the syringe was held in place for a minimum of 10 minutes before being withdrawn.

Subsequent KA injections and implantations were performed as for experiments with transgenic animals, with the KA injections performed a minimum of two weeks post virus injection. On-demand interventions were conducted a minimum of 6 weeks post viral injection.

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Post-operative care—For all surgical procedures, post-operative care consisted of recovery from anesthesia on a heating pad with regular visual inspection, followed by daily post-operative monitoring for a minimum of three days to inspect comfort level and healing of the surgical site. Neopredef powder was applied to the closed incisions as a topical antibiotic and analgesic. In the case of KA injection, no additional post-op analgesics were given. For viral injections and implantations, carprofen was administered subcutaneously (5mg/kg) during surgery. For implantations, post-operative ibuprofen was also administered orally (50-80mg/kg/day in water) for three days post-surgery as an additional analgesic.

Closed-loop seizure detection and interventions

After recovery from implantation, animals were placed in investigator managed housing for 24 hour video and LFP recordings. Electrographic hippocampal seizures were detected and on-demand optogenetic interventions were triggered as described previously (Armstrong et al., 2013; Krook-Magnuson et al., 2014). Briefly, animals were connected via electrical patch cords through an electrical commutator (PlasticsOne). Hippocampal LFP was amplified (Brownlee), digitized (National instruments), and analyzed in real time by custom MATLAB seizure detection software. Light interventions were delivered via optical patch cords (ThorLabs, Doric lenses) through an optical commutator connected to either LEDs (Plexon) or lasers (Shanghai Laser & Optics Century) of the appropriate wavelength (473 nm for ChR2, 589nm for HR). Power, measured post-hoc from the tip of implanted fibers, ranged between 0.2-2.4mW for ChR2 and 1.5-9.8mW for HR. There was no correlation between light power and effect on seizure duration for either blue light or amber light ($p > 0.05$, Spearman's correlations). Optogenetic interventions were triggered for 50% of events in a random fashion using custom closed-loop MATLAB software similar to that described previously and available for download (Armstrong et al., 2013). Three seconds of pulsed light was delivered unilaterally to the fastigial nucleus either contralateral or ipsilateral to the site of kainic acid injection. A variety of pulse lengths were tested, as discussed in the text.

Tissue harvesting and imaging of opsin expression

In order to confirm appropriate optical fiber targeting and viral expression, after on-demand interventions, mice were deeply anesthetized with 5% isoflurane and decapitated. Their brains were subsequently harvested and drop fixed in 4% paraformeldahyde. Sagittal brain sections of 50μm were collected in 0.1M phosphate buffer using a vibratome (Leica VT1000S). After sectioning, every third section was mounted with Vectashield mounting media with DAPI and covered with glass coverslips. Sections were visualized with epifluorescence microscopy (Leica DM2500). Confocal imaging of representative images was performed on an Olympus FluoView FV1000 BX2 upright confocal microscope (University Imaging Center, University of Minnesota).

Immunohistochemistry

In order to confirm specificity of the VGluT2-cre mouse line in targeting VGluT2-expressing neurons, VGluT2 immunohistochemistry was performed on brains of VGluT2-cre mice injected with cre-dependent virus for GFP expression. Tissue was harvested and fixed as described above. After sectioning, every third sagittal section containing the virally

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injected fastigial nucleus was transferred into 0.1M phosphate buffer and rinsed three times for 10 min. Slices were incubated for 1 hour at room temperature in a blocking solution containing 10% bovine serum and 0.5% triton diluted in TBS. This was followed by overnight incubation with primary antibody for VGluT2 (Millipore Sigma, 1:1000 diluted in TBS containing 2% bovine serum and 0.4% triton). Slices were subsequently rinsed 3 times for 10 minutes in TBS and incubated for 2 hours with Alexa fluor 594 anti-guinea pig (Jackson, 1:500 diluted in TBS containing 2% bovine serum and 0.4% triton). Sections were rinsed 3 times for 15 minutes in PB, mounted with Vectashield and visualized with epifluorescence microscopy (Leica DM2500) for cell counting. A total of three 50μm sagittal slices containing the fastigial nucleus were included for each animal, resulting in 352 total fastigial neurons counted between two mice. Confocal imaging of representative images was performed on an Olympus FluoView FV1000 BX2 upright confocal microscope (University Imaging Center, University of Minnesota).

Statistical analyses

Seizure duration after the time of trigger was analyzed off-line using a combination of manual and automated methods. Seizure events are processed automatically based on user-identified characteristics of spikes including amplitude, peak width, spike frequency and deflection (positive, negative, or both), and the resulting post detection seizure duration of all events were confirmed via manual inspection (Zeidler et al., 2018). No consistent effects on time to next seizure were noted. Reviewers were blinded to the light condition of triggers, and at least 100 events were analyzed per animal per condition. Seizure durations are normalized to percent duration relative to no-light condition. Comparisons of post-trigger seizure duration between light and no light conditions were compared in each animal using two-sample Kolmogorov-Smirnov and two-tailed Mann-Whitney tests. Effects on post-detection seizure duration were examined at the group level using Wilcoxon signed rank tests. A p value < 0.05 was considered statistically significant. Statistical analyses were conducted using MATLAB. Values are presented as mean ± SEM.

RESULTS

On-demand inhibition of the fastigial nucleus fails to attenuate hippocampal seizures

On-demand modulation of cerebellar Purkinje cells is highly effective at inhibiting hippocampal seizures (Krook-Magnuson et al., 2014). Intriguingly, either excitation or inhibition of Purkinje cells robustly terminated seizures, with Purkinje cell firing entraining to pulsed light delivery. Purkinje cells in the vermis project to, and inhibit, neurons in the fastigial nucleus. An important question is if similar on-demand inhibition of seizures can be achieved through direct modulation of neurons in the fastigial nucleus. Optogenetics also allows us to further ask whether inhibition, excitation, or simply a disruption of ongoing activity of fastigial neurons can provide seizure cessation similar to that previously seen with on-demand optogenetic modulation of Purkinje cells in the cerebellar cortex. We thus first sought to directly inhibit the fastigial nucleus in a similar on-demand fashion to determine whether this could attenuate seizures.

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In order to implement optogenetic inhibition of excitatory neurons in the fastigial nucleus, mice expressing Cre in VGluT2-expressing neurons were crossed with mice expressing the inhibitory opsin, Halorhodopsin (HR) in a Cre-dependent fashion as detailed in Materials and Methods (Fig. 1A). On-demand optogenetic inhibition was achieved in these animals via amber light (589nm) delivered through an optical fiber targeting the fastigial nucleus. Chronic seizure induction, monitoring, and on-demand interventions were implemented as described previously (Fig. 1B) (Armstrong et al., 2013; Krook-Magnuson et al., 2014). Using these methods, electrographic seizure events are detected in real time, with 50% of detected events randomly selected to receive light delivery (Fig. 1C).

Given the inhibitory input of Purkinje cells to neurons in the deep cerebellar nuclei, we hypothesized that direct inhibition of neurons in the fastigial nucleus would mimic the effects of cerebellar cortical stimulation. Previous work targeting Purkinje cells has shown that 3 seconds of long light pulses (1000ms on, 50ms off) as well as shorter pulses (50ms on, 100ms off) is highly effective at attenuating hippocampal seizures (Krook-Magnuson et al., 2014). We therefore tested these pulse paradigms targeting the fastigial nucleus.

Surprisingly, we found that 3 seconds of long pulses of light (589nm) delivered to the nucleus to inhibit fastigial neurons fails to attenuate hippocampal seizures (Fig 1C), with no significant difference between the durations of seizure events receiving light versus no light (Fig. 1D). Across the population, zero of six animals showed a significant effect of light delivery on post-detection seizure duration. There is no effect of fastigial inhibition on seizure duration regardless of whether the contralateral (Fig. 1E, $p = 0.094$, Wilcoxon signed rank test, $n = 6$ animals) or ipsilateral (Fig. 1F, $p = 0.219$, Wilcoxon signed rank test, $n = 6$ animals) fastigial nucleus is targeted.

We next examined whether shorter inhibitory pulses of amber light (589nm) at higher frequencies delivered to the fastigial nucleus could affect the duration of hippocampal seizures. As with longer light pulses, shorter 50ms pulses of light delivered at ~7Hz (50ms on, 100ms off; (Krook-Magnuson et. al, 2014)) had no significant effect on hippocampal seizure duration (Fig. 1G-I). Across the population, zero of six animals showed a significant reduction in seizure duration for either contralateral (Fig. 1H, $p = 0.687$, Wilcoxon signed rank test, $n = 6$ animals) or ipsilateral (Fig. 1I, $p > 0.99$, Wilcoxon signed rank test, $n = 6$ animals) fastigial inhibition. To further explore possible light parameters, we additionally examined 50ms on, 50ms off pulsed light delivery (10Hz) and found similar results (Fig. 1J-L). Zero of six animals showed a significant reduction in seizure duration for either contralateral (Fig. 1K, $p = 0.437$, Wilcoxon signed rank test, $n = 6$ animals) or ipsilateral (Fig. 1L, $p = 0.562$, Wilcoxon signed rank test, $n = 6$ animals) fastigial inhibition with this intervention approach. Together, these results suggest that on-demand direct inhibition of the fastigial nucleus is insufficient to disrupt hippocampal seizures.

On-demand excitation of the fastigial nucleus robustly attenuates hippocampal seizures

Given the inefficacy of direct inhibition of fastigial neurons, we next asked whether direct excitation of the fastigial nucleus could reduce the duration of hippocampal seizures. A similar transgenic approach was utilized for targeted opsin expression, with mice expressing Cre in VGluT2-expressing neurons crossed with mice expressing the excitatory opsin,

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Channelrhodopsin (ChR2) in a Cre-dependent fashion. Excitation was achieved via blue (473nm) light delivered to the fastigial nucleus (Fig. 2A), using the same light pulse paradigms as used in the previously described experiments.

Three seconds of pulsed blue light delivery (1000ms on, 50ms off) to the contralateral fastigial nucleus successfully terminated hippocampal seizure events (Fig. 2B-C), with a majority of events terminating within 1 second of stimulation onset (Fig. 2C, inset). Given that we saw no effect in VGluT2-HR expressing animals, the termination of seizures with light delivery in VGluT2-ChR2 animals is unlikely to be due to off-target effects of light delivery. Further supporting this, there was no effect of blue light delivery in an opsin negative control littermate (Fig. 2D), illustrating that excitation via opsin activation underlies anti-seizure effects. Across the population in opsin-positive VGluT2-ChR2 animals, on-demand excitation of the contralateral fastigial nucleus significantly reduced the duration of hippocampal seizures (Fig. 2E, 71 ± 13% reduction, $p = 0.016$, Wilcoxon signed rank test, $n = 7$ animals), with 6/7 animals showing a significant effect of light delivery. These results demonstrate that on-demand direct activation of the fastigial nucleus is able to attenuate hippocampal seizures.

Fastigial attenuation of hippocampal seizures occurs at multiple stimulation frequencies

We next tested whether longer pulses of blue light (1000ms on, 50ms off, for 3s) were necessary to disrupt seizures. The first additional stimulation parameter tested was 7Hz (50ms on, 100ms off), which produced robust seizure attenuation when targeting the cerebellar cortex (Krook-Magnuson 2014). As seen with longer light pulses, ~7Hz stimulation robustly attenuated hippocampal seizures, with a majority of seizure events terminating within one second of light delivery (Fig. 2F). Seizure disruption was not seen in an opsin negative control (Fig. 2G), again confirming that effects in opsin-positive animals were due to opsin activation rather than light delivery per se. Across the population of opsin-positive VGluT2-ChR2 animals, the duration of hippocampal seizures was significantly reduced with ~7Hz blue (473nm) light delivery (Fig. 2H, 67 ± 15 % reduction, $p = 0.031$, Wilcoxon signed rank test, $n = 7$ animals), with 6/7 animals showing a significant effect of light delivery.

To further assess the efficacy of fastigial stimulation in terminating hippocampal seizures, we also tested whether higher frequency (10Hz; 50ms on, 50ms off) blue light delivery to excite the fastigial nucleus could also be an effective intervention. We found that higher frequency 10Hz stimulation also robustly attenuated hippocampal seizures, with a majority of seizure events terminating within 1 second of blue light delivery (Fig. 2I). As seen with previous experiments, blue light delivery had no effect on seizure duration in an opsin negative control (Fig. 2J). Across the population, 10Hz stimulation produced a significant reduction in seizure duration in VGluT2-ChR2 opsin-positive animals (Fig. 2K, 63 ± 18% reduction, $p = 0.047$, Wilcoxon signed rank test, $n = 7$ animals), with 5/7 animals showing a significant effect of light delivery.

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Fastigial interventions are effective irrespective of the side relative to seizure focus

Ascending fastigial outputs cross the midline via the superior cerebellar peduncle, resulting in a largely contralateral organization relative to forebrain structures (Angaut & Bowsher, 1970). However, previous results indicate that inhibition of hippocampal seizures through modulation of the cerebellar cortex is not strongly lateralized (Krook-Magnuson et al., 2014). We thus tested whether excitation of the fastigial nucleus ipsilateral to the seizure focus could also disrupt hippocampal seizures. As with contralateral excitation, blue (473nm) light delivered to the ipsilateral fastigial nucleus successfully terminated seizures in VGlut2-ChR2 animals (Fig. 3A), across all stimulation paradigms tested. Seizure cessation induced by on-demand fastigial excitation is robust and rapid, with a majority of seizure events terminating within 1 second of light delivery (Fig. 3B). As with contralateral stimulation, no change in seizure duration was seen in an opsin negative control (Fig. 3C). Across the population in opsin-positive VGluT2-ChR2 animals, on-demand stimulation of the ipsilateral fastigial nucleus significantly reduced the duration of hippocampal seizures with long blue light pulses (Fig. 3D, 57 ± 12 % reduction, $p = 0.016$, Wilcoxon signed rank test, $n = 7$ animals), with 6/7 animals showing a significant effect of light delivery, shorter blue light pulses at ~7Hz (Fig. 3E, 54 ± 15% reduction, $p = 0.047$, Wilcoxon signed rank test, $n = 7$ animals), and shorter blue light pulses at 10Hz (Fig. 3F, 57 ± 12% reduction, $p = 0.016$, Wilcoxon signed rank test, $n = 7$ animals). These results indicate that on-demand excitation of fastigial neurons can disrupt hippocampal seizures regardless of the side targeted relative to the seizure focus.

Together, these results suggest that fastigial disruption of hippocampal seizures is not highly lateralized, and is not restricted to specific pulse durations or stimulation frequencies, but *is* dependent on excitation, rather than inhibition, of fastigial neurons.

Single pulse stimulation of the fastigial nucleus is an effective intervention

Given the diversity of stimulation frequencies and pulse durations that effectively terminate hippocampal seizures, and that the majority of seizure events stopped within 1 second of fastigial intervention, we next asked whether a single excitatory pulse of light delivered to the fastigial nucleus could also be effective. To test this, on-demand stimulation of either the contralateral and ipsilateral fastigial nucleus was implemented, with a single 50ms pulse of blue (473nm) light delivered at the time of seizure detection. We found that single blue light pulses to excite the fastigial nucleus are highly effective at reducing the duration of hippocampal seizures (Fig. 4A). As seen with previous interventions, there was no effect of light delivery in an opsin negative control (Fig. 4B). Across the VGluT2-ChR2 opsin-positive population, single pulses significantly reduced seizure duration when targeting either the contralateral (Fig. 4C, 47 ± 15% reduction, $p = 0.031$, Wilcoxon signed rank test, $n = 7$ animals) or ipsilateral (Fig. 4D, 44 ± 10% reduction, $p = 0.016$, Wilcoxon signed rank test, $n = 7$ animals) fastigial nucleus, with 6/7 animals and 5/7 animals showing a significant effect of light delivery for contralateral and ipsilateral stimulation, respectively. These results illustrate the powerful influence that even brief activation of the fastigial nucleus can exert over hippocampal seizure activity.

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Specific targeting of fastigial neurons using a viral approach

While VGluT2 is expressed by glutamatergic neurons of the fastigial nucleus (It , 1984), it is also found in the collaterals of both mossy and climbing fibers terminating in the deep cerebellar nuclei (Hioki et al., 2003). We thus used a viral approach to further increase the specificity of on-demand interventions to neurons of the fastigial nucleus. The contralateral fastigial nucleus was injected with viruses inducing the expression of ChR2. Subsequently, KA injections and implantations were performed as in previous experiments, with on-demand interventions implemented a minimum of 6 weeks post viral injection (Fig. 5A). Two separate viral targeting techniques were used to target fastigial neurons, as the fastigial nucleus contains glutamatergic, glycinergic and GABAergic neurons, all of which can be inhibited by Purkinje cells (Uusisaari & Knopfel, 2012)(Fig. 5B). In the first set of experiments, global targeting of fastigial neurons was achieved via injecting Black-6 mice with the virus AAV9-CAG-ChR2-GFP for the expression of channelrhodopsin (Fig. 5D), or, as a control, AAV9-CAG-GFP. We also selectively targeted glutamatergic neurons of the fastigial nucleus by injecting VGluT2-cre mice with a cre-dependent virus encoding channelrhodopsin (pAAV9-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA), or, as a control, AAV9-CAG-Flex-GFP (Fig. 5E). The selectivity of expression of virally-targeted glutamatergic neurons in VGluT2-cre mice was confirmed by performing immunohistochemistry against VGluT2 (Fig. 5C); greater than 99% of GFP expressing neurons (349 out of 352 neurons, n = 2 mice) were immunopositive for VGluT2.

A significant reduction in seizure duration was observed for both virally injected Black-6 (Fig. 5G) and VGluT2-cre (Fig. 5H) mice expressing channelrhodopsin with on-demand light delivery. Across the population of opsin-expressing virally injected Black-6 animals, on-demand excitation of the fastigial nucleus significantly reduced seizure duration (Fig. 5J, 39 ± 9% reduction, p = 0.031, Wilcoxon signed rank test, n = 6 animals), with 4/6 animals showing a significant effect of light delivery. On-demand selective excitation of glutamatergic fastigial neurons was also highly effective at reducing seizure duration (Fig. 5K, 66 ± 5% reduction, p = 0.031, Wilcoxon signed rank test, n = 6 animals), with 6/6 animals showing a significant effect of light delivery. Selective excitation of fastigial glutamatergic neurons produced a significantly greater reduction in seizure duration compared to exciting the fastigial nucleus more broadly (Fig. 5L, p = 0.026, Mann-Whitney test), suggesting that the improved cell-type targeting can provide greater seizure suppression benefits.

Single pulse stimulation also significantly reduced seizure duration in virally injected animals (39 ± 9% reduction, p = 0.016, Wilcoxon signed rank test, n = 3 Black-6 and n = 4 VGluT2 animals), illustrating that even brief activation of excitatory fastigial neurons is able to disrupt seizures.

As seen in our other experiments, opsin expression was required, as mice injected with control virus inducing the expression of only fluorescent protein showed no effect of light delivery on seizure duration (Fig. 5I, not significant in 4/4 animals). As an additional control, we also injected VGluT2-cre mice with a virus allowing cre-dependent expression of halorhodopsin (pAAV9-Ef1a-DIO-eNpHR 3.0-EYFP), to inhibit glutamatergic fastigial neurons. In support of earlier experiments, on-demand inhibition using a viral approach also

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had no effect on seizure duration, with 0/2 animals showing a significant effect of light delivery.

Together, these results show that excitation of fastigial glutamatergic neurons can robustly inhibit hippocampal seizures.

DISCUSSION

Using a combination of transgenic and viral approaches, this study reveals a powerful influence of fastigial neurons on hippocampal seizures. Seizures are robustly inhibited by multiple stimulation parameters, regardless of laterality of the fastigial nucleus targeted for stimulation. However, excitation, rather than inhibition, is required. Even a single excitatory light pulse delivered to the fastigial nucleus is sufficient to terminate seizures, emphasizing the profound influence of fastigial activation on hippocampal seizures. Viral targeting experiments reveal that selective activation of glutamatergic fastigial neurons provides attenuation of hippocampal seizures. Together, these results indicate that the fastigial nucleus could represent a promising target for therapeutic stimulation in TLE. Additionally, they highlight the importance of *excitation* in the deep cerebellar nuclei for cerebellar attenuation of seizures.

The finding that direct inhibition of the fastigial nucleus fails to affect hippocampal seizure duration was initially surprising. Given that Purkinje cells are inhibitory and that direct excitation of Purkinje cells robustly attenuates hippocampal seizures, a reasonable expectation is that direct inhibition of the fastigial nucleus should mimic the effects of Purkinje cell excitation. How then, can optogenetic excitation of Purkinje cells inhibit seizures, but not optogenetic inhibition of nuclear neurons? One possible explanation is the complex response to optogenetic manipulation of Purkinje cells themselves. During optogenetic excitation of Purkinje cells, cerebellar nuclei neurons show appropriately decreased firing rates (Chaumont et al., 2013; Witter et al., 2013; Brown & Raman, 2018). However, optogenetic excitation of Purkinje cells is subsequently followed by a pause in Purkinje cell activity (Krook-Magnuson et al., 2014; Lee et al., 2015; Brown & Raman, 2018) and a corresponding increase in firing in the cerebellar nuclei (Lee et al., 2015; Brown & Raman, 2018). Therefore, both optogenetic excitation or inhibition of Purkinje cells could result in excitation of nuclear neurons, and thereby inhibition of seizures. Our finding that on-demand excitation, but not on-demand inhibition, of fastigial neurons successfully terminates seizures strongly suggest that an increase in firing of nuclear neurons may be key for successful seizure intervention. Minimally, excitation of excitatory fastigial neurons is sufficient for seizure inhibition.

While our results provide strong support for a role of excitatory fastigial neurons in the suppression of seizures, our results do not rule out other potential contributors. For example, Purkinje cells can have direct projections to areas beyond the cerebellar nuclei (Schwarz et al., 2015; Hashimoto et al., 2018). The ability of these projections to inhibit seizures was not tested in this study. Additionally, the deep cerebellar nuclei have a diversity of cell types, including local interneurons and excitatory and inhibitory projection neurons (Uusisaari & Knopfel, 2012). While our results demonstrate that selectively targeting excitatory cells

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provides greater seizure control than broadly targeting fastigial neurons, selectively targeting other cell populations may also provide additional effects and interesting insights. For example, the fastigial nucleus contains a unique subpopulation of glycinergic projection neurons (Bagnall et al., 2009). The impact of selectively activating or inhibiting this population of neurons on seizure activity remains unknown. Similarly, while we have focused on the fastigial nucleus, previous work targeting the cerebellar cortex (Krook-Magnuson et al., 2014) suggests that other cerebellar nuclei may also be capable of producing seizure suppressive effects in temporal lobe epilepsy.

While the cerebellum is not typically associated with epilepsy, cerebellar impairments are often observed with epilepsy (Marcian et al., 2016; Boscolo Galazzo et al., 2018; Lee et al., 2018; Allen et al., 2019; Li et al., 2019), changes in cerebellar actvity are associated with seizure events (Niedermeyer & Uematsu, 1974; Mitra & Snider, 1975; Kandel & Buzsaki, 1993; Blumenfeld et al., 2009; Krook-Magnuson et al., 2014; Kros et al., 2017), and there have been noted cases of seizures originating in the cerebellum (Harvey et al., 1996; Mesiwala et al., 2002; Norden & Blumenfeld, 2002; Boop et al., 2013; Lascano et al., 2013; Martins et al., 2016). Indeed, the cerebellum was previously a target for seizure control of considerable interest and investigation (Cooke & Snider, 1955; Babb et al., 1974; Maiti & Snider, 1975). However, while early studies suggested that seizure inhibition with electrical stimulation of the cerebellum is possible, small clinical trials (and further animal studies) ultimately produced mixed results (for reviews, see (Miller, 1992; Fountas et al., 2010; Zhong et al., 2011)). As a result, the cerebellum fell out of favor. One reason for the discrepancy between these initial results and optogenetic stimulation of the cerebellum shown here and in previous work (Krook-Magnuson et al., 2014; Kros et al., 2015a)may be the closed-loop nature of the interventions. In on-demand interventions, stimulation is only applied on an ‘as-needed’ basis, limiting potential mal-adaptive plasticity or other negative effects of constant intervention; on-demand interventions, with their improved temporal alignment of intervention, may be inherently more effective (Good et al., 2009; Krook-Magnuson et al., 2015; Thomas & Jobst, 2015). Another benefit of optogenetic approaches is the cell type specificity, which may also aid in successful intervention (Krook-Magnuson & Soltesz, 2015). Here we find that cell-type specificity when targeting the fastigial nucleus for seizure control can indeed provide improved outcomes. Finally, as also demonstrated here, the direction of modulation can be crucial, raising the possibility that previous electrical stimulation efforts did not consistently produce excitation of the cerebellar nuclei.

While the cerebellum has long been considered a purely motor structure, increasing evidence suggests a role for the cerebellum in more cognitive functions (for reviews, see (Popa et al., 2014; Sokolov et al., 2017)). Mice with impaired cerebellar plasticity show disruptions in hippocampal place cells (Rochefort et al., 2011; Lefort et al., 2019), cerebellar activity can be synchronized with hippocampal oscillations (Wikgren et al., 2010; McAfee et al., 2019), and inhibition of Purkinje cells leads to functional activation of the hippocampus (Choe et al., 2018). However, while previous studies examining degenerating axons suggested a direct fastigial to hippocampal connection (Heath & Harper, 1974), evidence for a direct connection has not been replicated in later studies (Strick et al., 2009; Rochefort et al., 2013; Bohne et al., 2019), and we see no fibers from fastigial neurons in the hippocampus in our virally injected animals (data not shown). This suggests that other

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downstream pathways engaged by cerebellar stimulation must underlie hippocampal seizure attenuation (for a review, see (Yu & Krook-Magnuson, 2015)). Potential fastigial downstream targets of interest could include the reticular formation (Browning, 1985), superior colliculus (Soper et al., 2016), and thalamus (Fisher et al., 2010; Kros et al., 2015b; Salanova et al., 2015), but future experiments will be necessary to determine which fastigial targets are responsible. Notably, the vermis projects not only to the fastigial nucleus, but also the vestibular nuclei. Given our findings that direct stimulation of fastigial neurons is sufficient to inhibit seizures, vermal projections to the vestibular nuclei are unlikely to mediate seizure termination observed with optogenetic modulation of the vermis, or, minimally, are not required for it.

Our findings provide key insight into the mechanism by which modulation of the cerebellar cortex controls temporal lobe seizures. Specifically, we find that excitation of nuclear neurons is required, as inhibition of fastigial neurons had no effect. Excitation of fastigial neurons, in contrast, provided robust seizure control across a range of stimulation frequencies. In particular, excitation of fastigial excitatory neurons (which project to a number of downstream regions) was sufficient to terminate temporal lobe seizures. The deep cerebellar nuclei may be counterintuitively more accessible for intervention (Fountas et al., 2010; Wathen et al., 2018), as surgical complications including electrode displacement have been noted when targeting the cerebellar cortex (Wright et al., 1984; Fountas et al., 2010). The range of effective stimulation parameters and ability to successfully target either the fastigial nucleus contralateral or ipsilateral to the site of seizure focus add to its promise as a target for stimulation. Additionally, the ability to terminate hippocampal seizures with only a single light pulse is of therapeutic interest, as it provides seizure control while minimizing disruptions to ongoing cerebellar function. Therefore, targeting the fastigial nucleus for seizure control clinically should be revisited, as our data suggest it may be a powerful target for therapeutic intervention in temporal lobe epilepsy.

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Biography

Martha Streng received a B.A. in Neuroscience and Behavior from Mount Holyoke College and a Ph.D. in Neuroscience from the University of Minnesota, Twin Cities, where she studied the encoding of motor predictive and feedback information by Purkinje cells during online motor control tasks. Her current research focuses on the characterization of cerebellar circuitry and output channels in health and disease.

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KEY POINTS

- On-demand optogenetic inhibition of glutamatergic neurons in the fastigial nucleus of the cerebellum does not alter hippocampal seizures in a mouse model of temporal lobe epilepsy.
- In contrast, on-demand optogenetic excitation of glutamatergic neurons in the fastigial nucleus successfully inhibits hippocampal seizures. With this approach, even a single 50ms pulse of light is able to significantly inhibit seizures.
- On-demand optogenetic excitation of glutamatergic fastigial neurons either ipsilateral or contralateral to the seizure focus is able to inhibit seizures.
- Selective excitation of glutamatergic nuclear neurons provides greater seizure inhibition than broadly exciting nuclear neurons without cell-type specificity.

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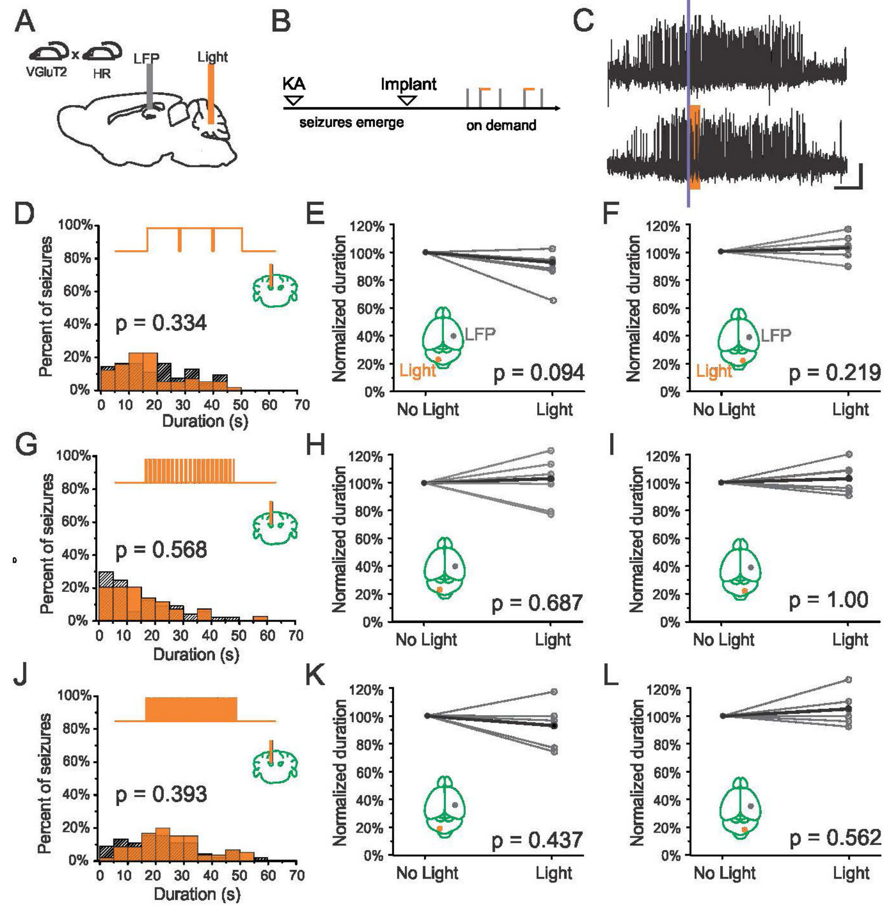

Figure 1.

Fastigial intervention in VGluT2-HR mice. A-B) Schematics of experimental design. Note that seizures are recorded from the hippocampus and light is delivered in an on-demand fashion to the fastigial nucleus during the chronic phase of the disorder. C) Example seizure events detected on-line (denoted by purple bar) that were either randomly selected not to receive light (top trace) or receive light (bottom trace, 3 seconds of light delivery denoted by amber box). Scale bar: 5s, 0.05mV. D-F) 3 seconds of long light pulses (1000ms on, 50ms off) to inhibit the fastigial nucleus produces no significant change in seizure duration (D, example animal; amber bars: events receiving light intervention; hashed bars: no-light internal controls; top trace illustrates pulsed light delivery paradigm) when light (589nm) is delivered to the contralateral (E) or ipsilateral (F) fastigial nucleus (each gray point represents data from one animal, black points represent mean). Similarly, 3 seconds of shorter light pulses (G-I) at 7Hz (50ms on, 100ms off), or (J-L) 10 Hz (50ms on, 50ms off), produce no significant change in seizure duration.

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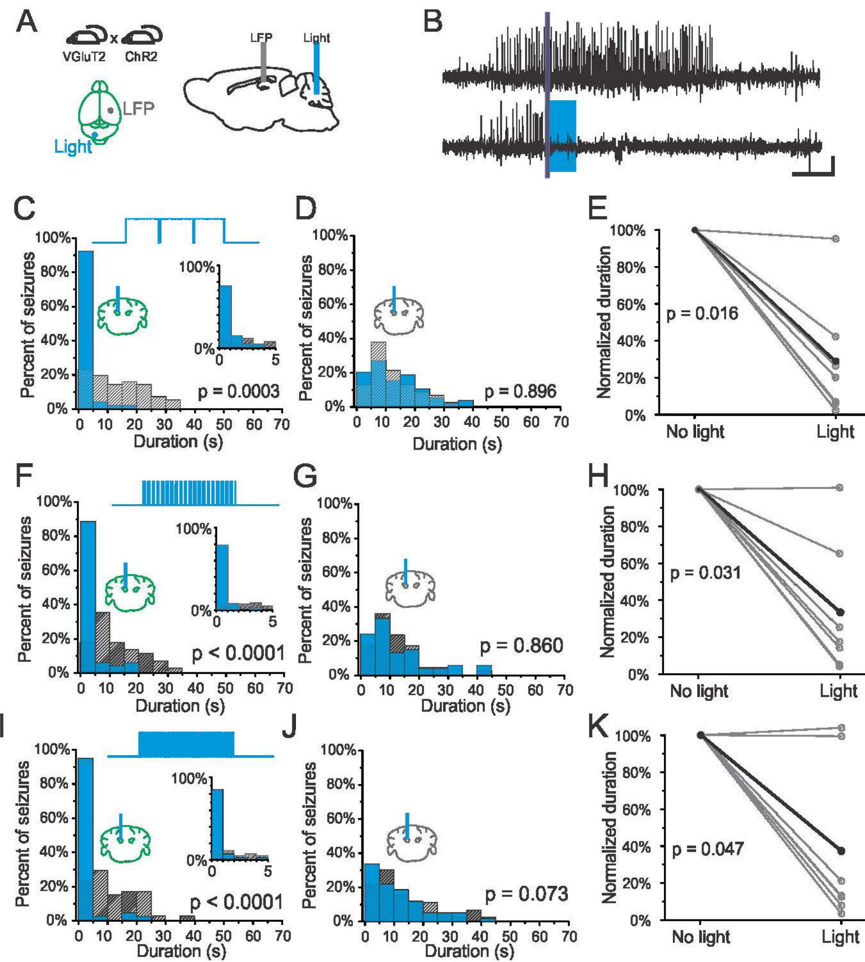

Figure 2.

On-demand optogenetic excitation of the fastigial nucleus contralateral to KA injection. A) Spontaneous seizures are recorded from the KA-injected hippocampus and blue (473nm) light is delivered in an on-demand fashion to the contralateral fastigial nucleus in VGluT2-ChR animals. B) Example seizure events detected on-line (denoted by purple bar) that were either randomly selected not to receive light (top trace) or receive light (bottom trace, 3 seconds of light delivery denoted by blue box). Scale bar: 5s, 0.05mV. Three seconds of pulsed light delivery (1000ms on, 50ms off) significantly reduces seizure duration. C) Post-detection seizure duration distributions for an example animal (93% reduction, $p < 0.001$, two sample Kolmogorov-Smirnov test). Blue bars: events receiving light intervention; hashed bars: no-light internal controls. Top trace illustrates pulsed light delivery paradigm. Inset: first 5s bin expanded, 1s bin size. D) No effect of light delivery on seizure duration in an opsin negative animal ($p = 0.896$, two sample Kolmogorov-Smirnov test). E) Light delivery produces a significant reduction of seizure duration in opsin positive VGluT2-ChR mice (each gray data point represents one animal, black data points represent mean). Similarly, 3 seconds of shorter light pulses (F-H) at 7Hz (50ms on, 100ms off), or (I-K) 10 Hz (50ms on, 50ms off), produce a significant reduction in seizure duration.

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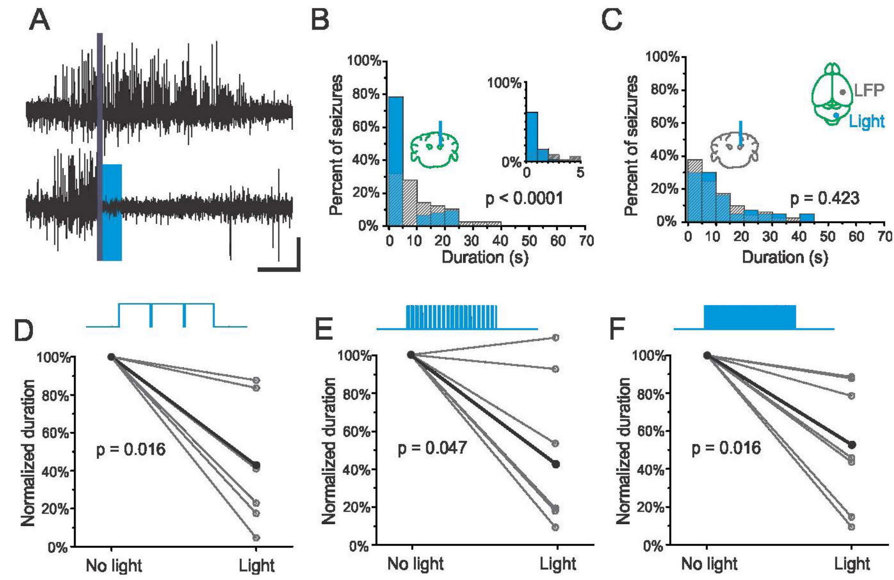

Figure 3.

On-demand optogenetic excitation of the fastigial nucleus ipsilateral to KA injection. A) Example seizure events detected on-line (denoted by purple bar) that were either randomly selected not to receive light (top trace) or receive light (bottom trace, 3 seconds of light delivery denoted by blue box). Scale bar: 5s, 0.05mV. Three seconds of pulsed blue light delivery (1000ms on, 50ms off) significantly reduces seizure duration. B) Post-detection seizure duration distribution for an example VGluT2-ChR animal; blue bars: events receiving light intervention; hashed bars: no-light internal controls; top trace illustrates pulsed light delivery paradigm (57% reduction, $p < 0.001$, two sample Kolmogorov-Smirnov test). Inset: first 5s bin expanded, 1s bin size. C) No effect of light delivery on seizure duration in an opsin negative animal ($p = 0.423$, two sample Kolmogorov-Smirnov test). D) Light delivery produces a significant reduction of seizure duration in opsin positive VGluT2-ChR mice (each gray data point represents one animal, black data points represent mean). Similar results are seen for 7Hz (E) and 10Hz (F) stimulation.

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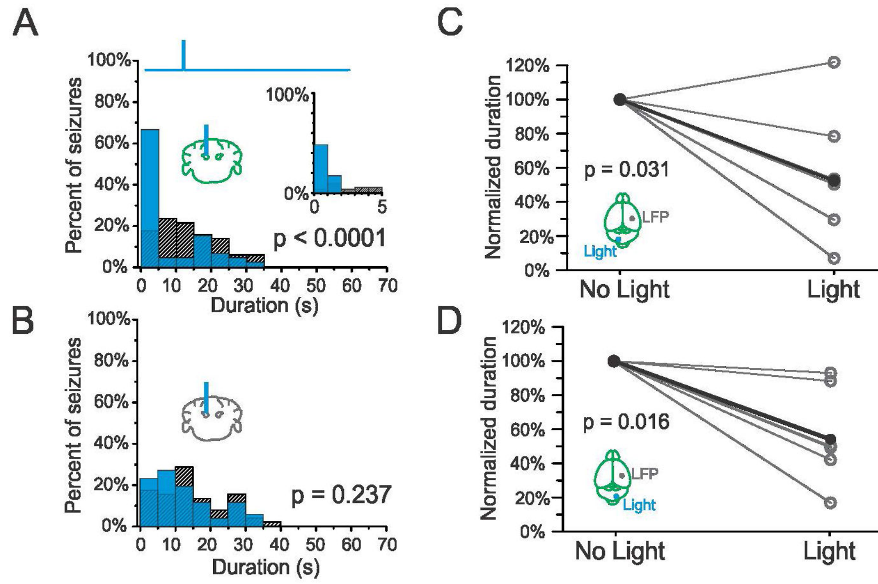

Figure 4.

A single 50ms pulse of light significantly reduces seizure duration in VGluT-ChR animals. A) Data from an example opsin-positive animal; blue bars: events receiving blue (473nm) light intervention; hashed bars: no-light internal controls; top trace illustrates pulsed light delivery paradigm (50% reduction, $p < 0.001$, two sample Kolmogorov-Smirnov test). Inset: first 5s bin expanded, 1s bin size. B) No effect of blue (473nm) light delivery in an opsin negative animal ($p = 0.237$, two sample Kolmogorov-Smirnov test). Single pulses of light in opsin positive VGluT2-ChR animals significantly reduce seizure duration when targeting either contralateral (C) or ipsilateral (D) fastigial nucleus (each gray data point represents one animal, black data points represent mean).

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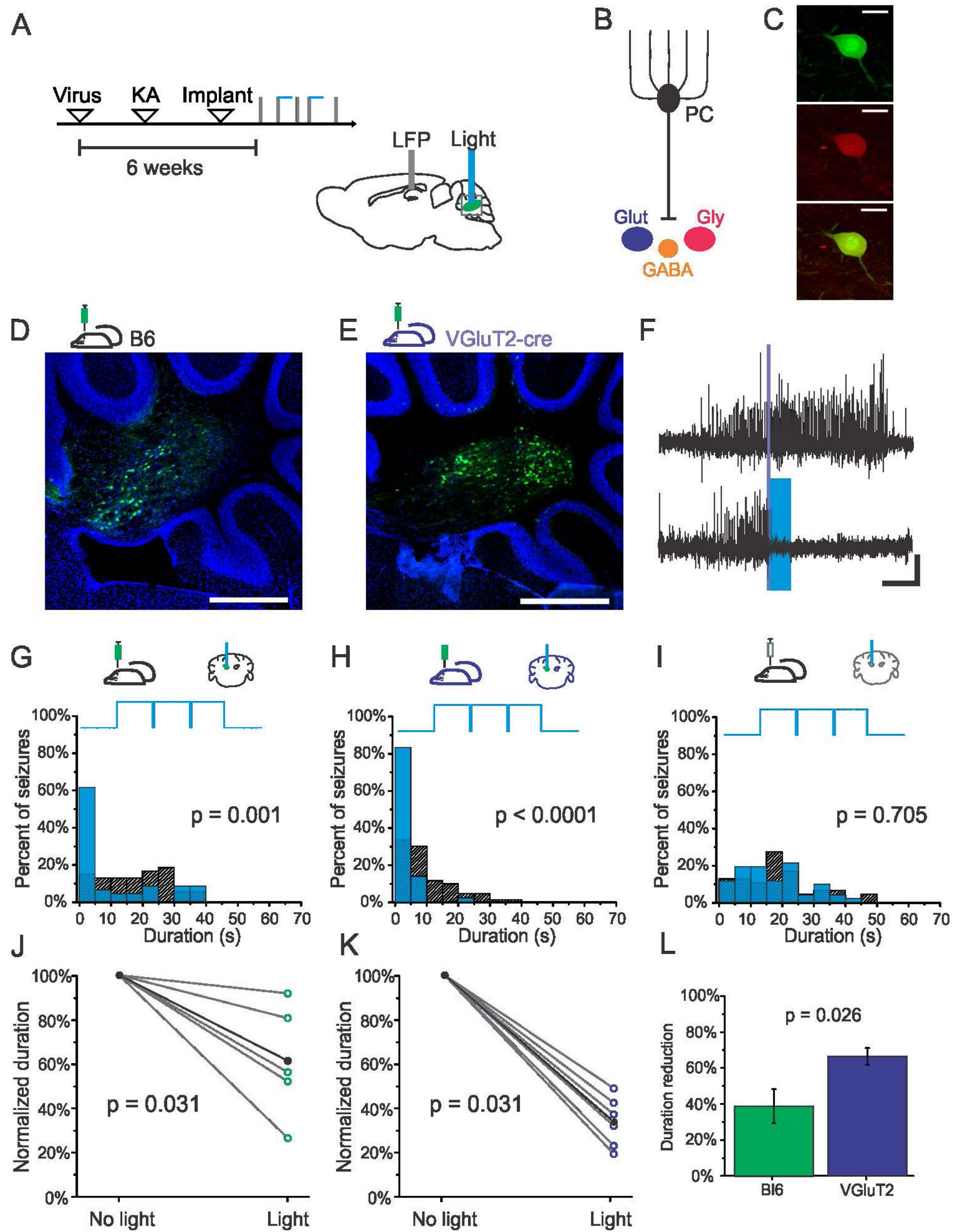

Figure 5. Selective excitation of fastigial glutamatergic neurons provides robust seizure control.
A) Schematic of experimental design and timeline. Viral approaches allowed for the targeting of nuclear neurons broadly, including nuclear glutamatergic, GABAergic, and glycinergic neurons (B), or selective targeting of glutamatergic nuclear neurons (shown in dark blue in the figure). (C) Selective expression in glutamategic neurons is achieved following injection of cre-dependent viruses in VGluT2-cre transgenic mice (Top- Green: GFP, Middle- Red: VGluT2 immunohistochemistry, Bottom- overlay, scale bar 70μm). D) GFP expression in nuclear neurons following injection in a Black-6 mouse. E) GFP expression in nuclear neurons following injection of cre-dependent virus in a VGluT2-cre mouse. Scale bars for D and E: 500μm. Green: GFP. Blue: DAPI. F) Example seizure events

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detected on-line (denoted by purple bar) that were either randomly selected not to receive light (top trace) or receive light (bottom trace, 3 seconds of blue (473nm) light delivery denoted by blue box). Scale bar: 5s, 0.05mV. G-H) Light delivery significantly reduces seizure duration in virally injected Black-6 (G, 44% reduction, $p = 0.001$, two sample Kolmogorov-Smirnov test) and VGluT2-cre (H, 81% reduction, $p < 0.001$, two sample Kolmogorov-Smirnov test) mice expressing channelrhodopsin. Blue bars: events receiving light intervention; hashed bars: no-light internal controls; top trace illustrates pulsed light delivery paradigm. I) No effect of light delivery in a mouse injected with AAV9-CAG-GFP control vector ($p = 0.705$, two sample Kolmogorov-Smirnov test). J-K) Stimulation significantly reduces the duration of hippocampal seizures across the population of channelrhodospin-expressing virally injected Black-6 (J) and VGluT2 (K) mice. Each open circle represents one animal. Black data points represent mean. L) Selective targeting of glutamatergic neurons in the fastigial nucleus produces significantly greater seizure attenuation than targeting fastigial neurons more broadly ($p = 0.026$, Mann-Whitney test).