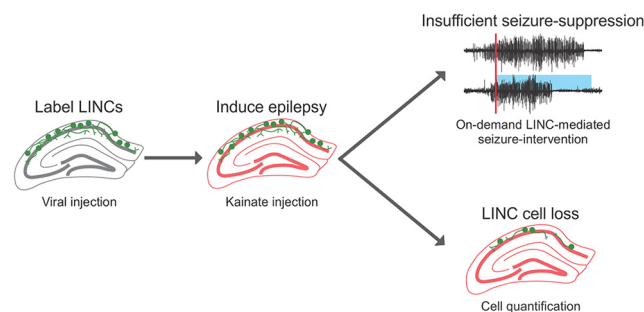


Disorders of the Nervous System

# LINCs Are Vulnerable to Epileptic Insult and Fail to Provide Seizure Control via On-Demand Activation

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## Visual Abstract



Temporal lobe epilepsy (TLE) is notoriously pharmacoresistant, and identifying novel therapeutic targets for controlling seizures is crucial. Long-range inhibitory neuronal nitric oxide synthase-expressing cells (LINCs), a population of hippocampal neurons, were recently identified as a unique source of widespread inhibition in CA1, able to elicit both GABA<sub>A</sub>-mediated and GABA<sub>B</sub>-mediated postsynaptic inhibition. We therefore hypothesized that LINCs could be an effective target for seizure control. LINCs were optogenetically activated for on-demand seizure intervention in the intrahippocampal kainate (KA) mouse model of chronic TLE. Unexpectedly, LINC activation at 1 month post-KA did not substantially reduce seizure duration in either male or female mice. We tested two different sets of stimulation parameters, both previously found to be effective with on-demand optogenetic approaches, but neither was successful. Quantification of LINCs following intervention revealed a substantial reduction of LINC numbers compared with saline-injected controls. We also observed a decreased number of LINCs when the site of initial insult (i.e., KA injection) was moved to the amygdala [basolateral amygdala (BLA)-KA], and correspondingly, no effect of light delivery on BLA-KA seizures. This indicates that LINCs may be a vulnerable population in TLE, regardless of the site of initial insult. To determine whether long-term circuitry changes could influence outcomes, we continued testing once a month for up to 6 months post-KA. However, at no time point did LINC activation provide meaningful seizure suppression. Altogether, our results suggest that LINCs are not a promising target for seizure inhibition in TLE.

### Significance Statement

Novel treatments are needed for temporal lobe epilepsy, and altering inhibitory signaling may provide seizure control. Recently, a previously uncharacterized hippocampal cell population, long-range inhibitory neuronal nitric oxide synthase-expressing cells (LINCs), was found to provide strong, widespread inhibition in healthy tissue. Despite being a novel source of powerful inhibition, and therefore a promising candidate for seizure control, on-demand activation of LINCs in a mouse model of temporal lobe epilepsy did not substantially suppress seizure activity. LINC numbers were decreased in epileptic tissue, indicating that LINCs are a vulnerable cell population. The heterogeneity of inhibitory signaling, and how it changes in epilepsy, are important factors to consider when developing new temporal lobe epilepsy therapies.

**Key words:** channelrhodopsin; closed-loop; GABAergic; interneuron; nNOS; responsive neurostimulation

## Introduction

Temporal lobe epilepsy (TLE), characterized by spontaneous recurrent seizures typically arising from the hippocampus, is the most common form of epilepsy in adults (England et al., 2012). With current antiseizure drugs, only 11–25% of TLE patients are seizure free (Zumsteg, 2006; Löscher and Schmidt, 2011; Bialer et al., 2020). Additionally, those who can find some relief may experience a range of negative side effects including nausea, liver dysfunction, bone degradation, and chronic cognitive impairments (Spuck et al., 2010; de Tisi et al., 2011; Sherman et al., 2011; Perucca and Gilliam, 2012; Duchowny and Bhatia, 2014; Laxer et al., 2014). Dysregulation of GABAergic signaling is a known key ictogenic factor (Hawkins and Saret, 1957; Benassi, 1962; Krnjević, 1983). However, the limited efficacy and negative side effects of antiseizure drugs that indiscriminately elevate inhibitory signaling emphasize the need to consider the timing of interventions, circuit changes in epilepsy, and the heterogeneity of GABAergic signaling to develop high-specificity treatments that can provide seizure control while limiting side effects (Muldoon et al., 2015; Christenson Wick and Krook-Magnuson, 2018; Farrell et al., 2019; Moxon et al., 2019; Dudok et al., 2022). There is incredible diversity of GABAergic signaling and GABAergic neurons (DeFelipe et al., 2013; Lovett-Barron and Losonczy, 2014; Pelkey et al., 2017; Wamsley and Fishell, 2017; Muñoz-Manchado et al., 2018). For example, in CA1, a sub-region of the hippocampus, there are >20 types of inhibitory neurons identified (Freund and Buzsáki, 1996; Klausberger and Somogyi, 2008). Recent work has identified and characterized additional CA1 populations, and genomic analysis suggests that even more populations remain

poorly characterized (Francavilla et al., 2018; Harris et al., 2018; Christenson Wick et al., 2019; Yao et al., 2021; Szabo et al., 2022). Each interneuron subtype has distinct properties and roles, subsequently forming unique microcircuits in healthy and epileptic tissue (Alexander et al., 2016; Booker and Vida, 2018; Shuman et al., 2020; Dudok et al., 2021; Righes Marafiga et al., 2021).

This heterogeneity in inhibitory cell populations provides both a challenge and an opportunity. It may be possible to harness the diversity of interneurons to identify novel and effective targets for seizure intervention. On-demand optogenetic seizure intervention (Armstrong et al., 2013; Paz et al., 2013) enables cell type-specific manipulations (Krook-Magnuson and Soltesz, 2015) and is a useful approach to study the potential for specific cell populations to control seizures (for review, see Tønnesen and Kokaia, 2017; Christenson Wick and Krook-Magnuson, 2018). This approach not only limits manipulation to specific cell populations, but also limits the timing of intervention to only when a seizure is detected (i.e., closed-loop stimulation, also known as responsive stimulation; Nagaraj et al., 2015).

Optogenetic approaches have been used to explore hippocampal circuit elements, and specifically, which populations may be able to curtail (or worsen or even induce) temporal lobe seizures (Krook-Magnuson et al., 2013; Osawa et al., 2013; Sukhotinsky et al., 2013; Berglind et al., 2014; Chiang et al., 2014; Krook-Magnuson et al., 2015; Ladas et al., 2015; Weitz et al., 2015; Wang et al., 2017; Bui et al., 2018; Chen et al., 2018, 2021; Tung et al., 2018; Botterill et al., 2019; Streng and Krook-Magnuson, 2020; Streng et al., 2021). This includes examination of inhibitory cell populations, such as parvalbumin-expressing cells (PV cells). Hippocampal PV cells encompass several inhibitory cell types and subtypes, including basket, axoaxonic, and bistratified cells (Woodson et al., 1989; Freund and Buzsáki, 1996; Varga et al., 2014). While the role of PV cells in seizures appears to be situation specific (Ellender et al., 2014; Sessolo et al., 2015; Assaf and Schiller, 2016; Lévesque et al., 2019; Magloire et al., 2019; Dudok et al., 2022; Wang et al., 2022), and may not be the same for all subtypes (Christenson Wick and Krook-Magnuson, 2019), previous work using on-demand optogenetic activation of PV cells at the onset of seizures in the intrahippocampal kainate (IHKA) mouse model of chronic TLE demonstrated successful hippocampal seizure inhibition (Krook-Magnuson et al., 2013, 2014b; Chen et al., 2021). This previous work supports the idea that *in vivo* activation of a select subpopulation of interneurons can be sufficient for the suppression of hippocampal seizures. However, the degree of seizure inhibition mediated by activation of PV cells was not as high as observed with some other closed-loop cell manipulations in the same TLE model (Krook-Magnuson et al., 2013, 2014b; Streng and Krook-Magnuson, 2020; Streng et al., 2021). Targeting different populations of hippocampal interneurons might be able to outperform on-demand optogenetic activation of hippocampal PV cells.

Received May 13, 2022; accepted December 19, 2022; First published January 30, 2023.

The authors declare no competing financial interests.

Author contributions: B.J.S., M.M.S., and E.K.-M. designed research; B.J.S. performed research; B.J.S., M.M.S., and E.K.-M. analyzed data; B.J.S., M.M.S., and E.K.-M. wrote the paper.

This work was funded by National Institutes of Health Grant R01-NS-104071 (to E.K.-M.), the University of Minnesota McKnight Awards (E.K.-M.), a University of Minnesota Informatics Institute-MnDRIVE (Minnesota's Discovery, Research, and Innovation Economy initiative) Informatics Graduate Assistantship (B.J.S.), and a MnDRIVE Research Fellowship in Neuromodulation (B.J.S.).

Acknowledgments: We thank Gord Fishell and his laboratory, including Robert Machold, Goichi Miyoshi, and Rowena Turnbull, for providing the Dlx5/6-Flpe and RCE:dual reporter mouse lines; and Karl Deisseroth and Charu Ramakrishnan for the intersectional virus. Additionally, we thank the members of the Krook-Magnuson Laboratory for their contributions to this work, in particular, Chris Krook-Magnuson, for maintenance and improvement of custom MATLAB software used for on-demand seizure intervention and offline analysis; and Bayla Brenningstall, Dylan Livingston, Ayan Sheikdon, Jane Yap, and Laurel Schuck for their help with offline analysis of seizure durations.

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<https://doi.org/10.1523/ENEURO.0195-22.2022>

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Recently, a previously uncharacterized source of widespread inhibition in the hippocampus was described—long-range inhibitory neuronal nitric oxide synthase (nNOS)-expressing cells (LINC; Christenson Wick et al., 2019). LINC is a GABAergic neuronal population in CA1, and, because of the following several unique features, we hypothesized that LINC would be in a privileged position to provide seizure control. (1) Despite being a relatively sparse inhibitory cell population, LINC provides widespread inhibition within CA1. Optogenetic activation of LINC in hippocampal slices resulted in postsynaptic inhibition of 80% of all patched cells in CA1, including both superficial and deep pyramidal cells (Christenson Wick et al., 2019). For comparison, PV cells primarily target deep pyramidal cells (Lee et al., 2014). (2) LINC is capable of altering hippocampal oscillations *in vivo* and therefore might also be effective in controlling aberrant network seizure activity (Christenson Wick et al., 2019). (3) LINC produces both GABA<sub>A</sub> and GABA<sub>B</sub> postsynaptic responses (Christenson Wick et al., 2019). Compared with GABA<sub>A</sub>, GABA<sub>B</sub>-mediated inhibition is not only longer lasting, but also independent of the intracellular chloride concentration (which may be altered in epilepsy, reducing the efficacy of GABA<sub>A</sub>-mediated inhibition; Cossart et al., 2005; Miles, 2012; Alfonsa et al., 2015; Wang et al., 2017).

Despite the widespread inhibition provided by LINC in healthy CA1, they have never been studied in the context of epilepsy (as they have only recently been described in healthy animals). Recent investigation of LINC (in healthy animals) was made possible by advances in viral vectors using intersectional genetic approaches (Fenno et al., 2014). LINC is identified in part by their expression of nNOS (Christenson Wick et al., 2019). However, nNOS is also expressed by excitatory neurons (Burette et al., 2002), which far outnumber LINC. Thus, it was only with a dual-targeting strategy that the investigation of LINC could be achieved: specifically, as done previously (Christenson Wick et al., 2019), we targeted LINC via their expression of both nNOS and Dlx5/6 (a transcription factor expressed by GABAergic neurons of the forebrain; Dimidschstein et al., 2016). While other interneurons can also express nNOS (Jinno et al., 2007; Tricoire et al., 2010; Krook-Magnuson et al., 2011; Armstrong et al., 2012), the viral vector approach taken, coupled with the location of injection, preferentially captures hippocampal LINC (Christenson Wick et al., 2019).

To determine whether LINC can influence seizure activity, we used the IHKA mouse model of chronic TLE (Bouilleret et al., 1999; Zeidler et al., 2018) and optogenetically activated LINC *in vivo* for on-demand seizure intervention. LINC-mediated seizure intervention was tested once a month, starting at 1 month post-epilepsy induction, for up to 6 months. We found that LINC activation did not substantially change seizure duration, even at late time points. We additionally found a substantial reduction in the number of LINC in epileptic tissue, suggesting epilepsy-related cell death. A reduction in the number of hippocampal LINC was observed even when the site of KA injection was moved to the amygdala. A propensity of LINC cell death in temporal

lobe epilepsy may prevent meaningful seizure inhibition via on-demand optogenetic activation of LINC and warrants future work examining the impact of this cell loss on hippocampal function.

## Materials and Methods

### Animals

All animal procedures were performed in accordance with the University of Minnesota animal care and use committee regulations. At all times, mice had *ad libitum* access to food and water and were on a 12 h light/dark cycle (low red light; zeitgeber time 0 = 8:00 A.M.). Both male and female mice, sexed at the time of weaning based on external genitalia, were used in all experiments. Mice were group housed in standard housing conditions in the animal facility at the University of Minnesota before and after viral injections. When needed, male mice were singly housed to avoid fighting after KA injections. Following implant surgery, all mice were singly housed to avoid damage to implants. Mice lived in investigator-managed housing during electrographic recordings.

For all experiments, mice were bred in-house. Using an intersectional genetic approach (Fenno et al., 2014), we virally labeled the nNOS-expressing GABAergic cell population LINC, as described in the study by Christenson Wick et al. (2019; Fig. 1B, Table 1). To enable this approach, two lines were crossed. One line expressed Cre recombinase in nNOS-expressing neurons and is referred to here simply as “cNOS”. The second line expressed Flp recombinase in GABAergic forebrain neurons via the *Dlx5/Dlx6* (*id6/id5*) intergenic enhancer region and beta-globin basal promoter, and here is referred to simply as “fDLX.” Crossing these lines resulted in “cNOS-fDLX” mice, which included offspring expressing both Cre and Flp recombinases in nNOS-expressing GABAergic neurons of the forebrain. Double-expressing cNOS-fDLX mice (Cre-positive and Flp-positive) were injected with virus and used as opsin-positive animals. Flp-negative cNOS-fDLX littermates underwent identical surgical procedures including viral injections. As viral expression requires both Cre and Flp expression, Flp-negative animals did not express channelrhodopsin and were used as opsin-negative controls. nNOS mice were purchased from The Jackson Laboratory (B6.129-Nos<sup>tm1(cre)Mgmj</sup>/J; stock #017526; Leshan et al., 2012) and were maintained as homozygotes. Founder fDLX mice were provided by the Fishell Laboratory, Harvard Medical School, Cambridge, MA, and are also available from The Jackson Laboratory [Tg(ml56i-flpe)39Fsh/J, stock #010815; Miyoshi et al., 2010]. fDLX breeders were backcrossed at least eight times with C57BL/6J mice (stock #000664; The Jackson Laboratory; bred in-house), and were maintained as hemizygotes. C57BL/6J mice were used for PV cell quantification (see below).

### Stereotactic surgeries

Mice underwent one to three stereotaxic surgeries conducted under 1–3% isoflurane anesthesia. Unless noted otherwise, mice received treatment with carprofen (5 mg/kg, s.c.), local bupivacaine at the start of surgery, and

postsurgical NeoPredef treatment around incisions, and recovered on a heating pad with appropriate observation.

### Viral injections

For all LINC-related experiments, mice first received a viral injection, starting at postnatal day 45 or greater (Fig. 1A, full timeline). In cNOS-fDLX-positive mice (described above), LINC were labeled with the excitatory opsin channelrhodopsin-2 (ChR2) fused to enhanced yellow fluorescent protein (eYFP) in a Cre-dependent and Flp-dependent manner [AAV-DJ-hSyn-Con/Fon-hChR2(H134R)-eYFP-WPRE; titers,  $4.4 \times 10^{12}$  and  $5.8 \times 10^{12}$ ; lot #AV6214C and #AV8403, UNC Viral Vector Core; Fig. 1B]. One microliter of virus was injected using a microliter syringe (Hamilton 2  $\mu$ l syringe, Neuros) into CA1 of the left dorsal hippocampus [distance from bregma: anteroposterior (AP),  $-2.0$  mm; mediolateral (ML),  $1.25$  mm left; dorsoventral (DV),  $1.35$  mm], at an approximate rate of  $200$  nl/min. The syringe was left in place for at least  $5$  min postinjection before being withdrawn. Virus injection with this approach into the stratum oriens preferentially labels LINC (Christenson Wick et al., 2019); Ivy and neurogliaform cells also express nNOS, but have a spatially restricted axonal plexus (Fuentelba et al., 2008; Armstrong et al., 2012), which likely limits virus uptake. However, any Ivy cells at the site of injection could potentially express eYFP-ChR2 with this approach. Any such off-target labeling should result in a very minimal number of Ivy cells expressing eYFP-ChR2, as they would need to be in close proximity to the injection site. Ivy cells are inhibitory and should, if anything, increase the efficacy of intervention if they inadvertently also expressed ChR2 and received light. Flp-negative littermates were injected in the same manner and with the same virus as cNOS-fDLX-positive mice. All on-demand experiments and tissue collection began at least 6 weeks post-viral injection.

### Epilepsy induction

We primarily used the IHKA mouse model of chronic TLE, in which KA is injected directly into the hippocampus (Bouilleret et al., 1999; Zeidler et al., 2018). In this model, weeks after KA injection, animals display spontaneous, recurrent seizures, which can be recorded electrographically from the hippocampus (Riban et al., 2002). The IHKA epilepsy model replicates key factors of pathology observed in TLE patients, including unilateral hippocampal sclerosis marked by partial cell loss, granule cell dispersion, and mossy fiber spouting (Suzuki et al., 1995; Bouilleret et al., 2000; Twele et al., 2016).

At least 13 d post-viral injection, animals received an intrahippocampal injection of KA. Specifically,  $90$ – $100$  nl of  $18.5$  mM KA in sterile saline (SA) was injected with a microliter syringe into the hippocampus (distance from bregma: AP,  $-2.8$  mm; ML,  $2.15$  mm left; DV,  $1.9$  mm). Note that this injection site is posterior to the viral injection to avoid direct overlap, and that KA injection was performed at a later time point to allow viral expression before this insult. For PV cell quantification (see below), C57BL/6J mice received an intrahippocampal KA injection in the same manner as cNOS-fDLX mice.

In a separate set of experiments, the intra-amygdala KA model of chronic temporal lobe epilepsy (Ben-Ari et al., 1980; Li et al., 2008; Mouri et al., 2008; West et al., 2022; BLA-KA) was used instead. In these cNOS-fDLX animals,  $200$  nl of  $18.5$  mM KA in sterile saline was injected into the basolateral amygdala (distance from bregma: AP,  $-1.0$  mm; ML,  $3.25$  mm left; DV,  $5.0$  mm). A subset of BLA-KA mice (three of five mice) underwent electrode and optic fiber implantation (see below). Hippocampal recordings from the three implanted BLA-KA mice all displayed spontaneous recurrent seizures, and one mouse displayed a high enough frequency of seizures to enable on-demand intervention; the majority of seizures detected in this animal during on-demand optogenetic seizure intervention were overtly behavioral.

An additional set of animals was used as controls for eYFP-positive cell counts and PV-immunohistochemistry experiments (see below). For this, opsin-positive cNOS-fDLX or C57BL/6J mice were injected with  $90$ – $100$  nl of  $0.9\%$  sterile saline in the same manner as IHKA injections.

For all epilepsy-induction and saline-control surgeries, mice received local bupivacaine, were rapidly recovered from anesthesia (within  $5$  min of injection; Twele et al., 2016), were treated with NeoPredef, and received no postsurgical analgesics to avoid interfering with epilepsy induction.

### Electrode and optic fiber implantation

At least 2 weeks post-epilepsy induction, mice were implanted with a recording electrode and an optic fiber. The hippocampal local field potential (LFP) was monitored by a bipolar twisted (local differential) microwire electrode ( $50$   $\mu$ m diameter stainless steel with polyimide insulation; P1 Technologies) implanted at  $2.4$  mm posterior,  $2.0$  mm left, and  $1.75$  mm ventral to bregma (IHKA mice), or  $3.1$  mm posterior,  $3.6$  mm left, and  $3.4$  mm ventral to bregma (BLA-KA mice). In all mice, an optic fiber, constructed in-house [diameter,  $200$   $\mu$ m;  $0.39$  numerical aperture (NA)], was implanted for light delivery at  $2.2$  mm posterior,  $1.2$  mm left, and  $1.35$  mm ventral to bregma. Mice received standard surgical treatments of carprofen ( $5$  mg/kg, s.c.), local bupivacaine at the start of surgery, and postsurgical NeoPredef treatment around the implant. Additionally, mice were treated with ibuprofen ( $50$ – $80$  mg/kg/d dissolved in drinking water, for 1 presurgical and 3 postsurgical days). Following implant surgery, mice recovered for at least 1 week before the start of electrophysiological recordings.

### Closed-loop seizure detection and optogenetic LINC activation

Simultaneous 24 h video LFP recordings of mice were used for on-demand optogenetic seizure intervention as described in the study by Armstrong et al. (2013). Hippocampal LFP was transmitted via electrical patch cables connected through an electrical commutator (P1 Technologies), amplified by an analog amplifier (model 410, Brownlee), digitized (National Instruments), and analyzed using custom MATLAB software. Online seizure detection

was tuned for each animal, based on ictal spiking properties such as spike amplitude, minimum and maximum spike width, and interspike interval. For each seizure detected, there was a 50% chance that blue light (wavelength ~473 nm; via laser (Shanghai Laser & Optics Century Co., Ltd.) or LED (Plexon)) would be delivered to the hippocampus via an optical patch cord (ThorLabs) through an optical commutator (Doric; Fig. 1C, example LFP traces). Thus, for each mouse about half of detected seizures received light, and half did not receive light, in a random manner, providing an internal within-animal comparison. Light was delivered either at 6.67 Hz with a 33.3% duty cycle (50 ms light pulses) for 30 s, or at 1 Hz with a 95% duty cycle (1 s light pulses) for 3 s, matching previous protocols (Krook-Magnuson et al., 2013, 2014b). Previous work supports that LINC is activated by a train of 50 ms light pulses, which elicits repeated postsynaptic inhibition (Christenson Wick et al., 2019). The power of light delivered through the tip of the implanted fiber, measured *post hoc*, was  $4.9 \pm 0.4$  mW. Across animals, there was no correlation between light power and the percentage of reduction in seizure duration with light ( $p = 0.60$ , Spearman's correlation).

On-demand testing in IHKA mice started 1 month post-KA injection. A subset of mice was tested for longer, for which we conducted on-demand testing once a month for up to 6 months post-KA injection. Another subset of mice started at a later time point and were tested monthly up to 6 months post-KA injection. At each time point, mice received two rounds of on-demand testing, one with each light stimulus (7 and 1 Hz stimulation). For each round of intervention, testing was conducted across both light cycles (i.e., across the entire circadian cycle).

### Tissue processing and cell counting

The specificity of the virally mediated intersectional genetics approach was confirmed, and quantification of eYFP-positive somata was conducted, via tissue processing and epifluorescence microscopy (microscope model DM2500, Leica). Tissue was collected immediately from all saline-injected mice after they were killed and, when possible, from KA-injected mice following on-demand testing. While the IHKA model is not traditionally considered a model of sudden unexpected death in epilepsy (SUDEP) per se, a number of animals died before the 6 month time point because of seizures. Brains from these mice were collected as soon as possible, but were only used to verify opsin expression and targeting. They were not used for cell quantification. All brains were drop fixed in 4% paraformaldehyde (PFA) and sectioned into 50  $\mu$ m coronal sections via vibratome (model VT1000S, Leica) that were mounted with DAPI (VECTASHIELD Antifade Mounting Media with DAPI, catalog #H-1200, Vector Laboratories) as a 1-in-4 series. With the exception of tissue from one mouse that was collected too long postmortem, a lack of eYFP expression was assessed and confirmed in all opsin-negative tissue ( $n = 19$  of 20 mice). Similarly, the expression of eYFP in opsin-positive tissue was confirmed from all but 3

opsin-positive mice ( $n = 22$  of 25 mice); these 3 animals had unusable tissue.

Tissue that was collected immediately after mice were sacrificed was used for quantification of eYFP-expressing cells. This included 10 brains from opsin-positive IHKA mice (5 following 1 month post-KA intervention; 5 following 6 month post-KA intervention), and 7 from opsin-positive saline-injected mice (1–6 months postinjection; cell count not correlated with time post-saline injection;  $p = 0.38$  Spearman's correlation). The number of eYFP-positive somata in the hippocampal formation spanning from ~1.0 to 3.8 mm posterior to bregma was counted manually in every fourth 50  $\mu$ m section (14 50  $\mu$ m sections total per animal) in all planes of focus at 20 $\times$  magnification (model DM2500 microscope, Leica), in a manner similar to that in the study by Christenson Wick et al. (2019). Representative images in Figure 2 were obtained via confocal imaging at 10 $\times$  and 40 $\times$  magnification on an Olympus FluoView BX2 (University Imaging Center, University of Minnesota).

To quantify KA-induced changes in the number of PV-immunopositive cells, at 1 month postinjection, tissue was collected from six and five intrahippocampal KA-injected and SA-injected C57BL/6J mice, respectively. Brains were drop fixed in 4% PFA for 2 d immediately following mice being sacrificed and then sectioned into 50  $\mu$ m sections. Matching quantification protocols for eYFP cells, a 1-in-4 series across the anterior–posterior extent of the hippocampus was used. To increase the penetration of antibodies, sections were suspended in 100% methanol for 3 h before free-floating PV immunostaining (1:1000; guinea pig anti-PV; GP72, Swant) in red. Sections were mounted with DAPI and imaged at 10 $\times$  magnification (total internal reflection fluorescence (TIRF) scope, Zeiss; University Imaging Center, University of Minnesota). In total, 14 slices per animal spanning ~1.0 to 3.8 mm posterior to bregma were used for the quantification of PV-immunopositive cells. Automated quantification of PV-immunopositive cells was conducted using background subtraction and additional thresholds, such as surface area, volume, and sphericity (Imaris software), followed by manual inspection of every slice and correction of cell counts, as necessary.

The approximate volume of tissue that would receive direct light stimulation was estimated using the Deisseroth Laboratory, Stanford University, Stanford, CA, online light transmission calculator (<https://web.stanford.edu/group/dlab/cgi-bin/graph/chart.php>). The optic fibers used had a NA of 0.39, a radius of 100  $\mu$ m, and a flat cleaved tip. The average power measured *post hoc* from the fiber tips (4.9 mW) was used. An index of refraction for brain tissue of 1.35 (Binding et al., 2011) and an irradiance value of 1 mW/mm<sup>2</sup> for ChR2 activation (Aravanis et al., 2007) were assumed. Accordingly, blue light delivery (473 nm) reached a depth of 0.9 mm. Given a half-angle of ~16.8°, the maximum radius of the cone of light was ~0.3 mm. Thus, with the optic fiber implanted at 2.2 mm posterior to bregma, light was directly delivered to a tissue volume expanding from ~1.9 to 2.5 mm posterior to bregma along the anterior–posterior axis.

## Statistical analyses

After detection, seizure duration was determined offline using a combination of manual and automated processes (Krook-Magnuson et al., 2013, 2014a; Streng and Krook-Magnuson, 2020; Streng et al., 2021). Similar to online detection, user-defined aspects of ictal spiking were defined and used for automated analysis of the duration of seizures following detection. All automated detections were subsequently manually inspected, and at all steps in the process experimenters were blinded to light condition. For IHKA mice, a minimum of 90 seizures per light condition detected within 48 h were used for analysis; datasets with fewer seizures were excluded. Because of a lower rate of seizures in BLA-KA mice compared with IHKA mice, a dataset of 60 seizures per condition detected over 12 d was used for analysis. Two-sample Kolmogorov–Smirnov (KS) and two-tailed Mann–Whitney (MW) tests were conducted to compare postdetection seizure duration between light and no-light events at the individual animal level. Seizure duration was considered statistically different with light only if both KS and MW  $p$ -values were  $<0.05$ .

Reduction in seizure duration was calculated at the individual level as a percentage of the duration reduction and as the difference of the average seizure duration of light and no-light seizures in seconds.

To determine whether light delivery changed seizure duration at the group level within a genotype, a paired Wilcoxon test was used to compare average no-light and light seizure durations. Cohen's  $d$  is one metric for assessing effect size, with a value of  $<0.2$  considered to indicate a small effect size (Cohen, 1977). For each experimental group (e.g., opsin-positive, opsin-negative animals), Cohen's  $d$  was calculated as the difference between average seizure duration with and without light delivery, divided by the pooled standard deviation ( $SD_p$ ; Eq. 1), as follows:

$$SD_p = \sqrt{((SD_1^2 + SD_2^2)/2)}, \quad (1)$$

where  $SD_1$  and  $SD_2$  are the SDs of each group.

For additional within-genotype group analysis, 90–100 events per condition were randomly sampled (via MATLAB) from each animal and used to create summative histograms to compare light and no-light seizure duration distributions via a two-sample KS test.

To assess the change in seizure reduction or duration over time, or with the power of light delivered, a Spearman's correlation was conducted.

To compare the percentage of reduction and the magnitude of seizure reduction in seconds between genotypes, a two-tailed MW test was used. To determine whether light-mediated reduction in seizure duration varied across genotype and either sex or stimulation parameter settings, a two-way ANOVA was used. To compare the proportion of animals with a statistically significant reduction in seizure duration with light across genotypes, the Pearson's  $\chi^2$  test was used.

To compare the number of eYFP-positive cells among saline, IHKA, and BLA-KA tissue, a Kruskal–Wallis ANOVA was used. To further compare the number of eYFP-positive cells between saline-injected and KA-injected tissue (IHKA

or BLA-KA), a two-tailed MW test was used. To compare the number of PV-immunopositive cells between saline and IHKA tissue, a two-tailed MW test was used. To compare the number of PV-immunopositive cells and eYFP-positive cells in IHKA within the volume of tissue receiving direct light stimulation, a two-tailed MW test was used. To assess eYFP cell count over time in saline-injected tissue, a Spearman's correlation was conducted.

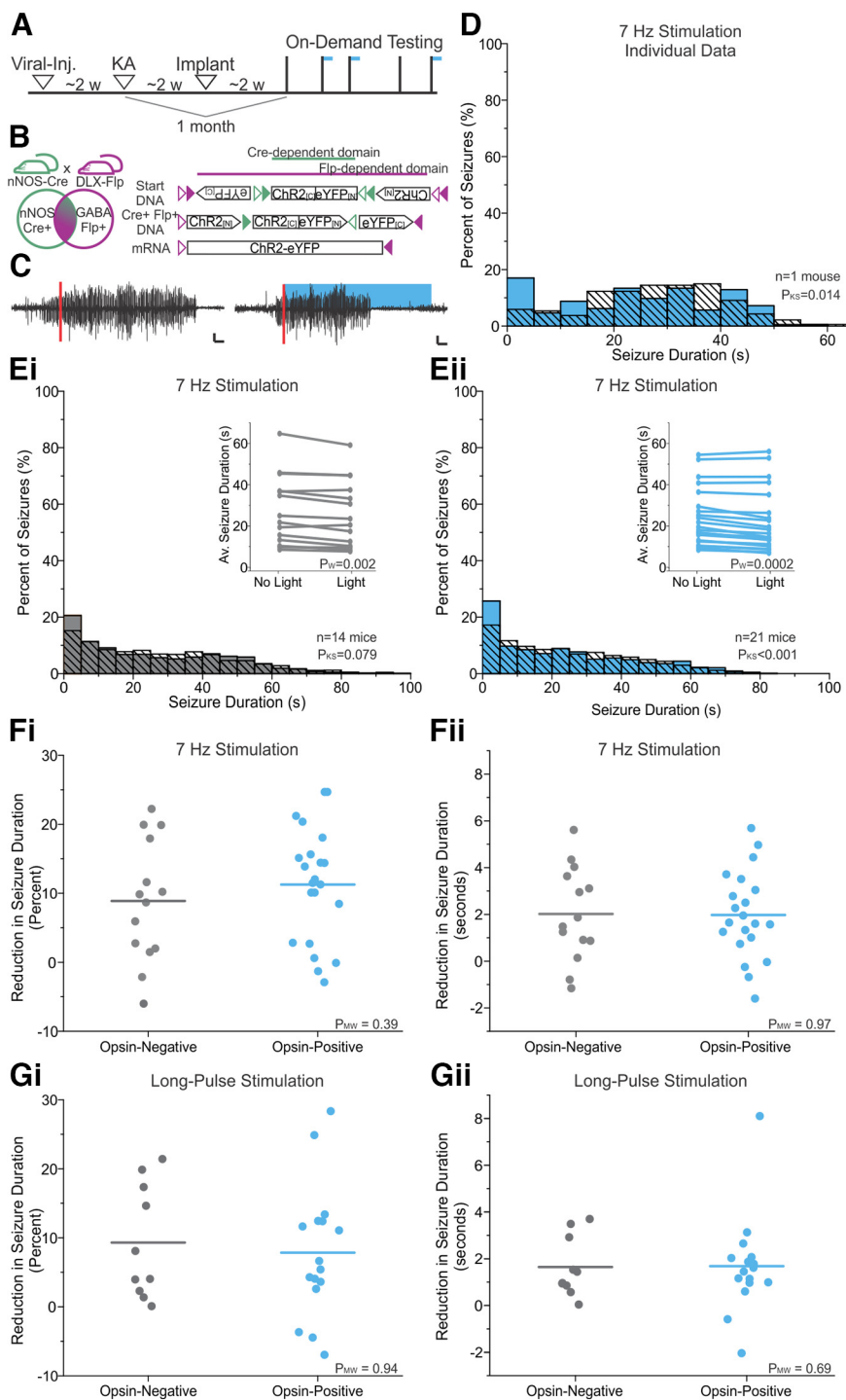
Statistical analyses were conducted using MATLAB (versions 2016–2021b) and OriginPro software (2016). For all statistical tests, a  $p$ -value of  $<0.05$  was considered statistically significant. All values are reported as the mean  $\pm$  SEM, unless noted otherwise.

## Results

### On-demand activation of LINC does not substantially influence seizure activity 1 month after epilepsy induction

LINC have been identified recently as a powerful and widespread source of inhibition in the hippocampus (Christenson Wick et al., 2019). We therefore examined whether activating LINC could provide seizure control using on-demand optogenetics to selectively activate LINC at the time of hippocampal seizures. As illustrated in Figure 1, A and B, we used a viral intersectional genetics approach to label and express ChR2 in LINC in CA1 of the hippocampus. We then induced epilepsy via intra-hippocampal KA injection and implanted a recording electrode (for seizure detection) and an optic fiber (for light delivery). Online seizure detection triggered light activation of LINC for 50% of detected events at random, providing a no-stimulation internal control (Fig. 1C, example trace). Opsin-negative animals served as an additional control and underwent identical experimental procedures. We first examined the impact of light delivery at  $\sim 7$  Hz with a 50 ms pulse width for 30 s; these stimulation parameters were chosen as they have previously been demonstrated to be effective for PV interneuron-mediated on-demand optogenetic seizure suppression in this same model of epilepsy (Krook-Magnuson et al., 2013).

At the individual animal level, the majority of opsin-positive animals showed no significant effect of light delivery; 13 of 21 ( $\sim 62\%$ ) opsin-positive animals had no significant effect of light delivery. In the remaining eight opsin-positive animals, light delivery statistically reduced seizure duration compared with control no-light seizures ( $p < 0.05$  for KS and MW tests in these animals). However, statistically significant seizure reduction in these animals did not correspond with sizeable suppression, as illustrated in Figure 1D, which shows seizure duration distributions for an example animal with a statistically significant reduction in seizure duration with light; note the lack of cluster effect. In this animal, seizure durations were reduced by only 12.0%, corresponding to a duration reduction of just 2.5 s. The maximum observed percentage of reduction across animals was only 24.7%, and this corresponded to a reduction in seconds of just 5.7 s (Fig. 1Eii, Fii). This indicates that even in individual animals where a statistically significant seizure duration reduction was achieved, effects remained small.



**Figure 1.** One month post-epilepsy induction, on-demand activation of LINC does not provide strong seizure control. **A**, Experimental timeline. On-demand seizure intervention starts at least 6 weeks post-viral injection and 1 month post-KA injection to induce epilepsy. During on-demand testing, the hippocampal LFP is continuously monitored and analyzed online for seizure detection (indicated by vertical lines), which triggers pulsed blue light delivery for 50% of seizures in a random manner (horizontal blue lines). **B**, An intersectional genetic viral vector approach is used to label LINC, an nNOS-expressing GABAergic cell population. Double-expressing offspring of nNOS-Cre and Dlx5/6-Flpe mice (left) are injected with a Cre-dependent and Flip-dependent virus (right) to mediate expression of eYFP-tagged channelrhodopsin for optogenetic activation and fluorescent labeling of LINC. Flip-negative littermates receive a viral injection in the same manner and are used as opsin-negative controls. Schematics were adapted from the studies by Christenson Wick et al. (2019) and Fenno et al. (2014). **C**, Example electrographic seizures detected (red vertical line) from an opsin-positive mouse, triggering on-demand seizure intervention (right trace; 7 Hz blue light stimulus for 30 s) or not receiving light (left trace; used as an internal control). Scale: 500  $\mu$ V, 2 s. **D**, Post-detection seizure durations during light (solid blue) and no-light (hashed black) conditions from an animal with a statistically significant—but still very minor—effect of light (seizure

continued

duration reduction: 12%, 2.5 s;  $p < 0.05$ , MW and KS tests). **Ei, Eii**, Postdetection seizure duration distributions at the group level. Solid gray bars (control animals; **Ei**) or blue bars (opsin-positive animals; **Eii**), seizures receiving 7 Hz light stimulation; black hashed bars, no-light seizures. Insets, Average seizure duration during no-light and light conditions by animal. **Fi, Fii**, Reduction in seizure duration by animal, as percentage of seizure duration reduction (**Fi**) or difference in seizure duration in seconds (**Fii**) for light (7 Hz stimulation) versus no light. Gray dots, control animals; blue dots, opsin-positive animals. **G**, Same as **F**, but for long-pulse light delivery (1 s on, 50 ms off, for 3 s). Note that while some individual animals showed a statistically significant response to light (**D**, example), and while there is a slight decrease in the overall seizure duration distribution in opsin-positive animals (**Eii**), there is no difference between opsin-positive and opsin-negative animals in effect size across animals (**F, G**), and no animal shows a strong response to light (maximum percentage seizure reduction is  $<30\%$ ; **Table 1**, summary of statistical analyses).

Further underscoring the lack of successful intervention in opsin-positive animals, changes in seizure durations reached statistical significance in a similar proportion of opsin-negative animals (5 of 14 for opsin-negative animals vs 8 of 21 for opsin-positive animals,  $p = 0.13$ , Pearson's  $\chi^2$  test), suggesting that the small, sometimes statistically significant, effects observed with opsin-positive animals are unlikely to reflect meaningful effects of LINC activation.

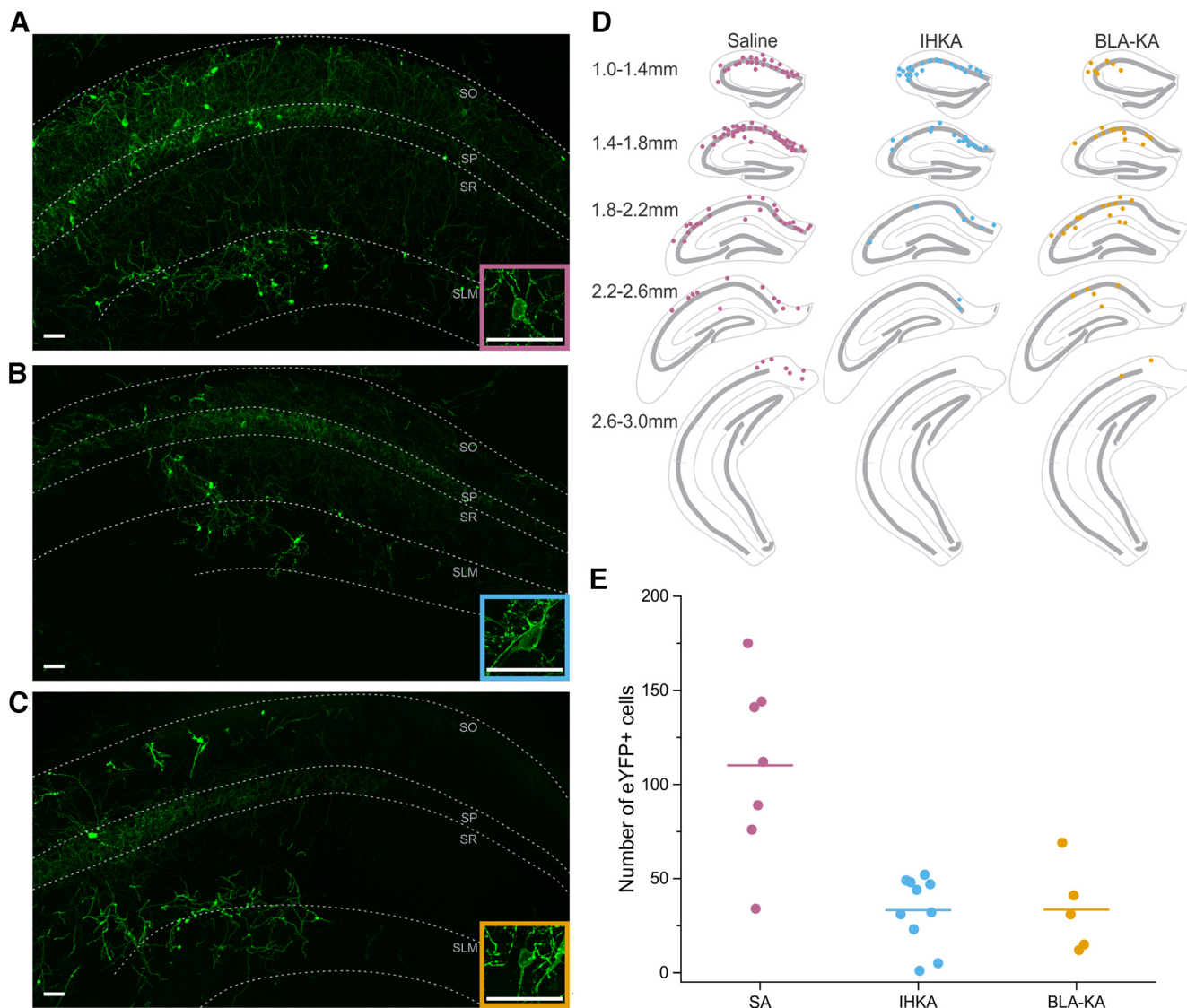
Across opsin-positive animals with 7 Hz stimulation, there was an average  $11.3 \pm 1.8\%$  decrease in seizure duration (**Fig. 1Fi**), corresponding to only a  $2.0 \pm 0.41$  s reduction in average seizure durations (**Fig. 1Fii**), and a small effect size of light delivery (Cohen's  $d = 0.14$ ).

These reductions were not significantly greater than those observed in opsin-negative animals (**Fig. 1F**;  $8.9 \pm 2.3\%$  decrease or  $2.0 \pm 0.53$  s decrease in seizure duration in opsin-negative animals; opsin-positive vs opsin-negatives:  $p = 0.39$ , MW test for percentage reduction;  $p = 0.97$ , MW for reduction in seconds), which also demonstrated a small effect of light delivery (Cohen's  $d = 0.12$ ). No apparent sex differences were observed across genotypes for either measurement of light-mediated reduction (percentage reduction: genotype,  $p = 0.34$ ; sex,  $p = 0.07$ ; interaction,  $p = 0.39$ ; reduction in seconds: genotype,  $p = 0.90$ ; sex,  $p = 0.20$ ; interaction,  $p = 0.55$ ; two-way ANOVAs).

**Table 1: Statistical analyses**

Reference	Test	<i>n</i>	<i>p</i> -value
Fig. 1D	Kolmogorov–Smirnov	194 light, 187 no-light seizures	0.014
Fig. 1Ei, inset	Paired Wilcoxon	14 mice	0.002
Fig. 1Ei	Kolmogorov–Smirnov	14 mice	0.079
Fig. 1Eii, inset	Paired Wilcoxon	21 mice	0.0002
Fig. 1Eii	Kolmogorov–Smirnov	21 mice	3.6889E-8
Fig. 1Fi	Mann–Whitney	14 opsin-negative, 21 opsin-positive mice	0.39
Fig. 1Fii	Mann–Whitney	14 opsin-negative, 21 opsin-positive mice	0.97
Fig. 1Gi	Mann–Whitney	10 opsin-negative, 16 opsin-positive mice	0.94
Fig. 1Gii	Mann–Whitney	10 opsin-negative, 16 opsin-positive mice	0.69
Fig. 1Fi–Gii	Two-way ANOVA	14 opsin-neg., 7 Hz stim; 21 opsin-pos., 7 Hz stim; 10 opsin-neg., long-pulse stim; 16 opsin-pos., long pulse stim mice	0.66; 0.66; 0.53
Results	Two-way ANOVA	6 opsin-neg., male; 8 opsin-neg., female; 12 opsin-pos., male; 9 opsin-pos., female mice	0.34, 0.07, 0.39
Fig. 2E	Kruskal–Wallis ANOVA	7 saline-injected, 6 kainate-injected (intra-hippocampal), 5 kainate-injected (intra-amygdala) mice	0.006
Fig. 2E	Mann–Whitney	7 saline-injected, 6 kainate-injected (intra-hippocampal) mice	0.004
Fig. 2E	Mann–Whitney	7 saline-injected, 5 kainate-injected (intra-amygdala) mice	0.015
Results	Mann–Whitney	5 saline-injected, 6 kainate-injected mice	0.008
Results	Mann–Whitney	6 C57BL/6J mice, 10 cNOS-fDLX mice	0.002
Fig. 3Ai	Spearman's correlation	46 on-demand intervention sessions; 21 mice	0.033
Fig. 3Aii	Spearman's correlation	65 on-demand intervention sessions; 21 mice	0.0015
Fig. 3Bi	Spearman's correlation	46 on-demand intervention sessions; 21 mice	0.39
Fig. 3Bii	Spearman's correlation	65 on-demand intervention sessions; 21 mice	0.28
Fig. 3Ci	Spearman's correlation	38 on-demand intervention sessions; 17 mice	0.0063
Fig. 3Cii	Spearman's correlation	51 on-demand intervention sessions; 19 mice	0.00014
Fig. 3Di	Spearman's correlation	38 on-demand intervention sessions; 17 mice	0.056
Fig. 3Dii	Spearman's correlation	51 on-demand intervention sessions; 19 mice	0.013
Materials and Methods	Spearman's correlation	24 opsin-positive, 20 opsin-negative mice	0.60
Materials and Methods	Spearman's correlation	7 opsin-positive saline-injected mice	0.38
Results	Spearman's correlation	46 on-demand intervention sessions; 21 mice	0.12
Results	Spearman's correlation	65 on-demand intervention sessions; 21 mice	1.95E-5
Results	Spearman's correlation	38 on-demand intervention sessions; 17 mice	0.078
Results	Spearman's correlation	51 on-demand intervention sessions; 19 mice	2.57E-5





**Figure 2.** Substantial reduction of LINC in epileptic tissue. eYFP-expressing somata were quantified from a 1-in-4 series of 50  $\mu$ m hippocampal slices from virus-injected cNOS-fDLX mice. **A–C**, Representative images of eYFP expression in CA1  $\sim$ 1.5 mm posterior to bregma. SO, Stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum moleculare. Scale bars, 50  $\mu$ m. **A**, Widespread eYFP expression in a representative saline-injected mouse [overall cell count in this animal, 112 (equal to the median of all saline animals)]. Inset, Single eYFP-expressing cell from the same animal. **B**, eYFP expression in an IHKA mouse (overall cell count in this animal, 48). Inset, Single eYFP-expressing cell from the same animal. **C**, eYFP expression in an intra-amygdala kainate (BLA-KA) mouse (overall cell count in this animal, 41). Inset, Single eYFP-expressing cell from the same animal. **D**, Example distributions of eYFP-positive somata along the hippocampal anterior–posterior axis in saline (left), IHKA (middle), and BLA-KA (right) mice (same animals as shown in **A–C**). **E**, The number of eYFP-positive cells was different between saline-injected, intrahippocampal kainate-injected, and intra-amygdala kainate-injected mice ( $p = 0.006$ , KW test, ANOVA). Control mice injected with saline have an average cell count of  $110 \pm 18.1$  (SA, left). IHKA epileptic mice (IHKA, middle) had a significant reduction in the number of eYFP-positive cells, with an average cell count of  $33 \pm 5.8$  (vs SA count,  $p = 0.004$ , MW test). Despite the site of initial insult being outside the hippocampus, BLA-KA animals (right) also had a significant reduction in the number of eYFP-positive cells in the hippocampus (average cell count,  $33.6 \pm 10.3$  vs SA count,  $p = 0.015$ , MW test). Dots represent individual mouse values, and lines represent average group values (Table 1, a summary of statistical analyses).

### Using a different stimulation pattern does not improve outcomes

To test whether the lack of LINC-mediated influence on seizure activity was possibly because of the pattern of light stimulation, we tested an additional set of stimulation parameters previously used to successfully inhibit seizures

through on-demand optogenetics (Krook-Magnuson et al., 2014b). Specifically, we tested 1,000 ms long pulses delivered for 3 s. At the group level, effects on seizure duration were small and not different between genotypes (Fig. 1G; opsin-positive:  $8.9 \pm 2.2\%$ ,  $1.7 \pm 0.5$  s reduction; opsin-negative:  $9.3 \pm 2.6\%$ ,  $1.6 \pm 0.4$  s reduction; opsin-positive

vs opsin-negative:  $p = 0.94$ , MW test for percentage reduction;  $p = 0.69$ , MW test for reduction in seconds; Cohen's  $d$  opsin-positive, 0.15; opsin-negative, 0.09). Overall, across genotypes the average and range of light-mediated seizure reduction with long-pulse stimulation was similar to 7 Hz stimulation (Fig. 1, compare  $F$ ,  $G$ ; percentage reduction: opsin-positive vs opsin-negative,  $p = 0.66$ ; long-pulse vs 7 Hz stimulation,  $p = 0.66$ ; interaction,  $p = 0.53$ ; reduction in seconds: opsin-positive vs opsin-negative,  $p = 0.99$ ; long-pulse vs 7 Hz stimulation,  $p = 0.51$ ; interaction,  $p = 0.93$ ; two-way ANOVAs). Therefore, regardless of the stimulation parameters used, there is little impact of LINC activation on seizures; at 1 month post-KA injection, LINC activation did not meaningfully influence seizure activity, despite LINC providing widespread inhibition in healthy tissue (Christenson Wick et al., 2019).

### A substantial reduction in the number of LINC is observed in epileptic mice

Given that LINC is a powerful source of inhibition in healthy tissue (Christenson Wick et al., 2019), we were surprised that LINC-mediated seizure suppression provided so little seizure control. Given the large circuit changes that can occur in temporal lobe epilepsy, we examined whether LINC persisted in the epileptic tissue. Cell counts were conducted from 10 opsin-positive animals that received intervention, including 5 animals that had received on-demand optogenetic intervention 1 month post-KA injection, and 5 that had received on-demand optogenetic intervention up to 6 months post-KA injection (see next section). For comparison, cell counts were also conducted for saline-injected opsin-positive animals, with tissue collected 1–6 months post-saline injection. Robust labeling of LINC was seen in saline-injected control animals (Fig. 2A,E), extending along the anterior–posterior extent of the hippocampus (Fig. 2D), as previously reported (Christenson Wick et al., 2019). A dramatic reduction in eYFP-labeled cells was observed in epileptic animals (Fig. 2B,D,E;  $p = 0.004$ , MW test), indicating that LINC is vulnerable in epileptic tissue. Note that viral labeling of LINC occurred before KA injection, and therefore the lower number of labeled LINC is unlikely to reflect differences in initial transduction. This substantial loss of LINC in epileptic mice may contribute to the inability to inhibit seizures via on-demand optogenetic activation of LINC.

Given that on-demand optogenetic activation of PV-expressing interneurons has previously been shown to inhibit hippocampal seizures in the IHKA model (Krook-Magnuson et al., 2013), we additionally examined the vulnerability of PV cells in our hands. While PV cells make up <5% of neurons in the hippocampus (Woodson et al., 1989; Freund and Buzsáki, 1996), PV-immunopositive cell numbers in saline-injected animals far outnumbered eYFP-labeled LINC (1800 ± 168 PV-immunopositive cells counted over 14 sections; one in four series). In IHKA animals, the number of PV-immunopositive cells was significantly reduced (825 ± 61 cells;  $p = 0.008$  vs saline controls, MW test; 54% reduction). This suggests that both PV cells and LINC are vulnerable populations.

While a number of additional factors will play critical roles in the ability of light delivery to impact seizures, we directly compared the relative numbers of PV-immunopositive cells and eYFP-ChR2-expressing LINC in the vicinity of light delivery in IHKA animals. Ignoring axonal activation, these cells are those that would be activated by light delivery. Given the calculated potential spread of light, we examined three sections centered around the location of the light source. In these sections, we counted 231 ± 34 PV-immunopositive cells in saline controls, which was reduced to 49 ± 17 cells in IHKA animals (representing a 79% reduction in cell numbers). In contrast, even in saline-injected controls, we found only 38 ± 6 eYFP-labeled LINC in these three sections, and this was further reduced to only 9 ± 2 eYFP-labeled cells in IHKA tissue. While this represents a similar percentage reduction (76% decrease), the result is a stark contrast in the raw number of remaining cells (nearly 50 vs <10;  $p = 0.002$  MW). Therefore, while both populations of interneurons are impacted in the IHKA model, the remaining number of targetable cells is much higher for PV interneurons than LINC.

### Moving the site of initial insult does not change outcomes

The noted LINC cell loss could have resulted from epilepsy itself (e.g., seizures) or could have been a consequence of injecting KA directly into the hippocampus. To begin to examine whether LINC loss is specific to the intra-hippocampal kainate model, and correspondingly whether LINC activation might be more efficacious in a different model, we turned to the intra-amygdala model of temporal lobe epilepsy. In this model, KA is not injected into the hippocampus, but is instead injected into the basolateral amygdala (BLA-KA; Ben-Ari et al., 1980; Mouri et al., 2008), and spontaneous hippocampal electrographic and behavioral seizures are observed during the chronic phase (Li et al., 2008). We tested on-demand optogenetic activation of LINC in one BLA-KA animal but found no significant change in seizure duration ( $p = 0.89$ , KS test). In fact, we saw a slight (5.8%) increase in average seizure duration with light in this animal. This suggests that the lack of LINC-mediated seizure control is not model specific. Additionally, despite a different site of initial direct injury, we found that there was still a significant reduction in the number of eYFP-positive cells in BLA-KA animals compared with saline-injected controls (Fig. 2E,  $p = 0.015$ , MW test; Fig. 2C, D, animal illustrated is the animal that received on-demand intervention). Thus, regardless of the site of KA injection, a large reduction in the number of LINC was observed in epileptic animals, which may impact the potential for LINC-mediated seizure suppression.

### Lack of LINC-mediated seizure suppression up to 6 months post-epilepsy induction

Axonal sprouting of inhibitory neurons in epilepsy has been observed (Zhang et al., 2009; Peng et al., 2013; Soussi et al., 2014), including sprouting that occurs 4–6 months post-KA injection (Christenson Wick et al., 2017). As axonal sprouting at later time points post-KA injection

could potentially compensate for inhibitory cell loss and allow more meaningful seizure control, we examined whether LINC-mediated influence on seizures would change over time. We tested animals with 7 Hz and long-pulse stimulation, as done at the 1 month post-KA injection time point (described above), each month for up to 6 months post-KA injection.

In opsin-positive animals, the average percentage reduction in seizure duration slightly increased over time with 7 Hz stimulation of LINC (Fig. 3Aii, Table 1;  $p = 0.0015$ ,  $r_s = 0.39$ , Spearman's  $\rho$  correlation). However, the percentage reduction remained small at all time points (Fig. 3). Moreover, when the reduction in seizure duration was measured in seconds, there was no such correlation with time since epilepsy induction (Fig. 3Bii;  $p = 0.28$ , Spearman's correlation). This suggests that the change in percentage reduction may reflect a difference in overall seizure durations (average seizure duration without light vs time:  $p = 1.95 \times 10^{-5}$ ,  $r_s = -0.50$ ; average seizure duration with light delivery versus time:  $p = 2.56 \times 10^{-5}$ ,  $r_s = -0.50$ ), rather than a change in the effectiveness of light per se. Note that not all animals have data from all time points. Importantly, LINC-mediated reduction in seizure duration remained modest even at 6 months: the maximum reduction in seizure duration with 7 Hz stimulation in opsin-positive animals was 34.7%, but this corresponded to only a 3.2 s reduction in average seizure duration in this animal. The maximum reduction in seconds was similarly modest, and was only 4.6 s (corresponding to 17.0%).

Further underscoring that no meaningful seizure control was ever achieved in opsin-positive animals, a similar slight increase in percentage reduction of seizure durations over time post-KA injection was also observed with 7 Hz stimulation in opsin-negative animals (Fig. 3Ai;  $p = 0.033$ ,  $r_s = 0.31$ , Spearman's correlation). This was accompanied by no change across time in absolute reduction in seconds (Fig. 3Bi;  $p = 0.39$ ,  $r_s = 0.13$ ) nor average seizure duration with or without intervention (average seizure duration without light:  $p = 0.12$ ,  $r_s = -0.23$ ; average seizure duration with light-delivery:  $p = 0.078$ ,  $r_s = -0.26$  Spearman's correlation).

Similar to 7 Hz stimulation, long-pulse stimulation showed a slight increase in percentage reduction over time, but this was again very modest and was observed in both opsin-negative and opsin-positive animals (Fig. 3C). A very slight increase over time was observed for seizure reduction measured in seconds in opsin-positive animals, but the impact of light remained modest and was never significantly greater than that seen in opsin-negative animals (Fig. 3D). Altogether, these results support the idea that, even at later time points post-KA injection, LINC activation is unable to substantially influence seizure activity.

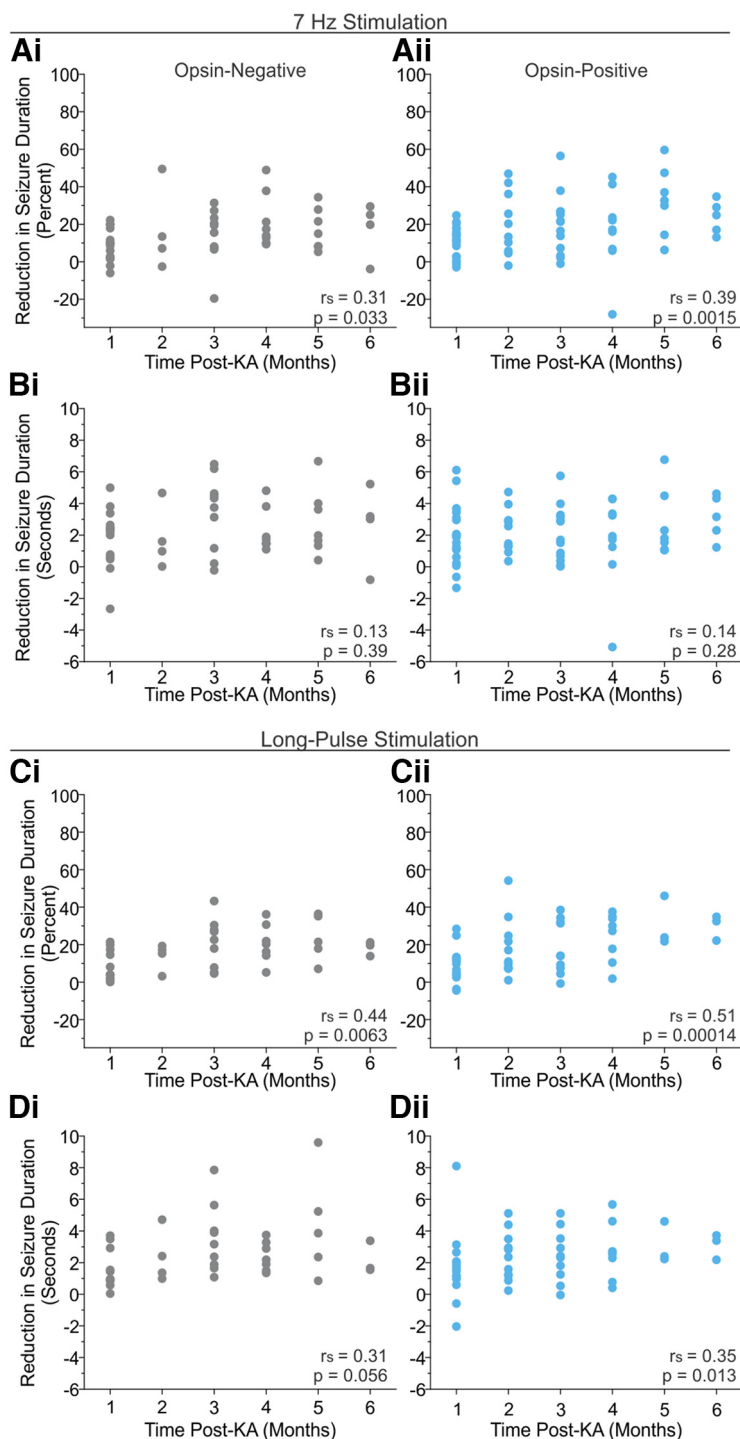
## Discussion

In healthy tissue, hippocampal LINC provide widespread inhibition mediated by both GABA<sub>A</sub> and GABA<sub>B</sub> postsynaptic receptors (Christenson Wick et al., 2019). We therefore hypothesized that they may be strong targets for seizure control using on-demand optogenetics.

However, regardless of the stimulation parameters chosen, or the time post-KA injection examined, no strong seizure control was ever achieved through activation of LINC. We noted a substantial reduction in the number of LINC in epileptic tissue, including when the site of initial insult was not in the hippocampus itself. This suggests that LINC are a vulnerable cell population in epilepsy, which may contribute to the lack of efficacy when targeting LINC for seizure control.

Optogenetics has been extensively used in the study of epilepsy (for review, see Christenson Wick and Krook-Magnuson, 2018; Cela and Sjöström, 2019; Walker and Kullmann, 2020). Previous work using on-demand optogenetics, with a variety of target strategies, has successfully inhibited seizures in the IHKA model of TLE (Krook-Magnuson et al., 2013, 2014b, 2015; Bui et al., 2018; Streng and Krook-Magnuson, 2020; Chen et al., 2021; Hristova et al., 2021; Streng et al., 2021). This includes work targeting populations in the hippocampus (Krook-Magnuson et al., 2013, 2015; Bui et al., 2018; Chen et al., 2021), indicating that this can be a successful approach—if the correct populations are manipulated in the correct way. On-demand optogenetics directly inhibiting principal cells in the hippocampus provided strong inhibition of electrographic seizures, with noted percentage reductions ranging from ~70% (Krook-Magnuson et al., 2013) to 75% (Krook-Magnuson et al., 2015). Previous work also found that on-demand optogenetic excitation of a different population of interneurons, PV-expressing interneurons, was able to inhibit seizures, although with less success than direct inhibition of principal hippocampal cells (Krook-Magnuson et al., 2013; Chen et al., 2021). The role of PV interneurons in seizures is complicated and depends on a range of factors, including the health of the tissue, whether seizures are acutely induced or spontaneously arising in epileptic tissue (and if induced, how and where), developmental stage and epilepsy type, and the relative timing of activation (Ellender et al., 2014; Sessolo et al., 2015; Assaf and Schiller, 2016; Lu et al., 2016; Shiri et al., 2016, 2017; Wang et al., 2017, 2022; Favero et al., 2018; Maguire, 2018; Lévesque et al., 2019; Magloire et al., 2019; Krook-Magnuson, 2020; Chen et al., 2021; Dudok et al., 2022; Kaneko et al., 2022).

Most relevant to the work presented here, however, is work using on-demand optogenetic excitation of PV interneurons in the hippocampus with the IHKA mouse model, which noted significant seizure inhibition, with an average percentage seizure duration reduction across animals of ~35–50% (Krook-Magnuson et al., 2013, 2014b; Chen et al., 2021). Notably, this was significantly greater than opsin-negative controls in those studies and is markedly higher than the average percentage seizure reduction achieved here with on-demand optogenetic activation of LINC. We found that LINC stimulation—with the same protocols used to successfully inhibit seizures via activation of PV cells—produced only an average 8.9–11% seizure duration reduction; these values were not significantly greater than opsin-negative controls. We initially hypothesized that on-demand optogenetic activation of



**Figure 3.** LINC activation does not substantially influence seizure duration for up to 6 months post-epilepsy induction. Reduction of seizure duration with light stimulation over time since the injection of KA to induce epilepsy. Note that, when possible, mice were tested at multiple time points, but some mice were excluded from some time points because of SUDEP or an insufficient number of events for analysis. **A, B**, The percentage reduction (**A**), but not the reduction in seconds (**B**), of seizure duration with 7 Hz light stimulation slightly increases over time. Light-mediated seizure suppression remains modest throughout. At 6 months post-KA, the maximum reductions are only 34.7% or 4.6 s, and at no point are effects notably larger in opsin-positive animals. **C, D**, Similar results occur with long-pulse light stimulation, with little change observed over time. At 6 months post-KA, the maximum reduction in seizure durations are only 34.8% or 3.7 s. The  $p$ -values correspond to Spearman's correlation ( $r_s$  = Spearman's Rho; Table 1, summary of statistical analyses).

LINCs would outperform seizure inhibition achieved by on-demand optogenetic activation of PV cells, as—in healthy tissue—LINCs provide strong inhibition to both deep and superficial CA1 pyramidal cells, and inhibition via both GABA<sub>A</sub> and GABA<sub>B</sub> mechanisms (Christenson Wick et al., 2019). In contrast, PV cells preferentially target deep pyramidal cells and primarily activate just GABA<sub>A</sub> receptors (Armstrong and Soltesz, 2012; Lee et al., 2014), which would be sensitive to a collapse in the chloride gradient (Dzhala and Staley, 2003; Cossart et al., 2005; Trevelyan and Schevon, 2013; Wang et al., 2017). However, the activation of LINCs failed to outperform the activation of PV cells and, indeed, failed to provide any meaningful seizure inhibition.

A number of factors may have contributed to the failure of on-demand optogenetic activation of LINCs to inhibit seizures. First and foremost, we noted a substantial decrease in the number of LINCs in epileptic tissue, resulting in very few remaining eYFP-ChR2-expressing cells. This, obviously, reduces the number of LINCs that can be activated by our on-demand optogenetic approach, limiting the potential impact of LINC activation. PV cells can also be lost in epilepsy (Maglóczy and Freund, 2005; Kuruba et al., 2011), including in the IHKA model of TLE (Marx et al., 2013). We found that the average number of PV-immunopositive cells in IHKA animals was reduced by 54% across the entire anterior–posterior axis, and by 79% specifically at the location light was delivered in this study. This percentage reduction at the site of light delivery was similar to the percentage reduction observed in eYFP-ChR2-labeled LINCs. How can PV cells then remain a viable target? First, the absolute number of remaining PV cells at the location of light delivery in this study was approximately five times that of labeled LINCs. Therefore, there would be substantially more cells available to potentially provide seizure control. Additionally, any axons in the path of light could additionally be activated. This is especially relevant, as it has been noted that inhibitory neurons can undergo axonal sprouting in epilepsy (Zhang et al., 2009; Peng et al., 2013; Soussi et al., 2014). Importantly, this includes sprouting of the axons of PV cells (Christenson Wick et al., 2017). In the IHKA model of TLE, long-range axonal sprouting from PV cells was noted 4–6 months post-KA injection (Christenson Wick et al., 2017). Axonal sprouting of surviving neurons may help compensate for cell loss. However, for LINCs, if axonal sprouting occurs, it appears to be insufficient to meaningfully impact outcomes: even at late time points, up to a full 6 months post-KA injection, on-demand optogenetic excitation of LINCs failed to provide meaningful seizure suppression (Fig. 3). Even without an epileptic insult, LINCs are a relatively sparse hippocampal neuronal population (Christenson Wick et al., 2019; compare also our saline cell counts), and sprouting of the axons of LINCs may be insufficient to overcome a further reduction in their numbers. Additionally, our experimental methods may not have allowed full capitalization of sprouting: most previous work targeting PV neurons in the IHKA model used a transgenic approach (Krook-Magnuson et al., 2013, 2014b), resulting in all PV neurons expressing channelrhodopsin. Therefore, any PV

cell now innervating the illuminated area would be able to contribute to seizure inhibition. In contrast to target LINCs, we had to take an intersectional genetic, viral vector-based approach. While we transfected LINCs for channelrhodopsin expression before the epileptogenic insult (to ensure robust transfection), and while we saw good viral spread with our approach (Fig. 2), areas more remote (including the contralateral hippocampus) would not be able to contribute to seizure suppression in the same manner. An additional difference between PV cells and LINCs is also worth noting: in addition to broadly inhibiting CA1 pyramidal cells, in healthy tissue LINCs also provide inhibition to inhibitory neurons. This could theoretically counteract the beneficial effects of inhibition they provide to pyramidal cells. However, many seizure medications increase inhibition broadly with at least some degree of success (Greenfield, 2013; Holtkamp, 2018; Kotloski and Gidal, 2022). Given the dramatic loss of LINCs in epilepsy, a more likely interpretation is insufficient inhibition, rather than poorly directed inhibition. However, a recent study did find greater on-demand optogenetic seizure control when targeting only hippocampal PV cells compared with broadly targeting hippocampal inhibitory interneurons (via DLX6/5; Chen et al., 2021).

We examined two different stimulation protocols (and neither worked), and we used light stimulation parameters that were previously successful when targeting other neuronal populations (Krook-Magnuson et al., 2013, 2014b). However, we cannot rule out that different experimental methods, including different light delivery paradigms, might be able to achieve greater seizure suppression via LINC activation. Previous work in healthy tissue demonstrated that a train of 50 ms light pulses produces repeated LINC-mediated postsynaptic inhibition, although, notably, there is substantial fatigue after the first light pulse (Christenson Wick et al., 2019).

Additionally, activation of LINCs *in vivo* revealed that 7 Hz stimulation alters hippocampal theta oscillations for several seconds, although, again, there was a decline in amplitude within the first 500 ms of light onset (Christenson Wick et al., 2019). Altogether, this provides evidence that LINCs can alter network activity over several seconds, with protocols similar to those used here, at least in healthy tissue. However, the substantial decrease in the amplitude of LINC-mediated inhibition over the course of the stimulation train may contribute to the poor seizure suppression observed. While it is unclear whether such fatigue could be overcome to allow for more successful intervention strategies, future work could potentially apply methods such as Bayesian optimization to examine whether LINC-mediated seizure suppression could be improved with different stimulation parameters (Stieve et al., 2023). Regardless of why LINC activation was unable to provide meaningful seizure control, and regardless of whether slightly better inhibition could have been achieved through different experimental methods, our results strongly indicate that on-demand activation of LINCs for seizure control is less robust than other approaches already demonstrated to be effective.

Our results also suggest that LINC<sub>s</sub> are a sensitive population, undergoing substantial cell loss in epileptic tissue. Importantly, moving the site of kainate injection did not rescue LINC numbers. Hippocampal cell death in BLA-KA mice is not atypical—pathologic changes in the hippocampus, including sclerosis marked by pyramidal cell loss, is observed following intra-amygdala kainate injections (Araki et al., 2002; Mouri et al., 2008). Preventing status epilepticus through the administration of diazepam before intra-amygdala KA injection did not alter the amygdalar lesion at the site of injection, but did result in a lack of hippocampal sclerosis (Ben-Ari and Lagowska, 1978). Thus, hippocampal sclerosis in BLA-KA mice is attributed to status and the propagation of seizures, further suggesting that seizures, rather than the (location of the) initial insult per se, caused LINC cell loss. As noted above, in healthy tissue, LINC<sub>s</sub> provide widespread inhibition within the hippocampus, and their loss therefore may contribute to phenotypes in epilepsy, including cognitive and affective phenotypes (Muldoon et al., 2015; Moxon et al., 2019; Shuman et al., 2020). LINC<sub>s</sub> also have long-range projections to areas outside the hippocampus, including the tenia tecta, the hypothalamus, and the medial septum (Christenson Wick et al., 2019). LINC<sub>s</sub> may play an important role in coordinating the activity between the hippocampus and these regions (Christenson Wick et al., 2019), and the loss of LINC<sub>s</sub> in epilepsy may also contribute to comorbidities and circuit dysfunction through the loss of this interregional communication. LINC<sub>s</sub> remain a poorly understood cell population, and a greater understanding of their role in healthy physiology and in epilepsy will be an important goal for future research.

There are a few important experimental aspects to this study to further consider. First, we examined the ability of LINC<sub>s</sub> to inhibit seizures in a model of chronic temporal lobe epilepsy that models not only seizures, but also hippocampus sclerosis (Suzuki et al., 1995; Bouilleret et al., 2000). Different results may have been achieved if we had instead used, for example, acute seizure induction in healthy tissue. Such results would then have failed to translate to situations with the (at times substantial) circuit changes that can happen in chronic temporal lobe epilepsy (de Lanerolle et al., 1992; Berg et al., 2009; Twele et al., 2016). The seizure model used—and subsequent differences in cytoarchitecture and pathology—has been associated with conflicting results across studies targeting other hippocampal inhibitory and excitatory cell populations. For example, the activation of mossy cells in the dentate gyrus was proconvulsant in an acute seizure model (Botterill et al., 2019), but anticonvulsant in the IHKA chronic epilepsy model (Bui et al., 2018). Second, even in the same model of temporal lobe epilepsy, there is considerable animal to animal variability. This highlights the importance of within-animal comparisons, but also the importance of considering subject dropout in results. Across time, animals that remained in our study showed, on average, a decrease in seizure durations with time. This resulted in an increase in the percentage of seizure reduction in time despite a relatively stable absolute reduction (in seconds)

of seizure duration. Importantly, some of the animals (presumably those with more severe initial epilepsy) were lost to SUDEP over the course of our experiments, which likely contributed to this observed change in seizure durations over time at the group level. A final important aspect to consider is that our experiments were well controlled, with both internal no-light controls and group comparisons to opsin-negative animals that received otherwise identical experimental procedures. The use of opsin-negative animals was critical, as it helped contextualize the small, but at times statistically significant, effects we did observe with light delivery. While some of this may be because of chance, and while effects in opsin-negative animals were always very small, it provided a key point of reference; without this context, we may have overinterpreted the occasional small, but significant, effect in opsin-positive animals.

Although light delivery always resulted in at most small effect sizes in opsin-negative animals, that any significant effect was observed is surprising. What may be driving this small, yet at times significant, effect? Light delivery alone can have impacts on neuronal systems through a variety of mechanisms. Light-induced tissue heating is one such possibility (Arias-Gil et al., 2016; Gysbrechts et al., 2016; Shin et al., 2016; Peixoto et al., 2020; Acharya et al., 2021). In the present study, following data collection, light power measured through the tip of the implanted optic fiber was an average of  $4.9 \pm 0.4$  mW. While this is not generally considered a high level of light for optogenetic studies, this amount of light could, in theory, have a small influence on neural activity via tissue heating (Arias-Gil et al., 2016; Acharya et al., 2021). However, we found that light power was not correlated with the percentage reduction in seizure duration with light ( $p = 0.60$ , Spearman's correlation), making it unlikely that tissue heating was the critical factor for the observed light-mediated effects. Another potential impact of light delivery alone is the alteration of neurologic activity via the visual pathway (Iaccarino et al., 2016; Jones et al., 2019; Chan et al., 2021). While optical connections were covered to mitigate external light delivery with stimulation, it did not prevent all of it. Therefore, a visual impact theoretically may have contributed to shorter average seizure durations with light. However, if mediated by a visual effect, it does not appear to be impacted by the frequency of light pulses, as the effects were similar between the two sets of stimulation parameters (opsin-negative percentage reduction, 7 vs 1 Hz;  $p = 0.44$ , MW test). Another potential source of light-mediated effects in opsin-negative animals is light-mediated alterations in blood flow (especially with blue light; Rungta et al., 2017). If light-mediated alterations in blood flow can alter seizures, this could be an interesting avenue for future investigations. However, the very small size of the noted effects is worth remembering. Any future investigations attempting to build on the finding would need to substantially increase the effect to have a meaningful impact on seizures. While any off-target effects of light were not large enough to be clinically relevant in our experiments, they are still an important experimental factor to consider when designing and

interpreting results. Consideration of all of these factors led us to the ultimate conclusion that on-demand optogenetic excitation of LINC neurons cannot provide robust seizure inhibition.

LINC neurons are an understudied hippocampal neuronal population, with a number of unique and exciting features (Christenson Wick et al., 2019). While they do not appear to be a strong candidate target for seizure interventions, a better understanding of their role in healthy physiology, and in turn the impact of LINC loss in epilepsy, will be important areas for future investigation.

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