Distinct Fastigial Output Channels and Their Impact on Temporal Lobe Seizures

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Despite being canonically considered a motor control structure, the cerebellum is increasingly recognized for important roles in processes beyond this traditional framework, including seizure suppression. Excitatory fastigial neurons project to a large number of downstream targets, and it is unclear whether this broad targeting underlies seizure suppression, or whether a specific output may be sufficient. To address this question, we used the intrahippocampal kainic acid mouse model of temporal lobe epilepsy, male and female animals, and a dual-virus approach to selectively label and manipulate fastigial outputs. We examined fastigial neurons projecting to the superior colliculus, medullary reticular formation, and central lateral nucleus of the thalamus, and found that these comprise largely nonoverlapping populations of neurons that send collaterals to unique sets of additional, somewhat overlapping, thalamic and brainstem regions. We found that neither optogenetic stimulation of superior colliculus nor reticular formation output channels attenuated hippocampal seizures. In contrast, on-demand stimulation of fastigial neurons targeting the central lateral nucleus robustly inhibited seizures. Our results indicate that fastigial control of hippocampal seizures does not require simultaneous modulation of many fastigial output channels. Rather, selective modulation of the fastigial output channel to the central lateral thalamus, specifically, is sufficient for seizure control. More broadly, our data highlight the concept of specific cerebellar output channels, whereby discrete cerebellar nucleus neurons project to specific aggregates of downstream targets, with important consequences for therapeutic interventions.

Key words: closed-loop interventions; fastigial nucleus; hippocampus; optogenetics; temporal lobe epilepsy

Significance Statement
The cerebellum has an emerging relationship with nonmotor systems and may represent a powerful target for therapeutic intervention in temporal lobe epilepsy. We find, as previously reported, that fastigial neurons project to numerous brain regions via largely segregated output channels, and that projection targets cannot be predicted simply by somatic locations within the nucleus. We further find that on-demand optogenetic excitation of fastigial neurons projecting to the central lateral nucleus of the thalamus—but not fastigial neurons projecting to the reticular formation, superior colliculus, or ventral lateral thalamus—is sufficient to attenuate hippocampal seizures.

Introduction
The cerebellum contains more than half of all total neurons in the CNS (Andersen et al., 1992), accounts for as much as 20% of the total oxygen consumption of the brain (Howarth et al., 2010), and is reciprocally connected with a large number of cortical and subcortical regions (Ramnani, 2006; Strick et al., 2009; Salmi et al., 2010; Ramnani, 2012; Kipping et al., 2013). Though canonically considered a motor control structure, mounting evidence indicates that the cerebellum is heavily involved in functions beyond this traditional framework (Schmahmann, 1996; Hilber et al., 1998; Leggio et al., 1999; Colombel et al., 2004; Popa et al., 2014; Yu and Krook-Magnuson, 2015; Schmahmann, 2019; Shipman and Green, 2020). Recent work has shown that the cerebellum can profoundly influence hippocampal function, with the ability to modulate hippocampal neuronal dynamics (Choe et al., 2018; Zeidler et al., 2020) and to alter hippocampal-dependent behavior (Rochefort et al., 2011; Lefort et al., 2019; Zeidler et al., 2020). The influence of cerebellar dynamics on hippocampal function, in addition to being a topic of great scientific interest, has the potential to meet an urgent translational need in temporal lobe epilepsy (TLE), a disorder characterized by chronic, spontaneous seizures typically arising in the hippocampal formation. TLE is the...
most common form of epilepsy in adults, but current treatment options have limited efficacy and carry the potential for problematic side effects, leaving 30–40% of epilepsy patients with uncontrolled seizures (Engel et al., 2012). We recently demonstrated that even very brief optogenetic interventions delivered to excitatory neurons in the cerebellar fastigial nucleus (FN) was highly effective at terminating hippocampal seizures in a mouse model of TLE (Streng and Krook-Magnuson, 2020a).

While recent work indicates a lack of a direct, monosynaptic, connection between the cerebellum and the hippocampus, at least in rodents (Rochefort et al., 2013; Bohe et al., 2019; Krook-Magnuson, 2020), the fastigial nucleus does project to >60 downstream targets (Fujita et al., 2020). This large number of output targets raises the possibility that seizure suppression via fastigial excitation requires the coordinated modulation of many areas (Ekelman Rooda et al., 2021). Alternatively, a specific output may be sufficient to inhibit seizures, and a long history of previous work suggests at least some degree of segregation of cerebellar outputs (Jansen and Jansen, 1955; Angaut and Bowsher, 1970; Noda et al., 1990; Fuchs et al., 1993; Teune et al., 2000; Zhang et al., 2016; Fujita et al., 2020). Fastigial targets of potential interest for hippocampal seizure control include the thalamus, superior colliculus (SC), and the reticular formation (Angaut and Bowsher, 1970; Batton et al., 1977; Bentivoglio and Kuypers, 1982; Andrezik et al., 1984; Angaut et al., 1985).

The cerebellum has numerous connections with thalamocortical networks (Middleton and Strick, 1998), and the fastigial nucleus in particular projects to several distinct thalamic nuclei (Haroon et al., 1981; Angaut et al., 1985; Fujita et al., 2020). Deep brain stimulation trials targeting the thalamus (albeit the anterior nucleus) have reduced seizures for some patients (Salanova et al., 2015), and activation of neurons in the deep cerebellar nuclei disrupt spike-and-wave discharges observed during thalamocortical absence seizures (Kros et al., 2015; Ekelman Rooda et al., 2021). Cerebellar connections to intralaminar and midline thalamic nuclei may be especially relevant for TLE, as they have been implicated in regulating limbic seizures (Wicker and Forcelli, 2016; Feng et al., 2017). While much of the current seizure literature has focused on the central lateral (CL) thalamus in the context of consciousness (Gummadavelli et al., 2015; Kundishora et al., 2017; Xu et al., 2020), the CL nucleus is of particular interest to us in the context of cerebellar-mediated seizure suppression: its activity is depressed (and bursty) during focal limbic seizures (Feng et al., 2017), and it provides a potential route (via the anterior cingulate) from the cerebellum to the hippocampus (Van der Werf et al., 2002; Rajasethupathy et al., 2015).

Another candidate region of interest is the SC, which, in addition to receiving fastigial input (Cohen et al., 1958; Roldan and Reinoso-Suarez, 1981; Fujita et al., 2020), is proposed to play a role in regulating seizure activity (Garant and Gale, 1987; Dean and Gale, 1989; Weng and Rosenberg, 1992) and can inhibit seizures in multiple animal models (Gale et al., 1993; Soper et al., 2016). The fastigial nucleus also has extensive projections to the reticular formation (Rasmussen, 1933; Andrezik et al., 1984; Zhang et al., 2016; Fujita et al., 2020), a collection of nuclei important for controlling brain states (Moruzzi and Magoun, 1949; Jones, 2003), which could potentially underlie seizure control (Ewell et al., 2015; Khan et al., 2018; Purnell et al., 2018; Streng and Krook-Magnuson, 2020b). Clearly, there are several strong candidate regions for mediating the seizure inhibition seen with on-demand fastigial stimulation, if concurrent excitation of multiple downstream targets is not required.

We therefore set out to examine fastigial outputs to the central lateral thalamus, the superior colliculus, and the medullary reticular formation, to determine (1) whether these areas are indeed targeted by separate fastigial neurons, (2) whether fastigial neurons targeting these areas also target other areas (and if so, which), and (3) whether modulation of any of these output channels, in isolation, is able to inhibit seizures. Items 1 and 2 provide important context for understanding any seizure outcome results. Using a dual viral targeting strategy, we labeled populations of fastigial neurons that project to the CL nucleus, SC, or reticular formation. Supporting previous literature, we find that these neurons represent largely distinct populations and project to generally segregating additional downstream areas. Using the intrahippocampal kainate mouse model of temporal lobe epilepsy and on-demand optogenetic manipulation of specific output pathways and online detection of spontaneous seizures (Krook-Magnuson et al., 2013; Armstrong et al., 2013), we further find that cerebellar control over hippocampal seizures can be achieved via activation of a specific output channel, rather than requiring broader network effects. Specifically, excitation of CL-projecting fastigial neurons, but not the other two output pathways examined, is able to robustly attenuate hippocampal seizures. Together, our results illustrate that different fastigial nucleus output channels carry different, therapeutically relevant, consequences.

Materials and Methods

Ethical approval

All experimental protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Animals

For all experiments, mice were bred in-house and had ad libitum access to food and water in all housing conditions. Black-6 mice (C57BL/6); stock #000664, The Jackson Laboratory) were used for initial examination of broad fastigial projections, for output channel-specific labeling and modulation, and for a subset of terminal stimulation experiments. Mice expressing Cre selectively in VGlut2-expressing neurons (B6.129S6(FVB)-Sk17atm1Wmrj; Mwar; stock #028863, The Jackson Laboratory; Vong et al., 2011) were also used for on-demand terminal stimulation experiments.

Animals were sexed at the time of weaning on the basis of external genitalia. Both male and female mice were used for all experiments. While experiments were not powered to test for sex differences, no trends of sex differences were observed. Until optical fiber and electrode implantation, animals were housed in standard group housing conditions in the Research Animal Resources animal facility at the University of Minnesota. Following implantation, animals were singly housed, and experiments were performed while housed in investigator-managed housing. In all conditions, animals were allowed ad libitum access to food and water, and were on a 12 h light/dark (low red light) cycle.

Stereotactic surgeries

Viral targeting. For all experiments, adeno-associated virus (AAV) serotype 9 was used for opsin expression in fastigial neurons because of its optimal expression in the fastigial nucleus with no apparent retrograde expression (Streng and Krook-Magnuson, 2020a). All injections were performed in adult mice (postnatal day 45 or later).

For initial characterization of fastigial fibers, Black-6 mice were injected with 120 nl of virus encoding green fluorescent protein (GFP) in a Cre-independent manner [AAV9-CAG-GFP; titer, 2 × 1013; lot #AV5221, UNC Vector Core (provided by Edward Boyden, Massachusetts Institute of Technology, Cambridge, MA)] via a Hamilton Neuros syringe into the left cerebellar fastigial nucleus (posterior, 6.48 mm; left, 0.75 mm; 3.7 mm ventral from bregma) under isoflurane anesthesia.

For experiments examining specific fastigial output channels, Black-6 mice were first injected with 120 nl of a retrograde virus encoding Cre [AAV9g-EF1a-mCherry-ires-Cre; titer, 1.37 × 1013; viral preparation
Fig. 5

Streng and Krook-Magnuson, 2020a
Klein et al., 2015
Streng and Krook-Magnuson, 2020a
Zeidler et al., 2018

... largely followed previously published protocols (mouse unilateral intrahippocampal kainic acid (KA) model of TLE [Armstrong et al. (2013); Bouilleret et al., 1999; Bragin et al., 1999; Krook-Magnuson et al., 2013, 2014; Gradinaru et al., 2007; Riban et al., 2002]. Animals that showed spontaneous hippocampal seizures weeks after KA injection were included in analyses (n = 1 animal). After every injection, the syringe was held in place for a minimum of 10 min before being withdrawn. On-demand interventions and/or characterization of fibers were conducted a minimum of 6 weeks post-viral injection.

For experiments targeting fastigial terminals, Black–6 mice were injected with virus encoding channelrhodopsin (ChR2) in a Cre-dependent manner [AAV9-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA; titer, 2.2 × 10^11; viral preparation #20198-AAV9, lot #V22125, Addgene (provided to Addgene by Karl Deisseroth); Gradinaru et al., 2007] consistent with our previously published methods for successful viral targeting of this nucleus (Streng and Krook-Magnuson, 2020a). Especially for injections targeting small structures, some virus may have reached neighboring structures; as noted in the Results section, in some instances, virus also reached the interposed nucleus (I_pb) in our fourth lateral off-target expression window excluded from analyses (n = 1 animal). After every injection, the syringe was held in place for a minimum of 10 min before being withdrawn. On-demand interventions and/or characterization of fibers were conducted a minimum of 6 weeks post-viral injection.

Epilepsy induction. Procedures for epilepsy induction using the mouse unilateral intrahippocampal kainic acid (KA) model of TLE largely followed previously published protocols (Cavalheiro et al., 1982; Bouilleret et al., 1999; Bragin et al., 1999; Krook-Magnuson et al., 2013, 2014; Gradinaru et al., 2007; Riban et al., 2002). Animals were removed from isoﬂurane a maximum of 5 min postinjection (Barkley et al., 2016). In this model, spontaneous recurrent electrographic seizures emerge from the damaged hippocampus, providing a strong model of pharmacoresistant (Ribon et al., 2002; Klein et al., 2015) temporal lobe epilepsy with hippocampal sclerosis (Bouilleret et al., 1999; Riban et al., 2002; Lévesque and Avoli, 2013; Zeidler et al., 2018). Only animals that showed spontaneous hippocampal seizures weeks after KA injection, as determined by video EEG monitoring, were included for on-demand optogenetic interventions.

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 (posterior, 2.6 mm; right, 1.75 mm; 1.6 mm ventral from bregma) for fastigial output channel-targeting experiments, mice were additionally implanted with optical fibers targeting the left fastigial nucleus (posterior, 0.6 mm; left, 0.75 mm; 2.5 mm ventral from bregma). For fastigial terminal targeting experiments, mice were implanted with optical fibers targeting the right central lateral nucleus of the thalamus (posterior, 1.34 mm; right, 0.75 mm; 2.75 mm ventral from bregma). Implants were secured to the skull using screws and dental cement following previous protocols (Armstrong et al., 2013), and mice were allowed to recover a minimum of 5 d before seizure monitoring and closed-loop interventions.

Postoperative care. For all surgical procedures, postoperative care consisted of recovery from anesthesia on a heated pad with regular visual inspection followed by daily postoperative monitoring for a minimum of 3 d to inspect comfort level and healing of the surgical site. Neo-Predef powder was applied to the closed incisions as a topical anti-biotic and analgesic. In the case of kainic acid injection, no additional postoperative analgesics were given. For viral injections and implantations, carprofen was administered subcutaneously (5 mg/kg) immediately before surgery. For implantations, preoperative and postoperative ibuprofen was also administered orally (50–80 mg/kg/d in water) as an additional analgesic.

Closed-loop seizure detection and interventions

A minimum of 5 d postimplantation, animals were placed in investigator-managed housing for chronic video and local field potential (LFP) recordings. Detection of electrographic seizures and on-demand optogenetic interventions generally followed previously published protocols (Krook-Magnuson et al., 2013; Streng and Krook-Magnuson, 2020a, Armstrong et al., 2013). Hippocampal LFP was recorded via electrical patch cords through an electrical commutator (PlasticsOne), amplified (Brownell), digitized (National Instruments), and analyzed in real time with custom MATLAB seizure detection software. A version of this software is available for download through Armstrong et al. (2013). Optogenetic interventions consisting of 3 s of pulsed light delivery (50 ms on, 100 ms off; Streng and Krook-Magnuson, 2020a) were triggered for 50% of events in a random fashion using this custom closed-loop MATLAB software and were delivered via LEDs (470 nm) through optical patch cords (THORLABS). Stimulation parameters were selected because of their efficacy in robustly attenuating hippocampal seizures when targeting the fastigial nucleus more broadly (Streng and Krook-Magnuson, 2020a) or the cerebellar cortex (Krook-Magnuson et al., 2014), and were originally selected to minimize movement side effects (Krook-Magnuson et al., 2014). Post hoc measurements of light delivery indicated an average LED power of 1.5 ± 0.6 mW at the tip of the implanted optical fiber.

Tissue harvesting and imaging of fibers

To characterize viral expression and confirm appropriate optical fiber targeting, after completion of on-demand interventions, mice were either deeply anesthetized with 5% isoﬂurane and decapitated or perfused with 0.1 M phosphate buffer followed by 4% paraformaldehyde. Brains were subsequently harvested and fixed in 4% paraformaldehyde. Sagittal brain sections of 50 µm were collected in 0.1 M phosphate buffer using a vibratome (model VT1000S, Leica). After sectioning, every third section was mounted with Vectashield mounting media with DAPI and covered with glass coverslips. Sagittal sections were initially visualized with epifluorescence microscopy (model DM2500, Leica), examining the entire brain in the one-in-three series, to identify brain regions with fibers and sections with structures of interest for confocal imaging. The approximate coordinates of the sagittal slices and structures of interest were determined using the third edition of the mouse brain atlas by Franklin and Paxinos (2007), in combination with DAPI and bright-field microscopy, which allowed for the discernment of areas of interest, including thalamic nuclei. Confocal imaging was performed on an upright confocal microscope (FluoView FV1000 BX2, Olympus; University Imaging Center, University of Minnesota), with ~400 × 400 µm fields of view used for subsequent quantification first selected using the DAPI signal to determine location (i.e., blinned to the specific enhanced yellow fluorescein- cent protein (eYFP) fiber locations/densities). Medial lateral coordinates for the structures of interest are as follows: SC, 0.72 mm right; MDv, 0.48 mm right; CL, 0.72 mm right; medial dorsal nucleus, lateral part (MDL), 0.72 mm right; medial dorsal nucleus, central part (MDC), 0.48 mm right; ventral medial (VM) nucleus, 0.72 mm right; VL nucleus, 0.72 mm right; parafascicular (PaF) nucleus, 0.6 mm right; zona incerta (ZI), 1.44 mm right; mesencephalic reticular (mRT) formation, 1.56 mm
right; laterodorsal tegmental (LDTg) nucleus, 0.48 mm right; IPAG, 0.84 mm right; ventral IPAG (vIPAG), 0.48 mm right; nucleus reticularis pontis caudalis (PrC), 0.48 mm right; parvo cellular reticular (PCRt) nucleus, 1.20 mm right; and spinal vestibular (SpVe) nucleus, 1.44 mm right. As noted in the Results section, this is a subset of areas in which fibers were seen. However, when an area is explicitly noted as not containing fibers, this is based not only on fiber quantification images, but also on careful viewing of the relevant area. Representative images were adjusted for brightness and contrast.

Quantification of fibers
After confocal images of eYFP-labeled fibers in structures of interest were taken from FN-SC, FN-MdV, and FN-CL mice (one image taken per location from 50 μm sagittal sections from three animals from each group), neurites within the imaged region of interest were measured using the Simple Neurite Tracer in Fiji by tracing each neurite and the corresponding branches as separate paths (note that quantification of fibers was performed for only a subset of areas showing expression). For the creation of a simplified summary schematic, the relative magnitude of fastigial fibers in a given location was assessed by comparing to the maximum observed in that area (for this, we additionally quantified fibers in a mouse injected with virus for broad expression of ChR2-GFP). Projections from a particular FN output channel to a region of interest were included in the summary schematic if the average total path length was at least 15% of maximum and was present at a minimum 15% level in at least two of the three animals quantified, with line thickness proportional to the percentage of the maximum of the average total path length for that output location. Every downstream area noted in the schematic had fibers present in each of the three animals quantified for that output channel.

Immunohistochemistry
Our procedures for VGluT2 immunohistochemistry largely follow our previously published protocol (Streng and Krook-Magnuson, 2020a). Briefly, every third sagittal section containing the downstream target of interest was blocked with 10% bovine serum and 0.5% Triton diluted in TBS, followed by overnight incubation with primary antibody for VGluT2 at 4°C (Millipore Sigma; 1:1000 diluted in TBS containing 2% bovine serum and 0.4% Triton). Tissue was then rinsed and incubated for 2 h with Alexa Fluor 594 anti-guinea pig (1:500 diluted in TBS containing 2% bovine serum and 0.4% Triton; Jackson ImmunoResearch). Following secondary incubation, tissue was mounted with Vectashield mounting media with DAPI and viewed for colocalization of virally labeled and immunolabeled fibers. For osteopontin (SPP1) immunohistochemistry, every third sagittal section of tissue containing the left fastigial nucleus was blocked with 5% bovine serum albumin for 1 h at room temperature before incubation in primary antibody goat anti-osteopontin overnight at 4°C (1:300; catalog #AF808, R&D Systems; RRID:AB_2194992). Tissue was then rinsed and incubated in donkey anti-goat Alexa Fluor 594 for 2 h at room temperature (1:500; catalog #A-11058, Thermo Fisher Scientific). Following secondary incubation, tissue was mounted with Vectashield mounting media with DAPI and viewed for colocalization of virally labeled and immunolabeled cell bodies using an epifluorescence microscope (model DM2500, Leica).

Statistical analyses
Seizure duration after the time of trigger and time to next seizure were analyzed offline using a combination of manual and automated methods consistent with our previous methods (Streng and Krook-Magnuson, 2020a). Software for automated analysis is available for download through GitHub (https://github.com/KM-Lab/Electrographic-Seizure-Analyzer; Zeidler et al., 2018). A minimum of 100 seizure events per animal per condition were processed automatically based on user-identified characteristics of spikes including amplitude, width, frequency, and deflection (positive, negative, or both), and the resulting postdetection seizure duration of all events were confirmed via manual inspection. Postdetection seizure duration distributions between light and no-light conditions were compared in each animal using two-sample Kolmogorov–Smirnov tests. Second, average postdetection seizure durations (one value per condition per animal) were compared, and, for visualization in figures, postdetection seizure durations with light intervention were normalized by dividing by duration without intervention (no-light internal controls for the same animal) and expressed as a percentage. A p value <0.05 was considered statistically significant. Statistical analyses were conducted using MATLAB. Values are presented as the mean ± SEM.

Results
Fastigial outputs target numerous downstream structures
On-demand excitation of the fastigial nucleus can robustly inhibit hippocampal seizures (Streng and Krook-Magnuson, 2020a). However, how the fastigial nucleus can produce such a profound influence on seizure activity is unclear. The fastigial nucleus projects to numerous downstream regions (Anguat and Bowsher, 1970; Batton et al., 1977; Bentivoglio and Kypers, 1982; Teune et al., 2000; Fujita et al., 2020). We first visualized fastigial projections to potential downstream targets of interest using a viral targeting strategy similar to our on-demand fastigial excitation work (Streng and Krook-Magnuson, 2020a). Specifically, we injected Black-6 mice with an AAV to achieve expression of the fluorescent protein GFP in neurons of the fastigial nucleus (AAV9-CAG-GFP; Fig. 1A). With this approach, we labeled fastigial neurons in both rostral and caudal portions of the fastigial nucleus (Fig. 1B,C), consistent with our previously published results (Streng and Krook-Magnuson, 2020a).

Matching previous reports (Haroon et al., 1981; Asanuma et al., 1983; Anguat et al., 1985; Gornati et al., 2018; Fujita et al., 2020), we found widespread axonal projection targets in numerous regions (Fig. 1), including the following three areas of particular interest regarding potential seizure suppression: the CL nucleus of the thalamus (Fig. 1Dii), SC (Fig. 1E), and MdV (Fig. 1Eii). As expected, fastigial terminals were visible in multiple other thalamic nuclei (Fig. 1D), including the ventral lateral (Fig. 1Dii), parafascicular (Fig. 1Dii), mediodorsal (Fig. 1Dv), ventral medial (Fig. 1Dvi), and zona incerta (Fig. 1Dvii) nuclei. In the midbrain, in addition to the superior colliculus (Fig. 1E), expression was also observed in other midbrain nuclei (Fig. 1F), including the mesencephalic reticular formation (Fig. 1Fii), lateral-dorsal tegmental nucleus (Fig. 1Fii), and both lateral and ventral lateral portions of the periaqueductal gray (Fig. 1Fiv,v). In the brainstem, fibers were observed in multiple regions of the reticular formation (Fig. 1Gii,iv,vii) and vestibular nuclei (Fig. 1Gii). We also observed fastigial fibers traveling caudally through the medulla, likely comprising cerebellospinal tracts (Fig. 1Gv). Only a subset of regions with GFP + terminals are presented here; we observed a large number of projection targets, consistent with a recent report identifying >60 downstream targets (Fujita et al., 2020). The expression pattern noted illustrates that broad labeling can be achieved with our injections and provides an important reference point for our next experiments looking at specific outputs using a dual virus approach and, ultimately, for our on-demand optogenetic experiments.

Fastigial outputs to the superior colliculus, medullary reticular formation, and central lateral nucleus comprise distinct populations
To examine whether neurons projecting to our three areas of interest (SC, MdV, and CL thalamus) represented distinct neuronal
Figure 1. Viral labeling illustrates the large number of downstream targets of fastigial neurons. A, Schematic of injection targeting, in which mice are injected with an AAV to induce the expression of GFP in fastigial neurons for broad labeling of fastigial outputs. B, C, GFP expression in fastigial neurons at two different magnifications. Dii–vii, Six weeks postinjection, GFP+ fastigial fibers are visible in the thalamus, including central lateral (ii), ventral lateral (iii), parafascicular (iv), medial dorsal (v), ventral medial (vi) and zona incerta (vii) nuclei. Blue box over white schematic indicates the approximate region of focus. Eii, iii, Fastigial fibers are also visible in the superior colliculus, including both deep and intermediate layers (ii, iii). Fii–iv, In the midbrain, fastigial fibers are observed in the mesencephalic reticular formation (ii), laterodorsal tegmental nucleus (iii), as well as both the lateral (iv), and ventral lateral (v) periaqueductal gray. Gii–iv, vi, vii, Extensive fastigial fibers are visible in the brainstem, including the medullary reticular formation (ii), vestibular nuclei, including the spinal vestibular nucleus (iv); and pontine reticular nuclei (vi, vii). Gv, Fibers are also visible in more caudal regions of the brainstem, likely traveling to the spinal cord. VM, ventral medial nucleus; PC, paracentral nucleus; scp, superior cerebellar peduncle; PaF, Parafascicular nucleus; SuG, superior gray layer; InG, intermediate gray layer; DpG, deep gray layer; LDTg, Laterodorsal tegmental nucleus; mRT, mesencephalic reticular nucleus; Gi, gigantocellular reticular nucleus; SpVe, Spinal vestibular nucleus; PCRT, Parvocellular reticular nucleus. All images are taken from sagittal sections; see Materials and Methods for approximate mediolateral coordinates for each region. Scale bars: B, 200 μm; C, 50 μm; D, G, 500 μm; E, Eii, F, 250 μm; Dii–vii, Eiii, Fii–iv, Gii–vi, 50 μm.

populations, as recently reported (Fujita et al., 2020), we used a dual viral strategy to achieve expression of ChR2-eYFP in populations of fastigial neurons defined by their projection targets. Specifically, mice were injected first in the downstream target of interest with an AAV designed to provide retrograde expression of Cre (retroAAV-Cre; Tervo et al., 2016), followed by injection of the contralateral fastigial nucleus with a Cre-dependent virus for ChR2-eYFP expression (Gradinaru et al., 2007; Fig. 2). The result of these dual injections is ChR2-eYFP expression only in fastigial neurons projecting to the downstream target of interest. We injected retroAAV-Cre into the SC (Figs. 2Av, 4Av, 4D), MdV (Fig. 2Bv), or CL thalamus (Fig. 2Cv), resulting in the labeling of fastigial neurons that project to the SC, MdV, or CL nucleus, henceforth referred to as FN-SC, FN-MdV, and FN-CL neurons, respectively.

FN-SC-labeled neurons (Fig. 2Ai) were located in the caudal portion of the fastigial nucleus (compare Fig. 2Ai, 1B, 3A). As expected, FN-SC neurons had strong labeling of terminals in the SC (Figs. 2Av, 4Av, D). Importantly, fibers from FN-SC neurons were largely absent from the MdV (Fig. 4Eh) and CL (Fig. 4L) nuclei. Conversely, FN-MdV neurons (Fig. 2Bi) were located throughout rostral and caudal portions of the fastigial nucleus (Figs. 2Bii, 3B), with some labeling extending into the interposed nucleus (Fig. 3B). Fibers from FN-MdV neurons were strongly present in the MdV (Figs. 2Bv, 4F, h), but not SC (Fig. 4Bv, D) or CL (Fig. 4L) nucleus. Similar to FN-SC neurons, FN-CL neurons (Fig. 2Ci) were preferentially located in the caudal portion of the fastigial nucleus (Figs. 2Ciii, 3C). Similar independence of expression was observed for FN-CL, with heavy labeling of terminals present in the CL (Figs. 2Cv, 4K), but not SC (Fig. 4Cd) or MdV (Fig. 4G, h). These results confirm that FN-SC, FN-MdV, and FN-CL cells represent distinct populations of fastigial neurons (Fujita et al., 2020). Note also that as all
three populations included at least some caudal labeling, full separation of these populations based solely on somatic location would not be possible.

As an additional step, we characterized whether FN-SC, FN-MdV, or FN-CL neurons expressed the molecular marker osteopontin (SPP1), which was recently characterized as a potential marker for subpopulations of fastigial neurons (Fujita et al., 2020). We found both SPP1+ and SPP1− FN-SC neurons were found. Bi, ii, iv, Same as for A, but for MdV injection targeting, in which fastigial cell bodies (i) are labeled after retrovirus injection in the MdV (ii, iv). Bi, v, FN-MdV cell bodies expressing ChR-eYFP are observed in both rostral and caudal portions of the fastigial nucleus (ii), and VGluT2+ fastigial fibers are present in the MdV (v). Cl–vi, Same as for A and B, but for CL retrovirus injection targeting (ii, iv), in which SPP1+ and SPP1− (vi) FN-CL neurons were labeled in the dorsal fastigial (ii) and eYFP terminals were located in the CL (vii). All images are taken from sagittal sections. Scale bars: Ai, Bi, Ci, 20 μm; Ai, ii, Cl, 100 μm; Ai, iii, Biii, Ciii, 200 μm; Av, vi, Biv, vi, Cv, vii, 10 μm; Av, Bv, Cv, insets, 2 μm.

Importantly, the location of fibers indicates that our dual-labeling approach effectively labels three distinct populations of fastigial neurons, in which fastigial neurons that project to the CL nucleus do not project to the SC or MdV, and so on. This is predominantly in keeping with recently reported findings (Fujita et al., 2020) and provided us with two important experimental benefits. First, given that distinct populations of neurons were labeled, this approach provided a means to directly target FN-SC, FN-MdV, or FN-CL somata for circuit dissection of seizure suppression, avoiding caveats associated with strategies optogenetically targeting terminals (e.g., insufficient light coverage). Additionally, this labeling approach allowed us to examine which (if any) other downstream regions each of these populations project to, providing greater insight into the circuit design of fastigial outputs.

We found that fastigial neurons that projected to the SC, MdV, or CL area did, indeed, also project to other areas and that, for many of these areas, segregation of these three output channels was largely maintained (Figs. 5-8).
Labeling of FN-SC neurons (Fig. 5) also resulted in labeled fibers in several thalamic nuclei, including the ventral lateral nucleus, and, to a lesser extent, the medial dorsal and ventral medial nuclei (Fig. 5B–D). Some expression was also observed in the parafascicular nucleus in one animal (Fig. 5E), and little to no expression was observed in the zona incerta (Fig. 5F). In other regions of the midbrain, expression was extremely limited (Fig. 5G–F). Similarly, essentially no fibers were observed in the pontine reticular formation (Fig. 5K) or vestibular nuclei, and we saw no evidence of FN-SC fibers traveling toward the spinal cord (Fig. 5L).

In contrast to FN-SC neurons, FN-MdV neurons (Fig. 6) send relatively limited inputs to the thalamus (Fig. 6B–F), with only the central portion of the medial dorsal nucleus showing dense FN-MdV fibers (Fig. 6B). Within the midbrain (Fig. 6G, H), strong fiber labeling was seen in the laterodorsal tegmental nucleus (Fig. 6H). The highest density of FN-MdV fibers was observed in the brainstem, with extensive terminals in regions of the pontine (in addition to medullary) reticular formation (Fig. 6I,J) and vestibular nuclei (Fig. 6K). This widespread brainstem expression also included the gigantocellular reticular nucleus, the oral part of the pontine reticular (PnO) nucleus, the medial vestibular nucleus, and the superior vestibular nucleus (not illustrated). FN-MdV fibers in the brainstem largely appeared limited to local terminals, as we did not observe strong evidence of FN-
MdV fibers traveling to the spinal cord (Fig. 6L). As we did note cell bodies in the interposed nucleus (IN; Fig. 3B), some of these fibers may be from IN-MdV neurons. The labeling of FN-CL neurons (Fig. 7) resulted in the labeling of fibers in additional thalamic nuclei, including the medial dorsal (Fig. 7B,C) and ventral medial (Fig. 7D) thalamic nuclei, with more limited projections to the parafascicular nucleus (Fig. 7F). FN-CL neurons also appear to send very sparse projections to the zona incerta (Fig. 7G). It should be noted that we cannot rule out limited direct viral labeling of fibers in thalamic nuclei neighboring the CL; however, we are able to see clear differences in thalamic fiber profiles between FN-CL, FN-SC, and FN-MdV injection schemes. FN-CL collaterals to the midbrain (Fig. 7H–K) and brainstem (Fig. 7L,M) were very limited. Finally, similar to FN-SC and FN-MdV neurons, and contrasting with what was observed with broad FN targeting (Fig. 1Gv), we did not observe evidence of FN-CL neurons sending projections to the spinal cord (Fig. 7M).

Quantification of fibers from FN-SC, FN-MdV, and FN-CL neurons further supports that these three populations send projections preferentially to largely segregated—but partially overlapping—thalamic, midbrain, and brainstem nuclei (Fig. 8). Overall, of these three output channels, FN-CL projecting neurons provided the strongest projections to the thalamus (Figs. 4L, 8A–F), with the notable exception of the VL (Fig. 8D), which received more input from FN-SC neurons, and the MDC (Fig. 8B), which received inputs from all three channels, but the heaviest came from FN-MdV neurons. In the midbrain, inputs from all three populations were largely limited and somewhat overlapping (Fig. 8G–J), with FN-MdV inputs to the laterodorsal tegmental nucleus being the most notable (Fig. 8H). While some fibers were found in the periaqueductal gray areas (Fig. 8I,J), these projections were extremely limited compared with the expression observed in animals with broad fastigial targeting (Fig. 1F). Finally, fastigial inputs to the brainstem were massively dominated by FN-MdV neurons, which send a high density of fibers throughout the medullary (Fig. 4) and pontine reticular formation (Fig. 8K,L), as well as the vestibular nuclei (Fig. 8M). Note that only a subset of targeted regions are illustrated in the figures and quantified in Figure 8.

Together, these results underscore that there are distinct output channels from the fastigial nucleus, and that FN-SC, FN-MdV, and FN-CL neurons comprise distinct populations, with different, largely segregated, sets of target regions. FN-SC and FN-CL neurons each send projections to unique thalamic nuclei, with more limited expression in the midbrain and brainstem (Fig. 8N,P). Conversely, FN-MdV neurons send numerous projections to several nuclei within the brainstem (Fig. 8O) and have extremely restricted projections to the thalamus. With this knowledge in hand, we were able to test the impact of optogenetic manipulation of these populations of neurons on seizures.

On-demand excitation of fastigial neurons projecting to the central lateral nucleus, but not SC or MdV, robustly attenuates hippocampal seizures

We used our dual viral strategy to implement on-demand optogenetic interventions in epileptic mice to determine whether fastigial influence over seizure activity can be achieved via FN-SC, FN-MdV, or FN-CL neurons. Chronically epileptic animals were implanted with recording electrodes in the hippocampus, and the local field potential signal was analyzed in real time to enable the detection of spontaneous electrophysiological seizure activity and subsequent closed-loop intervention (Krook-Magnuson et al., 2013; Armstrong et al., 2013). Light was delivered to the fastigial nucleus in animals expressing ChR2 selectively in FN-SC, FN-MdV, or FN-CL neurons. Light was delivered for half of detected

Figure 5. FN-SC neurons send collaterals to distinct thalamic and midbrain nuclei. A, Schematic of injection targeting for FN-SC neurons. B–F, In addition to strong fibers in the SC (Fig. 4A), FN-SC neurons display some fibers in the thalamus (B–F), especially in the VL nucleus (D). F, No fibers are observed in the ZI. G, H, J, In the midbrain, sparse fibers are observed in some regions including the mRT formation (G), LDTg nucleus (H), and vIPAG (J). I, K, Little to no fibers are observed in IPAG (I) or the brainstem, including the PnC (K). L, There is also no evidence of FN-SC neurons sending fibers to the spinal cord. All images are taken from sagittal sections. Abbreviations are as for Figure 1. Scale bars, 50 μm.
seizure events, in a random fashion, allowing each animal to serve as its own internal control.

Optogenetic excitation of FN-SC neurons (Fig. 9A) did not appear to have any effect on hippocampal seizures (Fig. 9B–D). Across the population, optogenetic excitation of FN-SC neurons failed to inhibit hippocampal seizures (Fig. 9D; \( p = 0.46 \), Kolmogorov–Smirnov test; Fig. 9D, inset: 8 ± 6% average reduction in seizure duration) with no significant effect of light
observed in any FN-SC animal tested (Fig. 9D, inset; not significant in six of six animals). We also observed no effect on the time to next seizure when modulating FN-SC neurons ($p = 0.31$, Wilcoxon signed-rank test; $n = 6$ animals).

Notably, because we targeted the somata of FN-SC neurons, we modulated the entire output channel with this approach. This would suggest that these fastigial outputs to other areas targeted by FN-SC neurons (e.g., ventral lateral thalamus) are also unlikely to contribute meaningfully to the seizure suppressive effects of fastigial modulation. To further explore this possibility, we used the same dual viral approach to target fastigial neurons traveling to the ventral lateral nucleus of the thalamus (Fig. 9E,F), which project to both the ventral lateral nucleus (Fig. 9G, left) and the superior colliculus (Fig. 9G, right). As with FN-SC modulation, on-demand excitation of FN-VL neurons failed to attenuate hippocampal seizures (Fig. 9H–J), with no significant effect of light delivery observed at the population (Fig. 9J, $p = 0.48$, Kolmogorov–Smirnov test; Fig. 9J, inset: average $8 \pm 13\%$ increase in seizure duration) or individual animal level (Fig. 9J, inset; not significant in four of four animals).

Together, these results suggest that the FN-SC/FN-VL outputs do not mediate the seizure suppression observed with broadly targeting FN output neurons.

We next assessed whether excitation of FN-MdV neurons would have any effect on hippocampal seizures (Fig. 10A). Similar to FN-SC neurons, optogenetic excitation of FN-MdV neurons did not robustly attenuate hippocampal seizures (Fig. 10B–D). Across the population, a modest, albeit statistically significant effect of light was observed (Fig. 10D: $p = 0.046$, Kolmogorov–Smirnov test; Fig. 10D, inset arrow, E). In this one animal, the measured reduction in seizure duration appeared to be more of a pause in seizure activity instead of a true attenuation (Fig. 10F–H), indicating that optogenetic excitation of FN-MdV neurons is insufficient to truly attenuate hippocampal seizures. As with FN-SC neurons, these results also suggest that other areas targeted by FN-MdV neurons (e.g., vestibular nuclei, MDC, pontine reticular formation) are unlikely to be driving fastigial control of hippocampal seizures.

In contrast to FN-SC and FN-MdV targeting, optogenetic excitation of FN-CL neurons was sufficient to attenuate seizures (Fig. 11A–C). Across the population, targeting FN-CL neurons robustly and consistently attenuated hippocampal seizures (Fig. 11D; $p < 0.001$, Kolmogorov–Smirnov test) with all animals (seven of seven; Fig. 11D, inset, average $34 \pm 17\%$ reduction in postdetection seizure duration) showing a significant effect of light delivery. These data indicate that on-demand activation of fastigial neurons projecting to the CL nucleus of the thalamus is sufficient for seizure inhibition. This finding is particularly striking given the lack of effect in either FN-SC or FN-MdV animals; note that FN-MdV animals not only had extensive fibers to numerous areas, but also had considerably more FN neurons.
labeled than FN-CL animals (Fig. 3), indicating that this effect cannot be explained by relative expression levels.

A potential benefit of pathway-specific modulation is a reduction in unwanted side effects. While a full examination of side effects, including motor effects, is outside the scope of this work, we did note a slight movement (i.e., a subtle "wobble") could be induced by broad FN optogenetic modulation (Movie 1) and that no such motor response was observed when stimulating FN-CL neurons (Movie 1).

In separate animals, we directly targeted the terminals of fastigial neurons in the CL (Fig. 11E,F). Despite caveats mentioned above associated with targeting terminals, we found significant inhibition of seizures with this approach (Fig. 11G: average 27 ± 9% reduction; p < 0.001, Kolmogorov–Smirnov test; Fig. 11G: inset, significant at the individual animal level in 7 of 11 animals). This further supports that the activation of FN-CL neurons is sufficient to attenuate hippocampal seizures.

Confirming that the effect of light delivery to the CL was mediated via opsin activation, no effect was observed for opsin–animals (injected with a virus for expression of GFP only) receiving light to the CL (Fig. 11Hf).
Figure 10. On-demand optogenetic excitation of FN-MdV neurons fails to attenuate hippocampal seizures. A, FN-MdV output channel and experimental schematic for targeting. B, Example seizure events detected (gray line) online that were randomly elected to either not receive light (top trace) or receive on-demand light delivery (bottom trace) to FN-MdV neurons. Blue bar indicates the timing of light delivery. C, Seizure durations from an example animal with targeting of FN-MdV neurons, illustrating no significant effect of light delivery ($p = 0.18$, two-sample Kolmogorov–Smirnov test). D, Population histograms of postdetection seizure durations across FN-MdV mice ($n = 600$ events, from six animals), showing a marginally significant effect of light delivery ($p = 0.046$, two-sample Kolmogorov–Smirnov test). Inset, One of six FN-MdV mice show a significant effect of light delivery (indicated by arrow). E, Histograms of postdetection seizure durations for events not receiving light (hashed bars) versus those receiving light (blue bars) in the one FN-MdV animal showing a significant effect of light delivery ($p = 0.04$, two-sample Kolmogorov–Smirnov test; 23% reduction in postdetection seizure duration with light in this animal). F, Example seizure events that were randomly elected either to not receive light (top trace) or to receive light delivery (middle and bottom traces) to FN-MdV neurons in that one animal, illustrating that the effect of light delivery, when present, is more of a pause in seizures than a true attenuation. G, Cumulative probabilities of the time to next seizure for seizure events receiving light (blue trace) and those not receiving light (gray trace), illustrating a trend toward a decrease in time to next seizure for events receiving light in this animal ($p = 0.05$, two sample Kolmogorov–Smirnov test; 13% reduction in time to next seizure). H, Seizure events that received light and stopped within 5 s tended to have a shorter time to next seizure (solid blue line) versus those that did not stop within 5 s (dotted blue line), again suggesting brief interruptions to seizures, rather than robust inhibition in this animal. Note that no light seizure events are not plotted, as only 12 no-light seizures lasted <5 s. Calibration: B, F, 5 s, 0.05 mV.

Figure 11. On-demand optogenetic activation of FN-CL neurons robustly attenuates hippocampal seizures. A, FN-CL output channel and experimental schematic for targeting. B, Example seizure events detected (gray line) online that were randomly elected to either not receive light (top trace) or to receive on-demand light delivery (bottom trace) to FN-CL neurons. Blue bar indicates the timing of light delivery. C, Seizure durations from an example animal with targeting of FN-CL neurons, showing a significant effect of light delivery ($p < 0.001$, two-sample Kolmogorov–Smirnov test). D, Population histograms of postdetection seizure duration events across FN-CL mice ($n = 700$ seizure events, from seven mice), showing a significant effect of light delivery ($p < 0.001$, two-sample Kolmogorov–Smirnov test). Inset, Normalized seizure duration for light versus no light across FN-CL mice, with seven of seven mice (100%) showing a significant reduction at the animal level. Black dot denotes average. E, To directly target FN fibers in the CL nucleus, mice were injected in the fastigial nucleus with virus encoding channelrhodopsin, followed by intrahippocampal kainic acid for the induction of epilepsy, and then implanted with electrodes in the hippocampus and optic fibers targeting the central lateral nucleus for on-demand interventions. F, Example detected seizure events that were randomly elected either to not receive light (top trace) or to receive light delivery (bottom trace) to fastigial fibers in the CL nucleus. Blue bar indicates the timing of light delivery. G, Population histograms of postdetection seizure duration events across FN-CL mice ($n = 1100$ seizure events, from 11 mice), showing a significant effect of light delivery ($p < 0.001$, two-sample Kolmogorov–Smirnov test). Inset, Normalized seizure duration for light versus no light across CL fiber targeting mice, with 7 of 11 mice showing a significant reduction at the animal level. Black dot indicates average. H, No effect of light delivery to the CL is observed in control animals ($n = 300$ seizure events from 3 mice, $p = 0.14$, two-sample Kolmogorov–Smirnov test). Calibration: B, F, 5 s, 0.05 mV.
Across the populations, in contrast to the effects seen in an FN-MdV animal, there was no effect on the average time to next seizure when targeting FN-CL neurons ($p = 0.297$, Wilcoxon signed-rank test), or when targeting fastigial terminals in the CL ($p = 0.240$, Wilcoxon signed-rank test). At the individual animal level, only one animal showed a significant effect of light delivery on the time to the next seizure, but it was an increase in time to next seizure with light (rather than a decrease). This suggests that FN-CL neurons produce a true seizure cessation (rather than a brief pause).

Together, these results show that fastigial attenuation of hippocampal seizures can be achieved by selectively exciting FN-CL neurons, but not by exciting FN-SC/FN-VL or FN-MdV neurons. Our findings argue against the need for broad modulation of fastigial outputs, as the activation of fastigial outputs to the CL thalamus, selectively, is sufficient to inhibit seizures. More broadly, our results underscore the concept of distinct fastigial output channels (Fig. 8N–P) and highlight that the existence of such distinct output channels can have important, therapeutically relevant, consequences (Figs. 9-11).

**Discussion**

Using viral approaches coupled with neuroanatomical tracing and on-demand optogenetics, this study reveals that a specific fastigial output channel is sufficient for the attenuation of hippocampal seizures. While the fastigial nucleus projects to a great many different target regions, we found that the organization of these outputs into segregated channels could be harnessed for selective modulation and seizure control. Specifically, fastigial neurons that project to the SC also project to other regions, including heavy projections to the ventral lateral thalamus; however, activation of this output stream did not provide seizure control. Cerebellar nuclear neurons projecting to the MdV also target central regions of the medial dorsal thalamic nucleus, and send dense, widespread projections to brainstem reticular and vestibular nuclei; activation of these neurons did not provide seizure control. Finally, fastigial neurons that project to the CL thalamus also project to the ventral medial and medial dorsal thalamic nuclei, and, to a lesser extent, areas including zona incerta and the parafascicular nucleus; excitation of this output channel was able to inhibit seizures. Together, these results indicate that fastigial control of hippocampal seizures does not require broad, concurrent manipulation of several fastigial output channels, as excitation of FN-CL neurons is sufficient. Notably, these findings also illustrate the functional relevance of distinct fastigial output channels, and the potential usefulness of understanding cerebellar circuitry at this level.

We previously demonstrated that fastigial excitation can robustly attenuate hippocampal seizures (Streng and Krook-Magnuson, 2020a). However, the lack of direct projections to the hippocampus (Strick et al., 2009; Rochefort et al., 2013; Watson et al., 2019; Krook-Magnuson, 2020) requires that seizure cessation be mediated via connections with at least one intermediate region. Despite being densely connected with a very large number of downstream targets (>60 total, as estimated by Fujita et al., 2020), we demonstrate that the fastigial output channel to the CL nucleus of the thalamus is sufficient for seizure suppression. An intralaminar nucleus considered to be part of the higher-order thalamus (Saalmann, 2014), the CL nucleus has been implicated in attention (Schiff et al., 2013), working memory (Wyder et al., 2004), and arousal (Van der Werf et al., 2002). Especially relevant in the context of epilepsy and seizures, it is hypothesized that the intralaminar thalamic nuclei serve to synchronize—and desynchronize—the cerebral cortex to coordinate the activity of cortical neurons and networks (Saalmann, 2014). Focal limbic seizures modulate neuronal firing in the CL nucleus, causing both an overall decrease in their activity levels and an increase in bursting (Feng et al., 2017). Additionally, electrical stimulation of the CL nucleus during acutely evoked hippocampal seizures can induce cortical desynchronization and improve measures of consciousness and arousal (Gummadavelli et al., 2015; Kundishora et al., 2017; Xu et al., 2020), suggesting that this nucleus can play an active role in both participating in and influencing seizure networks. Our data illustrate that perturbing activity in the CL nucleus may be able to inhibit seizures, as activation of FN-CL neurons attenuated spontaneous hippocampal seizures (Fig. 11). Notably, we recorded seizures in the hippocampus near the previous site of kainic acid injection and the presumed seizure focus, likely indicating that the CL is ultimately able to impact “upstream” hippocampal seizure activity. This may be through broad impacts on cortical synchrony (as noted above) or through specific effects—e.g., on the anterior cingulate cortex (Van der Werf et al., 2002), which is reported to have direct projections to the hippocampus (Rajasethupathy et al., 2015).

While our work used the intrahippocampal kainate mouse model of temporal lobe epilepsy, the ability of the cerebellum to influence seizures extends to regions outside the hippocampus (for review, see Streng and Krook-Magnuson, 2020b). It remains unknown whether cerebellar influence over different seizure networks is also mediated by neurons projecting to the CL. In a recent study examining thalamocortical absence seizures, activation of cerebellar nuclei terminals in the thalamus was able to attenuate spike-and-wave discharges, but little appeared to be mediated via the CL nucleus in particular (Eelkman Rooda et al., 2021). Indeed, that work tentatively concluded that cerebellar inhibition of absence seizures was mediated via multiple nuclear outputs (channels) working together. This is in stark contrast to our findings; differences in outcomes may be because of epilepsy type, the cerebellar nucleus targeted, or experimental methods (i.e., we primarily targeted somata within the cerebellar nucleus to avoid difficulties with selective and sufficient terminal activation).

It is important to note that our experiments examine the FN-CL output channel, which has collaterals to additional areas.
Even for our fiber-targeting experiments, in which light was delivered directly to the CL, we cannot rule out potential antidromic activation. As such, other areas that FN-CL neurons project to, including the VM nucleus of the thalamus, may contribute to the antiseizure effects shown here. The VM nucleus projects to cortical areas, including anterolateral motor cortex (Guo et al., 2018) and infralimbic prefrontal cortex (Sieveritz and Arbuthnot, 2020), and can target inhibitory neurons (and, in particular, layer 1 neurogliaform cells; Armstrong et al., 2012; Overstreet-Wadiche and McBain, 2015; Anastasiades et al., 2021). Neurons in the VM are highly modulated by spike-and-wave discharges during absence seizures (Paz et al., 2007), and targeting cerebellar terminals in the VM can provide some inhibition of spike-and-wave absence seizures (Eiklmam Roorda et al., 2021). However, the VM is also targeted by FN-SC neurons (Fig. 8C), albeit to a lesser extent, and the activation of FN-SC neurons did not inhibit hippocampal seizures (Fig. 9). Additionally, we cannot rule out that our CL viral injections did not result in some direct labeling of fastigial fibers in nearby nuclei, such as the PaF nucleus and MD. While the MD is of potential interest regarding seizure control (Cassidy and Gale, 1998; Bertram et al., 2008; Sloan et al., 2011; Wicker and Forcelli, 2016, 2021), the MD also receives input from FN-SC and FN-MdV, which were ineffective in inhibiting seizures. Either the VM nucleus and MD are playing little to no role in the seizure inhibition effects of FN-CL neurons, or FN-SC/FN-MdV projections to the VM nucleus/MD are insufficient to achieve seizure inhibition, and/or FN-SC/FN-MdV neurons and FN-CL neurons project to different populations of VM/MD neurons.

It is important to note that our results firmly show that not all thalamic nuclei that receive fastigial input provide seizure inhibition effects. Specifically, we show that the activation of FN-SC neurons, which have collaterals to the VL thalamus, does not inhibit seizures. We additionally directly targeted FN-VL neurons and, again, saw no inhibition of seizures. Therefore, fastigial inhibition of seizures is not mediated by fastigial-thalamic projections in a generic sense, but rather by FN-CL neurons specifically.

In this regard, in addition to identifying key fastigial outputs necessary for seizure cessation, our results provide important evidence as to which targets may not be involved in mediating the antiseizure effects of direct fastigial modulation. Given that direct optogenetic modulation of the SC is highly effective in disrupting seizures in several rodent models of different types of epilepsy (Soper et al., 2016), we initially reasoned that fastigial projections to the SC might be mediating fastigial inhibition of seizures. However, on-demand optogenetic excitation of FN-SC neurons failed to inhibit hippocampal seizures.

We also show that excitation of fastigial projections to the medullary reticular formation fails to attenuate seizures. This argues against the hypothesis that seizure suppression is mediated by a reticular formation-induced brain state change (Pfaff et al., 2012; Ewell et al., 2015; Khan et al., 2018; Purnell et al., 2018; Sakai, 2018; Streng and Krook-Magnuson, 2020b). Given the extensive fibers observed with FN-MdV labeling, as well as the distribution of cell bodies throughout the fastigial nucleus, the FN-MdV output channel may actually be composed of multiple subchannels. These subchannels may contain different aggregates of additional downstream targets, such as those described previously (Teune et al., 2000). None appear to mediate seizure inhibition. Our results additionally indicate that other regions receiving strong input from FN-MdV neurons, including the vestibular nuclei, are unlikely to be significantly contributing to the suppression of hippocampal seizures.

While we cannot rule out that other FN output channels not tested in this study may also be able to inhibit seizures, the robust effect of FN-CL modulation, coupled with the lack of an effect when modulating either FN-SC or FN-MdV output channels, strongly argues against the suppression of hippocampal seizures requiring coordinated changes across a large number of fastigial targets. Rather, there are particular pathways—starting with the FN-CL output channel—by which the cerebellum influences the hippocampus and hippocampal seizures. While a different pathway and/or output channel may be at play in healthy animals, it is worth noting that temporally precise modulation of hippocampal activity is observed with stimulation of cerebellar neurons in non epileptic animals (Choe et al., 2018; Zeidler et al., 2020). Additionally, multisynaptic rabies tracing, following injections into the hippocampal dentate gyrus (again, in non epileptic animals), results in labeling of neurons in the fastigial nucleus (Watson et al., 2019), further supporting a (multisynaptic) connection between these structures. Notably, this rabies labeling is seen in the caudal portion of the fastigial nucleus—the same region in which we observe FN-CL neurons (Fig. 3C)—suggesting that hippocampal activity is influenced by this subpopulation of fastigial neurons.

A long history of work has suggested the segregation of outputs, based largely on somatic location within the fastigial nucleus (Angaut and Bowsher, 1970; Bentivoglio and Kuypers, 1982; Noda et al., 1990; Fuchs et al., 1993; Teune et al., 2000), and, conversely, fiber collaterals allowing the same nuclear neuron to project to multiple downstream targets (Bentivoglio and Kuypers, 1982; Gonzalo-Ruiz and Leichnetz, 1987; Lee et al., 1989; Ruirok and Teune, 2014). However, we are just beginning to appreciate the full diversity and complexity of fastigial projections. For example, an excellent recent study examined output from fastigial neurons by subregion within the fastigial nucleus, and suggested five distinct excitatory output channels (Fujita et al., 2020), three of which bear many similarities to the populations we characterize here, including the following: (1) neurons in the caudal fastigial nucleus (and to some extent the caudal dorsolateral protuberance) projecting to areas including the CL, MD, and VM thalamus (similar to our FN-CL channel); (2) neurons in the caudal dorsolateral protuberance (and to some extent caudal fastigial) projecting to areas including the VL and PaF thalamus, zona incerta, and SC (somewhat in keeping with our FN-SC channel); and (3) neurons in more rostral portions of the fastigial, projecting to areas including the medullary reticular formation, vestibular nuclei, and spinal cord (apparently encompassing some of our FN-MdV channel and additional channels, as our FN-MdV neurons were located rostrally and caudally but did not project to the spinal cord). Fujita et al. (2020) identified a region of fastigial neurons that contains projections to the CL, ventral medial, and medial dorsal nuclei, and we specifically show that (some) fastigial neurons that project to the CL have collaterals to one or more of these additional targets. Given the partial blurring of anatomic regions within the fastigial seen by Fujita et al. (2020), it is difficult to know from those studies if the output channels identified are nonoverlapping. Indeed, our work suggests that while the FN-CL, FN-SC, and FN-MdV populations are distinct, there are areas of overlap that receive input from multiple output channels (e.g., the MD thalamus). Fastigial output channels therefore can send collateral projections to both convergent and divergent downstream targets. The finding that all three of the output channels we examined impinge on the MD thalamus warrants future study, including examination of whether additional output channels (e.g., FN neurons projecting...
to the spinal cord) may also send collaterals to the MD. Such a finding could explain previous work using dual retrograde injections with large target areas, which suggested that (some) FN neurons projecting to the spinal cord also ascend, to either the mesencephalic tegmentum or diencephalon (Bentivoglio and Kuypers, 1982). Our results suggest that the FN-SC and FN-CL output channels do not underlie these previous findings, and instead, that different locations (not SC and not CL) are the target of these ascending fibers. As very few colocalized fastigial cells were identified in the study by Bentivoglio and Kuypers (1982) after retrograde mesencephalic tegmentum injections paired with retrograde spinal cord injections, it is also possible that very sparse descending fibers in our FN-SC animals were missed (e.g., they were sufficiently limited as to be missed by examining a one in three series) or that there are subtle mouse versus rat differences. It is also possible that a very specific subset of FN-SC or FN-CL neurons, which our injection scheme failed to label, has projections to the spinal cord. In our opinion, this is a less likely scenario, but one that cannot currently be ruled out. Future work taking a dual viral approach similar to the one used here, but targeting the spinal cord, could provide insight into collaterals of fastigial-spinal fibers.

Together, our results underscore the complexity of fastigial outputs and projections, and highlight the relevance of this complexity. Rather than requiring broad modulation of fastigial outputs, or even broad activation of fastigial outputs to the thalamus, we show that seizure suppression is achievable via selective activation of FN-CL neurons (but not FN-SC, FN-VL, or FN-MdV neurons). This information provides important potential new avenues for therapeutic interventions.

References


