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The Efficacy of Ventral Pallidum- Deep Brain Stimulation in Rat models of Epilepsy

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Abstract

Antiepileptic drugs have been a primary option for patients with epilepsy worldwide, however, about one-third of patients do not respond to pharmacotherapy. For these individuals, resective surgery can be performed but seizures are still reported in some cases. With that in mind, neuromodulation or deep brain stimulation (DBS) is a plausible alternative to provide seizure freedom for refractory individuals. Vagus nerve stimulation (VNS), anterior thalamus DBS and responsive neurostimulation (RNS) are FDA approved as neuromodulatory approaches for epilepsy. They reduce and delay seizures but do not prevent or abolish seizures. In the previous study, the Shin lab showed that DBS of the ventral pallidum (VP) with a frequency of 50 Hz prevented partial and secondarily generalized seizures in the temporal lobe epilepsy rat model. While the data underscored a therapeutic potential for this brain area as a novel target for DBS for epilepsy, it was derived from the acute seizure-inducing paradigm. In this study, we hypothesize DBS of the VP prevents spontaneous seizures, which better aligns with human epilepsy. The study will investigate whether the adverse effects of VP-DBS are present. Overall, the study has clinical merit and impact since the quality of life may be improved with VP-DBS with fewer co-morbidity.

Acknowledgments

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Introduction

Epilepsy, a common neurological disorder affecting people regardless of age, is marked by recurrent and sudden episodes of seizures as well as related health problems (Epilepsy Foundation). It affects approximately 65 million individuals globally and 3.4 million individuals in the United States (Epilepsy Foundation). In the US, every 1 in 26 people is predicted to develop epilepsy in their life with a various range of causes including genetic factors, strokes, tumors and head injuries (Epilepsy Foundation & Epilepsy Foundation of Metropolitan New York). Generalized seizures and partial (focal) seizures are the two subdivided groups of seizures (Epilepsy Foundation of Metropolitan New York). Generalized seizures affect both sides of the brain at the same time and partial seizures affect a localized area (Epilepsy Foundation of Metropolitan New York). There is a series of motor symptoms during seizures including sustained rhythmical jerking movements, weak muscles and splitting legs, tense and rigid muscle during tonic state and unilateral or/and bilateral limb movements (Epilepsy Foundation & Epilepsy Foundation of Metropolitan New York).

There are several treatments and interventions for those affected by epilepsy in a life spanning antiepileptic drugs, resective surgery, and neuromodulation. Specifically, patients with epilepsy are first treated with antiepileptic drugs. However, 30-40% of patients remain refractory after this approach (Duncan, Sander, Sisodiya, & Walker, 2006) with drug resistance reported as a recurring problem in patients with temporal lobe epilepsy (TLE) (Sørensen & Kokaia, 2012). In these cases, a subsequent therapeutic strategy involving non-pharmacological treatments using resective surgery to remove the epileptic foci is employed [Mayo Clinic & Duncan et al., 2006]. Yet, even after surgery is performed, 30-40% of these individuals still have seizures (Duncan et al., 2006). Neuromodulation (Al-Otaibi, Hamani, & Lozano, 2011) and deep brain stimulation

(DBS) (Hamani et al., 2010) are considered to be alternative approaches to reduce epileptic seizures in these patients. The former includes vagus nerve stimulation (VNS) whereas the latter comprises of anterior thalamus (ANT) DBS and responsive neurostimulation (RNS); all are FDA approved as electrical stimulation approaches for epilepsy, but they have shown to only delay the appearance of seizures or reduce the frequency of these events in the majority of cases. Therefore, there are no DBS or neuromodulation of current targets that can prevent or mitigate seizures in epilepsy; albeit some seizure-freedom have been reported with ANT-DBS in about <20% of individuals. With that said, finding a novel DBS target that provides more potent seizure control than current applications has strong clinical utility.

With the above considerations in mind, we looked towards the ventral pallidum (VP), a basal ganglia structure for limbic and affective function, since animals injected with GABA-A receptor antagonists in the VP caused seizure suppression in an absence model of epilepsy in rats (Deransart, Vercueil, Marescaux, & Depaulis, 1998). Given that pharmacological modulation of the VP can alter seizure symptomology, the Shin lab applied DBS at 50 Hz, 300 µA and 90 µs pulse width bilaterally in the VP and reported that this prevented partial and secondarily generalized seizures in a rat model of temporal lobe epilepsy and generalized tonic-clonic seizures (Mahoney et al., 2018). From these findings, DBS in VP is thought to be an effective method to prevent seizures; moreover, because of its marked ability to accomplish this for brainstem seizures, it was also postulated that VP DBS could potentially mitigate cardiovascular and/or respiratory dysfunction by inhibiting epileptiform activity from invading into brainstem structures controlling these functions.

In this study, the effects of VP-DBS on acute seizures are further delineated by examining how neural network excitability is attenuated by this neuromodulatory approach to control seizures.

In addition, this study tests whether VP-DBS prevents or diminishes spontaneous seizures since human epilepsy is spontaneous rather than acute. Past research in the Shin lab employed acute seizure animal models generated by injecting a chemoconvulsant and observing resulting seizures for up to 4 hours after. Findings from this study will help to substantiate the utility of VP-DBS as the next-in-line alternative for preventing seizures in epilepsy. Current options for neuromodulation have had limited efficacy in this regard. To accomplish the goals of this proposal, we employ the pilocarpine rat model of acute and spontaneous seizures and apply VP-DBS. Our overarching hypothesis is that this therapeutic treatment will prevent or reduce seizing episodes by attenuating excitability in critical brain areas involved in the epileptogenic formation and/or propagation. After, the location of bilateral stimulating electrodes will be confirmed in the VP with post-mortem brain immunohistological analyses. Lastly, we assess changes in c-FOS expression in the brains of seizing animals with and without VP-DBS as a marker for neuronal hyper-activity to reveal the underlying neural networks affected for seizure control (Bullitt, 1990).

Materials and Methods

Bilateral electrodes implantation

Naïve rats at least 200 grams body weight were anesthetized with 5% isoflurane in an induction chamber and then placed into a stereotaxic apparatus with 2% isoflurane. Bupivacaine (0.1 mL) was injected at the incision site subcutaneously (subQ). A hole was drilled in the S1 cortex at -4.3mm anterior-posterior (AP) and -3.0 mm medial-lateral (ML) from bregma. Two smaller holes were drilled to target the VP at -0.3 mm AP, +/-2.2 mm ML and -7.3 mm dorsal-ventral (DV) from bregma. After, we inserted two 12 mm stimulating twisted wire electrodes at 125 µm diameter (each wire) (Plastics One, Roanoke VA). Four holes were drilled near the vertical axis of bregma in the first, third and fourth quadrants to implant two electrocorticography (ECoG) electrodes; one in the nose bone as a reference electrode and the other in the S1 cortex. The other holes were used to insert anchor screws for support. Connecting pins from all the electrodes (stimulating and recording) were all inserted into a cap and this was super glued and dental cemented to the top of the scalp for stabilization of electrodes. Saline at 5 mL was injected subQ for post-surgery hydration, 1 mL of buprenorphine was administered subQ and a Rimadyl tablet containing analgesics was given to the rats in their home cage after surgery for two consecutive days to relieve pain and reduce inflammation.

Acute seizure induction and deep brain stimulation paradigm

At least one week after rats were implanted with bilateral electrodes, animals were placed in a Plexiglas cylinder (width x height: 12 inches x 12 or 24 inches) with the cap attached to the stimulation apparatus via a commutator cable. Prior to attaching the cap to the stimulator, the top

of the dental cemented ensemble was cleaned with water and air-dried to prevent blood clots formation. Then, an intraperitoneal (IP) injection of 1mg/kg scopolamine, a muscarinic receptor antagonist, was administered to reduce piloerection and other peripheral symptoms from muscarinic receptor activation (Yu, Cai, Liu, Chu, & Su, 2007). Immediately after, VP-DBS stimulation was turned with frequency at 50 Hz, pulse width at 90 µs, and current at 300 µA as done previously in the lab. Twenty minutes later, we IP-injected 400 mg/kg pilocarpine to elicit forebrain and brainstem seizures acutely (Curia, Longo, Biagini, Jones, & Avoli, 2008). Stimulation was left on continuously for 4 hours with seizure behavior and simultaneous ECoG electrode recordings monitored during the whole experiment.

In vivo electrophysiological recordings

With our ECoG electrode recordings, oscillatory activity or local field potentials (LFPs) were obtained from the primary S1 cortical area in the differential configuration (Model 3000, A-M Systems, Sequim, WA). Signals were sampled at 1 kHz, high- and low-passed at 1 Hz and 300 Hz, respectively, and digitized (MiniDigi 1B, Molecular Devices, CA, USA).

Immunocytochemistry

After DBS and completion of experiments, rats were anesthetized with 5% isoflurane in the induction chamber and IP-injected with 1 mL of urethane (1.2-1.5 g/kg). Once unresponsive, animals were perfused transcardially with injections of 60 mL of heparin and 60 mL of 4% paraformaldehyde (PFA) at a steady rate to the apex of the left ventricle. The brains were extracted, immersed in PFA overnight and switched to 4% sucrose next day. Whole brains were sliced into

40 to 60 µm thick sections using a Microm HM-500 cryostat or Thermofisher cryotome (Pittsburgh, PA) for c-Fos immunocytochemistry and immersed in a 6-well plate containing 0.1M phosphate buffer solution (PBS). Brain sections containing the VP were set aside and placed on glass slides for post-mortem confirmation staining (described below).

For c-Fos immunocytochemistry, the sections were washed in a 6-well plate with 0.04% PBS/Triton X 3 times for 10 minutes, 3% hydrogen peroxide for 10 minutes, and then 0.04% PBS/Triton 4 times for 10 minutes in the room temperature. Sections were placed in 1.5 mL Eppendorf tubes containing 1 mL of blocking solution (100 µL of normal goat serum and 900 µL of 0.04% PBS/Triton X) for an hour. Sections were reacted with 1 µL of primary antibody solution and incubated overnight at 4°C. Next day, sections were washed in another 6-well plate with 0.04% PBS/Triton X 3 times for 10 minutes in the room temperature. Sections were reacted with 1 mL of secondary antibody solution in another tube (100 µL of normal goat serum, 900 µL of 0.04% PBS/Triton X and 2 µL of Secondary antibody) and incubated for an hour in the room temperature. Sections were washed with 0.04% PBS/Triton X 3 times for 10 minutes, incubated in AB mixture for an hour and washed in PBS 4 times for 10 minutes. Diaminobenzidine (DAB) solution was added to the section, Sections were washed with PBS again and then placed on a glass slide with a cover slide. A Zeiss Axio Imager M2 microscope (Zeiss, Washington, DC) with Neurolucida system was used to collect bright field (color) images and images were analyzed on Biolucida.

To confirm the correct placement of stimulating electrodes, the slides with sliced brains containing bilateral VP were stained with cresyl violet (CV). For this, the sections were rehydrated tissue in 95% EtOH for 10 minutes, 70% EtOH for 3 minutes, 40% EtOH for 3 minutes and in dH2O for 3 minutes. Sections were dipped into staining dishes containing cresyl violet for 3 minutes and de-stained in dH2O for 3 minutes, 40% EtOH for 2 minutes, 70% EtOH for 2 minutes,

95% EtOH for 2 minutes, 100% EtOH for 3 minutes, and Xylene for 5 minutes. A coverslip was placed on top with Permount. After drying on a slide warming apparatus, the brain slices were scanned using pathscanner IV device (Electron Microscopy Sciences, Hatfield PA).

Data analyses of acute seizure behavior and c-Fos immuno-reactivity expression

The total number of seizures at each stage, the cumulative duration of each stage of seizures and the average duration of each stage of seizures were quantified. In rodents, both acute and spontaneous seizures can be divided into partial forebrain seizures showing head movements and some unilateral forelimb movements, generalized forebrain seizures showing bilateral forelimb movements, and generalized brainstem seizures showing uncontrolled muscle contraction in the whole body comprising of jumping, wild running, and tonic extensions. Therefore, in our analyses we scored seizure behavior using the commonly used and well-documented Racine Scale (Mahoney et al., 2018). Specifically, seizures are generally divided into 7 stages with staring and mouth clonus denoted as stage 1; head nodding occurring in stage 2; unilateral forelimb movements in stage 3; bilateral forelimb movements in stage 4; bilateral forelimb movements with falling characterized in stage 5; wildly running and jumping in stage 6 and tonic-clonic extensions in stage 7 (Mahoney et al., 2018).

Animals after having undergone acute seizures with or without VP-DBS were sacrificed and brains fixed, removed and sectioned for c-Fos immuno-staining. This was to identify which areas of the brain were attenuated by VP-DBS and the magnitude of alteration. The intensity of c-Fos expression in forebrain and midbrain was assessed by counting a number of visible c-Fos particles. Sections with no c-Fos particles were labeled with an “x’. In contrast, * indicates a

number less than 10 particles, ** indicates a number between 10-20 particles, *** indicates a number between 20-40 particles and **** indicates c-Fos immuno-reactivity in a brain area with more than 40 particles. c-Fos particles were visualized by using the NDP reviewer 2 (Hamamatsu, Bridgewater, NJ).

Induction of spontaneous seizure and analyses

We follow a commonly performed protocol for eliciting spontaneous seizures by first inducing acute seizures. First, naïve rats were IP-injected with 1mg/kg scopolamine in 1mL saline. After 20-30 minutes, 400 mg/kg pilocarpine in 1mL saline was IP-injected. Once rats exhibited stage 4 bilateral forelimb clonus, which is a proxy for status epilepticus, we monitored animals for 90 minutes and then terminated the acute seizures by IP-injecting 20 mg/kg diazepam in 1mL 15% tween/saline solution. Afterward, rats were monitored for 48 hours and animals that survived this procedure were implanted stimulating electrodes bilaterally in the VP and reference and ECoG electrodes in the nose bone and S1 cortex, respectively, the next day. With a 90 minute period of status epilepticus, the mortality noted is about 20-30% in our hands. After surgery, rats were placed in a clear plexiglass cylinder and their headcap was connected to the stimulators and electrophysiological acquisition systems (A-M Systems, Sequim, WA) via a commutator. Food and water were made available ad libitum. A video electroencephalogram (EEG) system was used to monitor the rats' behavior and ECoG recordings almost continuously 24 hours a day consecutively for 4 weeks. Rats that survive induced acute seizures with status epilepticus lasting 90 minutes that are terminated with an anticonvulstant (e.g., diazepam) will start to exhibit spontaneous seizures lasting weeks in an unpredictable and sporadic manner. We intend to monitor these animals over 4 weeks. Afterward, the rats were later perfused transcardially with injections

of heparin and PFA. The whole brain was sliced and processed for c-Fos immunocytochemistry and CV staining as described above. Behaviors of rats during seizures were quantified using the Racine Scale in a similar manner to assessing and characterizing acute seizure. A timeline of the experimental design for both acute and spontaneous seizure induction and monitoring period is presented in fig. 2.

Chemicals and drugs

All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) whereas items for immunohistochemistry such as antibodies were purchased from Santa Cruz Biotechnology (CA, USA), Novus Biologicals (Littleton, CO, USA) or Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Data analysis and statistics

Parametric data were analyzed by one-way ANOVA with Bonferroni post-hoc multiple comparisons using GraphPad Prism (Version 6.0, LaJolla, CA). We could not perform two-way ANOVA statistics due to the limited and unequal sample sizes at this time. For all statistical analyses, data were considered significant at $p<0.05$ and shown on figures with asterisks. For c-Fos immuno-expression, we employed a qualitative approach as described above with particle counts.

Results

Bilateral electrode implantation in the VP

As mentioned before, we confirmed the placement of the stimulating electrodes with CV stained brain slices. The placement was considered to be on target when the bilateral electrodes were implanted in both hemispheres of the VP as shown in Fig. 1 in coronal (A) and sagittal (B) orientations. Example of a targeted VP brain is showed in Fig. 3 without (A) and with (B) CV stain with a nearly equal distance of the electrodes from the midline and electrodes located below the median preoptic nucleus (MnPO). From 18 rat brains, 4 of them were confirmed with the correct location bilaterally in the VP; 3 animals were still unprocessed and therefore unconfirmed and 11 animals were denoted as having misplaced DBS stimulating electrodes in the VP (either one or both).

VP-DBS in acute seizure rat models

To determine the effect of VP-DBS in acute seizure, we measure the following metrics: the total number of seizures (fig. 3A), cumulative duration of seizures (fig. 3B) and average duration of seizures (fig. 3C) of VP-stimulated rats, stimulated rats but off-target in the VP and unstimulated animals but administered pilocarpine. Throughout a 4-hour monitoring period, VP-DBS rats showed the least number of seizures (p=0.0019, n=3), which was not significantly different than animals with mis-targeted VP-DBS (n=1). Interestingly, the latter off-target implant group was also found to have a significantly lower number of seizures than un-stimulated seizing rats (p=0.0094, n=5). For the other two parameters, we did not see a significant difference between any of the animal groups with cumulative duration of seizures and the average duration of seizures

(figs. 4B, C). However, this could arise from the low sample size since the trend appears to show that animals with VP-DBS exhibit less cumulative seizure durations (fig. 4B) and average seizure durations (fig. 4C) than both un-stimulated and off-target animals When we focused on specific seizure phenotypes, we noted that the rats without any stimulation showed the greatest number of stage 3 and 4/5 seizures (fig. 5A) and cumulative seizure durations (fig. 5B), whereas the rats with VP-DBS had the least number of seizures with only stage 4 seizures noted (fig. 5A; on-target group and black histogram). The rats that were misplaced outside the VP tended to show a variety of seizures spanning stage 3, 4/5 and 6/7 seizures; particularly for average seizure durations (fig. 5C).

c-Fos protein expression

c-Fos is an immediate early gene protein which has been commonly used as a marker to visualize hyper-excitability in neural networks. For our application, we aimed to see if VP-DBS provided anti-seizure efficacy by diminishing activity in certain brain areas. For that reason, we selected some of the forebrain and midbrain structures thought to be epileptogenic for c-Fos analyses (Figs. 6). At this stage of the study, we had two animals with acute seizures having c-Fos immuno-expression and both showed similar c-Fos particle spatial and abundant patterns in structures of forebrain and midbrain with a small deviation between them (Fig 6A). For instance, there were high levels of c-Fos particle expression in both acutely seizing animals in the piriform cortex (Pir), the paraventricular thalamic nucleus (PV) in the forebrain areas. Low expression in c-Fos was seen in both animals in the dentate gyrus (DG) and the three amygdaloid areas, intermediodorsal thalamic nucleus (IMD) and cingulum (Cg) (fig. 6A, B). In midbrain structures, we noted high levels of c-Fos immuno-expression in the posteromedial cortical amygdaloid nucleus (PMCo), TeA: temporal association cortex (TeA), V2L: secondary visual cortex (lateral

area) (V2L) and retrosplenial agranular cortex (RSA) (fig. 6A, C). Interestingly, there were deviations between acutely seizing animal #1 and #2 in c-Fos expression. In the forebrain, central amygdaloid nucleus (CeM) was expressed in large amounts in one animal, but not the other (fig. 6A, B). In the midbrain, there were differences between animals in the amygdalopiriform transition area (Apir) and dorsal lateral geniculate nucleus (DLG) (fig. 6A, C).

VP-DBS in a spontaneous seizure rat model

To better align with human epilepsy comprising of spontaneous, not acute, seizures, we had placed seizing animals with these types of seizures in individual cylinders with a 24-hour video-EEG acquisition system. At this point in the project, we have only analyzed 3 days of spontaneous seizure data from 4 animals. The ones with identifications DZA9, DZA10 and DZA13 are animals with spontaneous seizures without VP-DBS; DZA11 and DZA14 are rats that underwent the pilocarpine spontaneous seizure induction protocol, but also have VP-DBS throughout the monitoring period (fig. 7). The number of seizures is presented for each animal in figure 7A-E separated into the different seizure phenotypes. From these analyses, we can see that the rats without DBS exhibited a greater number of seizures consisting of various seizure stages (3, 4, and 5) (fig. 7A-C) with animal DZA13 having the most diverse seizures. Conversely, the animals with VP-DBS had the lowest number of seizures and the type of seizures was only stage 4 seizures (fig. 7D-E). ECoG traces from spontaneous seizing rats were recorded and presented in fig. 8. Smaller amplitude and faster frequencies of oscillations can be seen coinciding with stage 4 and 5 seizures (fig. 8). Conversely, we can easily distinguish these epileptiform activities from the larger amplitude, sharper deflected but lower frequency oscillations that are seen when rats are exploring in their containers or grooming (fig. 8). When we consolidated the data from the 3

seizing rats without VP-DBS and compared this to the 2 animals with VP-DBS, we found that most of the rats in the former group had more numerous seizures (fig. 9, top graphs) and higher cumulative seizure durations (fig. 9, middle graphs) during the night time hours (7PM to 7AM) than in the daytime (7AM to 7PM) (fig. 9). The average seizure duration appeared to be the same regardless of the time of day. We did observe that the seizure numbers and cumulative seizure durations tended to decrease at day 3. Notably, rats receiving VP-DBS had much lower seizure numbers (fig. 9, top graph), cumulative seizure durations (fig. 9, middle graph) and average seizure durations (fig. 9, lower graph) than unstimulated animals. Furthermore, stimulated animals showed almost no difference in the number and duration of seizures regardless of whether it was night or daytime hours and the (fig. 9).

FIGURES

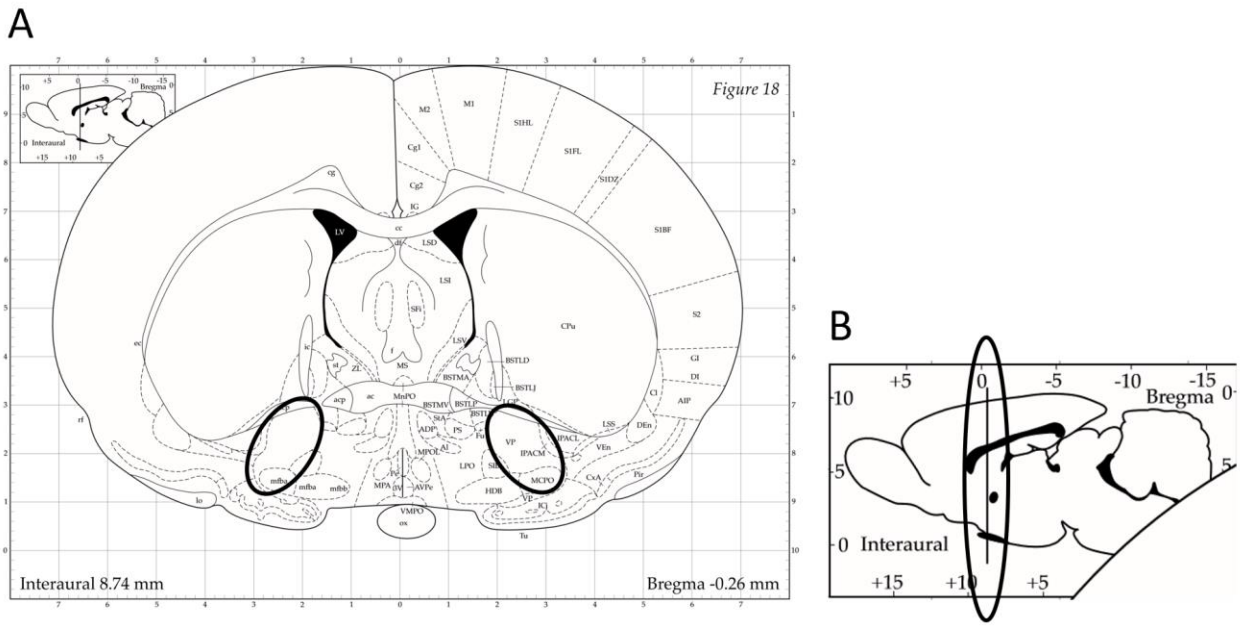


Figure 1. Ventral pallidum in rat brain. VP is a forebrain structure closer to the ventral side of both sides of the brain. Pictures are retrieved from (Paxinos & Watson, 1998) and the black circles denote this area on the right and left hemispheres in in the coronal orientation (A) and the anterior-posterior location of the VP in the sagittal plane (B).

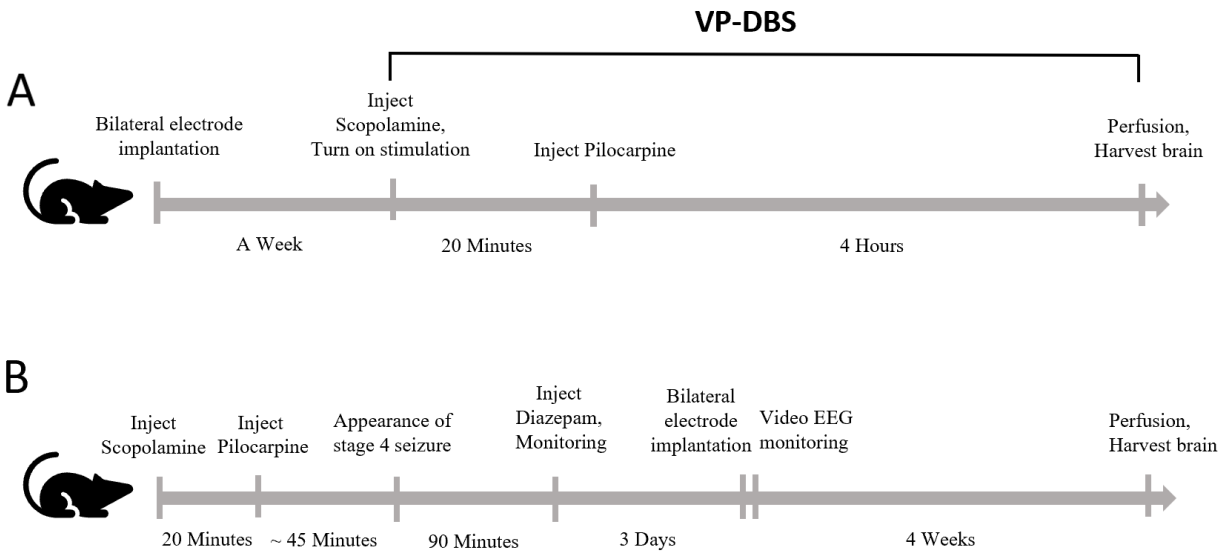


Figure 2. Timeline. Acute seizure-inducing paradigm shown in (A). Rats received scopolamine and pilocarpine injections a week after receiving bilateral electrode implantation surgery. After 4 hours of VP-DBS or no stimulation, the rats were sacrificed, and the brains were preserved. In (B), a timeline is provided for inducing spontaneous seizures. Rats were injected with scopolamine, pilocarpine and acute seizures were terminated with diazepam. They were monitored for 3 days post-diazepam administration to identify animals that survived. These animals received bilateral electrode implantation in the VP and put in a 24-hour video EEG monitor system for 4 weeks. Spontaneous seizures manifest during this time period in animals unless mitigated by treatment. Afterwards, rats are sacrificed, and post-mortem analyses of brain tissue is conducted.

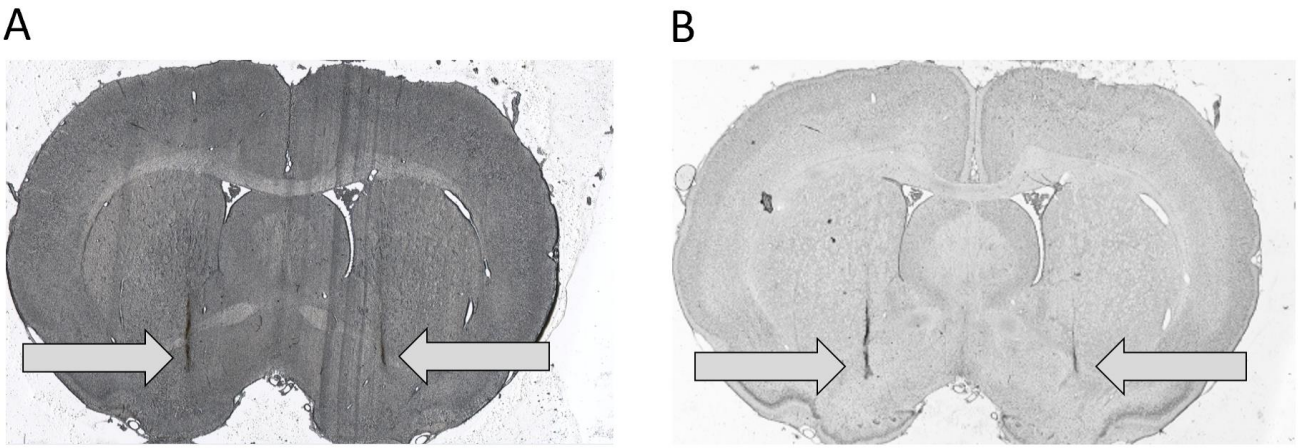


Figure 3. Bilateral electrodes in the VP-DBS rat models. Implanted stimulating electrodes are labeled with gray arrows. A. Slice shows placement with electrodes without any staining in (A) and the same slice shows placement with electrodes after cresyl violet (CV) stain in (B).

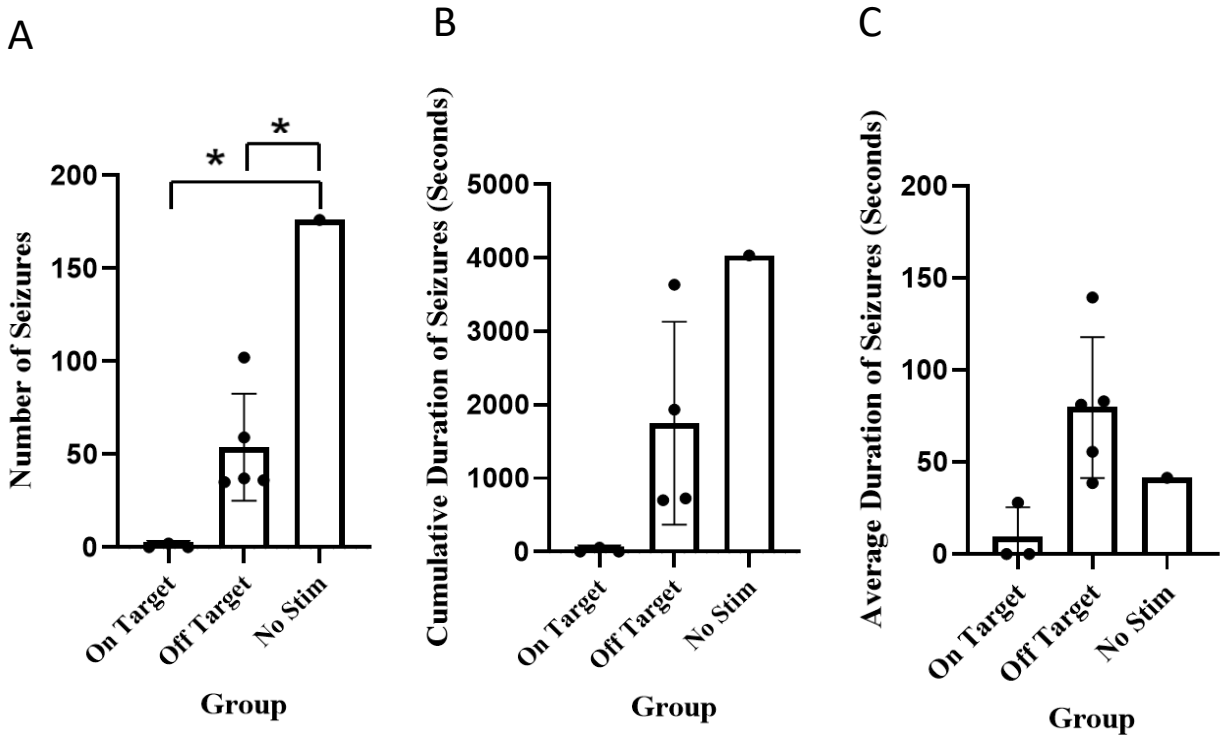


Figure 4. Acute seizures in pilocarpine-treated rats with or without VP-DBS. A total number of seizures in rats with VP-DBS (on target), misplaced stimulating electrodes either in one or both VP (off target). Rats without stimulation showed the most seizure episodes than those with VP-DBS. In (B), cumulative duration of seizures is shown with unstimulated rats exhibiting the longest duration of seizures than those receiving VP-DBS or off target implants. In (C), the average duration of seizures in the three animal groups show that rats with off-target stimulation had the longest average seizure durations. N=3 for on target; N=5 for off target; N=1 for no stimulation.

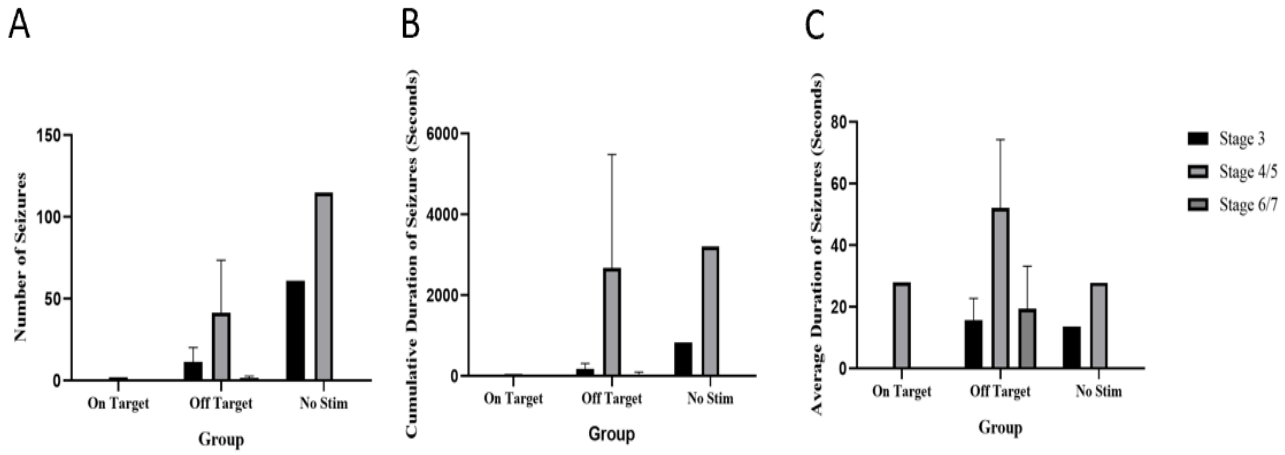


Figure 5. Specific stages of acute seizures in pilocarpine-treated rats with or without VP-DBS. The rats without VP-DBS had the greatest number of stage 3 and 4/5 seizure (A) and longest cumulative duration of stage 3 seizures (B). Rats that had misplaced electrodes in one or both VP hemispheres exhibited the longest average duration of stage 4/5 seizures. Notably, stage 4/5 seizures occurred the most in animal groups, but at different magnitudes with the smaller values in rats with VP-DBS. N=3 for on target; N=5 for off target; N=1 for no stimulation.

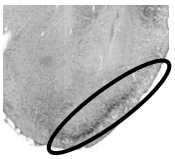
A

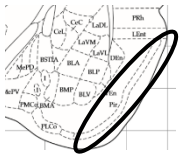
	Forebrain							Midbrain						
	Pir	Cg	CeM	PV	IMD	MeAD, MePD, MePV	DG	PMCo	Apir	TeA	V2L	RSA	DLG	SNR
Animal 1	****	*	***	****	*	*	*	****	**	****	****	****	****	*
Animal 2	****	X	*	**	X	**	**	***	****	****	****	****	**	**

B

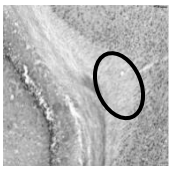
Forebrain

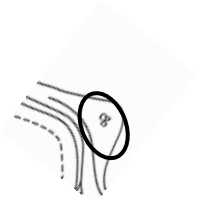
Pir



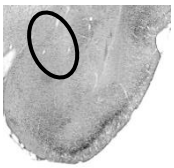


Cg

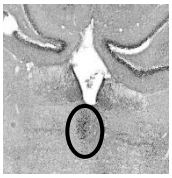


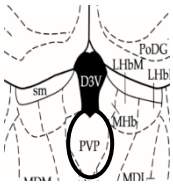


CeM

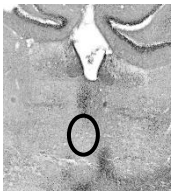


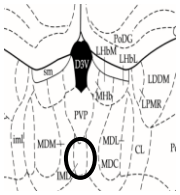
PV





IMD

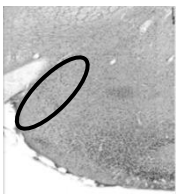


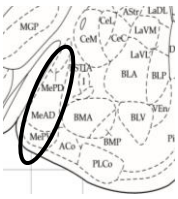


MeAD

MePD

MePV

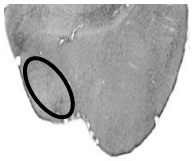




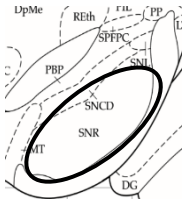
C

Midbrain

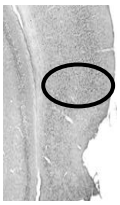
PMCo

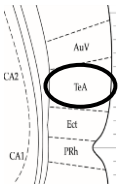


SNR

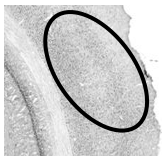


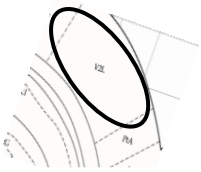
TeA



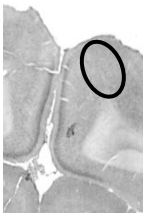


V2L

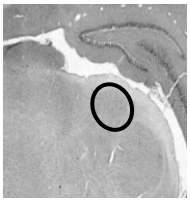


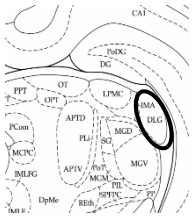


RSA



DLG





For figure on previous page: Figure 6. C-Fos protein expression in forebrain and midbrain. C-Fos immuno-expression from two acute seizing rats in various forebrain and midbrain areas are summarized in (A). The symbols X, *, **, ***, **** indicate the number of c-Fos particles in the brain area. Specifically, X= no particles; *= <10 particles; **= 10-20 particles; ***= 20-40 particles and ****= >40 particles. The forebrain structures are as follows: piriform cortex (Pir); cingulum (Cg); central amygdaloid nucleus (medial division) (CeM); paraventricular thalamic nucleus (PV); intermediodorsal thalamic nucleus (IMD); medial amygdaloid nucleus (anterodorsal part) (MeAD); medial amygdaloid nucleus (posterodorsal part) (MePD); medial amygdaloid nucleus (posteroventral part) (MePV) and dentate gyrus (DG). The midbrain structures are as follows: posteromedial cortical amygdaloid nucleus (PMCo); amygdalopiriform transition area (Apir); temporal association cortex (TeA); secondary visual cortex (lateral area) (V2L); retrosplenial agranular cortex (RSA); dorsal lateral geniculate nucleus (DLG); substantia nigra (reticular part) (SNR). In (B), raw images with c-Fos particle expression is presented for each forebrain (left column) and midbrain (right column) areas described in (A). The part and orientation of the rat atlas specific to the raw images are shown beside each other and circled in black for reference.

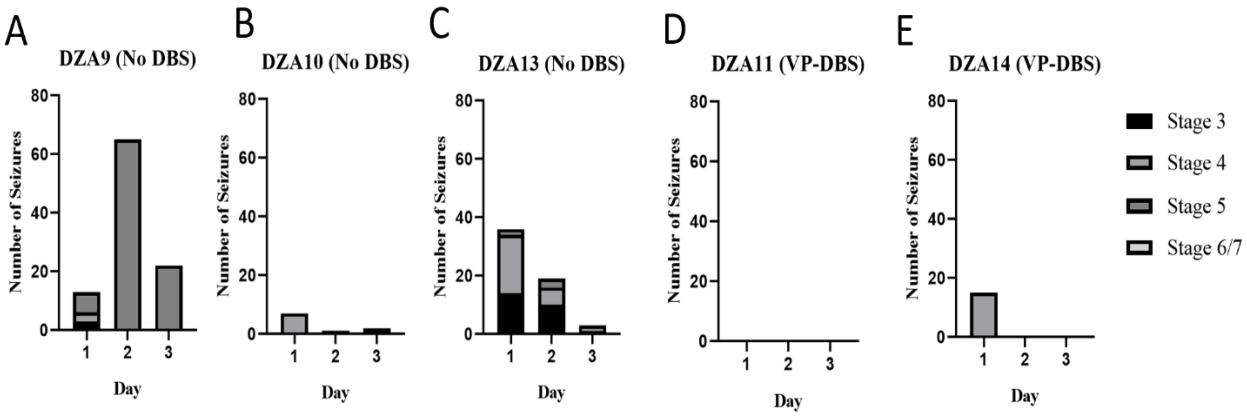


Figure 7. Stages of spontaneous seizure in rats with or without VP-DBS. Number of seizures are reported from each rat separately with 3 unstimulated animals (A: DZA9, B: DZA10, C: DZA13) and 2 with VP-DBS (D: DZA11, E: DZA14). Stage 3, 4, 5 and 6/7 are all shown for the first 3 days of the spontaneous seizure monitoring period.

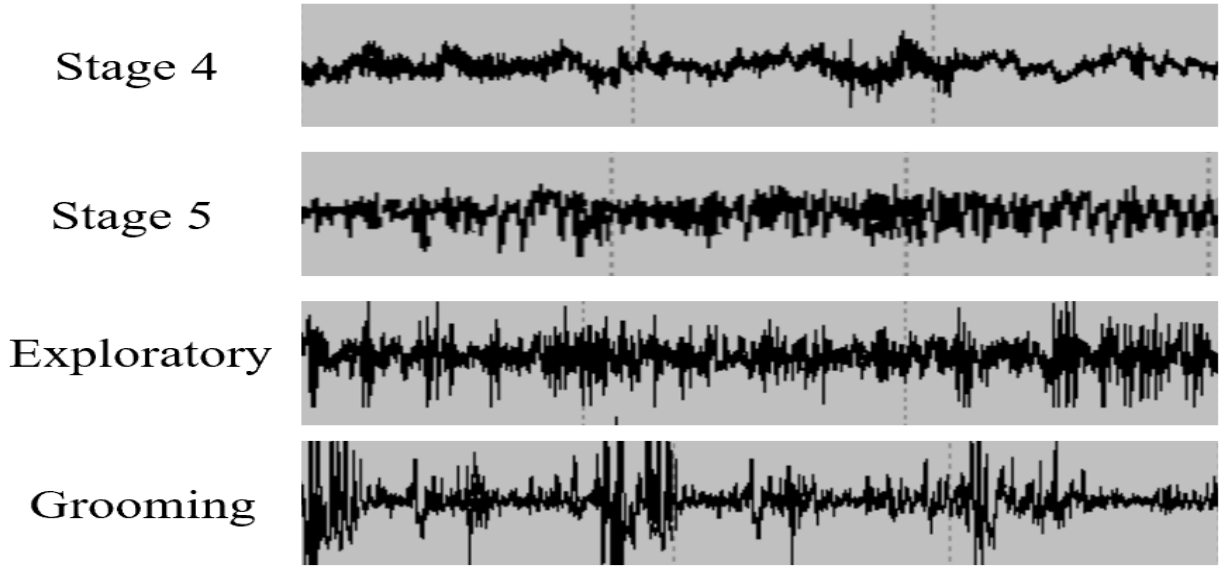


Figure 8. ECoG traces from rats during spontaneous seizure monitoring period. ECoG traces during stage 4 and 5 seizure are characterized by smaller amplitude and fast oscillations whereas larger amplitude and sharp waves but with low frequencies are seen during exploratory and grooming behavior.

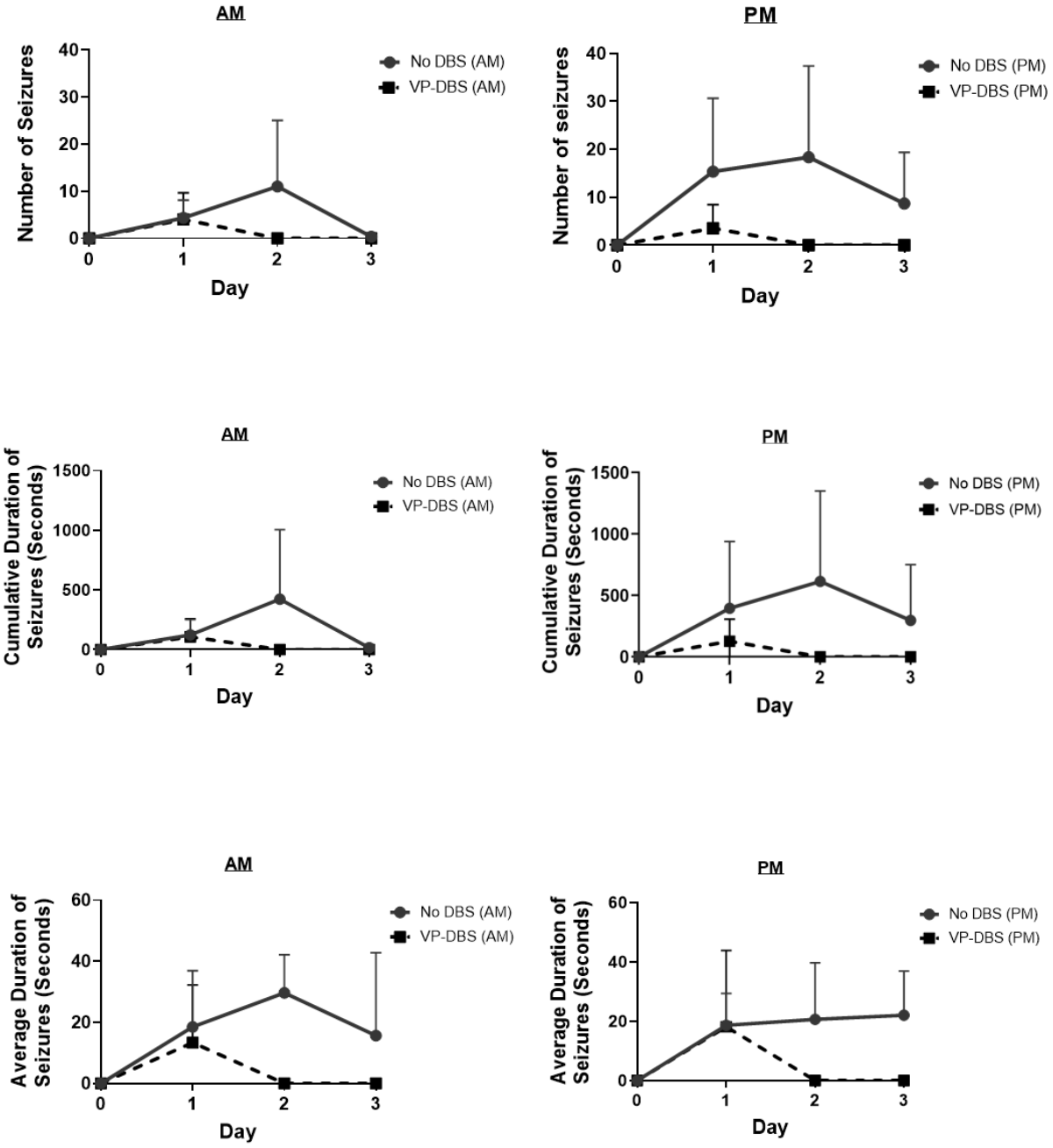


Figure 9. Spontaneous seizure in rats with or without VP-DBS over first three days of monitoring. Spontaneous seizure numbers (top row), cumulative duration of seizures (middle row) and average seizure durations (bottom row) are shown for rats with (black squares) or without VP-DBS (black circles) over the first 3 days of spontaneous seizure monitoring. The analyses are separated into seizures noted during the daytime (AM) and night-time (PM). N= 3 for unstimulated rats; N=2 for VP-DBS animal.

Discussion

Overall, our findings from acute and spontaneous seizure models revealed that rats with VP-DBS exhibited a smaller number and duration of seizures, particularly those at stage 4. It indicates that VP-DBS can possibly control seizures from escalating into more severe stage 5, 6 and 7 seizures, which are transitions from generalized forebrain seizures to generalized brainstem seizures. From the observations of the acute seizing animals, the rats without any electrical stimulation exhibit stage 1 and 2 with head nodding and mouth clonus. Next, animals show stage 3 and 4/5 seizures which are partial to generalized forebrain seizures, respectively, 20 minutes after pilocarpine administration and these appear with various latencies between seizure stages. Stage 6/7 would occur later in the 4-hour seizure period. Yet, in contrast to the emergent of these seizure stages, rats that did receive bilateral VP-DBS remained within stage 1, 2, 3 with occasional stage 4 seizures for the whole 4-hour period. Even though these were seen, it should be noted that the number and duration of these seizures were significantly less than those observed in unstimulated animals.

In the spontaneous seizure models, unstimulated rats had stage 4 seizures around 45 minutes after injecting pilocarpine. Some rats with stage 6/7 brainstem generalized seizures would die. In fact, our duration for stage 4 seizures and status epilepticus results in a high mortality rate up to 48 hours post-pilocarpine injection. The rats that survive this insult and do not have VP-DBS had numerous spontaneous but unpredictable seizures with most occurring during night time. For rats with VP-DBS, they showed stage 1, 2, 3, and 4 seizures only with no difference in a number of seizures regardless of the day or night.

There are a few key limitations of our proposal at this time that prevents us from making strong conclusions about the data. A major point is our small and incomplete sample size. Due to

the technically difficult nature of properly implanting stimulating electrodes bilaterally in VP, there were a significant number of animals with off-target placements. Furthermore, the placement of electrodes has not yet been confirmed for a number of rats that underwent acute and spontaneous seizure experiments. Also, the c-Fos experiments and results stemmed from animals with pilocarpine-induced acute seizures, but we have not analyzed the data from animals with VP-DBS yet. We also did not apply a quantitative metric to assess changes in c-Fos expression patterns and abundance. In the future, we can employ FIJI ImageJ software to digitize images to obtain measurable particle count numbers. Alternatively, we can also use a non-bias stereology approach. Moreover, we plan to adopt a new protocol where we will co-stain c-Fos with vGLUT1 or vGLUT2 (glutamate transporters) and visualize the immuno-staining expression patterns and overlap of both markers with fluorescent microscopy. This design will better inform us about whether the excitatory glutamatergic neuronal activity is diminished with VP-DBS and where in different brain areas. For our spontaneous seizing animal experiments, we only analyzed 3 days of data. While the number and cumulative seizure durations appear to decrease at the 3-day period and may suggest that these animals will not undergo more seizures, it is important to highlight that this random and unpredictable seizure occurrence is typical for this seizure model. In fact, we expect that the unstimulated animals will have high and low expression of spontaneous seizures over the 4-week period. However, as our preliminary data would indicate for the animals with VP-DBS, there is optimism that this trend will persist throughout the entire monitored period.

In summary, our findings posit that VP-DBS can hold potential as an effective neuromodulatory approach for epilepsy. While the data is preliminary and at an early stage in development, there are promising indications that increasing sample size and analyzing data encompassing 4 total weeks of video-EEG will substantiate the results here. Ultimately, VP-DBS

may fill in the critical need in preventing seizures in individuals with epilepsy. Current FDA approved targets, while still effective, reduce seizure frequency numbers but have limited utility in preventing these from occurring.

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