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Supplementary Information for

Descending projections from the *substantia nigra pars reticulata* differentially control seizures.

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SI Methods

Animals

Sprague-Dawley (SD) rats (280-300 g at the start of the study) were purchased from Envigo/Harlan (Frederick, MD). Adult female GEPR-3s (~ 6 weeks of age at the time of surgery) were obtained from our colony Georgetown University Medical Center. Long Evans (LE) glutamic acid decarboxylase (GAD)-cre positive transgenic rats were obtained from the Rat Resource and Research Center and bred with wild-type LE (Charles River, Wilmington, MA) to produce heterozygous GAD-cre rats. All rats were group-housed in a temperature and humidity controlled room in the Division of Comparative Medicine at Georgetown University with food (Lab Diet #5001) and water available *ad libitum*. All procedures were performed during the light phase of the light-dark cycle (0600–1800, lights on). This study was approved by the Georgetown University Animal Care and Use Committee.

Surgery

Rats were anesthetized and placed into a Kopf stereotaxic frame (Tujunga, CA). For all experimental groups using SD rats or GEPR-3s, rAAV8-CAG-ArchT-GFP (UNC Vector Core) was microinjected into the SNpr bilaterally through a 30-gauge dental needle as we have previously described (1). For control experiments, rAAV-CAG-GFP (UNC Vector Core) was used. For GAD-cre rats, rAAV8-FLEX-ArchT-tdTomato (UNC Vector Core) was used. The injection coordinates for the SNpr were: 3.2mm posterior to bregma, 2.5mm lateral to midline and 8.5mm ventral to the dura with the incisor bar 5.0 mm above the interaural line. 1.5 µl of virus was injected sequentially into each SNpr at a rate of 0.2 µl/min; the injection needle was left in place for at least 5 min to allow virus diffusion before retraction. Following the microinjections, an optical fiber (200 µm core, 0.22NA) was implanted either 0.2 mm dorsal to each injection site or in the DLSC: 5.0 mm posterior to bregma, 2.5 mm lateral to midline, and 4.5 mm ventral to the dura, or PPN: 7.7 mm posterior to bregma, 2 mm lateral to the midline, and 7.5 mm ventral to the dura. Implants were held in place with jeweler's screws and dental acrylic. Prior to surgery, efficiency of each optic was tested and recorded for use in behavioral testing.

Electroencephalography. EEG screw electrodes were implanted through holes in the skull such that the bottom of each screw was in contact with the dura. Each animal was implanted with electrodes bilaterally over the parietal lobe, bilaterally over the frontal lobe, and two over the cerebellum (ground and reference). EEG wires were routed into a plastic pedestal (PlasticsOne, Roanoke, VA) and held in place with dental acrylic. Data were recorded using LabChart 8 (AD instruments, Colorado Springs, CO) with a 50 Hz low pass filter. Electrographic traces are derived from frontal leads referenced to the cerebellum. As described below, EEGs were performed for the pentylenetetrazole, Area Tempestas, and gamma butyrolactone models.

Optogenetics.

Behavioral testing commenced 3 weeks post-surgery to allow for transgene expression. The implanted optical fibers were connected to fiber-coupled diode pumped solid state lasers with a wavelength of 532 nm. Power loss was calibrated at the time of testing to deliver 10 mW out the tip of the implanted fiber. Unmodulated light delivery was continuous and 100 Hz delivery was pulsed with a 50% duty cycle. Light power in tissue was modeled using MonteCarloLight and LightHeatPlotter as described by Stujenske and colleagues (2). Rats were connected to fiber optics prior to seizure induction; stimulation was initiated immediately following or concurrent with chemoconvulsant administration or acoustic stimulation and lasted the entire observation period. Experiments were performed using a repeated measures design, with each animal serving as its own control (i.e., each rat was tested with and without optogenetic stimulation). To minimize order effects, some rats were tested with optogenetic stimulation as the first data point analyzed, and others with baseline as the first point.

Pentylenetetrazole (PTZ)

Pentylenetetrazole is a GABA-A receptor antagonist, and one of the primary models used to screen anti-seizure medications (3). Depending on the dose used, this drug evokes electrographic seizures, followed by clonic seizures similar to those seen in models of temporal lobe epilepsy, followed by wild running and tonic extension, which models secondarily generalized seizures in human temporal lobe epilepsy. We measured the severity of the seizures (described below), latency to seizure onset (as a control for drug injection), and electrographic seizure duration in a subset of animals.

PTZ (Sigma) was dissolved in 0.9% saline at a concentration of 10 mg/ml and administered via intraperitoneal (i.p.) injection at an initial dose of 25 or 30 mg/kg. To maximize the utility of each animal, this dose was adjusted based on individual variations in response to PTZ as we have previously described (1). The same dose was used both for optogenetic testing and for baseline testing sessions.

PTZ seizures were scored using a modified seizure scale as we have previously described (1): 1 = single myoclonic jerk; 2 = multiple myoclonic jerks, unilateral forelimb clonus; 3 = bilateral facial and forelimb clonus (FFC); 3.5 = FFC with a body twist; 3.75 = FFC with a full body roll; 4 = FFC with rearing; 4.5 = FFC with rearing and a body twist; 5 = FFC with rearing and loss of balance; and 6 = running bouncing seizure +/− tonic forelimb extension.

Tests with PTZ were separated by at least 48 hours and the order of testing (i.e., with and without stimulation) was balanced to avoid potential confounds associated with repeated dosing with PTZ. Due to the nature of the stimulation (i.e., flashing light) it was impossible to blind the observers to the nature of the experimental session. Detailed behavioral observations were made in real-time by trained observers. After data collection, scores were assigned by a blinded observer based on the observation notes. For optogenetic testing, the light source was activated immediately after administration of PTZ and rats were observed for 30 minutes after the time of injection. Ten SD and all GAD-Cre LE were used for EEG monitoring and seizure confirmation.

Area Tempestas

Area Tempestas is a functionally-defined ictogenic region of the anterior piriform cortex of rodents,(4) with a homologous site reported in human temporal lobe epilepsy (5, 6). Area Tempestas-evoked seizures are phenotypically similar to those evoked in other models of temporal lobe epilepsy, such as kindling and status epilepticus. Seizures are characterized by repeated bouts of bilateral clonus with rearing and loss of balance, and reflect the complex partial seizures observed in human temporal lobe epilepsy.

A stainless steel guide cannula was stereotaxically implanted above the left AT (4.0 mm anterior to bregma, 3.5 mm lateral to midline, 5.5 mm ventral to the dura) (1). At the time of drug infusion an internal cannula was placed to extend an additional 2 mm to reach the target site. Bicuculline methiodide (Sigma) was dissolved in 0.9% saline at a concentration of 1 mM. Initially, 100 pmol of bicuculline was microinjected into the AT at a rate of 0.2 µl/min (1, 4); microinjection procedures follow those we have previously described (7). For optogenetic testing, the light source was turned on when the injection was completed and rats were observed for 60 minutes.

Bicuculline dose was increased as needed to evoke a seizure score between 3-5, using a seizure scale, as previously described (1): 0.5 = jaw clonus; 1 = myoclonic jerks of the contralateral forelimb; 2 = forelimb clonus (with or without facial clonus); 3 = bilateral facial and forelimb clonus; 4 = rearing plus bilateral facial and forelimb clonus; 5 = loss of balance in addition to rearing and bilateral facial and forelimb clonus. Behavioral observations were made in real-time by trained observers and scores were assigned after data collection based on the observation notes by a blinded observer. We report both the seizure severity and the number of Stage 2 or greater seizures observed as our primary outcome measures as these reflect the degree of network recruitment and the propensity to initiate seizure activity, respectively.

Audiogenic seizure (AGS) testing

The Genetically Epilepsy Prone Rat (GEPR-3) is a model of inherited epilepsy. GEPR-3 animals have a genetic predisposition to generate seizures in response to loud noise. This form of

reflex epilepsy engages the same brainstem networks that generate tonic and tonic-clonic seizures in humans.(8)

Animals were presented with acoustic stimulation, which consisted of 105-110 dB pure tones (Med Associates, St. Albans, VT) or 110 dB mixed sounds (delivered via an electrical bell). Acoustic stimulation presented until a seizure was elicited, or for 60 sec if no seizure activity was observed. Sixty min after baseline AGS, GEPR-3s were bilaterally stimulated at 100 Hz starting ~ 30 s prior to presentation of AGS-inducing stimuli.

Convulsive behavior was scored as: 0 = no seizure response, 1 = one episode of wild running seizures (WRS), 2 = two or more WRS episodes, 3 = one WRS episode followed by tonic-clonic seizures characterized by bouncing clonus, and tonic dorsiflexion of the neck and shoulder. AGS severity, latency to seizure onset and seizure duration were scored in real-time by two observers and recorded using a video monitoring system (Med Associates, St. Albans, VT).

Gamma butyrolactone (GBL) testing

The gamma butyrolactone model of absence seizures produces spike-and-wave discharges that have high face, construct and predictive validity in rats for human absence seizures (9). GBL acts as a prodrug for gamma hydroxybutyrate, which in turn triggers repeated spike-and-wave discharges accompanied by behavioral arrest.

GBL (Sigma) was dissolved in 0.9% saline at a concentration of 100 mg/ml and administered via i.p. injection (100 mg/kg).

Rats were monitored for the occurrence of 'spike-and-wave'-like discharge (SWDs) activity for 20 min after the time of injection for each of three session types: GBL alone, GBL in combination with 100 Hz optogenetic stimulation, and GBL in combination with unmodulated stimulation. Unmodulated stimulation was used to determine if patterned stimulation was required for anti-seizure activity. SWDs were assessed offline using LabChart 8 by a treatment-blind observer. Signal was filtered (band pass 2–50 Hz) and SWDs were differentiated from normal runs of alpha rhythm based on amplitude, as SWDs showed peak-to-peak amplitude that was > 2x the background activity.

Multiunit Recording

Six male SD rats were used for these experiments. Rats underwent surgery, as described above, but received only virus injection into the SNpr. After three weeks, rats were anesthetized with 1.5 g/kg of urethane (0.3 mg/ml) and placed in a stereotaxic frame. A 0.5 cm x 0.5 cm craniotomy was made 3.0 mm posterior to bregma, bilaterally. The dura was incised with a needle and retracted. A tungsten microelectrode (2.5 megaOhm resistance, MicroProbes, Gathersburg, MD) and an optical fiber were first positioned and recorded in the SNpr. Electrode and fiber optic were then moved to the DLSC. Data were acquired using a AM Systems Model 3000 differential amplifier.

Histology

Following testing, rats were deeply anesthetized with equithesin and perfused with 10 mM phosphate buffered saline followed by 4% paraformaldehyde. Implants were carefully retracted from the brain after perfusion. Brains were removed and cryoprotected in sucrose (30% w/v) until cryosectioning.

SD and GEPR slides were blocked (3.75% normal goat serum, 2% bovine serum albumin, 0.3% Triton X-100 in TBS). Slides were incubated for up to 24 hours with primary antibody (1:2000, rabbit anti-GFP, Life Technologies, Washington, DC) at 4°C and then incubated for 90 min with secondary antibody (1:1000, goat anti-rabbit, AF498, Invitrogen, Carlsbad, CA) at room temperature. Slides were washed and then coverslipped with Cytoseal-60. Fluorescent photomicrographs were collected on a Nikon 80i microscope with a QImaging QIClick camera.

GAD Immunofluorescence

GAD brain sections on slides were dried at 40C for 1 hour, washed 3x10 min in 1X PBS, and quenched in 1mM glycine for 30 min. Sections were then washed again 3x10 min in 1X PBS and blocked for 2 hours in 1X PBS containing 0.3% Triton X-100 and 5% normal goat serum (Sigma Aldrich AB7481). Sections were incubated with mouse anti-GAD67 (MAB5406, Fisher Scientific, 1:1000) diluted in blocking solution (5% normal goat serum in 1X PBS with 0.3% Triton) at 4°C for 1 day. Sections were next washed with 3x10 min in 1X PBS and then incubated with Alexa 594

conjugated goat anti-mouse secondary antibody, diluted 1:1000 in blocking solution, at room temperature for 2 h. Following secondary antibody incubation, tissue was washed 3x10 min with 1X PBS, and coverslipped with DAPI fluoromount (Vector Laboratories H-1500).

GAD+ Neuron colocalization

Z stack images through the SNpr were taken on a Leica SP8 confocal microscope using a 20X oil-immersion lens (Leica SP8). Neurons transduced with GFP+ were quantified for colocalization with cell bodies of histologically stained GAD+ neurons. FIJI software was used for quantification and median intensity Z projections were quantified using the cell counter plug-in.

Statistics and data analysis.

Statistical analyses were performed in GraphPad Prism (GraphPad Software, Inc, La Jolla, CA). Non-parametric data (e.g., seizure severity, seizure frequency, seizure count) were analyzed using Wilcoxon Matched Pairs test for paired data. Parametric data (e.g., GBL experiments) were tested for normality using the Kolmogorov-Smirnov normality test. Unless otherwise noted, all GBL experiments passed the normality test and were analyzed using paired t-tests or ANOVA. The threshold for statistical significance was set at $P < 0.05$. A trend was considered to be a $P < 0.075$, but > 0.05.

Supplemental References

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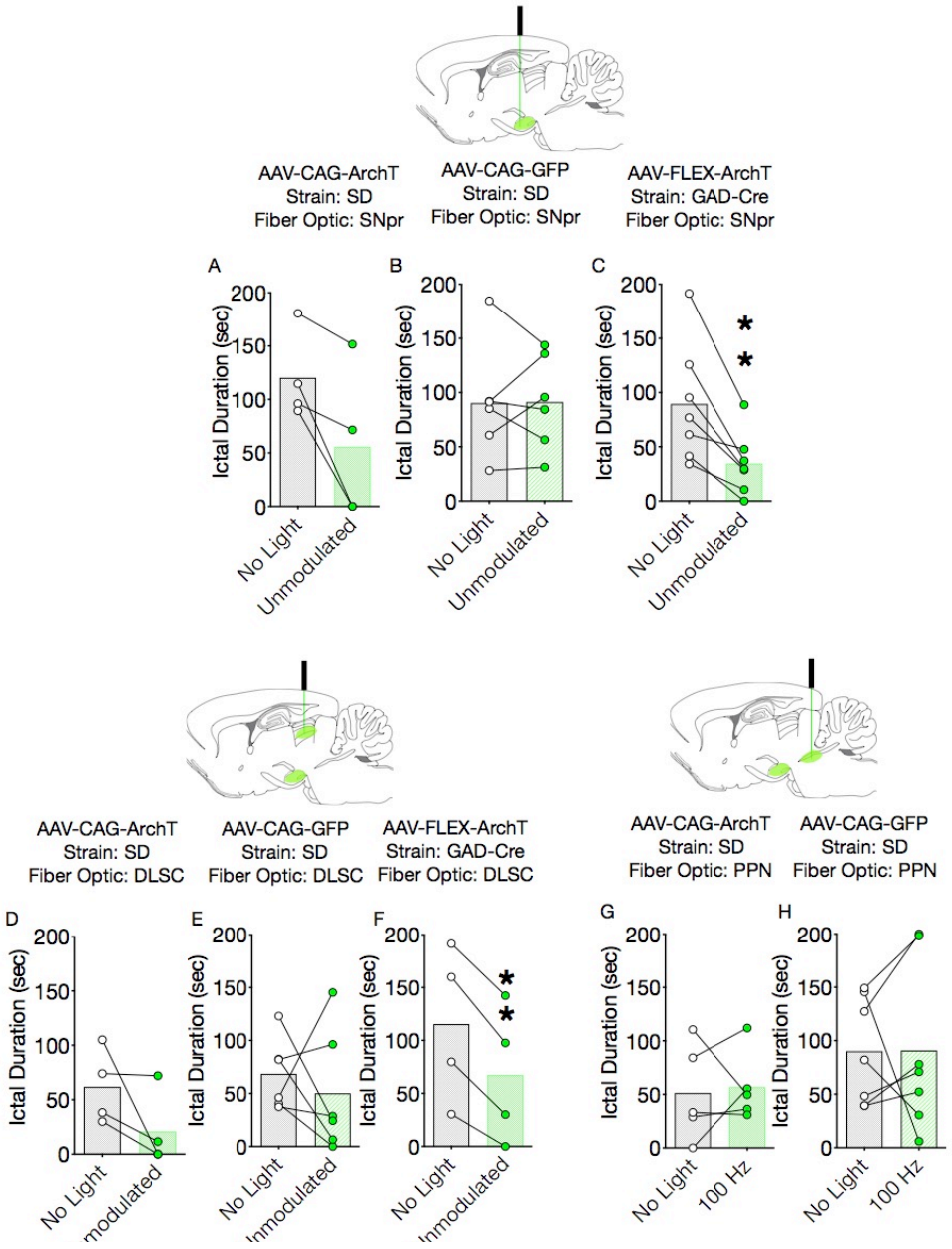

Fig. S1. Quantification of electrographic fast ictal activity in the PTZ model. (A) Duration of fast ictal electrographic activity under no light and unmodulated light delivery conditions in wild-type (SD) rats expressing ArchT in the SNpr. While duration of activity was numerically decreased in all animals, it did not reach the level of statistical significance. (B) Duration of fast ictal activity in control (AAV-CAG-GFP) animals was unchanged as a function of light delivery. (C) Duration of fast ictal activity was significantly decreased by light delivery in GAD-Cre rats expressing ArchT in the substantia nigra (paired t-test, t=4.418, df=6, p=0.0045). (D) Silencing nigrotectal terminals in wild-

type (SD) rats reduced fast ictal activity in 3 of 4 animals, but did not reach the level of statistical significance. (E) Light delivery to nigrotectal terminals was without effect in control (AAV-CAG-GFP) animals. (F) Nigrotectal silencing in GAD-cre animals significantly reduced the duration of fast ictal activity (paired t-test, $t=7.314$, $df=3$, $p=0.0053$). Light delivery to nigrotegmental terminal fields was without effect on the duration of fast ictal activity in ArchT (G) and control vector (H) expressing animals. ** = $P<0.01$

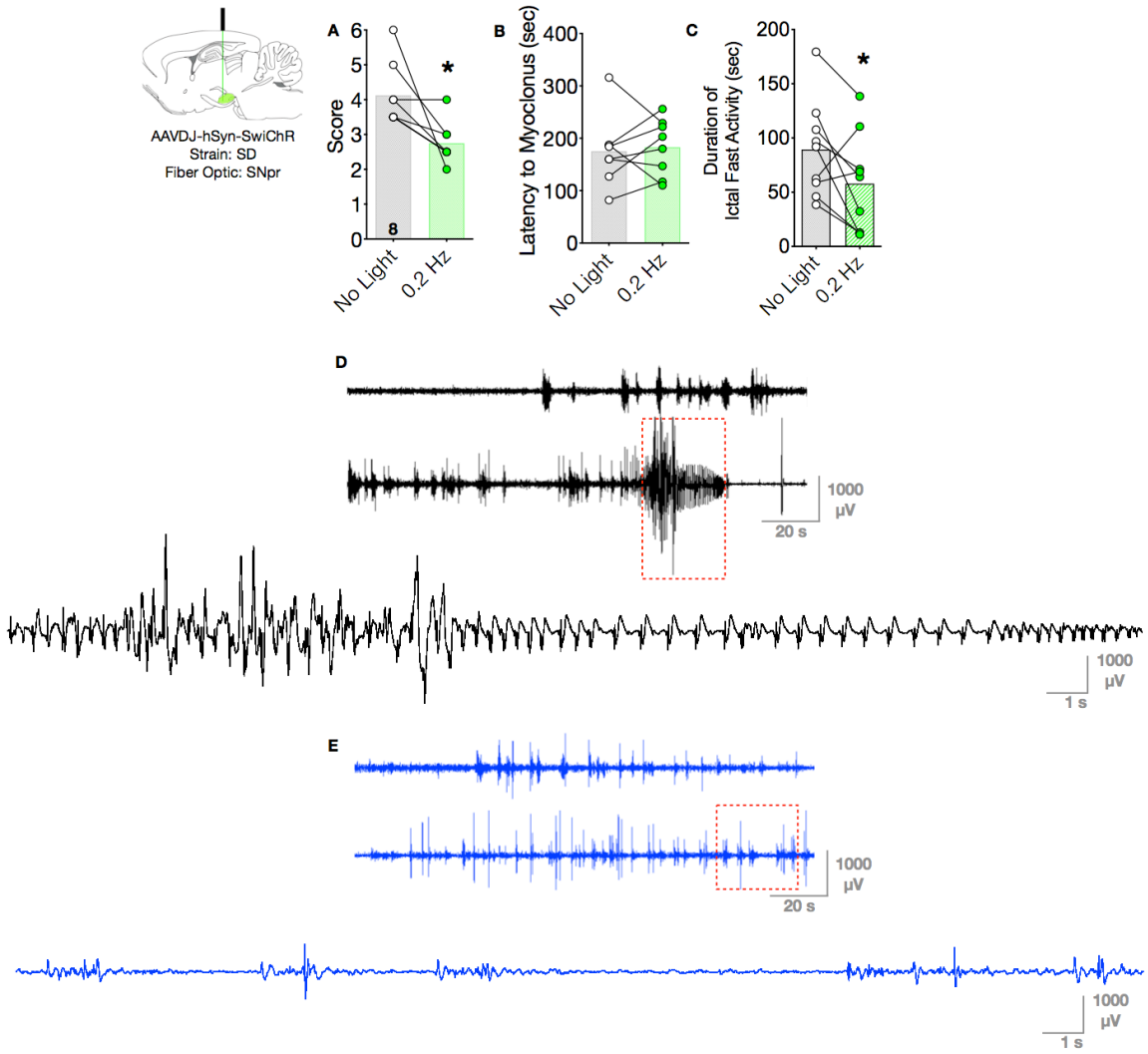

Fig. S2. Optogenetic silencing of SNpr via step-function opsin attenuates seizures evoked by pentylenetetrazole. Optogenetic inhibition of cell bodies in the SNpr significantly attenuated the severity of PTZ-induced seizures (A; p=0.0156, Wilcoxon paired sign-rank test) without impacting latency to seizure onset (B; (paired t-test, t=0.3485, p=0.7377)). Total duration of fast ictal electrographic activity was significantly reduced (t=2.46, df=8, p=0.039) following light delivery, as is also evident in the cortical EEG traces (compare D to E). (D) Cortical EEG of a PTZ seizure in the absence of light delivery, (E) cortical EEG of the same subject in C during a PTZ seizure with optogenetic silencing of SNpr. Bars indicate means with individual animal data indicated by circles and lines. Red boxes indicate the segment displayed with an expanded timescale. * = P<0.05

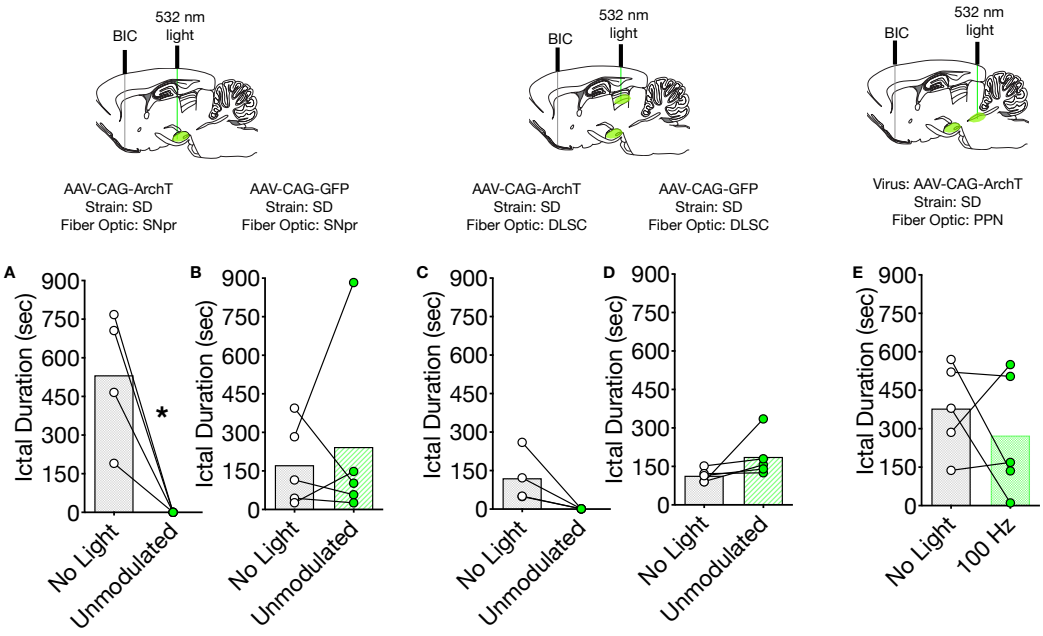

Fig. S3. Quantification of electrographic fast ictal activity in the Area Tempestas model. Fast ictal electrographic activity was abolished following light delivery to the SNpr in ArchT-expressing animals (A, paired t-test, t=4.051, df=3, p=0.027), but not in animals expressing GFP (B, paired t-test, t=0.4771,df=4, p=0.6582). (C) Optogenetic silencing of nigrotectal terminal fields abolished fast ictal activity in all recordings analyzed, but did not reach the level of statistical significance (paired t-test, t=2.429, p=0.0934). (D) Light delivery to nigrotectal terminals was without effect in rats expressing a control vector. (E) Light delivery to nigrotegmental terminals was without effect on the duration of fast ictal activity. * = P<0.05

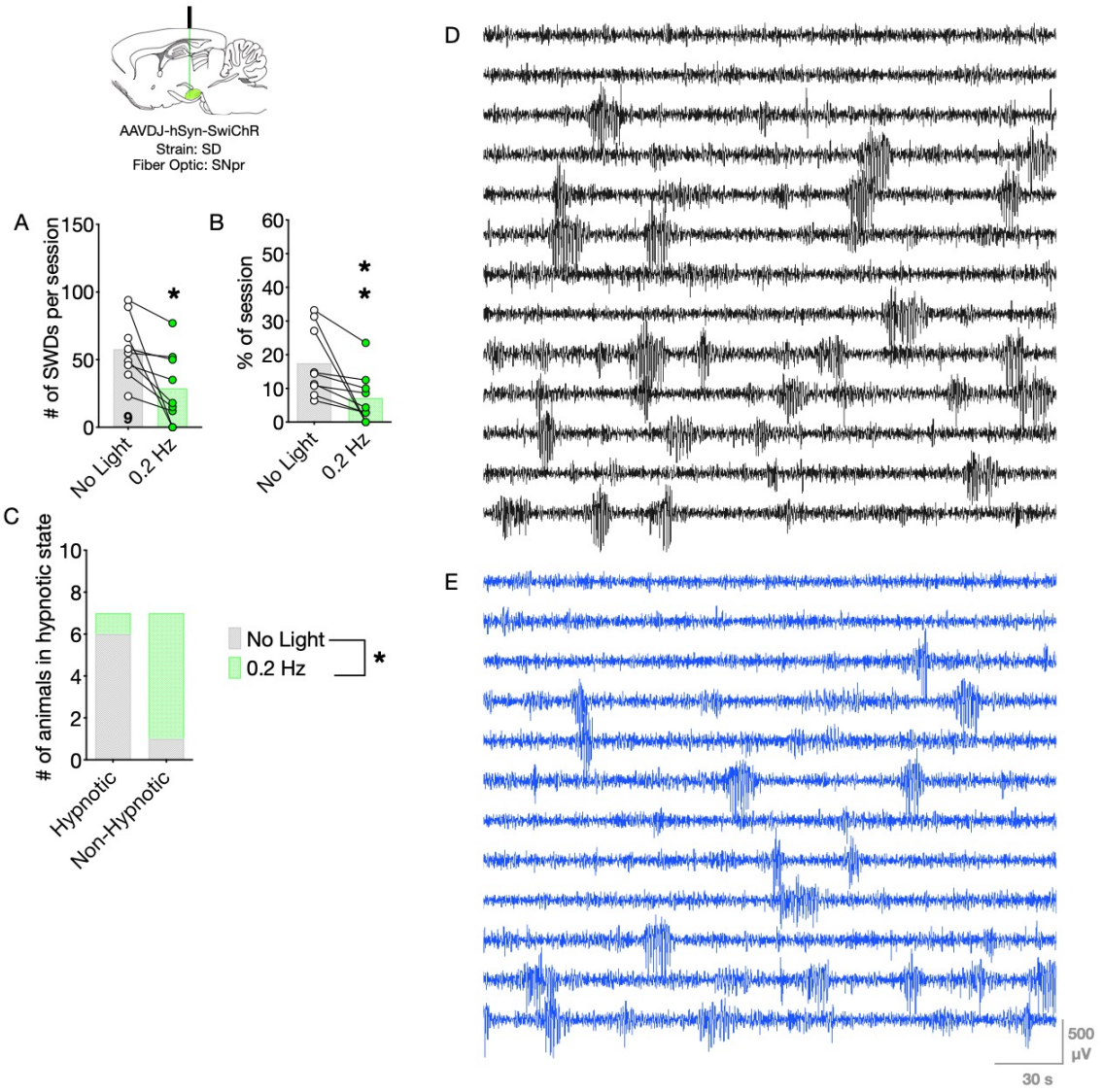

Fig. S4. Optogenetic silencing of SNpr via step-function opsin attenuates spike-and-wave discharges evoked by GBL. Optogenetic inhibition of cell bodies in the SNpr significantly reduced (A) the total duration of SWDs and (B) the number of SWDs evoked by GBL. (C) Incidence of GBL-evoked hypnotic state was reduced by optogenetic silencing of SNpr. (D) Cortical EEG of GBL seizures in the absence of light delivery, (D) cortical EEG of the same subject in C during a GBL session with optogenetic silencing of SNpr. Red squares indicate SWDs. Bars indicate means with individual animal data indicated by circles and lines. * = P<0.05, **= P<0.01.