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Preparation and Implantation of Electrodes for Electrically Kindling VGAT-Cre Mice to Generate a Model for Temporal Lobe Epilepsy --Manuscript Draft--

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TITLE:

Preparation and Implantation of Electrodes for Electrically Kindling VGAT-Cre Mice to Generate a Model for Temporal Lobe Epilepsy

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SUMMARY:

This report describes the methods to generate a model of temporal lobe epilepsy based on the electrical kindling of transgenic VGAT-Cre mice. Kindled VGAT-Cre mice may be useful in determining what causes epilepsy and for screening novel therapies.

ABSTRACT:

It was discovered that electrical kindling of VGAT-Cre mice led to the spontaneous motor and electrographic seizures. A recent paper focused on how unique VGAT-Cre mice were used in developing spontaneous recurring seizures (SRS) after kindling and a likely mechanism – insertion of Cre into the VGAT gene – disrupted its expression and reduced GABAergic tone. The present study extends these observations to a larger cohort of mice, focusing on key issues such as how long the SRS continues after kindling and the effect of the animal's sex and age. This report describes the protocols for the following key steps: making headsets with hippocampal depth electrodes for electrical stimulation and for reading the electroencephalogram; surgery to affix the headset securely on the mouse's skull so that it does not fall off; and key details of the electrical kindling protocol such as duration of the pulse, frequency of train, duration of train, and amount of current injected. The kindling protocol is robust in that it reliably leads to epilepsy in most VGAT-Cre mice, providing a new model to test for novel antiepileptogenic drugs.

INTRODUCTION:

Epilepsy is a major neurological disorder with significant economic and human burdens. NINDS estimates there are 3 million Americans with epilepsy. Approximately 0.6 million of these patients have temporal lobe epilepsy (TLE)¹. Unfortunately, medical treatment of TLE fails in one-third of the patients because of ineffectiveness, development of drug resistance, or

intolerance to side effects². Clearly, there is a significant need to develop novel therapies for TLE, a conclusion shared by the American Epilepsy Society Basic Science Committee, the International League Against Epilepsy Working Group for Preclinical Epilepsy Drug Discovery, and the National Advisory Neurological Disorders and Stroke Council^{3,4}.

Current animal models of temporal lobe epilepsy use either chemoconvulsants (e.g., kainate, pilocarpine) or prolonged electrical stimulation to induce a long-lasting status epilepticus^{5–7}. Many animals die during the procedure (10%–30% in rats, up to 90% in mice⁸). Animals that survive and develop epilepsy show extensive neuronal death throughout the brain^{9,10}. This death triggers a cascade of responses, beginning with the activation of microglia, astrocytes, and infiltrating monocytes. Neuronal responses include circuit reorganization (e.g., mossy fiber sprouting), birth of new neurons that fail to integrate properly into circuits (e.g., ectopic granule cells), and intrinsic changes that lead to hyperexcitability (e.g., upregulation of Na⁺ channels). An epilepsy model without significant neuronal death will facilitate the search for new antiepileptic drugs.

While testing the GABA hypothesis of epilepsy, it was discovered that treating VGAT-Cre mice with a mild electrical kindling protocol led to the spontaneous motor and electrographic seizures¹¹. In general, the electrical kindling of rodents does not lead to spontaneous seizures that define epilepsy, although it can, in cases of over-kindling¹¹. VGAT-Cre mice express Cre recombinase under the control of the vesicular GABA transporter (VGAT) gene, which is specifically expressed in GABAergic inhibitory neurons. It was found that insertion of Cre disrupted the expression of VGAT at the mRNA and protein levels, thus impairing GABAergic synaptic transmission in the hippocampus. It was concluded that kindled VGAT-Cre mice could be useful to study the mechanisms involved in epileptogenesis and for screening novel therapeutics¹¹. The present report provides the methods used in generating the model in detail.

PROTOCOL:

Animal use followed ARRIVE¹² guidelines and was approved by the Animal Care and Use Committee of the University of Virginia.

1. Making headsets with two bipolar electrodes (Figure 1)

[Place Figure 1 here]

1.1. Cut 3.5 cm of stainless polytetrafluoroethylene-coated stainless-steel wire.

1.2. Strip about 1 mm of the insulation coat off the wire on both ends. Do not have too much of the wire stripped.

1.3. Put two pins on a vise holder with the bottom part of the pin that has a longer slit facing down.

1.4. Apply flux onto the stripped ends of the wire and onto the tops of the pins.

89
90 1.5. Tin the stripped part of the wire with just enough solder to coat it.

91
92 1.6. Add a minimal amount of solder to the top of the pin without overflowing onto the
93 sides.

94
95 1.7. Place one end of the stripped ends of the wire into the pin as deep as it will allow while
96 the solder is melted.

97
98 NOTE: There is a side hole where the wire can come out – do not let the wire come out of the
99 pin. All of the stripped wire must stay within the pin.

100
101 1.8. Repeat steps 1.6–1.7 for the second pin, now with the other stripped ends of the wire.

102
103 1.9. Let the pins sit for 2–3 min to dry, remove them from the vise holder, and then pull on
104 them to make sure that the connection between the wire and the pins is strong and holds.

105
106 1.10. Rinse the pins in cold water, and then dry.

107
108 1.11. Verify conductance between pin 1 and pin 2 using an ohmmeter.

109
110 1.12. Bring the pins at the ends of the wire together; hold them parallel and close. Clamp a
111 hemostat to the center of the wire. Then, rotate the hemostat, so the wire twists around itself
112 fairly tight. Remove the hemostat.

113
114 1.13. Clamp a forceps onto the twisted wire 2 mm below the pins and bend the wire at 90°.

115
116 1.14. Push the same wire again 90° back over the forceps creating another bend 1 mm from
117 the first.

118
119 1.15. Cut the twisted wire at a 45° angle below the bend at 3.5 mm with small sharp scissors.

120
121 1.16. Prepare two of these bipolar (double-pin twisted) electrodes for each headset (optional,
122 second is back-up in case of electrical problems with the first).

123
124 1.17. Prepare one single reference electrode by cutting a wire with pins soldered on both
125 ends into two (steps 1.1–1.17, **Figure 1A**).

126
127 1.18. Cut the wire at 7 mm.

128
129 1.19. Bend the end 1 mm below the tip.

130
131 1.20. Then, cover the bent 1 mm tip of the wire with forceps and rotate the wire tight around
132 the forceps to create a small loop (1 mm in diameter).

1.21. Bend the loop perpendicular to the straight part of the wire to make the wire end-point outward again.

1.22. Assemble the two bipolar electrodes and the single reference electrode into the six-pin pedestal in a way that the bipolar electrodes are side by side with 6 mm distance between them, and that the reference electrode is placed in the middle outer hole (**Figure 1B**).

NOTE: An alternative method is to implant the electrodes, cement them in place, and then insert their pins into the pedestal.

2. Stereotaxic electrode implantation

2.1 Sterilize all surgical tools by autoclaving before the surgery. A sterile surgical field must be maintained during the surgery, and sterile surgical gloves must be used.

2.2 Use 8-week-old VGAT-Cre mice (age-matched, both male and female) for surgeries 4 weeks after weaning. Record the weight of the animal before surgery to allow the measurement of post-surgical weight loss.

2.3 Use a certified isoflurane vaporizer or a low-flow anesthesia system equipped with a precision syringe pump, integrated digital vaporizer, and feedback heat pad.

NOTE: Low-flow systems are capable of delivering anesthesia at low flow rates proportionate to the animal's size into either an induction chamber or through a nose cone on the stereotaxic frame (70 mL/min, isoflurane concentration in air is 4% for induction and 2% for surgery). Using less anesthesia not only benefits the animal during surgeries but also reduces the risk of lab personnel's exposure to isoflurane.

2.4 Place the anesthetized animal on a heated pad warmed to 37 °C to keep it warm during surgery. If using a feedback-controlled temperature system, insert the temperature probe into the rectum of the animal for temperature monitoring during surgery.

2.5 Mount the animal onto the stereotaxic frame by gently placing ear bars into the ears and the front upper teeth into the incisor bar. Make sure that the head is leveled and centered and cannot be moved when slightly probed.

2.6 Subcutaneously inject 0.5 mL of normosol for hydration.

2.7 Apply ocular lubricant to prevent corneal drying.

2.8 Monitor for the depth of anesthesia by the absence of the withdrawal reflex after pinching a hindlimb paw, and then decrease isoflurane to 1.5%–2.0% during surgery.

2.9 Trim the scalp hair around the surgical area with scissors and disinfect the skin with three cycles of alternating application of iodine and ethanol, finishing with iodine.

2.10 Make an incision on the skull, cutting out a part of the skin with scissors, exposing the skull. Pushing the skin aside, using a cotton swab, clean the skull from all the muscles and underlying tissues obstructing the view.

NOTE: To stop accidental bleeding, apply pressure over the bleeding site with a sterile cotton swab until it stops.

2.11 Clean the skull with hydrogen peroxide using sterile cotton swabs to make the skull sutures and both bregma and lambda visible.

2.12 Dry the skull thoroughly, and then apply one drop of self-etching dental adhesive using its applicator. Brush it into the skull, wait 60 s, and cure with a dental UV light for 40 s. A glossy surface indicates that the adhesive has been effectively cross-linked with the skull.

NOTE: This step is critical for secure attachment of the headset.

2.13 Use a 0.031" drill bit (0.79 mm) to drill two burr holes bilaterally for implantation of hippocampal depth electrodes (approximately 5,000 rpm). Drill one extra burr hole for the reference electrode in the cerebellum behind the lambda.

NOTE: When drilling, take care to lower the drill slowly and avoid drilling into the brain.

2.14 The coordinates for the electrodes are as follows (from bregma in mm): hippocampal electrodes at 3 mm posterior, 3 mm lateral, and 3 mm depth; and cerebellar reference electrode at 6 mm posterior, 0 mm lateral, and 0 mm depth (subdural).

2.15 To increase the accuracy, use a stereotactically mounted drill, zero the stereotactic manipulator X/Y axis when touching bregma – this is the point of reference for the coordinates.

2.16 Assemble a headset by inserting all the electrodes in the six-pin pedestal, making sure the pins are pushed all the way into the pedestal. Mount the pedestal into the electrode holder on a stereotactic frame.

2.17 Align electrodes above the corresponding burr holes. Stereotactically implant twisted bipolar stainless-steel wire electrodes in the right and left hippocampus and reference electrode into the cerebellum by slowly lowering the headset and guiding the electrodes into the burr holes.

2.18 When the hippocampal twist electrode is right above the hole, zero the Z axis and slowly lower to -3.0 mm.

2.19 Cover the skull surface and electrodes with dental cement and fill in the space between skull surface and the bottom of the pedestal. Wait for it to dry and harden. Then detach the electrode holder from the stereotaxic arm and remove the holder from the pedestal.

2.20 Inject 0.1 mL of ketoprofen (IP) for analgesia and a second dose of 0.5 mL of normosol for hydration and remove the animal from the stereotaxic frame.

2.21 Place a heated pad in the vivarium cage and move the animal onto the pad. Once it is fully awake, return it to the vivarium. Animals are housed singly from this point on to prevent chewing on each other's headset.

2.22 Feed the animal some soft food for 72 h after the surgery, monitor for weight loss, and subcutaneously inject 0.5 mL of normosol if the animal is losing weight and ketoprofen if there are signs of pain.

3. Electrical kindling protocol

3.1 Hook-up the mice to the EEG recording system using a flexible cable that fits the sockets on the mouse head and commutator (see the **Table of Materials** list).

3.2 Connect both leads to the stimulating electrode to the output of a constant current stimulator.

NOTE: It is very helpful to have a circuit board that switches these electrodes away from the EEG recorder and to the stimulator.

3.3 Set the stimulator to deliver 1 ms pulses at 50 Hz for a 2 s train duration.

3.4 Set the output of the stimulator at 10 microamps (μA) and deliver the 1st pulse.

3.5 Monitor the EEG for a characteristic after-discharge of high-frequency spikes that outlast the electrical stimulation pulse.

3.6 If no discharge is observed, then increase the amount of current injected in 20 μA increments until an after-discharge is triggered. The amount of current required is the After-Discharge Threshold (ADT).

3.7 Typical ADTs are 20–50 μA . If no discharge is observed even after increasing to 200 μA , then troubleshooting electrical connections and headset wiring with a high sensitivity ohmmeter is required. If the problem is in the stimulating electrode, then try stimulating with the other depth electrodes.

3.8 Animals are kindled by stimulating either 2x or 6x per day using a current that is 1.5x the ADT value for that mouse.

3.9 Monitor the behavioral response to the stimulation, which rises from the change of state to bilateral tonic-clonic seizures with falling. Score using a modified Racine class system¹¹.

REPRESENTATIVE RESULTS:

Animals

The model was originally developed using VGAT-Cre mice (*Slc32a1*^{tm2(cre)Lowl/J})¹³ on a mixed background. However, it has also been applied to the VGAT-Cre strain that is congenic with C57BL/6J. No difference has been observed in epilepsy that develops between the strains. Both strains express Cre recombinase under the control of the vesicular GABA transporter promoter. These mice were generated by knocking in an IRES-Cre cassette after the stop codon in the *Vgat* gene. These mice breed normally, so they are maintained as homozygotes. To prevent genetic drift of the colony, purchase breeders and only use F1 generation mice for experiments. All mice were housed in an AAALAC-accredited vivarium. Mice were given free access to food and water, 12 h light/dark cycles, and an enriched environment. For electroencephalographic (EEG) recording, mice were individually housed in clear plastic cages (homemade), allowing for concurrent video monitoring. Use both male and female mice for experiments. No difference in terms of kindling rate or seizure frequency has been observed between the sexes. The majority of these studies used 8-week-old mice at the time of headset surgery. However, one can also use mice that are between 4–20 weeks old. At 4-weeks, the mouse skull is ~90% of its final size¹⁴, so affixing a headset has minimal effect on growth. The time between surgery and commencement of the kindling protocol is not critical, although mice do need to be observed carefully during recovery from surgery for 72 h.

Kindling parameters

The electrical stimulation protocol was developed by Lothman and coworkers and described in detail in their 1989 paper¹⁵. In brief, the hippocampal electrode is connected to a constant current stimulator, and a 1 ms biphasic square wave pulse is delivered at 50 Hz over 2 s. Various parameters of the kindling protocol have been tested¹⁶. Use a current intensity that is 1.5x the minimal amount of current required to trigger an electrographic seizure for that mouse (After-Discharge Threshold, ADT). Mice are electrically stimulated twice a day every other day (2x, mid-morning and early afternoon). **Figure 2A** shows an overview of the kindling protocol and key seizure properties. However, a fast-kindling protocol is also effective, herein mice are stimulated six times a day delivered every other day (6x, one-hour between stimulations). Interestingly, kindling rates are similar between 2x and 6x protocols in terms of the number of stimulations required to achieve the kindled state (**Figure 2B**: 2x, 15 ± 1 , $n = 46$; 6x, 13 ± 1 , $n = 12$, mean \pm SEM, $P = 0.3$). The mice are considered fully kindled when stimulations evoke five tonic-clonic seizures with loss of postural control, which is a behavioral score of 5 on a modified Racine scale¹⁷. Kindling beyond this level, so-called over-kindling, can be performed but carries the risk of a fatal tonic seizure or SUDEP¹⁸. Mortality in this VGAT-Cre protocol is ~20% (15 out of 119 mice); this includes mice of either sex that died after either evoked or spontaneous seizures (8 male, 7 female).

Seizure properties

The recording system used in these studies has been discontinued. Alternative suppliers of EEG recording set-ups that are in use at the University of Virginia Rodent Epilepsy Monitoring Unit are provided in the **Table of Materials**. **Figure 2A** shows an overview of the kindling protocol and key seizure properties.

[Place **Figure 2** here]

VGAT-Cre mice typically reach the kindled state criterion after 15 stimulations (pooled 2x and 6x protocols, mean \pm SD of 7.4 stimulations, **Figure 2B**). As can be seen by the violin plot of the data, many animals require less stimulations (10) and many require more (18). It has not been possible to find a factor that determines the difference, excluding sex, age, ADT value, or electrode placement. Within a few weeks of being kindled, most mice develop multiple spontaneous seizures (90%), which defines epilepsy. The latency to spontaneous recurring seizures (SRS) is 10.7 ± 6.3 days (**Figure 2C**). A fraction of mice develop SRS before reaching the kindled state. As noted previously¹¹, VGAT-Cre mice are not spontaneously epileptic and require electrical stimulation to develop epilepsy. Once seizures begin, they occur at a frequency of 1.3 ± 0.6 seizures per day (**Figure 2D**). All electrographic seizures are accompanied by tonic-clonic motor seizures. The initial studies were short-term (spontaneous seizure frequency measured 1–2 weeks), but when the recording period was extended it was discovered that seizure frequency diminished with time. Using an arbitrary cut-off of 5 consecutive days without a seizure, reliable seizures occur for 23 ± 11 days. Taken together, this defines the period kindled VGAT-Cre mice are useful for drug screening campaigns. Power analysis using these SD and an effect size of 50% reduction shows 16 mice per group are required for statistically significant effects on stimulations to kindle, seizure frequency, and epilepsy duration.

A feature of the kindled VGAT-Cre model that distinguishes it from post-status epilepticus models is the absence of neuronal death. This was assayed using two accepted methods (**Figure. 3**): one, by counting nuclei in the CA1 layer stained with anti-NeuN antibody; and two, by measuring the amount of Fluoro-Jade C staining in hippocampal subfields that are vulnerable to chemoconvulsant-induced cell death (dentate, CA1, and entorhinal cortex).

[Place **Figure 3** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Key steps in EEG headset fabrication. (A) Appearance of the electrodes at the various steps in the protocol (numbers in the left match step). (B) A picture of the final product mounted in a homemade holder that fits the stereotaxic frame. Note, the holder ends with a collar pin assembly that fits into the headset pedestal.

Figure 2: Seizure properties of kindled VGAT-Cre mice. (A) Schematic diagram of the time course of an experiment. (B) The distribution of the number of electrical stimulations required to reach the fully kindled state. The kindled state is achieved when stimulations evoke five bilateral tonic-clonic motor seizures. (C) The distribution of latency to first spontaneous seizure

after the last electrical stimulation. **(D)** The distribution of observed seizure frequencies, which is calculated as the total number of seizures divided by the number of days recorded. **(E)** The distribution of how long mice were epileptic as defined by spontaneous seizures occurring with an inter-seizure interval below 5 days. All the graphs use violin plots where the median is shown with a dark line and 25th and 75th quartiles are shown with light lines. The number of animals in each plot is shown on the X-axis.

Figure 3: Neuronal death as assayed by either anti-NeuN staining or Fluoro-Jade C staining.

(A) Plot of anti-NeuN-positive neurons in various sub-fields of the hippocampus and entorhinal cortex. Abbreviations are as follows: DGC, dentate granule cell layer; hilus refers to the dentate hilus, CA1, cornu ammonis pyramidal layer I; and EC, entorhinal cortex; L2, layer 2; and L3, layer 3. Two horizontal brain slices corresponding to -4 mm below bregma were stained from each animal (Control naive mice, n = 7; epileptic VGAT-Cre, n = 13; see Straub et al. for details¹¹). Data were normalized to average neuronal counts determined in naive VGAT-Cre mice in the respective subfield. Images of Fluoro-Jade C staining from an epileptic VGAT-Cre mouse **(B)** and from a post-status rat (Li/pilocarpine model, see Dey et al. for details and analysis¹⁰). Analysis of the Fluoro-Jade C staining of VGAT-Cre mice was presented in Straub et al.¹¹).

DISCUSSION:

This report describes a protocol where electrical kindling of mice leads to epilepsy. Since the stimulating electrode is placed in the hippocampus, this is a focal limbic epilepsy that models temporal lobe epilepsy (TLE) in patients. A critical step in this protocol is to use VGAT-Cre mice, which due to insertion of an IRES-Cre recombinase cassette in the *Vgat* gene, shows impaired inhibitory GABA currents¹¹. C57BL/6 do not develop epilepsy after kindling with this protocol, although it is possible that other strains of mice will.

The protocol was developed using placement of the stimulating depth electrodes in the hippocampus. The coordinates provided the target with the perforant path projections from the entorhinal cortex as they enter the CA1 subfield. The electric field induced by electrical stimulation has not been defined, therefore, the precise location of the electrodes is not critical. In fact, rodents can be electrically kindled anywhere in the limbic circuit, e.g., amygdala and piriform cortex¹⁹. The following are critical steps in the protocol: one, proper soldering of the EEG headsets to ensure low resistance to the stimulating current; two, using a dental adhesive that is in use in dental clinics to bind to the skull and provide a surface for adhesion of the dental cement; and three, the use of a constant current amplifier to deliver the described electrical pulses.

Trouble-shooting is typically limited to checking electrical connections, which include the connections in the headset, the headset to the cable, the cable to the commutator, and the commutator to the recording device. Using an ohmmeter with high sensitivity is critical.

Limitations to the technique include the requirement of appropriate expertise and equipment to perform the surgeries and record the EEG.

An advantage of the model over existing animal models of TLE is that there is minimal death of neurons¹¹. The other TLE models are post-status epilepticus models, which trigger extensive neuronal death¹⁰. This death leads to extensive activation of microglia, astrocytes, and infiltration by circulating monocytes. Taken together, it becomes difficult to distinguish mechanisms that cause epilepsy from mechanisms triggered by prolonged status epilepticus. It is anticipated that kindled VGAT-Cre mice will be useful for developing novel therapies that are both anti-seizure and anti-epileptic. This report provides key metrics and power analysis that can guide future drug development efforts using these mice.

Another advantage to the kindled VGAT-Cre model is the high percentage of animals that develop epilepsy (90%) and the regularity of the spontaneous seizures. Disadvantages of the model are a relatively low seizure frequency (1.5/day) and that seizure frequency declines after 3 weeks, and in some cases seizures seem to stop. Efforts are underway to address these issues.

ACKNOWLEDGMENTS:

The authors thank John Williamson for helpful discussions on this protocol. This work was supported by NIH/NINDS grant NS112549.

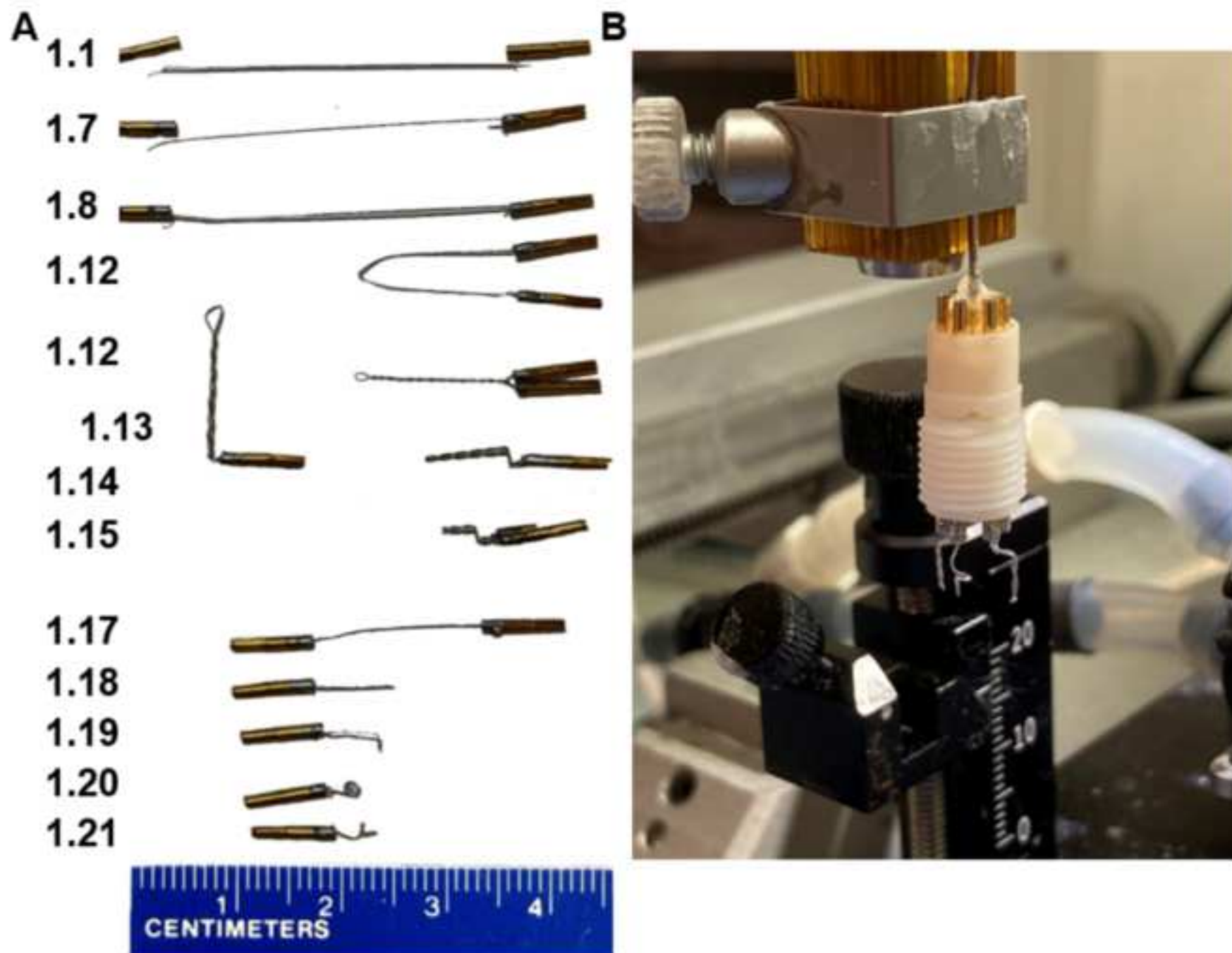
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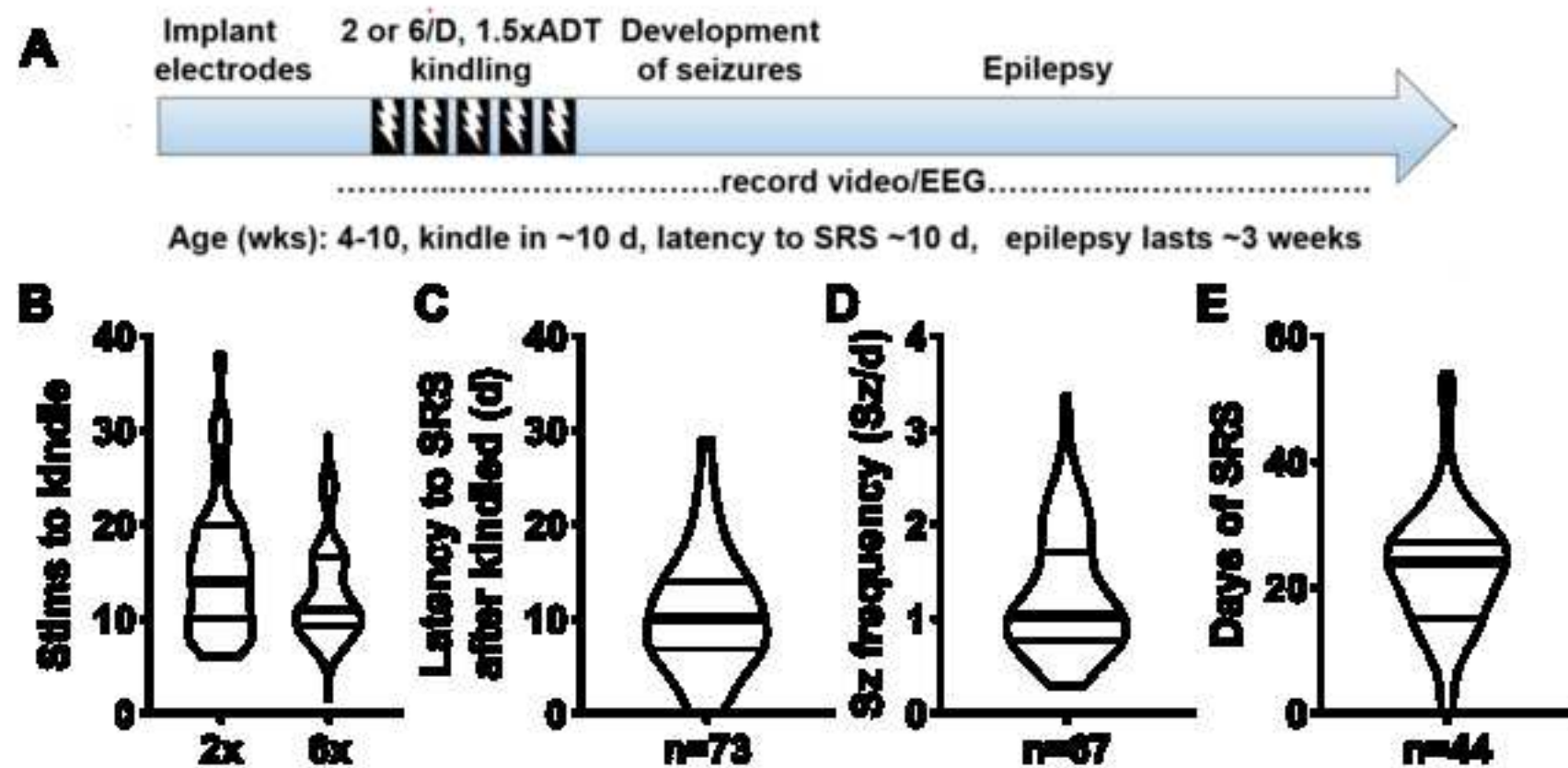
The authors have no conflicts to disclose.

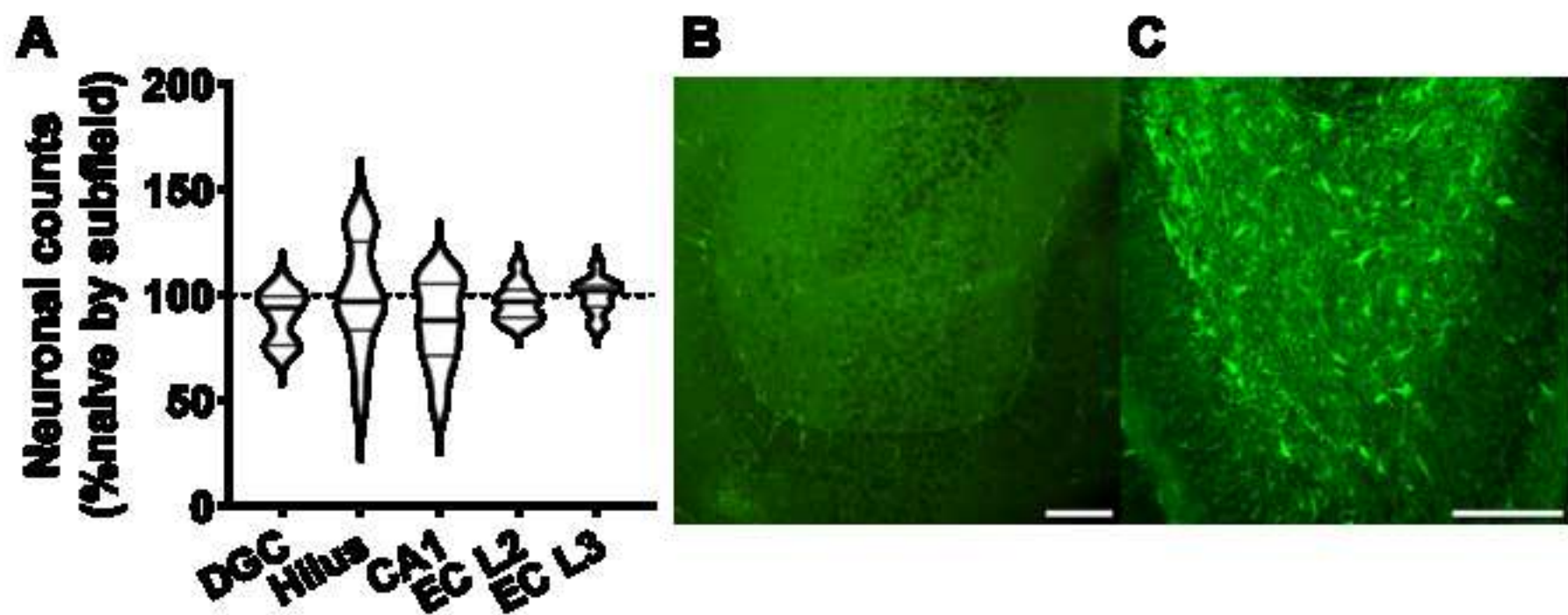
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Response to comments on 62929 R1

Line 1: We have gotten approval for filming the live animal procedure. Our ACUC has one stipulation and that is that they reserve the right to review the video before dissemination.

Line 68 added Ethics statement: "Animal use follows ARRIVE guidelines¹ and was by the Animal Care and Use Committee of the University of Virginia."

This resulted in adding one more reference:

- 1 Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M. & Altman, D. G. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* **8** (6), e1000412, doi:10.1371/journal.pbio.1000412, (2010).

Line 169, depth of anesthesia defined by adding "by the absence of the withdrawal reflex after pinching a hindlimb paw"

Comment A4 asks for Fig. 3 details, and the following was added at the end of the Representative Results section: "A feature of the kindled VGAT-Cre model that distinguishes it from post-status epilepticus models is the absence of neuronal death. This was assayed using two accepted methods (Fig. 2): one, by counting nuclei in the CA1 layer stained with anti-NeuN antibody; and two, by measuring the amount of Fluoro-Jade C staining in hippocampal subfields that are vulnerable to chemoconvulsant-induced cell death (dentate, CA1 and entorhinal cortex).

[Place **Figure 3** here]"

Comment A4 asks how these data show this is a model of temporal lobe epilepsy. This point is covered in the 2nd sentence of the Discussion.

Comments A4, A6, A7, corrected Fig. numbers.

Comment A5, added graph to figure 1B and updated text.