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TITLE:

A Behavioral Screen for Heat-Induced Seizures in Mouse Models of Epilepsy

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KEYWORDS:

epilepsy, febrile seizures, heat-induced seizures, GEFS+

SUMMARY:

The goal of the method is to screen for hyperthermia or heat-induced seizures in mouse models. The protocol describes the use of a custom-built chamber with continuous monitoring of the body temperature to determine whether elevated body temperature leads to seizures.

ABSTRACT:

Transgenic mouse models have proved to be powerful tools in studying various aspects of human neurological disorders, including epilepsy. The *SCN1A*-associated genetic epilepsies comprise a wide spectrum of seizure disorders with incomplete penetrance and clinical variability. SCN1A mutations can result in a large variety of seizure phenotype ranging from simple, self-limited fever-associated febrile seizures (FS), moderate-level genetic epilepsy with febrile seizures plus (GEFS+) to more severe Dravet Syndrome (DS). Although FS are commonly seen in children below 6–7 years of age who do not have genetic epilepsy, FS in GEFS+ patients continue to occur into adulthood. Traditionally, experimental FS have been induced in mice by exposing the animal to a stream of dry air or heating lamps, and the rate of change in body temperature is often not well controlled. Here, we describe a custom-built heating chamber, with a plexiglass front, that is fitted with a digital temperature controller and a heater-equipped electric fan, which can send heated forced air into the test arena in a temperature-controlled manner. The body temperature of a mouse placed in the chamber, monitored through a rectal probe, can be increased to 40–42 ˚C in a reproducible manner by increasing the temperature inside the chamber. Continual visual monitoring of the animals during the heating period demonstrates induction of heat-induced seizures in mice carrying an FS mutation at a body temperature that does not elicit behavioral seizures in wild-type litter mates. Animals can be easily removed from the chamber and placed on a cooling pad to rapidly return body temperature to normal. This method provides for a simple, rapid, and reproducible screening protocol for the occurrence of heat-induced seizures

in epilepsy mouse models.

INTRODUCTION:

Epilepsy, the fourth most common family of neurological disorders in the United States[1], are characterized by an imbalance of excitatory and inhibitory drive in the CNS that leads to recurrent seizures. Febrile seizures (FS) or fever associated seizures can occur in the general population, most often in children as early as 3 months up through 6 –7 years of age. However, in some individuals with genetic mutations, most often in a sodium channel gene, FS can persist beyond the age of 7 years into adulthood. This condition is referred to as febrile seizures plus or FS+. Rapid advances in genome sequencing have identified over 1,300 mutations in the human sodium ion channel gene *SCN1A*, making it a hotspot for epilepsy mutations. *SCN1A* mutations have been linked to a wide spectrum of seizure disorders, including febrile seizures (FS), genetic epilepsy with febrile seizures plus (GEFS+), and Dravet Syndrome (DS)[2–6]. About 20% of SCN1A missense mutations leads to GEFS+[5,7,8]. Pediatric history of complex or prolonged FS in childhood can subsequently develop into more debilitating forms of epilepsy such as temporal lobe epilepsy (TLE)[9–11]. Dravet Syndrome arises due to truncation mutations or loss of function mutations in SCN1A and is a severe form of intractable epilepsy, with childhood onset of febrile seizures that develop into refractory seizures, and is often associated with cognitive, developmental, and motor impairments[2,5,12]. Since many individuals with GEFS+ and/or DS exhibit febrile seizures, it becomes imperative to develop novel therapies to better combat these seizure disorders.

Animal models of SCN1A associated epilepsy have proven invaluable in characterizing different types of seizures (febrile vs generalized) and dissecting the neuronal mechanism of seizure generation[13–18]. While the study of spontaneous seizures via EEG/EMG recordings in rodent brains is well established and is a very useful tool, only a few studies have attempted to mimic febrile seizures in mouse models[14,16,19–23]. Previous studies have used a jet of heated dry air, or a methacrylate cylinder fitted with a thermal system, or heat lamps with a temperature controller in enclosed test arenas[9,16,21–24] to induce seizures via hyperthermia. In order to increase body temperature in a more controlled environment, the protocol described here uses a custom-built chamber with a temperature-controlled heating system that allowed reproducible rates of increase in the body temperature of a mouse inside the chamber. The heat chamber was constructed from wood (length 40 cm x width 34 cm x height 31 cm) and was fitted with a digital temperature controller with a K thermocouple. A small axial fan equipped with a heater at the back panel of the chamber directs heated air into the chamber regulated by a digital temperature controller. This forced air heating system enables one to control the rate at which the chamber temperature increases. (**Figure 1A,B**). The K thermocouple located inside the wooden heat chamber sends feedback to the digital temperature controller, to maintain constant temperatures inside of the box during the assay. Setting the temperature on the digital temperature controller, enables the electric fan to send heated forced air through vents to uniformly heat the chamber (**Figure 1A**). The front panel of the heat chamber is a clear plexiglass sheet to enable easy video recording of the trials.

Adult (P30–P40) mice, heterozygous for a missense mutation in SCN1A that causes GEFS+ and an equal number of wild-type litter mates to serve as the control group, were selected for each

experiment. Animals, both male and female, used in these studies weighed at least 15 g as wild-type mice weighing less were more sensitive to heat-induced seizures than heavier animals of the same age. In the pilot study, both mutant and wild-type mice were observed to seek out the cooler corners of the chamber at the back and remained there for prolonged periods of time. To circumvent this, effective floor size inside the heat chamber test arena was reduced to length 16.5 cm x width 21.5 cm x height 27.5 cm by placing a wooden block B (dimensions 20 cm x 8cm x 7.2 cm) at the right side of the chamber (**Figure 1A**). The temperature of the heat chamber was initially set at 50 ˚C and preheated for at least 1 h before the start of the experiment, to ensure uniform heating inside the chamber. Each mouse was fitted with a rectal thermometer for continuous monitoring of body temperature throughout the experiment. A single mouse was placed in the chamber at a time and the temperature was kept at 50 ˚C between 1st–10th minute. The temperature was then raised to 55 ˚C for 11th–20th minute, and finally raised to 60 ˚C for 21st–30th minute. This resulted in a reproducible rate of increase in the mouse body temperature (**Figure 2A**). Each trial was video-taped and behavioral analysis was conducted offline.

The heating protocol can be easily modified to change the initial temperature of the heat chamber and the rate that the chamber is heated, which in turn changes how quickly the body temperature of the mouse is elevated during the assay. Thus, this method provides more flexibility over traditional methods in setting up the behavioral screens involving heat-induced seizures. The heat-induced seizure protocol can also be used to screen for anti-epileptic drugs that make mutant mice more resistant to heat-induced seizures or increase the threshold temperature at which seizures are observed. Similarly, beneficial effects of restrictive diet regimes such as keto diet on heat-induced seizures can be examined in normal chow-fed vs keto-fed mice.

[Place **Figure 1** here].

PROTOCOL:

All animal procedures were performed in accordance with the guidelines of Institutional Animal Care and Use Committee (IACUC) at University of California, Irvine.

1. Preparation for the heat-induced seizure assay

1.1 Switch on the **Power On** button on the heat chamber, followed by the **Heat On** button.

1.2 Set the temperature of the heat chamber at 50 °C using the keypad on the digital temperature controller.

1.3 Wait for a minimum of 1 h to preheat the chamber at 50 ˚C before introducing the first mouse into the chamber. Preheating ensures uniform heating inside the chamber.

1.4 Line the floor of the mouse heat chamber with cob bedding.

1.5 Mount a video recording camera in front of the heat chamber for recording each heat-induced seizure assay trial.

1.6 Line a 140 mm diameter Petri dish with thick layers of tissue paper and place it on ice to serve as a cooling pad.

NOTE: At the end of the assay, individual mouse will be transferred on the prechilled cooling pad to help bring down their elevated body temperature.

2. Preparing the mouse for heat-induced seizure assay

2.1 Select 10 adult mice (P30–P40), 5 that carry the epilepsy causing mutation and 5 of the wild-type litter mates for heat-induced seizure screening assay.

NOTE: Wild-type mice, not harboring any epilepsy causing mutation do not exhibit heat-induced seizures at temperatures below 44 ˚C and serve as the control group.

2.2 Weigh each mouse to be used for the screening assay and record its body weight. Only mice weighing 15 g or more should be used for the assay.

2.3 Screen one mouse at a time in the mouse heat chamber.

2.4 Briefly anesthetize the mouse using a few drops of isoflurane in a bell jar for 10–15 s.

2.5 Take the animal out of the bell jar and place it on a paper towel.

2.6 Ensure that the mouse is completely anesthetized by checking that the mouse is unresponsive to a noxious toe pinch.

2.7 Coat the metal tip of the rectal temperature probe with a lubricant (such as petroleum jelly) and gently insert it into the mouse.

2.8 Secure the rectal probe to the mouse's tail with tape, so the probe does not come out during the assay.

NOTE: Alternatively, place the animal in a mouse restrainer cone and insert the rectal temperature probe. Secure it by taping to the tail.

2.9 Ensure the rectal probe is connected to a multimeter that displays internal body temperature of the mouse.

2.10 Place the animal in a fresh cage lined with cob bedding, i.e., the recovery cage.

2.11 Start a timer and wait for 5 min. Observe the mouse until it has completely recovered from anesthesia and the mouse is active and grooming.

2.11.1 Simultaneously, monitor the core body temperature of the mouse till it stabilizes at 35–36 ˚C.

2.12 At the end of 5 min, note the body temperature of the mouse. This is the initial body temperature at time "0" min.

NOTE: If the core body temperature of the mouse is below 35 ˚C, wait for additional time for the animal to recover from anesthesia-induced hypothermia.

2.13 Quickly, transfer the individual mouse into the preheated mouse chamber. This marks the START of the experiment trial. Only one mouse is screened at a given time.

3. Heat-induced seizure assay

3.1 After gently placing the mouse on the floor of the pre-heated mouse heat chamber, start the camera for video recording the experiment.

3.2 Start the stopwatch. Record the body temperature of the mouse from the rectal thermometer at 1 min intervals for the duration of the experiment.

3.3 At regular intervals, increase the temperature of the mouse heat chamber such that the body temperature of the mouse increases at a rate of 0.25 ˚C /min.

NOTE: It is important that the body temperature of the mouse should rise at a rate of 0.25 ˚C/min. More rapid increases in body temperature can lead to heat stroke or death and should be avoided.

3.4 Following this protocol, increase the temperature of the mouse heat chamber by 5 ˚C every 10 min as shown in **Figure 2A**.

3.5 At 9.5 min, set the temperature of the heat chamber to 55 ˚C, to stabilize the temperature of the heat chamber to 55 ˚C by the 10^{th} min as shown on the digital temperature display.

3.6 Similarly, increase the temperature to 60 ˚C at 19.5 min to stabilize the temperature of the heat chamber to 60 ˚C by the 20^{th} min. Each seizure screening trial lasts for 30 min.

3.7 If the mouse has a seizure (vocalizes, shows head nodding, forelimb clonus, hindlimb extension, falls on its side, or experiences generalized tonic/clonic convulsions), record the following information.

3.7.1 Record the body temperature of the mouse during the seizure (seizure threshold temperature) from the rectal temperature thermometer.

3.7.2 Record the seizure behavior characteristics such as head nodding, forelimb clonus, hind limb extension, falling on side, and/or generalized tonic/clonic seizures (GTCS) displayed by the mouse.

3.8 Quickly but gently pick up the mouse from the chamber and place it on the cooling pad prepared in step 1.6.

3.9 Wait for the mouse body temperature to come down to 36–37 °C, before transferring it to a recovery cage. Only one mouse is placed in a recovery cage at a time.

NOTE: Do not mix mice that are yet to be used for heat-induced screening with the mouse that has already experienced the heat-induced seizure experiment trial.

3.10 Gently and carefully, cut the tape between the mouse tail and rectal probe wire with a pair of scissors to remove the rectal probe from the mouse.

3.11 Wipe clean the metal tip of the rectal probe with 70% alcohol and soft tissue wipes to keep it ready for the next trial.

3.12 Continue to observe the mouse in the recovery cage until it resumes normal activity (walking, grooming, etc.), before returning the mouse to its home cage. This marks the END of the experiment trial for this mouse.

3.13 Record the animal status after the assay—alive and recovered from the test session or dead. High intensity seizures involving uncontrolled jumping and generalized tonic/clonic seizures can sometimes result in the death of the mouse.

3.14 If a mouse does not experience heat-induced seizures within the 30 min observation period or the body temperature of the mouse reaches 44 ˚C, remove the mouse from the heat chamber and place on the cooling pad till the body temperature of the mouse returns to 36–37 ˚C.

3.15 Reset the temperature of the mouse heat chamber to 50 ˚C and allow it to equilibrate till the display temperature on the digital temperature controller shows 50 ˚C.

3.16 Prepare the next mouse for screening trial as described in section 2 and repeat the steps from section 3.

4. Euthanizing the animals

4.1 After concluding the screening on all the mice individually for heat-induced seizures following the 30 min trial, euthanize all the mice as per the institution's IACUC guidelines.

5. Analyzing the heat-induced seizure data

5.1 After completing the screening of a cohort of animals, calculate the percentage of mice in a given genotype showing seizures using the following formula:

$$\% \text{ of mice in showing seizures} = \frac{\text{Number of mice that exhibit heat seizures}}{\text{Total number of mice screened}}$$

5.2 Estimate the mean seizure threshold temperature of mice within a given genotype by averaging the seizure threshold temperature of all mice (noted in step 3.7) in that genotype that exhibit heat-induced seizures.

5.3 Perform statistical analysis to determine whether the percentage of mice and mean seizure threshold temperature values are significantly different between mutant and wild-type mice, using Student's *t*-test, at $p < 0.05$.

5.4 While still being blind to the identity and genotype, replay the video recordings of each of the mouse during the heat-induced seizure assay screening on a computer screen to score severity of seizure bouts.

5.5 Give scores to individual mouse exhibiting heat-induced seizure behavior by using the modified Racine scale[13] as described by previous studies[13,14]. See **Table 1** for details.

[Place **Table 1** here]

5.6 If a mouse, while experiencing heat-induced seizures, only shows head nodding, give it a score of 2. If a mouse starts a seizure episode with head nodding but also exhibits forelimb clonus, falling over, and/or jumping give it a score of 5.

5.7 Record the maximum score for each mouse using the modified Racine scale[13] as described above.

5.8 Plot a scatter graph of maximum Racine scores exhibited by all mice in a given genotype.

5.9 Statistically compare maximum Racine scores among different mouse groups as a method to determine the severity of behavioral seizures such as heat-induced seizures.

NOTE: Racine scores are helpful to compare seizure characteristics between different mutant mice groups or genotypes. It is expected that the wild-type mice would not undergo heat-induced seizures and would not have to be considered for Racine score comparisons.

REPRESENTATIVE RESULTS:

Animal models with febrile seizure mutations are expected to undergo heat-induced seizures at elevated body temperatures that do not induce seizures in the wild-type litter mates. SCN1A mutations have been linked with febrile seizures, including K1270T GEFS+ patients, who display both febrile and afebrile generalized seizures[7]. We screened CRISPR generated SCN1A K1270T GEFS+ mutant mice recently described in a study[14] for the occurrence of heat seizures in two genetic backgrounds – seizure resistant 129X1/SvJ (129X1) and seizure susceptible C57BL/NJ (B6N) backgrounds. Age matched wild-type litter mates in the mouse heat chamber which do not harbor any GEFS+ mutations and thus, are not expected to exhibit heat-induced seizures, served as the control group. The rate of the body temperature change over time was evaluated by plotting mean body temperature of mice recorded every minute during the assay. There was no difference in the rate of change of body temperature between heterozygous mutant mice and wild-type litter mates tested in respective 129X1 and B6N genetic backgrounds (**Figure 2B,C**). This suggests that thermoregulation is not altered in K1270T GEFS+ heterozygous mutant mice.

All heterozygous mutant mice from 129X1 (n = 15) or B6N (n = 9) genetic backgrounds exhibited heat-induced seizures (**Figure 2D**). None of the wild-type mice in the 129X1 enriched background (n = 13) exhibited heat-induced seizures (**Figure 2D**). In contrast, a third of the mice tested (n = 3 out of the 9 mice) in the seizure sensitive B6N background exhibited heat-induced seizures. Statistical comparison shows that percentage of heterozygous mutant mice exhibiting heat-induced seizures was significantly higher than their respective wild-type counterpart mice in both the 129X1and B6N genetic backgrounds (**Figure 2D**, Fisher's exact test, 129X1 $p < 0.0001$; B6NJ $p = 0.009$). The average seizure threshold temperature between the heterozygous mutant mice in 129X1 and B6N genetic backgrounds was similar. 129X1 mutant mice have a mean seizure threshold temperature of 42.6 ± 0.20 ˚C, which was not significantly different from the mean seizure threshold temperature of 42.7 ± 0.06 ˚C seen in B6N mice (**Figure 2E**; two-tailed unpaired Student's *t*-test, $p = 0.782$). It is important to note that the mean seizure threshold temperature of three B6N wild-type mice that exhibited heat-induced seizures was 43.7 ± 0.08 ˚C and significantly higher than the mean seizure threshold of 42.7 ± 0.06 ˚C displayed by B6N heterozygous mutant mice (**Figure 2E**, two-tailed unpaired Student's *t*-test, $p < 0.0001$).

The plexiglass front of the chamber makes it possible to do continuous video recordings during the assay that can be used later to score for seizure severity in each mouse on a modified Racine scale as described previously[14,20]. During a typical assay, heterozygous mutant mice would display heat-induced seizures with vocalization and/or head-nodding (Racine score 2), and rapidly transition to forelimb clonus, falling on side, jumping, hindlimb extension, and/or generalized tonic/clonic seizures (Racine scores 3–5) when body temperature reached about 42 ˚C. The maximum Racine score represents the most severe heat-induced seizure behavior among the mutant mice. The maximum Racine score of heterozygous mutant mice in 129X1 enriched background (n = 15) is not different from heterozygous mutant mice in B6N (n = 9) genetic background (**Figure 2F**; Mann-Whitney test, $p > 0.9999$). This suggests that heat-induced seizure behavior characteristics in K1270T GEFS+ mutant mice are independent of strain background.

Taken together, the data demonstrates that all mutant mice exhibit heat-induced seizures with

similar frequency, seizure threshold temperature, and behavioral seizure severity in a strain-independent manner. The majority of wild-type litter mates do not exhibit such seizures at or below 44 ˚C. About one-third of the wild-type control mice in a seizure sensitive B6N background did display heat-induced seizures (possibly due to genetic background effects) but the seizure threshold temperature was significantly higher compared to mutant mice in the same background. These results suggest that mutant mice in B6N genetic background are susceptible to heat-induced seizures at lower temperature thresholds due to the *SCN1A* GEFS+ mutation they harbor. Thus, using this protocol, one can evaluate heat-induced seizures in epilepsy mutant mice and distinguish from wild-type litter mate mice, which either do not undergo heat-induced seizures or display heat seizures at significantly higher temperatures.

FIGURE AND TABLE LEGENDS:

Figure 1: Description of the custom-built mouse heat chamber. (**A**) The front panel of the wooden mouse heat chamber shows the side control panel containing Power ON/OFF switch that turns on digital temperature controller, K thermocouple, fan heater's ON/OFF switch and heat indicator. The outer dimensions of the box and the inner test arena are shown in cm. A wooden block B used to effectively reduce test arena surface is also shown. The bottom of the test arena is covered with cobb bedding to prevent mice from directly coming in contact with heated wooden surfaces. (**B**) The back panel of the heat chamber shows the fan mounted on the top air vent and the power cord to supply electricity to the chamber. This figure is modified from Figure 3 in Das et al., 2021, eNeuro[14].

Figure 2: Mutant mice exhibit heat-induced seizures. (**A**) The heating protocol for behavioral screening of heat-induced seizures in mice. (**B–C**) Mean body temperature of mice across time in wild-type (*Scn1a*$^{+/+}$ - black triangles) and heterozygous mutant (*Scn1a*$^{KT/+}$ - orange circles) mice in two genetic backgrounds 129X1 and B6N, respectively. (**D**) Percentage of mice showing heat-induced seizures in both genetic backgrounds. Wild-type (*Scn1a*$^{+/+}$) and heterozygous (*Scn1a*$^{KT/+}$) mice are represented by black and orange bars, respectively. Heterozygous mutants in 129X1 and B6N backgrounds are shown in orange solid bars and orange bars with black stripes, respectively. (**E**) Seizure temperature threshold to heat-induced seizures in wild-type (*Scn1a*$^{+/+}$) and heterozygous mutant (*Scn1a*$^{KT/+}$) mice in both strains. (**F**) Scatter distribution of maximum Racine scores of heat-induced seizures exhibited by heterozygous (*Scn1a*$^{KT/+}$) mice in both genetic backgrounds. Each dot represents maximum Racine score in a single mouse. Number of animals in each genotype is shown within parentheses. Data shown in panels B–F are mean ± S.E.M. This figure is modified from Figure 3 in Das et al., 2021, eNeuro[14].

Table 1: Racine scores.

DISCUSSION:

We describe a simple and effective protocol to screen for occurrence of heat-induced seizures in mice, the behavioral equivalent of febrile seizures in human patients. The assay evaluates several parameters, including the percentage of mice showing seizures, seizure threshold, severity of seizures on a Racine scale, in order to compare sensitivity of control and test mice groups to

increases in body temperature.

A critical step in this protocol involves increasing the heat in the chamber while continuously monitoring the body temperature of the mouse. It is imperative that the maximum body temperature the mice will experience in these assays is 44 ˚C because wild-type animals can undergo heat-induced seizures at body temperatures >44 ˚C. All procedures should be approved by the institution's IACUC committee. To ensure continuous monitoring of core body temperature of the mouse during the assay, securely tape the rectal temperature probe to the tail of the mice. If during the assay, the mouse body temperature is found to remain unchanged for prolonged periods of time even after increasing the temperature of the mouse chamber, ensure that the rectal temperature probe has not come out of the mouse or is attached loosely to the tail.

Genetic background of mouse models can affect sensitivity to the SCN1A mutation and pharmacologically induced seizures[18,25–27]. As discussed in the results above, the genetic background of the mice can influence their susceptibility to heat-induced seizures. *Scn1a* K1270T GEFS+ mutant mice were tested in two genetic backgrounds – 129X1 and B6NJ, and a small percentage of wild-type mice (33%) in the seizure sensitive B6NJ background, were also observed to undergo heat-induced seizures. However, in comparison to the heterozygous mutant *Scn1a*$^{KT/+}$ mice, the B6NJ wild-type mice experienced heat-induced seizures at a significantly higher temperature threshold. This confirms that the genetic mutation (*Scn1a* K1270T) that was introduced by CRISPR knock-in makes the mutant mice more susceptible to hyperthermia-induced seizures.

There are several advantages of adopting this protocol, which are summarized below. First, unlike the use of stream of dry air or heated lamps, a temperature-controlled forced air set up within an enclosed space provides the experimenter more control over heating up the test arena at a desired rate. The steps in the heating protocol can be easily modified to increase/decrease the starting temperature, duration of each step, etc. to screen older mice that are heavier or larger rodents such as rats. Second, continuous monitoring of mouse body temperature via the attached rectal probe, gives valuable information about the rate of body temperature change in individual mouse, throughout the assay. This allows the experimenter to closely observe that the rate of temperature change in the mouse does not exceed 0.25 ˚C/min (which might be stressful for the animals), when adapting this protocol to other test arenas. Importantly, the rate of change of body temperature across time in different mice groups can shed light on their ability to thermoregulate and could be helpful to understand whether febrile seizure causing mutations also alter thermoregulation in mice. Third, continuous body temperature monitoring ensures that the seizure threshold temperature measurements using this protocol are accurate, since they are recorded concurrently with the first bout of seizure experienced by the mouse. If the body temperature of the animal is not continuously monitored or seizure threshold temperature is measured after taking the animal out of the test arena, seizure threshold values can vary due to the time taken to handle the mice post seizures. Finally, this method circumvents the need to use invasive methods to induce fever (by injecting pathogens) in mice to mimic febrile seizures in human patients.

One of the limitations of this protocol is that it is difficult to screen juvenile (less than P30 in age) mice for heat-induced seizures. The protocol was developed to screen for sensitivity of adult mice (P30–P40 and above) to heat- or hyperthermia-induced seizures. In our experience, the younger wild-type mice, especially those weighing below 15 g, are more likely to undergo heat-induced seizures, which could be due to underdeveloped thermoregulation mechanisms, physiological thermal stress, or a combination of both. Hence, it is not ideal to perform the heat-induced seizure screen on juvenile mice using this protocol.

Future studies that combine EEG monitoring while subjecting the mouse to heat-induced seizures can shed light on EEG seizure patterns of heat-induced seizures, similar to a previous study[19]. Neuronal activity in specific areas in the mouse brain can be traced by combining optogenetic approaches and immunohistochemistry-based studies after harvesting the brain tissue. Also, effects of restrictive diets such as keto diet on reducing febrile seizures can be evaluated by subjecting keto-fed mice and normal chow-fed mice to heat-induced seizure protocol. Similarly, epilepsy drug screening paradigms can be developed to test and identify candidate anti-epileptic drugs that ameliorate or suppress heat-induced seizures in drug-fed or treated mice when compared to vehicle-fed or control mice.

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DISCLOSURES:

The authors declare no conflicts of interest.

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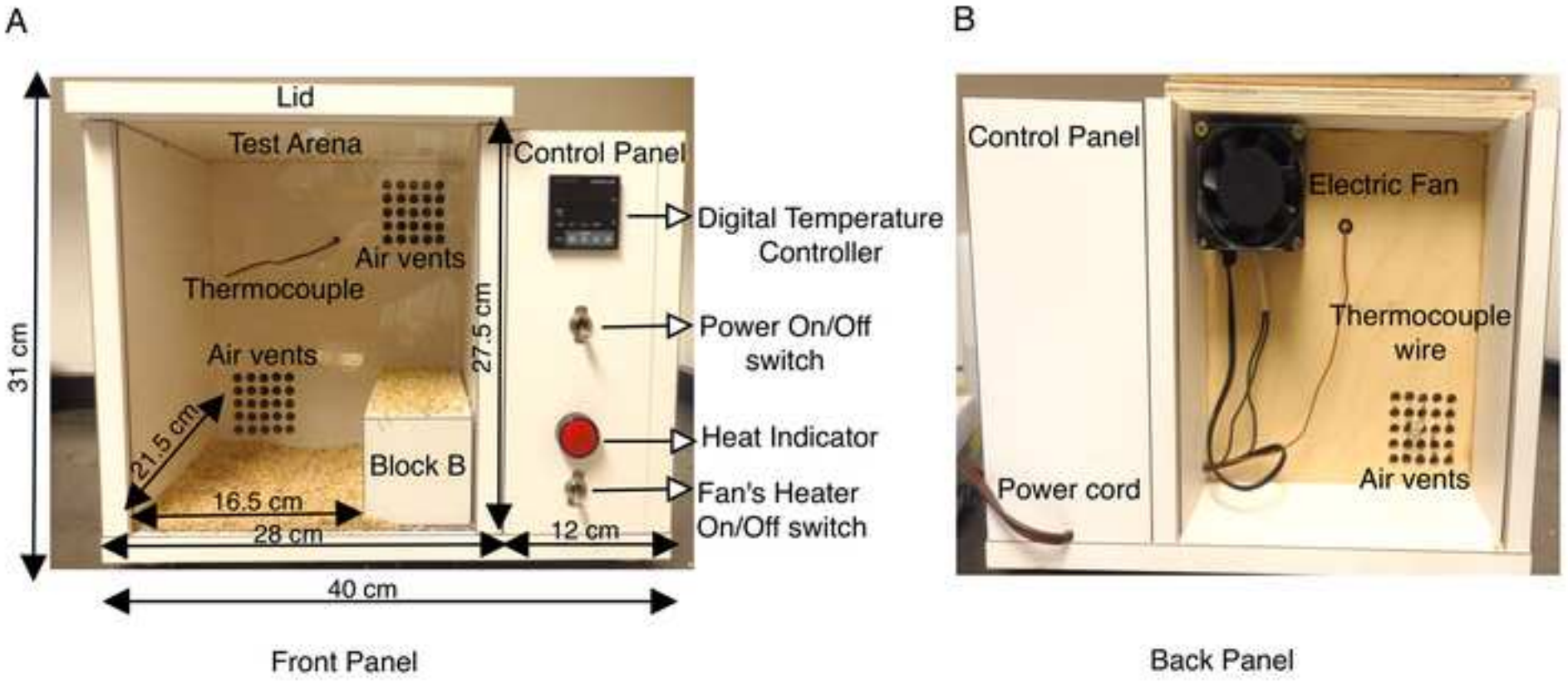

Figure 1.

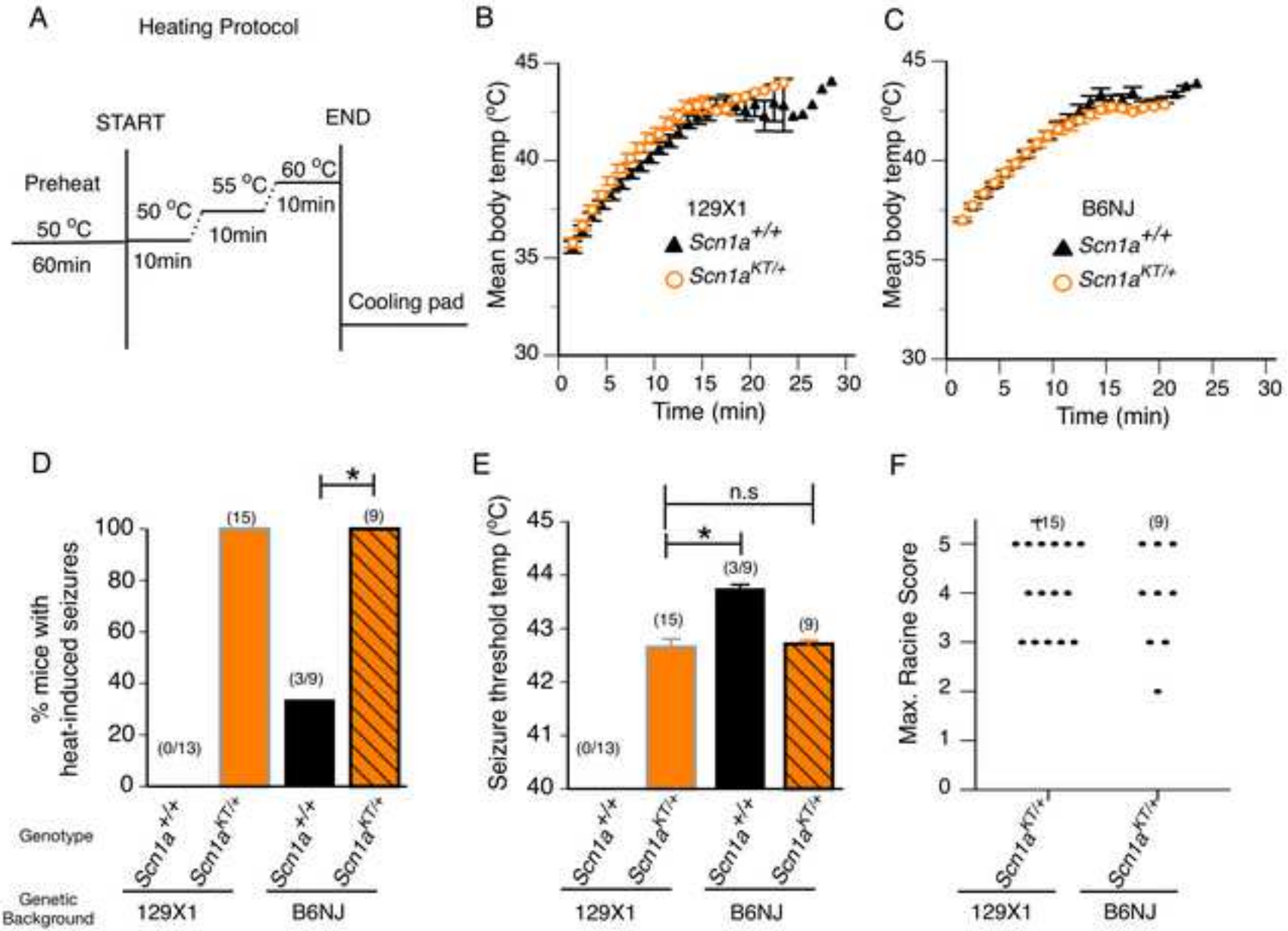

Figure 2.

Table

Racine Score	Seizure characteristics
0	No seizures
1	Mouth and facial movements
2	Head nodding
3	Forelimb clonus, usually one limb
4	Forelimb clonus with rearing
5	Generalized tonic-clonic seizure, rearing, jumping, falling over

Response to Reviewers

Dear Dr. Das,

Your manuscript, JoVE62846 "A behavioral screen for hyperthermia-induced seizures in mouse models of epilepsy," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually.

Your revision is due by **May 17, 2021**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Vineeta Bajaj, Ph.D.
Review Editor
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We thank both the reviewers and the reviewing editor for their helpful comments and suggestions to a previous version of our manuscript. The manuscript was modified to address the concerns raised by the reviewers and adhered to the formatting suggestions as per JoVE's publishing guidelines. A detailed point by point response is provided below. A colored font is used for responses to provide better visibility.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
The manuscript has been proofread for spelling and grammar issues.

2. Please provide an email address for each author.
An email address is provided for all authors.

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) without brackets and in order.
The references have been formatted as suggested.

4. Please do not include references in the abstract section.
References have been removed from the abstract section.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript

and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Inkbird digital, ITC-100RH, Farnam custom products AF20-200-120-xx10-3.1, RET-3, Braintree Scientific, Inc, ThermoWorks, Braintree Scientific, Inc, etc.

Commercial language has been removed from the manuscript.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.
Protocol numbering has been revised as per instructions.

7. Please revise the following lines to avoid overlap with previously published work: 210-214, 217-219.
The text has been modified to avoid overlap with previous publication.

8. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
The ethics statement has been moved from the end of the Protocol section to the top, as suggested.

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
The text in protocol section is now written in the imperative tense.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.
The text in the protocol has been modified to avoid large paragraphs.

11. The Protocol should contain only action items that direct the reader to do something. Please move other details to the intro/discussion section as appropriate. Please ensure the actions are described in order.
The detailed descriptions have been moved out of the protocol and included in the introduction or discussion sections.

12. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
The text has been revised to remove the usage of personal pronouns.

13. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

14. A: how do you set the chamber temperature -please include button clicks, knob turns, etc.
Detail has been added in step 1.1 on page 3.
“1.1 Switch on the Power On button on the heat chamber, followed by Heat On button.”

15. How many mice can be added to one chamber at one time?
One mouse is placed in the chamber at a time. This is mentioned in the introduction section on page 3, line 107 – “A single mouse was placed in the chamber and the temperature was set at 50 ^{o}C between 1st-10th minute”

and under the protocol section on steps 2.3 and 2.13.

“2.3 Screen one mouse at a time in the mouse heat chamber.”
“2.13 Quickly, transfer the individual mouse into the preheated mouse chamber. This marks the START of the experiment trial. Only one mouse is screened at a given time.

16. B4: How do you check the depth of anesthesia?
Step 2.6 now explains that depth of anesthesia should be checked by lack of response to a noxious toe pinch.
“2.6 Ensure that the mouse is completely anesthetized by checking that the mouse is unresponsive to a noxious toe pinch.”

17. B8: How do you check the body temperature of mouse every min?
The rectal probe is attached to a thermometer that displays internal body temperature of the mouse. By keeping track of time using a stopwatch, one needs to read the core body temperature of the mouse, every minute, during the 30 minute assay.

This is explicitly mentioned in the following protocols steps:
“2.9 The rectal probe is connected to a multimeter that displays internal body temperature of the mouse.”

“3.2 Start the stopwatch. Record the body temperature of the mouse from the rectal thermometer at 1 minute intervals for duration of the experiment.”

18. B11: Does this cause any temperature shock?

Elevating the core body temperature of the mouse to 44 ^{o}C at a rate not exceeding 0.25 ^{o}C/ minute has not caused any temperature dependent shock or stress in the wild-type mice tested in 129X1/SvJ or C57Bl/6NJ genetic backgrounds. However, it is important to note that heating up the chamber at a rapid rate or to elevating the body temperature to higher than 44 ^{o}C can lead to heat stress and should be avoided. It is important to control the rate of heating as emphasized in the protocol and discussion sections. These critical steps minimize exposure of the animals to stressful conditions.
The protocol section mentions the following:

"3.3 At regular intervals, increase the temperature of the mouse heat chamber such that the body temperature of the mouse increases at a rate of 0.25 ^{o}C/minute.
Note: It is important that the body temperature of the mouse should rise at a rate of 0.25^{o}C/minute. More rapid increases in body temperature may lead to heat stroke or death and should be avoided."

On page 10 under the discussion section, the following text has been added:
"A critical step in this protocol involves increasing the heat in the chamber while continuously monitoring the body temperature of the mouse. It is imperative that the maximum body temperature the mice will experience in these assays is 44 ^{o}C because wild-type animals can undergo heat-induced seizures body temperatures > 44 ^{o}C. All procedures should be approved by the institution's IACUC committee."

19. B16: Racine Scale needs a citation? How do you use this?
The original submission referred to a study by Cheah et al., 2012 for the use of Racine scale for mouse seizure behavior described here. New text has been added to elaborate on how to score seizure characteristics using the Racine scale under section 5 - Analyzing the heat-induced seizure data on pages 7-8.

20. B17: Do you euthanize the mouse at this point? If yes how? Please include all specific details associated with your experiment. how do you dissect the brain?
Yes, all mice should be euthanized at the end of the study. The text was edited to explain this in detail in the protocol subsection 4:

"4. Euthanizing the animals

4.1 After concluding the screening on all the mice individually for heat-induced seizures following the 30min trial, euthanize all the mice as per institution's IACUC guidelines."

The method of euthanasia selected by individual researchers should be approved by the IACUC committee of the experimenter's affiliated institution or university. The animals included in the present study were euthanized mice by anesthetizing them with an overdose of CO2 gas followed by cervical dislocation as per UC Irvine's IACUC guidelines.

The text pertaining to the mouse brain dissection and immunohistochemistry studies have been moved to the discussion, as it is more appropriate to discuss anatomy-based studies as a follow up experiment. Post seizure brain anatomy studies is a distinct protocol and is not required to be combined with heat seizure assay described in this protocol.

21. Please clearly state what does "control" refer to in this experiment – Mouse exposed to the chamber but not to heat? Please include details.

Age matched wild-type litter mate mice which do not harbor any epilepsy-causing

mutations, and are exposed to the heat chamber following the identical protocol used for mutant mice. These are referred to as “control” mice. Since they do not have any epilepsy causing mutations, they are not expected to undergo heat induced seizures, which are associated with mutations that increase the sensitivity of the organism to heat induced or febrile seizures.

We mention this now on page 4 lines 153-157:
“2.1 Select 10 adult mice (P30-P40), 5 that carry the epilepsy causing mutation and 5 of the wild-type litter mates for heat-induced seizure screening assay.

Note: Wild-type mice which do not harbor any epilepsy causing mutation do not exhibit heat-induced seizures at temperatures below 44 ^{o}C and serve as the “control” group.”

22. C2: How do you check for all these?
We have now included detailed text on pages 7-8 to explain how to use the Racine scale for scoring the seizure videos under section 5 “Analyzing the heat-induced seizure data.”

23. Please include a single line space between each step of the protocol.
Done

24. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
The essential steps of protocol to be included in the video have been highlighted.

25. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Both the figure legend 1 and 2 carries the following sentence “This figure is modified from Figure 3 in Das et al., 2021, eNeuro” on lines 388 and 403 respectively.
The previous publication referred to is an open access publication. We have attached the email from eNeuro publication team and a link to their editorial policy explicitly mentioning that they are an open source journal and use of materials in other publications is permitted.
eNeuro journal’s policy on copyright is highlighted below:

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26. Please do not make points for the discussion section. Please use paragraph style instead.
Discussion text has been modified as suggested.

27. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The discussion section has been modified to include the above topics in paragraphs.

28. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.
Reference style has been formatted as suggested.

29. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the table in alphabetical order.
Table of essential supplies has been modified as suggested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Antara Das et al built up a Transgenic mouse model with SCN1A mutations. They describe a custom-built heating chamber, with a plexiglass front, that is fitted with a digital temperature controller and a heater-equipped electric fan which can send heated forced air into the
test arena in a temperature-controlled manner.

Major Concerns:
How could this procotol be used for general epilepsy animal model?

Response: This assay would not be particularly useful for mouse models of epilepsy that are not associated with induction of heat-induced seizures. However, there are literally thousands of mutations in just the SCN1A and SCN8A sodium channel genes, associated with Dravet Syndrome and GEFS+, seizure disorders that result in increased sensitivity to febrile seizures in patients. As new mouse lines carrying one or more of these mutations are generated, the protocol described in this study could be used to define the heat-induced seizure characteristics and to screen for therapeutics that reduce or eliminate the seizures. It would also be possible obtain EEGs or optogenetic recordings while conducting the heat-induced seizure assay. This would shed light on the nature of the EEG patterns of a heat-induced seizure and by selectively activating specific parts of the mouse brain through optogenetic studies, one could identify brain areas involved during a seizure. We elaborate on this in the discussion section on pages 11-12:

“Future studies that combine EEG monitoring while subjecting the mouse to heat-induced seizures can shed light on EEG seizure patterns of heat-induced seizures, similar to a previous study[6]. Neuronal activity in specific areas in the mouse brain can be traced by combining optogenetic approaches and immunohistochemistry based studies after harvesting the brain tissue. Also, effects of restrictive diets such as keto diet on reducing febrile seizures can be evaluated by subjecting keto fed mice and normal chow fed mice to heat-induced seizure protocol. Similarly, epilepsy drug screening paradigms can be developed to test and identify candidate anti-epileptic drugs that ameliorate or suppress heat-induced seizures in drug-fed or “treated” mice when compared to vehicle-fed or “control” mice.”

Reviewer #2:

Manuscript Summary:

This manuscript explains a useful method of FS induction using as a model for researches in the field of experimental epilepsy. In general, here the authors have planned a device to use an electric heating fan for blowing heated air in to a wooden chamber and will be monitored the chamber and animal core temperature during induction of FS. There is a detailed time table for temperature elevation periods to control the process.

Major Concerns:

No.

Minor Concerns:

In general I think that this device and method will be interesting and useful for researchers. I could not understand the feedback system for turning the fan off when the temperature reaches to the final Degree of Centigrade. If there has planed its Ok, If not, you can think about this concern.

Response: The axial fan works in synchrony with the digital temperature controller, such that the digital temperature controller dictates how much heated air the fan pushes into the chamber. The temperature on the digital temperature controller is set by the

experimenter. We have provided a more complete description of the heating system in the introduction on pages 2-3:

“This forced air heating system enables one to control the rate at which the chamber temperature increases and the maximum temperature. (Fig.1A and 1B). The K thermocouple located inside the wooden heat chamber sends feedback to the digital temperature controller, to maintain constant temperatures inside of the box during the assay. Setting the temperature on the digital temperature controller, enables the electric fan to send heated forced air through air vents to uniformly heat the chamber (Fig.1A).”

Email from eNeuro office

eNeuro <eNeuro@sfn.org>
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To: Antara Das

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Please proceed with submitting your manuscript to JoVe.

Regards,

Vince Carmona
Central Office
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Sent: Thursday, April 15, 2021 12:06 PM
To: eNeuro <eNeuro@sfn.org>
Subject: permission to use modified figure from eNeuro paper

Hi eNeuro,

We recently published our work in eNeuro "Interneuron dysfuntion is a new mouse model of SCN1A GE FS+" DOI: 10.1523/ENEURO.0394-20.2021, and I am the first author on the paper.

We would like to use a modified version of Figure 3 in the above paper to describe the heat-induced seiz ure protocol that we developed in a new publication in JoVE journal. We understand that our publication is open access, and we have duly cited the publication in eNeuro in our JoVE manuscript.

We wanted to confirm if we can go ahead and submit the manuscript to JoVE. If you could send us an em ail confirmation to us that would be great. Please let us know if you have any questions for us.

Thank you

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