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Seizure Suppression by High Temperature via cAMP Modulation in Drosophila

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ABSTRACT Bang-sensitive (BS) Drosophila mutants display characteristic seizure-like activity (SLA) and paralysis after mechanical shock. After high-frequency electrical stimulation (HFS) of the brain, they generate robust seizures at very low threshold voltage. Here we report an important phenomenon, which effectively suppresses SLA in BS mutants. High temperature causes seizure suppression in all BS mutants (parabss1, eas, scla) examined in this study. This effect is fully reversible and flies show complete recovery from BS paralysis once the temperature effect is nullified. High temperature induces an increase in seizure threshold after a brief pulse of heat shock (HS). By genetic screening, we identified the involvement of cAMP in the suppression of seizures by high temperature. We propose that HS induces adenylyl cyclase which in turn increases cAMP concentration which eventually suppresses seizures in mutant flies. In summary, we describe an unusual phenomenon, where high temperature can suppress SLA in flies by modulating cAMP concentration.

Drosophila bang-sensitive (BS) mutants with epilepsy-like phenotypes are used as an animal model to study seizure disorder. BS mutants are known phenotypically for their characteristic seizure-like activity (SLA) and paralysis behavior. In adult stage BS mutants, SLA can be induced by mechanical shock or evoked by high-frequency electrical stimulation (HFS). The behavioral phenotype is complex with several distinguishable phases: seizure, initial paralysis, tonic–clonic-like activity and recovery seizure (Benzer 1971; Ganetzky and Wu 1982; Pavlidis et al. 1994; Pavlidis and Tanouye 1995; Lee and Wu 2002; Zhang et al. 2002; Parker et al. 2011a,b; Burg and Wu 2012).

Numerous BS genotypes have been reported including easily shocked (eas), slamdance (sda), and paralyticbas1 (parabss1) (Pavlidis et al. 1994; Zhang et al. 2002; Parker et al. 2011a,b). The parabss1 mutant is the strongest seizure-prone mutant and hardest to suppress by suppressor mutation and drug, and has been presented as a model for human intractable epilepsy. It is a gain-of-function mutation of the Drosophila voltage-gated Na+ channel that resembles especially the human SCN2A mutation p.Arg1882Gly responsible for neonatal epilepsy with late-onset episodic ataxia (Parker et al. 2011a,b; Schwarz et al. 2016).

BS mutants also represent a unique class of temperature-sensitive paralysis mutants. Recently it has been shown that BS mutants also display temperature-induced seizures upon exposure to either low or high temperature; however, different brain anatomical foci are required for seizure induction by either mechanical or temperature stressors (Burg and Wu 2012). In particular, eas and parabss1 undergo seizures by exposure to low temperature (8°C) whereas bang-sensitive (bas) and technical knock-out (tko) exhibited temperature-induced-seizures at high temperature (39°C). The allele parabss1 gives rise to temperature-dependent SLA (Sun et al. 2012). Several loss-of-function para alleles, including para1570 and para11057, are conditional temperature-sensitive paralytic mutations causing immediate, but reversible paralysis of adult flies when they are shifted from permissive to restrictive temperature (Suzuki and Grigliatti 1971; Siddiqui and Benzer 1976; Ganetzky and Wu 1982; Lee and Wu 2002; Burg and Wu 2012). In another temperature-sensitive mutant, mle-sup (maleless no action potential, temperature-sensitive) (Kernan et al. 1991), axonal conduction fails at high temperature. Synaptic transmission is impaired at restrictive temperatures, which leads to rapid paralyses in larvae and adults (Wu et al. 1978).

In Drosophila, various other channel types and second messengers are also involved in temperature-sensitive behavior, which include not only transient receptor potential (TRP) ion channels but also cAMP. For example, painless, one of the TRP channel superfamly members, is required for sensing nociceptive stimuli at temperatures over 38°C (Tracey et al. 2003). Pyrexia, another TRP channel, protects flies from high temperature stress over 40°C (Lee et al. 2005). In contrast, dTRPA1, a
heat-activated TRP family ion channel, participates in the temperature selection by opening at warm temperatures (24°–29°) (Rosenzweig et al. 2005). Moreover, in mushroom bodies (MB), CAMP signaling modulates temperature preference behavior (TPB) in Drosophila (Hong et al. 2008).

The adenylyl cyclases (ACs) are enzymes with key regulatory roles in all cells. ACs catalyze the conversion of adenosine triphosphate (ATP) to 3',5'-cyclic AMP (cAMP). In Drosophila, the best studied AC is rutabaga (rut) which encodes a Ca2+/calmodulin-responsive adenylyl cyclase (Levin et al. 1992). Flies carrying the rut- allele have low levels of adenylyl cyclase activity, especially Ca2+/CAMP-stimulated activity (Livingstone et al. 1984; Dudai et al. 1985). cAMP is a second messenger, and used for intracellular signal transduction in numerous physiological and developmental processes, including associative learning (Quinn et al. 1974; Livingstone et al. 1984).

In the present study, using behavioral assays and electrophysiology, we describe an unusual phenomenon where high temperature causes suppression of seizures. We find that at 38°, SLA is reduced in Drosophila BS mutants. This suppressive effect of temperature requires only a brief pulse of HS, with the effect fully reversible when flies are returned back to room temperature. We show that high-temperature seizure suppression depends on cAMP: suppression does not occur under conditions of low [cAMP] due to adenylyl cyclase loss-of-function.

MATERIALS AND METHODS

Fly stocks

Drosophila strains were maintained on standard cornmeal-molasses-agar medium at room temperature (24°). The paralytic (par) gene is located at map position 1–53.5 and encodes a voltage-gated Na+ channel (Loughney et al. 1989; Ramaswami and Tanouye 1989). The allele used here is the bang-sensitive (BS) paralytic mutation, parabut, previously named bas-1. It is the most seizure-sensitive of fly mutants, the most difficult to suppress by mutation and by drug, and is a model for human intractable epilepsy (Ganetzky and Wu 1982; Parker et al. 2011a,b). The parabut allele is a gain-of-function mutation caused by a substitution (L1699F) of a highly conserved residue in the third membrane-spanning segment (S3b) of homology domain IV (Parker et al. 2011a,b). The easily shocked (eas) gene is located at 14B on the cytological map and encodes an ethanolamine kinase (Pavlidis et al. 1994). The BS allele used in this study is cas3+, which is caused by a 2-bp deletion that introduces a frame shift; the resulting truncated protein lacks a kinase domain and abolishes all enzymatic activity (Pavlidis et al. 1994). The slamdance (sda) gene is located at 97D and encodes an aminopeptidase N. The allele used in this study is sdains+ 7.8 caused by a 2-bp insertion in the 5' untranslated region (Zhang et al. 2002). The rutabaga (rut) gene is located at 12F5-7 and encodes an adenylyl cyclase (Livingstone et al. 1984; Levin et al. 1992). The allele used in this study, rut-1, is a loss-of-function mutation caused by an amino acid substitution (G1026R; Levin et al. 1992). The rut-1 mutant flies have low levels of adenylyl cyclase activity, especially Ca2+/CAMP-stimulated activity (Livingstone et al. 1984). The rut-1 and UAS-rutRNAi lines were obtained from the Bloomington Drosophila Center. The insert for UAS-rutRNAi is located on the 3rd chromosome.

BS behavior and HS

Behavioral testing for BS paralysis was performed on flies 3 d after eclosion, as described previously (Kuebler and Tanouye 2000). Flies were anesthetized with CO2 before collection and tested the following day. For testing, 10 flies were placed in a clean food vial and stimulated mechanically with a VWR vortex mixer at maximum speed for 10 sec. The parabut, eas, and sda mutants ordinarily show 100% penetrance of BS paralytic behavior. Recovery from BS paralysis was determined by counting the number of flies standing at different intervals following stimulation. Recovery time was the time where 50% of flies had recovered. For genotypes that display partial penetrance of BS paralysis, only those flies that displayed paralysis were used for recovery time analysis. For BS behavioral analysis, pools of flies are combined for each genotype from among the separate trials (in total, n ≈ 100 for each genotype). For analyses using HS, 10 flies were placed in a clean food vial and tested the following day. The vial was submerged in a water bath (38° for 30 sec), and then tested for BS behavioral paralysis. The time between HS and behavioral testing was typically 30 sec.

Electrophysiology

In vivo recording of SLA and seizure threshold determination in adult flies was performed as described previously (Kuebler and Tanouye 2000; Lee and Wu 2002). Flies 2–3 d posteclosion were mounted in wax on a glass slide, leaving the dorsal head, thorax, and abdomen exposed. Stimulating, recording, and ground metal electrodes were made of uninsulated tungsten. Seizure-like activity was evoked by high-frequency electrical brain stimulation (0.5-msec pulses at 200 Hz for 300 msec) and monitored by dorsal longitudinal muscle (DLM) recording. During the course of each experiment, the giant fiber (GF) circuit was monitored continuously as a proxy for holobrain function. For seizure-threshold determination in HS flies, one fly/food vial was placed and tested the following day. Prior to recording, they were HS for 30 sec at 38° and mounted in wax on a slide and used for electrophysiological recordings. The time between delivery of the HS and electrophysiological recording was typically 2–3 min. For each genotype tested n ≥ 5.

Data analysis

All error bars shown represent the standard error of the mean. Chi-square tests were used to compare the penetrance of seizures. Student’s t-test and ANOVA were used to compare recovery times and seizure thresholds across genotypes, as appropriate. For ANOVA analysis, where the null hypothesis was rejected by the overall F ratio, multiple comparisons of data sets were performed by Fisher’s least significant difference with t-test significance set at P < 0.05.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS

High temperature suppresses seizures in BS mutants

To investigate the effect of temperature on seizure-sensitivity in BS mutants, we used a brief HS protocol. The BS mutants were HS at 32° for 3 min in the water bath. We found that BS paralysis behavior in parabut mutants was decreased in both males and females up to 40% compared to control non-HS parabut mutants, which showed 100% BS paralysis. To test if HS decreases BS paralysis in other BS mutants, we checked eas and sda mutants under the same HS protocol. We observed a significant decrease in BS paralysis in both eas and sda mutants. In eas, BS paralysis was decreased to 60–80% (males and females) whereas in sda, it was reduced to 40–80% (in males and females) compared to 100% BS at room temperature (Figure 1, A and B). Thus, regardless of genotype, HS suppresses BS paralysis in all BS mutants studied here.

Dependence of seizure suppression by HS on temperature and time

We sought to determine the optimum conditions for HS at which maximum seizure suppression can be achieved in all BS mutants.
examined in this study. We hypothesized that both the range of temperature and time duration for HS may affect the seizure susceptibility. To determine the optimum temperature for HS to maximally suppress BS paralysis, we used the same HS protocol but varied the range of temperature for HS. HS was given that varied from 30 to 38°C temperature range for 3 min. We found a strong correlation between temperature and reduction in BS paralysis. BS paralysis was correspondingly decreased from lower to higher temperature. All the BS mutants at room temperature exhibited 100% BS paralysis behavior, but from 30 to 38°C BS paralysis behavior was decreased from 100 to 0% (Figure 1, A and B). At 38°C BS paralysis was decreased to ~0% compared to controls without HS; therefore, we decided to perform subsequent experiments using HS at 38°C.

Next we varied our HS protocol to determine the optimum time duration dependence of HS. HS was at 38°C for different times, ranging from 10 sec to 3 min. We found that the reduction in BS paralysis was strongly dependent on the time duration of HS. From 10 sec to 3 min of HS at 38°C, we observed a decrease in BS paralysis from 100 to 0% (Figure 1, C and D). Maximum reduction in BS paralysis was observed by 30 sec HS pulses. For BS paralysis behavior, from 30 to 38°C BS paralysis behavior was decreased from 100 to 0% (Figure 1, A and B). At 38°C BS paralysis was decreased to ~0% compared to controls without HS; therefore, we decided to perform subsequent experiments using HS at 38°C.

To investigate how long HIBPS persists in BS mutants, we analyzed BS paralysis behavior in BS mutants at different time intervals following HS, ranging from 15 sec to 1 hr. We found that HIBPS decreased with longer time duration and displayed genotype bias. In parabss1 and eas BS male and female mutants, nearly 80% of flies showed a decrease in HIBPS within 2 min, whereas in sda this decrease in HIBPS was slower and required at least 20 min. Also, sda mutants displayed gender-dependent BS paralysis behavior, with female mutants showing a faster decrease in HIBPS (100% BS after 20 min) compared to male mutants (100% BS after 45 min) (Figure 2, A and B). Therefore, these findings indicate that HIBPS is dependent on time interval and BS mutants show complete reversion in HIBPS within an hour after the HS.

HS alters seizure threshold in BS mutants
Seizure threshold (the minimum voltage of an HFS required to induce a seizure) is a quantitative measure of a fly’s seizure susceptibility. In particular, seizure-sensitive BS mutants have a low seizure threshold (Kuebler and Tanouye 2000; Lee and Wu 2002; Zhang et al. 2002; Parker et al. 2011a,b) whereas a seizure suppressor usually increases the seizure threshold in BS mutants (Kuebler et al. 2001; Lee and Wu 2006; Song and Tanouye 2006, 2007; Song et al. 2007; Howlett and Tanouye 2013). To check if HS alters the seizure threshold in BS mutants, we performed electrophysiological recordings to determine threshold in BS mutants after HS treatment. We stimulated concurrently the GF circuit using single pulse stimulation and recorded evoked DLM potentials. The GF circuit acts as a proxy for the state of the entire nervous system during and after SLA. Since in parabss1 and eas mutants HIBPS reverts back very quickly (within 5 min), it was not feasible to determine the effect of HS on seizure threshold in these mutants. Therefore, we used sda single mutants to study the effect of HS on seizure threshold. HS significantly changed the seizure threshold for seizures in sda. At room temperature, SLA can be induced in sda at low seizure threshold (7.3 ± 0.41 V HFS, mean ± SEM, n = 5; P < 0.0001, ANOVA.
test; Figure 2C and E). However, after HS, the seizure threshold became nearly threefold higher (20.5 ± 6.28 V HFS, mean ± SEM, n = 8; P < 0.0001, ANOVA test; Figure 2D and E). Thus, suppression of BS paralysis appears to be due to an increase in seizure threshold after HS in BS mutants.

Reverse genetic RNA interference (RNAi) screening to identify the underlying mechanism in the suppression of BS by HS

To elucidate the molecular mechanism of seizure suppression by HS, we carried out reverse genetic RNAi screening. We hypothesized that any identified mutant that abolishes the HIBPS, i.e., even after HS flies show BS paralysis, should be involved in the seizure suppression mechanism. Using the GAL4/UAS binary system, in particular elav^{155}-GAL4 para^{bas} and UAS-RNAi lines, 14 genes were screened (pyrexia (pyx), nanchung (nan), transient receptor potential cation channel A1 ortholog (dTrpA1), synatobrevin (Syb), synaptosomal-associated protein 24kDa (Snap24), synaptosomal-associated protein 25kDa (Snap25), Rab3 interacting molecule (Rim), Rim binding protein (Rbp), syntaxin 1A (Syx1A), painless (pain), histamine-gated chloride channels subunit 1 (hisCl1), ora transientless (ort), cyclic nucleotide-gated ion channel-like (cng), and I_{h} channel (I_{h}). These genes were selected based on the assumption that genes involved in thermoregulation or synaptic transmission might be involved in HIBPS. However neither approach produced any reversion in BS paralysis by HS.

Recently, it has been shown that in the Drosophila MB, cAMP signaling modulates TPB (Hong et al. 2008). Flies with low levels of cAMP prefer a lower temperature and flies with high levels of cAMP prefer a high temperature. We tested whether the cAMP signaling pathway may be involved in BS paralysis suppression by HS. When elav^{155}-GAL4 para^{bas} female flies were crossed with UAS-rut RNAi males, the double mutant males with genotype elav^{155}-GAL4 para^{bas/Y};UAS-rut RNAi/+ showed a significant increase in BS paralysis.
Figure 3  HIBPS in BS mutants requires rutabaga function. (A) Pan-neuronal expression of UAS-rut RNAi reduces the HS-induced suppression in BS paralysis behavior. In double mutants [blue striped bar, genotype $elav^{155}$-GAL4 $parabss1/Y; UAS-rutRNAi/+ (HS)] expression of rutRNAi driven by $elav$-GAL4 produces 24% BS paralysis following HS ($n = 120$) compared to 0% BS paralysis in control $elav^{155}$-GAL4 $parabss1/Y$ hemizygous males ($n = 80$, white bar) after HS. Flies with same genotype [blue crosshatched bar, $elav^{155}$-GAL4 $parabss1/Y; UAS-rutRNAi/+ (RT)] at RT showed 100% BS paralysis ($n = 120$). (B) Pan-neuronal expression of UAS-rutRNAi also reverts the HS-induced suppression in eas paralysis behavior. Double mutants [green striped bar, genotype $elav^{155}$-GAL4 eas/Y; UAS-rut RNAi/+ (HS)] displayed 26.2% BS paralysis behavior ($n = 42$) following HS compared to 0% BS paralysis in single hemizygous mutants $elav^{155}$-GAL4 eas/Y ($n = 50$, white bar). Flies with same genotype [green crosshatched bar, genotype $elav^{155}$-GAL4 eas/Y; UAS-rutRNAi/+ (RT)] at RT showed 100% BS paralysis ($n = 45$). (C) HIBPS is reduced in sda by the $rut^{1}$ mutant. Hemizygous male double mutants (genotype, $rut^{1}/Y; sda$) show 27% BS paralysis following HS ($n = 502$, red vertically striped bar, $P = 0.0001$, Fisher’s exact test). Double mutant $rut^{1}; sda$ female flies show 54% BS paralysis following HS ($n = 398$, red diagonally striped bar), whereas at RT flies with the same genotype ($rut^{1}; sda$) were 90% BS ($n = 209$, red crosshatched bar).

**Reversion of HIBPS of BS paralysis by $rut^{1}$ mutant**

To further explore and to validate $rut$ as the gene involved in suppression of BS paralysis by HS, we used the $rut^{1}$ mutant. We generated double mutant $rut^{1}; sda$ flies by crossing $rut^{1}$ mutant in the $sda$ background. When $rut^{1}; sda$ double mutants, we observed a significant increase in BS paralysis. The BS paralysis after HS was ~40% (including both males and females) ($n = 900$, $P = 0.0001$, Fisher’s exact test; Figure 3C) compared to control mutant $sda$ single mutants which showed 0% BS paralysis after HS. However, we observed some gender-biased change in BS paralysis. $rut^{1}; sda$ double mutant females showed a higher increase in BS paralysis after HS than males. BS paralysis in $rut^{1}; sda$ double mutant female flies was ~54% ($n = 398$) after HS, whereas in double mutant $rut^{1}; sda$ male flies BS paralysis was only 27% ($n = 502$, Figure 3C). Together, these results indicate that $rut^{1}$ is involved in suppression of BS paralysis by HS.

Next, we sought to determine the change in seizure threshold due to $rut^{1}$ in $rut^{1}; sda$ double mutants. Since $rut^{1}$ decreased the suppression of BS paralysis by HS, we anticipated that $rut^{1}; sda$ double mutants should show a reduction in seizure threshold after HS compared to HS $sda$ single mutants. We used electrophysiological recordings to determine seizure threshold for $rut^{1}; sda$ double mutants and $sda$ single mutant flies after HS. The seizure threshold for $rut^{1}; sda$ double mutant flies was reduced sixfold ($3.7 \pm 0.1$ V HFS, mean $\pm$ SEM, $n = 5$; similar to $sda$ single mutants at room temperature) compared to $sda$ single mutant HS flies ($20.5 \pm 2.81$ V HFS, mean $\pm$ SEM, $n = 8$, $P < 0.0001$, unpaired Student’s $t$-test; Figure 4). This reduction in seizure threshold is correlated with the reversal of BS paralysis to the normal level. Thus, $rut$ and hence cAMP may be involved in seizure suppression by HS in BS mutants by reducing the seizure threshold.

**DISCUSSION**

Human seizure phenotypes arise from interactions between genetic and environmental factors (Ottman et al. 1996; Szyf 2015). Every individual has the potential to have a seizure; however, some are more likely to have seizures than others. These individuals have a low level of resistance to seizures; a low seizure threshold. Seizure threshold is thought to be mainly due to genetic factors coming from familial inheritance (Kjeldsen et al. 2001; Pelitto et al. 2014), but environmental factors can also modulate seizure threshold and make seizures more likely, for example severe head injury or infection (Ottman et al. 1996; Szyf 2015). Numerous environmental stresses are found to enhance seizure-sensitivity or trigger seizure events. For example, seizures can result from exposure to drugs and poisons, such as antidepressants, lead poisoning, nicotine, and carbon monoxide. Other environmental stresses triggering seizures include stress, lack of sleep, alcohol consumption, light flashes, and hormonal imbalance (Herman 2002; Wood et al. 2004; Jett 2012; Alexandre et al. 2015). Although genetic and environmental factors are both known contributors to seizure phenotypes, little is known about the mechanisms responsible for how they might interact.
Drosophila is attractive for evaluating genetic and environmental contributions to seizure susceptibility. The biggest advantage comes from a good collection of neurological mutations with known and quantifiable effects on seizure susceptibility that includes seizure-sensitive, seizure-resistant, seizure-suppressor, and seizure-enhancer mutations. Thus, genetic background can be well controlled with single mutants and double mutant combinations with all individuals of a given genotype having similar seizure thresholds. The present study examined the effect of temperature, as an environmental stressor, on seizure susceptibility in Drosophila. We find here and in two recent studies (Kroll et al. 2015; Saras and Tanouye 2016) that interactions between temperature and genetic background are complex in their effects on seizure susceptibility, and differ depending on the different mutations contributing to the genotype.

We show here that high temperature can indeed alter BS mutant seizure susceptibility. However, our initial assumption was found to be incorrect; this environmental stressor does not exacerbate seizure sensitivity. Instead of promoting or enhancing BS phenotypes, high temperature acts as a suppressor. BS paralytic behaviors are significantly reduced following HS in all three BS mutants examined in this study (parabss1, eas, and sda). HIBPS of parabss1 is especially notable because its phenotypes have proven difficult to suppress by suppressor mutations and drugs (Parker et al. 2011a,b). Only a brief pulse of HS is required for HIBPS. When the flies are allowed to recover at room temperature, HIBPS itself quickly reverts and flies regain bang-sensitivity indicating that seizure suppression is transient.

Suppression of behavioral BS paralysis by HS appears to result from an increase in seizure threshold. During the period corresponding to HIBPS for sda, there is a transient increase in seizure threshold of about threefold as observed in electrophysiological recordings. Because of this seizure threshold increase, sda seizure-sensitivity, ordinarily low at room temperature, is brought close to the wild-type range. This is sufficient to account for the loss of the BS paralytic behavior for sda. Thus, we suggest that HS from the environment is capable of interacting with the sda nervous system; this interaction causes an increase in seizure threshold and a suppression of BS mutant phenotypes.

A challenge is to determine mechanisms for how environmental factors, such as high temperature, interact with the nervous system to alter seizure threshold. For this, genetic screening in Drosophila provides a promising approach. We can utilize mutations to gain molecular access to the challenge and use continued genetic analysis to elucidate the mechanism. We undertook genetic screening using RNAi in our initial attempt to identify an interaction mechanism for HIBPS. RNAi for candidate genes involved in thermoregulation or synaptic transmission were tested and found not to be effective in altering HIBPS.

In contrast, RNAi for the adenylyl cyclase gene, rut, resulted in significant levels of bang-sensitivity following HS. A role for rut in bang-sensitivity for this system is evident since rut RNAi was similarly effective for two BS mutants parabss1 and eas and the rut mutation was effective for sda. Furthermore, electrophysiology shows that the change in HIBPS is correlated with a decrease in seizure threshold. This approach has resulted in a sixfold decrease in seizure threshold is sufficient in magnitude to completely account for the change in HIBPS affected by rut.

Figure 4 Electrophysiology of seizure thresholds in sda and rut backgrounds after HS. (A) Electrophysiological recording from a rut1; sda fly DLM fiber evoked by a 4 V HFS stimulation (0.5-msec stimuli at 200 Hz for 300 msec) following HS. The stimulation evokes seizure-like electrical activity (SLA), indicating that the voltage is at or above seizure threshold. (B) At room temperature, a rut1; sda double mutant fly exhibits the same seizure threshold (4 V HFS). Horizontal calibration is 1 sec for (A–C). Vertical calibration is 20 mV. (C) Histogram compares seizure thresholds in sda and rut1; sda genotypes. Seizure threshold is low in sda control flies (red bar, 7.3 ± 0.41 V HFS, n = 5). Following HS, sda seizure threshold is substantially increased (red checkered bar, 20.5 ± 2.81 V HFS, n = 7). In the double mutant rut1; sda seizure threshold following HS is low (red striped bar, 3.7 ± 0.1 V HFS, n = 5) even after the HS.

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Author contributions: AS and MAT designed the study and experiments, and wrote the manuscript. AS performed behavior and electrophysiology experiments, analyzed the data, and performed statistical analysis. All authors edited the manuscript.

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