Increased persistent Na\(^+\) current contributes to seizure in the slamdance bang-sensitive Drosophila mutant

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Marley R, Baines RA. Increased persistent Na\(^+\) current contributes to seizure in the slamdance bang-sensitive Drosophila mutant. J Neurophysiol 106: 18–29, 2011. First published March 30, 2011; doi:10.1152/jn.00808.2010.—There is clinical need to extend the understanding of epilepsy and to find novel approaches to treat this condition. Bang-sensitive (bs) Drosophila mutants, which exhibit reduced thresholds for seizure, offer an attractive possibility to combine tractable genetics, electrophysiology, and high-throughput screening. However, despite these advantages, the precise electrophysiological aberrations that contribute to seizure have not been identified in any bs mutant. Because of this, the applicability of Drosophila as a preclinical model has not yet been established. In this study, we show that electroshock of bs slamdance (sda) larvae was sufficient to induce extended seizure-like episodes. Whole cell voltage-clamp recordings from identified motoneurons (termed aCC and RP2) showed synaptic currents that were greatly increased in both amplitude and duration. Current-clamp recordings indicated that these inputs produced longer-lived plateau depolarizations and increased action potential firing in these cells. An analysis of voltage-gated currents in these motoneurons, in both first and third instar larvae, revealed a consistently increased persistent Na\(^+\) current (\(I_{\text{Na,p}}\)) and a reduced Ca\(^{2+}\) current in first instar larvae, which appeared normal in older third instar larvae. That increased \(I_{\text{Na,p}}\) may contribute to seizure-like activity is indicated by the observation that feeding sda larvae the antiepileptic drug phenytoin, which was sufficient to reduce \(I_{\text{Na,p}}\), rescued both seizure-like episode duration and synaptic excitation of motoneurons. In contrast, feeding of either anemone toxin, a drug that preferentially increases \(I_{\text{Na,p}}\) or phenytoin to wild-type larvae was sufficient to induce a bs behavioral phenotype. Finally, we show that feeding of phenytoin to gravid sda females was sufficient to both reduce \(I_{\text{Na,p}}\) and synaptic currents and rescue the bs phenotype in their larval progeny, indicating that a heightened predisposition to seizure may arise as a consequence of abnormal embryonic neural development.

aCC; motoneuron; phenytoin; RP2; synaptic current

Epilepsy is a common syndrome manifesting a range of symptoms that affects ~1% of the global population. Although antiepileptic drugs (AEDs) are available, only two-thirds of patients report relief; the remaining one-third of patients are relatively insensitive to drug intervention (Schmidt 2002). Because of this, there is a need for alternative treatments based on novel targets.

A large majority of idiopathic epilepsies undoubtedly arise as a result of genetic mutations. Indeed, there is now a considerable list of single gene mutations that reduce seizure thresholds in both rats and mice (Frankel 2009). There are also, albeit fewer, rodent strains that spontaneously develop an increased prevalence of seizure [e.g., DBA and EL mice and GAERS rats (Frankel 2009)]. However, the use of rodents for such studies is not without limitations. Chief among these, perhaps, is that rodents are labor and facility intensive to maintain. Second, epilepsy is a complex trait that is significantly influenced by genetic background, and it is challenging to control for this in complex animals (Schauwecker and Steward 1997).

Drosophila melanogaster offers tractable genetics and genotypes that readily and reliably display significantly reduced seizure thresholds. These genotypes, which were first identified by Benzer in 1971, are termed bang-sensitive (bs) mutants due to their heightened response to mechanical shock (Benzer 1971). A subsequent study (Pavlidis et al. 1994) demonstrated that bs mutants exhibit lower thresholds for electroshock-induced seizures than wild-type (WT) controls. This study used high-frequency electroshock to the adult brain and recordings from flight muscle to report motor activity in the central nervous system (CNS). Such stimulation of the brain results in a discharge of excitatory potentials in flight muscle followed by an extended period of synaptic failure before a final recovery seizure, characterized by a volley of excitatory potentials, is observed; the whole episode lasts up to a few minutes (Zhang et al. 2002). The susceptibility to “seize” is defined by the voltage threshold required to induce the seizure-like response.

The underlying neuronal aberrations that reduce threshold for seizure induction in any bs mutant remain unknown. Analysis of motoneuron axonal excitability indicate that the threshold for excitation is significantly reduced in bs mutants, which is consistent with the reduced thresholds for seizure induction (Ganetzky and Wu 1982). However, whether the central motoneurons are also hyperexcitable remains to be shown. Although more than adequate for the determination of seizure threshold, the adult preparation is not the best suited to allow for a more rigorous understanding of the underlying abnormalities in individual neurons. In contrast, relatively sophisticated electrophysiology can be applied to central neurons in Drosophila larvae (Baines and Bate 1998; Rohrbough and Broadie 2002). In particular, two motoneurons, termed aCC and RP2, have been extensively characterized (Baines et al. 1999; Baines et al. 2001). This characterization includes a comprehensive description of intrinsic membrane conductances and synaptic currents, with the latter reflecting network properties in central motor circuitry (Baines 2004).

Seizures in bs mutants exhibit sufficient similarities with humans to suggest that the underlying neuronal abnormalities are analogous and that the results derived have clinical relevance (Kuebler and Tanouye 2002; Reynolds et al. 2004; Tan

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et al. 2004). Moreover, many bs mutants have been well characterized with respect to both gene and protein function; for example, slandance (sda) is a mutation in the Drosophila homolog of human aminopeptidase N (APN) (Zhang et al. 2002). APN (also known as CD13) is a glycoprotein that is widely expressed in animal tissues, including the nervous system (Inagaki et al. 2010). This enzyme, which catalyzes the removal of neutral and basic amino acids from the NH2-termini of peptide substrates, has been proposed to function in a wide spectrum of roles, including acting as a receptor to mediate cell adhesion, antigen processing, and, in particular, cancer progression (for a review, see Zhang et al. 2002). How this mutation is able to reduce the threshold for seizure activity in Drosophila is unknown. It also remains unknown whether mutations in mammalian APN result in seizure-like activity.

In this study, we report the first detailed electrophysiological characterization of central neuron function in larvae of a defined bs mutant. We show that sda mutant larvae exhibit prolonged bouts of seizure-like episodes induced by electroshock. Our electrophysiology shows that synaptic currents in aCC/RP2 motoneurons are increased in both amplitude and duration, an observation that may suggest heightened network excitability. We also report changes in both voltage-gated Ca2+ and Na+ conductances in sda aCC/RP2 motoneurons. In particular, we show that the voltage-gated persistent Na+ current (I\textsubscript{Na,persistent}) is increased, whereas the transient Na+ current (I\textsubscript{Na,t}), which is mediated by the same ion channel (Lin et al. 2009), is not affected. This is consistent with a change in the functional properties of this ion channel to increase the apparent ratio of I\textsubscript{Na,persistent}. Here, we provide evidence to show that the ratio between these two components is an important determinant of seizure-like behavior. We confirmed our findings in sda mutants through pharmacological treatment of WT flies and, moreover, provide evidence to suggest that the aberrations observed in the sda mutant may arise as a consequence of altered embryonic neural development.

**MATERIALS AND METHODS**

**Fly stocks.** Flies were maintained on standard corn meal medium at 25°C. Embryos were collected by allowing females to lay on grape agar (Dutscher, Essex, UK) plates supplemented with a small amount of live yeast paste at 25°C. Flies used were Canton-S WT and slandance\textsuperscript{enu\textsubscript{7.8}} (sda) flies (Zhang et al. 2002).

**Seizure induction in adult flies.** Adult flies (1–2 days posteclosion) were anesthetized by CO2 and placed in empty plastic fly vials (10 flies/vial). Flies were allowed to recover for at least 2 h and were then subjected to 10 s of mechanical shock delivered via vortex (Vortex genie 2, Scientific Industries, Bohemia, FL). The time for each fly to recover, defined as standing, was scored, and a mean value was taken for each vial (termed “mean recovery time”). Any flies that did not recover were disregarded from the analysis (this number was minimal).

**Seizure induction in third instar larvae.** Before stimulation, third instar larvae were washed to remove food residue and gently dried using paper tissue. Larvae were then allowed to recover on agar plates until normal crawling behavior resumed. A stimulator was constructed composed of two tungsten wires fixed to a nonconducting rod. The tips of the two wires (0.1 mm diameter) were set ~1–2 mm apart and were placed across the anterior-dorsal surface, over the approximate position of the CNS. A direct current pulse (50 V, 3 s), generated by a Grass S88 stimulator (Grass Instruments), was applied. The animal responded by tonically contracting and ceasing normal motile behavior. The time to resumption of normal behavior was determined (mean recovery time). Normal crawling was defined as a sustained, whole body length peristaltic wave that resulted in forward motion.

**Drug feeding of third instar larvae.** A stock solution of phenytoin was prepared in DMSO (4 mg in 50 μl, both from Sigma, Poole, UK), and this was then added to 10 ml double-distilled H2O with added 5% dried yeast extract to produce a final phenytoin concentration of 0.4 mg/ml. This solution (2 ml) was then placed on to the surface of a grape agar plate (50 mm diameter) and left to dry on a warm plate (~30°C) for 3–4 h. In each case, control plates were made that contained only the vehicle (DMSO), which never exceed 0.02% (vol/vol), and yeast extract. Anemone toxin (rATXII) was diluted with double-distilled H2O to stock aliquots of 10 μM and further diluted to the working concentration as needed: 150 μl of the final working concentration were coated onto a well of a 24-well plate (Costar 3524, Corning) that had previously had each well one-third filled with grape agar. The rATXII-coated plate was then left to dry on a warm plate for 1–2 h. In both cases, larvae were allowed to feed on these plates for 24 h before being tested.

**Drug feeding of adult flies.** Mated adult females were fed on phenytoin (0.4 mg/ml) for 2 days by adding flies to food vials containing the drug. Flies were then transferred to nondrug-containing vials and allowed to lay for a further 2 days before being removed. These drug-free vials were left at 25°C, and wall-climbing third instar larvae used for analysis. To determine the levels of phenytoin ingested by female flies and transfer to oocytes and third instar larvae, the added phenytoin was spiked with 14C-labeled phenytoin (0.074 MBq/ vial, Perkin Elmer, Cambridge, UK), and counts were normalized to milligrams of protein (Bio-Rad, Hemel Hempstead, UK).

**Electrophysiology.** Recordings were performed in both young first instar larvae (1–4 h posthatching) and wall-climbing third instar larvae at room temperature (22–24°C). First instar larvae were dissected with sharpened tungsten wires as previously described (Baines and Bate 1998). The third instar CNS was dissected free with fine forceps and fixed to Syrgald (Dow Corning)-coated coverslips with cyanoacrylate glue (Vetbond, WPI, Stevenage, UK). Rupture of the neurolemma surrounding the CNS was as previously described (Baines and Bate 1998) but with the larger third instar CNS requiring a slightly larger bore micropipette.

Whole cell voltage- and current-clamp recordings were achieved using thick-walled borosilicate glass electrodes (GC100F-10, Harvard Apparatus, Edenderry, UK) fire polished to resistances of between 15 and 20 MΩ (first instar larvae) and 15 and 15 MΩ (third instar larvae). aCC and RP2 motoneurons were initially identified based on both size and dorsal position in the ventral nerve cord. Unequivocal identification was determined after recording by labeling with 0.1% Alexa fluor 488 hydorazide, sodium salt (Molecular Probes, Eugene, OR), which was included in the patch saline solution. Recordings were made using a Multiclamp 700B amplifier controlled by pCLAMP (version 10.2, Molecular Devices, Sunnyvale, CA). Recordings were sampled at 20 kHz and filtered online at 10 kHz. Capacitance was measured by integrating the area under the capacitance transient resulting from a step protocol from ~60 to ~90 mV.

Synaptic currents showing multiple peaks were quantified as a single event; the amplitude was taken from the largest peak, which invariably was the the first one. Such multiple events are rare in both WT and sda flies (see Fig. 2). A more detailed explanation of how we measured both amplitude and frequency is provided in Stacey et al. (2010). To measure synaptic current amplitude, the change from prefiring baseline to peak current amplitude was determined using MiniAnalysis (Synaptosoft, Decatur, GA). The duration of each synaptic current was defined as the time from current initiation until the return to baseline (±3 pA), which was determined with Clampfit (version 10.2). For all histograms shown, synaptic current parameters were determined for each cell recorded, and an average value was calculated. This was repeated for multiple cells, and cumulative averages ± SE were calculated and are shown. For cumulative
probability plots (Fig. 2, F and G), all synaptic current amplitudes or durations obtained from multiple cells are shown. Because we observed a change to \( I_{\text{Isup}} \), and not to \( I_{\text{dat}} \), we determined (and report) the ratio of these two conductances, which are mediated by the same ion channel. Normalization of our data to a ratio additionally compensated for the fact that the magnitude of both components showed variability between genotypes (and, to a lesser extent, within genotypes).

**Electrophysiological solutions.** The external saline solution for dissection and normal whole cell recording of synaptic currents consisted of (in mM) 135 NaCl, 5 KCl, 4 MgCl\(_2\), 2 CaCl\(_2\), 5 TES, and 36 sucrose. K\(^+\) conductance was isolated using a saline solution that consisted of (in mM) 135 NaCl, 5 KCl, 4 MgCl\(_2\), 2 CaCl\(_2\), 5 TES, 36 sucrose, and 2 \( \mu \)M TTX. Ca\(^{2+}\) conductance saline solution consisted of (in mM) 50 NaCl, 6 KCl, 50 BaCl\(_2\), 50 tetraethylammonium chloride (TEA), 10 4-aminopyridine, 10 MgCl\(_2\), 10 HEPES, and 10 glucose. Na\(^+\) conductance saline solution consisted of (in mM) 100 NaCl, 6 KCl, 50 TEA, 10 4-aminopyridine, 10 MgCl\(_2\), 10 HEPES, and 10 sucrose. All solutions were pH 7.4.

The internal saline solution consisted of (in mM) 140 KCH\(_3\)SO\(_3\), 5 KCl, 2 MgCl\(_2\), 11 EGTA, and 20 HEPES (pH 7.4). When either Ca\(^{2+}\) or Na\(^+\) conductances were recorded, CsCH\(_3\)SO\(_3\) was substituted for KCH\(_3\)SO\(_3\) and CsCl\(_2\) was substituted for KCl. TTX and rATXII were obtained from Alomone Labs (Jerusalem, Israel); KCl, KOH, NaOH, NaCl, sucrose, and glucose were obtained from Fisher Scientific (Loughborough, UK); HEPES, MgCl\(_2\), and CaCl\(_2\) were obtained from BDH (Poole, UK); and all remaining chemicals and drugs were obtained from Sigma.

**Statistics.** All values are shown as means \( \pm \) SE. Significance was determined using a nonpaired \( t \)-test. Results were deemed significant at \( P < 0.05 \) or \( P < 0.01 \).

**RESULTS**

Although an increased susceptibility to seizure in bs mutants, including \( sda \), has been previously characterized in adult flies (Zhang et al. 2002), the precise underlying electrophysiological defects in central neurons have not been described. This is primarily because the adult CNS has not, until recently, been considered suitable for electrophysiological recordings from identified neurons. In contrast, whole cell patch-clamp recordings from larval central neurons are now routine, and, as such, there exists a considerable body of background literature describing, for example, the voltage- and ligand-gated conductances present in larval motoneurons (Baines 2004; Baines et al. 2001; Choi et al. 2004; Pym et al. 2006; Rohrbough and Broadie 2002). To exploit this, we developed an electrophoshock assay to test for increased susceptibility for seizure-like phenotypes in third instar bs mutants.

**\( sda \) larvae exhibit prolonged seizures after electroshock.** It has previously been shown that adult bs mutants, including \( sda \), exhibit an initial seizure-like phenotype followed by a period of paralysis and then a short recovery seizure in response to a mechanical stimulus (Zhang et al. 2002). The total duration of this seizure-like episode can be measured by the mean recovery time, which is defined as the time taken for all flies to be standing after the stimulus. We repeated this determination for \( sda \) adults, which showed a significantly longer mean recovery time compared with WT flies (Fig. 1A). Increased mean recovery time in adults correlates with a decreased seizure threshold (i.e., increased seizure sensitivity) and, as such, is routinely used to determine the predisposition to “seize” in flies (Song and Tanouye 2008).

**Fig. 1.** Both  slamdance (\( sda \)) adult flies and larvae display a bang-sensitive (bs) phenotype. A: seizure-like activity can be induced in adult flies using mechanical shock. The mean recovery time in \( sda \) flies was, however, significantly longer compared with wild-type (WT) flies (0.64 \( \pm \) 0.3 vs. 40.5 \( \pm \) 3.6 s, \( n = 190 \) and 120). B: electroshock of third instar larvae (L3; 50 V, 3 s) was sufficient to elicit seizure-like episodes. The mean recovery time was, again, significantly increased in \( sda \) L3 (24.9 \( \pm \) 3.4 vs. 164.9 \( \pm \) 20 s, \( n = 40 \)).

Whereas bs behavior is well documented in adults, how the larval stages of \( sda \), or other bs mutants, respond to similar extreme stimulation is unknown. Because larvae are not suited to mechanical shock testing, we instead applied a brief electric shock to the dorsal cuticle. Such stimulation of WT third instar larvae resulted in a full body paralysis (seizure/paralysis) that lasted, on average, 24.9 \( \pm \) 3.4 s (\( n = 40 \)) before full recovery was observed (Fig. 1B). Full recovery was scored as sustained peristaltic contractions travelling the whole body length of the larva that resulted in sustained forward motion. Partial recovery was frequently observed in body segments that had not been directly stimulated (often posterior to the site of stimulation). This partial recovery would often take the form of the larva attempting, but failing, to resume full body motile behavior. On occasion, a failure of body lifting during the peristaltic wave was also observed. This behavior, in combination with myogenic mouth hook flexing, would cause the larva to move forward (via the mouth hooks) and then backward (via failed body lifting during the peristaltic wave), producing a rocking motion. Both of these behaviors were discounted in terms of “full” recovery. In contrast to WT larvae, identical electroshock of \( sda \) larvae resulted in a significantly increased mean recovery time (164.9 \( \pm \) 20.0 s, \( n = 40 \); Fig. 1B), indicative of a heightened predisposition to seizure-like behavior. Videos showing typical WT and \( sda \) responses to electroshock are available in the Supplemental Material.\(^1\) Identical stimulation was sufficient to increase mean recov-

\(^{1}\) Supplemental Material for this article is available at the Journal of Neurophysiology website.
Sporadic excitation of motoneurons is increased in sda mutants. Our electroshock data indicated that sda larvae, similar to adults, display an increased duration of seizure-like activity in response to extreme stimulation. To determine a possible mechanistic basis for this, we focused our attention on larval motoneurons. These cells, which lie within the CNS, project axons to the body wall muscles that are responsible for the seizure-like activity we observed after electroshock.

Larval motoneurons receive cholinergic excitatory synaptic drive from presynaptic interneurons (Baines 2003). Voltage-clamp recordings (holding potential: $-60 \text{ mV}$) are sufficient to reveal these currents, termed spontaneous rhythmic currents (SRCs) (Rohrbough and Broadie 2002), which occur at a robust frequency, amplitude, and duration. To allow direct comparisons to be made, we restricted our recordings to two motoneurons (aCC and RP2) that receive identical synaptic drive (Baines et al. 2001). The mean cell capacitance of both WT and sda motoneurons (a measure of cell size and potentially cell development) was not statistically different ($12.1 \pm 0.7 \text{ vs. } 11.9 \pm 0.8 \text{ pF}, \text{ WT vs. } \text{ sda motoneurons}, P > 0.05$). Voltage-clamp recordings of SRCs in third instar larvae showed a significant increase in both amplitude ($55.6 \pm 3.2 \text{ vs. } 68.9 \pm 5.4 \text{ pA/pF}$) and duration in sda motoneurons ($1.79 \pm 0.3 \text{ vs. } 2.7 \pm 0.2 \text{ s;} \text{ Fig. 2, A–D, F, and G}$). Analysis of minis (i.e., synaptic currents that remain in the presence of TTX) showed no significant difference in amplitude ($7.5 \pm 1.6 \text{ vs. } 6.2 \pm 0.7 \text{ pA/pF}, \text{ WT vs. } \text{ sda motoneurons, respectively, } P < 0.05$). This suggests that the increased amplitude and duration seen in SRCs is due to increased transmitter release from presynaptic interneurons. In contrast, the frequency of SRCs was significantly reduced in sda motoneurons ($14.4 \pm 2.1 \text{ vs. } 6.0 \pm 1.4 \text{ min}^{-1}; \text{ Fig. 2E}$). Analysis of larval crawling showed that third instar sda larvae are significantly slower than their WT counterparts, reflecting the reduced frequency of motor excitation ($3.2 \pm 0.1 \text{ vs. } 6.2 \pm 0.2 \text{ cm/min peak speed, } P < 0.01$) (W. Ockert and R. A. Baines, unpublished observations). Unfortunately, our attempts to combine simultaneous electroshock with patch recording from motoneurons, in an attempt to determine what happens to network activity during a seizure-like episode, did not prove to be possible (the shock caused a loss of seal resistance on our patch recording). We conclude from these data that network activity in the motor pattern generator that underlies movement in Drosophila larvae is constitutively increased in strength but reduced in frequency in the sda mutation.

To determine how the increased magnitude SRCs in motoneurons influenced action potential firing, we repeated our

![Fig. 2. sda motoneurons are exposed to increased synaptic excitation. A and B: Typical examples of spontaneous rhythmic currents (SRCs) recorded in aCC/RP2 motoneurons (traces are from a RP2 motoneuron in this instance) in WT and sda L3. Scale bars – 200 pA/2 s. C: the mean amplitude of SRCs recorded was significantly greater in sda motoneurons (55.6 ± 3.2 vs. 68.9 ± 5.4 pA/pF). D: the duration of SRCs was also significantly increased in sda motoneurons (1.79 ± 0.3 vs. 2.7 ± 0.2 s). E: the frequency of SRCs observed was significantly reduced in sda motoneurons (14.4 ± 2.1 vs. 6.0 ± 1.4 min$^{-1}$). For all means, n = 8. F and G: cumulative probability plots that better show the distribution of individual current amplitudes (F) and duration (G) for the averaged WT and sda data shown in C and D, respectively.](http://jn.physiology.org/attachment/?aid=10.1152/jn.00342.2010&download=1)
Motoneurons in sda mutants show altered membrane conductances. The increased SRC amplitude and duration we recorded (with voltage clamp) in motoneurons in sda motorneurons are indicative of increased transmitter release by presynaptic interneurons. Our current-clamp recordings also showed larger and longer sustained depolarizations, which were the result of enhanced synaptic excitation. We also considered, however, the possibility that motoneurons are also intrinsically more excitable in sda flies. Consistent with this, a previous study (Ganetzky and Wu 1982) has reported that motor axon excitability in another bs mutant, bang senseless, is increased.

We again used voltage clamp to determine whether changes in membrane conductances also occurred in sda aCC/RP2 motoneurons [peak membrane conductances in these two neurons are near identical, and data were pooled (Baines et al. 2001)]. Initially, we isolated and recorded voltage-gated \( I_{\text{Na}} \) (\( I_{\text{Na}} \) and \( I_{\text{Nat}} \)), \( K^+ \) currents [fast (\( I_{\text{Kf}} \)) and slow (\( I_{\text{Ks}} \)) voltage-dependent \( K^+ \) currents], and \( Ca^{2+} \) current (\( I_{\text{Ca}} \)) in first instar larvae because they provide better space clamp (first instar sda larvae showed the same changes in SRC amplitude, duration, and frequency as seen in third instar larvae; data not shown).

Values for each current component were as follows: \( I_{\text{Kf}} \) 84.1 \pm 5.9 pA/pF, \( I_{\text{Ks}} \) 64.0 \pm 5.5 pA/pF, \( I_{\text{Ca}} \) -21.2 \pm 2.7 pA/pF, \( I_{\text{Nat}} \) -24.1 \pm 2.7, and \( I_{\text{Nap}} \) -4.6 \pm 0.4 pA/pF in WT controls; and \( I_{\text{Kf}} \) 77.4 \pm 14.2 pA/pF, \( I_{\text{Ks}} \) 66.6 \pm 12.0 pA/pF, \( I_{\text{Ca}} \) -13.9 \pm 1.8 pA/pF, \( I_{\text{Nat}} \) -25.5 \pm 2.1 pA/pF, and \( I_{\text{Nap}} \) -7.3 \pm 1.0 pA/pF in sda larvae. Figure 4 shows normalized values for each current investigated, with the comparative WT value set at 100%. Any current value below 100% is reduced, and values above 100% are increased. A particularly striking observation was that \( I_{\text{Nap}} \) was significantly increased in sda larvae (159%), whereas there was no change in \( I_{\text{Nat}} \) (Fig. 4C). This is notable because both current components are mediated by the same ion channel (Paralytic; see DISCUSSION). We also observed a significant reduction in \( I_{\text{Ca}} \) in sda larvae (Fig. 4, A and D) but no change in \( I_{\text{Kf}} \) and \( I_{\text{Ks}} \) (Fig. 4, A and B). To confirm that these changes were maintained in later third instar larvae (which we used for electroshock testing), we repeated our voltage-clamp determinations of both \( I_{\text{Nap}}, I_{\text{Nat}}, \) and \( I_{\text{Ca}} \) in aCC/RP2 motoneurons at this later developmental stage. Despite the poorer space clamp, we found that \( I_{\text{Nap}} \) was again significantly elevated in sda larvae (14.2 \pm 1.2 vs. 7.5 \pm 0.8 pA/pF, \( P < 0.01 \)), whereas \( I_{\text{Nat}} \) remained unchanged from WT controls (see Fig. 5, A and B). Significantly, however, \( I_{\text{Ca}} \) in third instar sda larvae was not different from third instar WT larvae (\(-50.9 \pm 7.8 \) vs. \(-48 \pm 8.2 \) pA/pF; data not shown). While the reason that \( I_{\text{Ca}} \) was only reduced in first instar sda mutants remains unclear, it may be because of differential expression of \( Ca^{2+} \) channels between young and older aCC/RP2 motoneurons. In embryos, the predominant \( I_{\text{Ca}} \) is sensitive to amiloride, indicative of it being similar to vertebrate T-type channels (Baines and Bate 1998). However, by third instar, L-type \( I_{\text{Ca}} \) encoded by \( Ca^{2+} \) channel protein \( \alpha _{1}\)-subunit D (Dmca1D) predominates (Worrell and Levine 2008). Even though the influence of sda on \( I_{\text{Ca}} \) remains to be fully explained, the consistent increase in \( I_{\text{Nap}} \) is indicative that this current underlies heightened seizure activity rather than the reduction in \( I_{\text{Ca}} \), which we observed only in first instar larvae.

To show experimentally how these changes might affect neuronal excitability, we used current clamp to inject depolarizing current and counted the action potentials fired (i.e.,

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**Fig. 3.** Endogenous synaptic drive elicits greater excitation in sda motoneurons. A and B: typical current-clamp recordings, in which the membrane voltage was free to change, showing the sustained depolarizations that arise in aCC/RP2 motoneurons due to presynaptic excitatory input (recordings are shown from an aCC motorneuron in this instance). Notably, these depolarizations were slightly larger and significantly longer in duration and fired considerably more action potentials (arrows) in sda motoneurons (B) compared with their WT counterparts (A). Scale bar = 10 mV/100 ms.
conclude that aCC/RP2 motoneurons, in sda, have an intrinsically reduced membrane excitability. However, whether this reduction is due to the increase in $I_{Na}$ in these neurons remains to be determined (see DISCUSSION). Regardless of the precise mechanism, the reduction in intrinsic excitability in sda motoneurons (as evidenced from controlled injections of constant current; Fig. 4E) is clearly insufficient to fully compensate for the heightened excitation that results from the increased endogenous synaptic drive we observed in this mutation (cf. Fig. 3).

Reduction of $I_{Na}$ rescues bang sensitivity. Our electrophysiology results indicated that motoneurons in sda are exposed to increased synaptic excitatory drive. Drosophila motoneurons, similar to mammalian neurons, are able to compensate for increased synaptic excitation by reducing intrinsic membrane excitability (Baines 2003; Mee et al. 2004). In the case of Drosophila, the increased synaptic excitation of motoneurons is countered, at least in part, by a homeostatic reduction in the expression of voltage-gated Na$^+$ channels, which manifests as significantly reduced $I_{Na}$ in voltage-clamp recordings (Mee et

input-output determination). We performed these experiments in both third (Fig. 4E) and first (data not shown) instar larvae and observed the same results. Injection of constant current into sda aCC/RP2 neurons resulted, on average, in fewer action potentials being fired compared with WT controls. Thus, we
Phenytoin is significant for the behavioral phenotype associated with sda that an increased \( I_{\text{sNap}} \) has already been implicated in human seizure and, moreover, is the target of certain AEDs, including phenytoin (Lampl et al. 1998; Segal and Douglas 1997). To test whether phenytoin affects this, and other, current(s) in Drosophila, we repeated our voltage-clamp analysis of conductances in third instar aCC/RP2 motoneurons with the added presence of phenytoin (30 \( \mu \)M). We found that this amount of phenytoin was sufficient to inhibit \( I_{\text{sNap}} \) by \(-43\% (7.0 \pm 1.0 \text{ vs. } 4.0 \pm 0.5 \text{ pA/pF}, \text{control vs. phenytoin}, n = 5, P \leq 0.05)\). In contrast, this amount of phenytoin did not significantly reduce \( I_{\text{sNa}} \) or \( I_{\text{Ca}} \) (\( I_{\text{Nat}} \) 34 \pm 5.7 vs. 30 \pm 5.5 pA/pF and \( I_{\text{Nat}} \) 48 \pm 8.2 vs. 61 \pm 4.7 pA/pF, WT vs. \text{sda} motoneurons, respectively, \( P > 0.05 \)).

Having established that an acute exposure to phenytoin is sufficient to selectively block \( I_{\text{sNap}} \), we tested whether this AED might influence this current in intact larvae and, by doing so, reduce seizure severity in \text{sda} larvae. To do so, we fed phenytoin to third instar larvae (0.4 mg/ml for 24 h) and initially used voltage clamp to determine the efficacy of action. Figure 5A shows that feeding phenytoin to \text{sda} larvae again had no effect on aCC/RP2 \( I_{\text{Nat}} \). As expected, however, phenytoin ingestion resulted in a significant decrease in \( I_{\text{sNap}} \) in \text{sda} larvae (Fig. 5B). Analysis of \( I_{\text{Ca}} \) showed no effect of phenytoin feeding on this conductance (50.9 \pm 7.8 vs. 48.4 \pm 2.6 pA/pF, vehicle control vs. \text{sda}, respectively, \( P > 0.05 \)). We also noted that feeding phenytoin to WT third instar larvae paradoxically resulted in a significant increase in \( I_{\text{sNap}} \) (with no significant change in \( I_{\text{Nat}} \), Fig. 5B). We next calculated the \( I_{\text{sNap}} \)-to-\( I_{\text{Nat}} \) ratio (\( I_{\text{sNap}}/I_{\text{Nat}} \) with and without phenytoin, which is shown in Fig. 5C. We did this to normalize our data because the absolute values of both \( I_{\text{sNat}} \) and \( I_{\text{sNap}} \) varied from cell to cell. After exposure to phenytoin, \( I_{\text{sNap}}/I_{\text{Nat}} \) in \text{sda} larvae was reduced from 45% to 21%, which was approximately equivalent to that in WT larvae. Treatment of WT larvae with phenytoin increased \( I_{\text{sNap}}/I_{\text{Nat}} \) to 50.7%. To determine whether the electroshock-induced seizure episode induced in \text{sda} larvae could also be ameliorated by treatment with phenytoin, we measured the mean recovery time in identically treated third instar larvae. Feeding phenytoin to \text{sda} larvae significantly reduced the mean recovery time (Fig. 5D). Thus, treatment of \text{sda} with phenytoin not only reduced \( I_{\text{sNap}} \) to near control, non-bs, levels but also reduced the duration of the seizure episode induced by electroshock. We also measured the mean recovery time of WT larvae fed phenytoin, which increased \( I_{\text{sNap}} \) (see above). WT larvae fed with phenytoin exhibited a significantly longer mean recovery time to electroshock, indicating that phenytoin, when fed to WT larvae, acts as a proconvulsive. The same phenomenon has also been reported in rats (Callaghan and Schwark 1980; Rundfeldt et al. 1990) Taken together, our data are consistent with \( s\text{Na} \) being an important contributor to seizure-like behavior in this Drosophila bs mutant.

**Phenytoin rescues synaptic currents.** We showed above that feeding of phenytoin ameliorated seizure-like behavior in \text{sda} larvae. To test whether feeding of phenytoin suppresses mean recovery time by reducing network activity, SRCs of drug-fed third instar \text{sda} larvae were recorded. Third instar larvae were allowed to feed on phenytoin (0.4 mg/ml for 24 h), and SRCs were then recorded from aCC/RP2 motoneurons. Figure 6A shows the effects on SRC amplitude: significant reductions were seen in both WT and \text{sda} larvae when fed phenytoin. Similar marked reductions were seen in synaptic current duration (Fig. 6B), whereas no significant changes were observed for frequency (Fig. 6C). We conclude that feeding phenytoin to \text{sda} larvae reduced SRC amplitude and duration to below that seen in WT controls. This correlates well with the marked recovery time.
reduction we observed in mean recovery time to electroshock in this genotype (Fig. 5D). Moreover, the fact that feeding phenytoin to WT larvae, which is proconvulsive, increased \( I_{\text{Nap}} \) but also reduced SRC amplitude and duration is consistent with \( I_{\text{Nap}} \) contributing to seizure and not increased synaptic excitation.

Increasing \( I_{\text{Nap}} \) generates a bs phenotype in WT larvae. To provide additional support for our hypothesis that seizure-like severity in \( sda \) flies is primarily influenced by the magnitude of \( I_{\text{Nap}} \), we artificially increased this current component in WT third instar motoneurons and tested the effect that this had on mean recovery time to electroshock. To increase \( I_{\text{Nap}} \) we fed third instar larvae rATXII, a toxin known to selectively potentiate this current component in neurons (Hartung and Rathmayer 1985; Mantegazza et al. 1998), including \( Drosophila \) motoneurons (Mee et al. 2004). In contrast, acute (added to recording saline solution) or chronic (24-h feeding) exposure to rATXII had no effect on \( I_{\text{Nat}} \) as measured in third instar larvae (48 ± 8.2 vs. 46.3 ± 16.2 and 42.8 ± 14.4 pA/pF in WT control vs. rATXII acute and rATXII chronic, respectively, \( P > 0.05 \)). Serial dilutions of rATXII were added to grape agar, and WT third instar larvae were allowed to feed freely for 24 h before the electrophysiological determination of \( I_{\text{Nap}} / I_{\text{Nat}} \) and mean recovery time to electroshock. Again, we determined \( I_{\text{Nap}} / I_{\text{Nat}} \) to normalize our data to compensate for any changes in \( I_{\text{Nat}} \) that may also result from the ingestion of this toxin (although this was minimal).

Our results show that there was a strong correlation between \( I_{\text{Nap}} / I_{\text{Nat}} \) and mean recovery time (Fig. 7). For example, feeding rATXII at 40 nM and above resulted in a significant increase in \( I_{\text{Nap}} / I_{\text{Nat}} \) (\( I_{\text{Nat}} \) was unchanged, and, therefore, \( I_{\text{Nap}} / I_{\text{Nat}} \) increased) and also a correspondingly significant increase in mean recovery time. Larvae fed rATXII below this dose (i.e., 10 nM) were borderline statistically different from controls with respect to both \( I_{\text{Nap}} / I_{\text{Nat}} \) and mean recovery time (\( P = 0.05 \)). This dose response indicates that the threshold for a seizure-like phenotype in WT larvae lies close to an \( I_{\text{Nap}} / I_{\text{Nat}} \) of ~35%. Above this level, mean recovery time is significantly increased. This correlation provides strong support for a causal relationship between elevated \( I_{\text{Nap}} \) and an increased seizure-like phenotype. Thus, taken together, our data indicate that elevated \( I_{\text{Nap}} \) contributes to increased seizure-like activity in \( sda \) flies. Therefore, this bs mutant may offer an attractive model for understanding the role of this current in the aetiology of those human epilepsies with which it has been implicated.

**Does a predisposition for seizure-like behavior arise from altered neural development?** Recent studies, in both rats and flies, have provided support for the possibility that at least some forms of epilepsy are due to aberrant early neural development (Blumenfeld et al. 2008; Hekmat-Scafe et al. 2005). This possibility is significant because it offers a potential to cure these epilepsies through early drug intervention. To determine whether the seizure-like phenotype in \( sda \) flies might also arise as a consequence of altered embryonic neural development, we attempted to influence this early stage. To do so, we allowed gravid \( sda \) females to feed continually on food containing phenytoin (0.4 mg/ml in DMSO). We collected the eggs laid by these females on phenytoin-free agar plates and allowed development to third instar larvae to occur in the absence of drug. Our rationale for doing this was to effect the transfer of phenytoin to the developing embryo only (see below). Remarkably, when tested for responses to electroshock at the third instar, the offspring of such phenytoin-fed \( sda \) females showed a complete rescue of mean recovery time relative to vehicle (DMSO)-fed \( sda \) controls (Table 1). Because these third instar larvae were grown in conditions that did not include drug for the duration of larval development, we tentatively conclude that early treatment is sufficient to prevent the bs phenotype characteristic of this mutant. In contrast, feeding phenytoin to gravid WT females resulted in offspring that showed greatly increased mean recovery times (Table 1), which recapitulated the effect of feeding WT larvae this drug for 24 h (cf. Fig. 5). Although we have yet to fully understand this effect, this observation is consistent with disturbed patterns of activity during neurogenesis increasing the likelihood of seizures.

We consider it highly unlikely that phenytoin fed to gravid females would persist in appreciable amounts in larvae during development. However, to measure how much might persist...
I rescued the mean recovery time to electroshock. However, while the actual values for amplitude, duration, and frequency were 113.2 ± 0.9 in WT controls, the progeny of sda treated females effectively rescued the mean recovery time to electroshock when fed directly to sda third instar larvae (data not shown). Thus, we conclude that the amount of phenytoin present in third instar larvae, the stage at which our mutation is significant because this current has already been implicated in human epilepsy and has long been used as a target for drug intervention (George 2005; Rhodes et al. 2004). Our finding serves to validate the use of (at least this) bs mutant as an appropriate model to further our understanding of human epilepsy and, excitingly, for the possible development of novel treatments.

Table 1. Maternal drug feeding suppresses seizure in sda larvae

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean Recovery Time, s</th>
<th>n</th>
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<tbody>
<tr>
<td>WT</td>
<td>31.2 ± 3.8*</td>
<td>40</td>
</tr>
<tr>
<td>WT + DMSO</td>
<td>86.5 ± 3.8*</td>
<td>15</td>
</tr>
<tr>
<td>WT + PHY</td>
<td>187 ± 26.6*</td>
<td>20</td>
</tr>
<tr>
<td>sda</td>
<td>203.5 ± 18*</td>
<td>40</td>
</tr>
<tr>
<td>sda + DMSO</td>
<td>267.3 ± 41.4†</td>
<td>15</td>
</tr>
<tr>
<td>sda + PHY</td>
<td>86.5 ± 12.0‡</td>
<td>20</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of flies tested. Feeding phenytoin (PHY; 0.4 mg/ml) to gravid females resulted in progeny that did not display increased seizures. Progeny were electroshocked at the third instar and were never exposed to PHY other than what might have been transferred to them from the adult female. Wild-type (WT) and slamdance (sda) larvae fed vehicle only (DMSO) exhibited increased mean recovery times, indicating that DMSO has a proconvulsive effect. WT larvae fed PHY showed a greatly increased mean recovery time, indicating that this compound is proconvulsive when fed to normal nonbang-sensitive animals (see RESULTS for more detail). Pair-wise significance is indicated as follows: *‡ P ≤ 0.01 and †P ≤ 0.05.

DISCUSSION

Experimental validity of using Drosophila for human epilepsy research is incomplete. This is primarily because we do not have a good understanding of the specific neurological defects that underlie heightened seizure-like episodes in the many bs mutants that are increasingly being used for such research. To address this shortfall, we exploited a larval preparation that allows unprecedented access to characterize seizure behavior and neuronal function in these mutant backgrounds. Our demonstration that \( I_{\text{Nat}} \) is elevated in sda mutants is significant because this current has already been implicated in human epilepsy and has long been used as a target for drug intervention (George 2005; Rhodes et al. 2004). Our finding serves to validate the use of (at least this) bs mutant as an appropriate model to further our understanding of human epilepsy and, excitingly, for the possible development of novel treatments.

![Fig. 8. Maternal feeding of PHY reduces the ratio of \( I_{\text{Nat}} \) to \( I_{\text{Nat}} \) in F1 progeny. A: feeding gravid sda females PHY (0.4 mg/ml) reduced \( I_{\text{Nat}}/I_{\text{Nat}} \) recorded in aCC/RP2 motoneurons in their L3 progeny. From the point of egg laying to recording, the progeny were not exposed to exogenous PHY. The plots show the distribution of ratios determined for WT L3, sda L3, and sda L3 born from PHY-treated mothers (sda treated). The vertical lines show the population means (21%, 45, and 29%, n = 8, 7, and 15, respectively). sda treated L3 were significantly different from sda L3 (P ≤ 0.01) but not from WT L3 (P > 0.05). B: magnitudes of both \( I_{\text{Nat}} \) and \( I_{\text{Nat}} \) recorded in 15 cells from sda treated L3 shown in A. Magnitudes were compared with sda L3 born from untreated mothers (values for both currents were set to 100%). In 7 of 15 cells (data group 1), \( I_{\text{Nat}} \) was reduced relative to \( I_{\text{Nat}} \). In 7 of 15 cells (data group 2), \( I_{\text{Nat}} \) was increased relative to \( I_{\text{Nat}} \). Open circles show recordings from aCC motoneurons, whereas solid circles show recordings from RP2 motoneurons.

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Although extensively characterized in terms of behavior and genetics, the underlying causes of reduced seizure thresholds and prolonged mean recovery times in bs mutants are unknown (Song and Tanouye 2008). This is arguably because the adult fly has, until recently, been considered intractable for detailed electrophysiology of central neurons. By comparison, application of whole cell recording techniques has been possible in both the embryo and larvae of Drosophila for more than a decade (Baines and Bate 1998). This possibility offered us the opportunity to characterize, in detail, the neuronal abnormalities present in bs larvae. However, it was first essential that we convincingly show that bs larvae share the bs phenotype of their adult counterparts. To do so, we used electroshock, which clearly demonstrated that sda mutants exhibited seizure-like episodes that lasted significantly longer than WT controls. It should be noted, however, that these episodes can be also induced in WT larvae, much as they can in WT adults, but that their severity is much reduced. In bs adult flies, seizure-like activity can be elicited at lower stimulus intensity than in WT adults (Kuebler and Tanouye 2000).

Our analysis of sda shows clear alterations in membrane conductances in, and increased synaptic excitation of, motoneurons. It is tempting to speculate that these events are the cause and effect, respectively. The increased synaptic excitation of motoneurons indicates that the presynaptic interneurons that drive the motor output are more excitable, perhaps due to a similarly increased $I_{\text{Nap}}$ in these cells (although because these interneurons remain unidentified, this is not yet testable). Voltage-gated $I_{\text{Na}}$ in most neurons comprises both rapidly inactivating transient component ($I_{\text{Nap}}$) and a smaller but persistent component ($I_{\text{Nat}}$) (Catterall 2000). While our functional understanding of the former is comprehensive (underlying the depolarizing phase of the action potential), the function of the latter is less clear. $I_{\text{Nap}}$ is activated in the subthreshold voltage range and is believed to contribute to plateau generation, pacemaker activity, and increased firing frequencies (Li and Bennett 2003; Li et al. 2004; Nikitin et al. 2006; Tazerart et al. 2008).

Why then are sda motoneurons, which have increased $I_{\text{Nap}}$, not more excitable than WT motoneurons? Our previous work has identified homeostatic mechanisms in Drosophila motoneurons that are capable of regulating intrinsic membrane conductances to compensate for changing synaptic excitation (Baines et al. 2001). In particular, Drosophila motoneurons dynamically regulate the expression of voltage-gated Na$^+$ channels through the control of translation of Paralytic mRNA (which encodes the sole voltage-gated Na$^+$ channel in flies) to dampen or potentiate action potential firing (Mee et al. 2004; Muraro et al. 2008). Based on these observations, we would predict that exposure to heightened synaptic excitation (observed in sda) would be compensated for by a reduction in ability to fire action potentials in response to a fixed depolarizing input (Baines 2003; Baines et al. 2001). Our observations of reduced action potential firing in these motoneurons in response to injection of fixed depolarizing current is, therefore, validation of such homeostatic mechanisms. Unexpectedly, however, in sda motoneurons we observed no change in $I_{\text{Nat}}$ and, indeed, increased $I_{\text{Nap}}$. Regardless of these uncertainties, analysis of endogenous synaptic drive in sda aCC/RP2 motoneurons clearly showed longer-lived plateau depolarizations that elicited increased action potential firing. We conclude that the homeostatic mechanism that mediates the apparent reduction in membrane excitability in sda motoneurons, if present, is not sufficient to fully compensate for the greatly increased synaptic drive that these neurons are exposed to in this mutant.

This raises the interesting question of whether bs behavior in sda motoneurons is due to a failure of homeostatic compensation. While this remains unclear, it seems likely that such mechanisms, which act to fine tune neuronal excitability, are particularly important during embryonic development when neural networks first form. Alterations in the balance of excitation and inhibition at these early stages may initiate, through self-reinforcing cycles of abnormal activity, inappropriate network activity that results in an increased incidence of seizure-like activity postembryonically (Blumenfeld et al. 2008). Our observations that the presence of phenytoin during embryogenesis had either antiepileptic (sda) or proconvulsive (WT) consequences provides support for such a hypothesis. It is possible that the reduction of $I_{\text{Nap}}$ in backgrounds where it is elevated (i.e., sda) is sufficient to reduce abnormal activity, whereas a reduction below normal levels (i.e., WT) might conceivably evoke such an imbalance of activity. In the sda background, at least, the mechanism of action of phenytoin is seemingly cell type specific. aCC and RP2 motoneurons differ in a number of ways: target muscle, size of synaptic terminal (1b/1s), resting potential, threshold, and delay to first spike (Schaefer et al. 2010). Thus, it is perhaps not surprising that each neuron achieves a reduction in $I_{\text{Nap}}/I_{\text{Nat}}$ through a different mechanism: a reduction of $I_{\text{Nap}}$ in aCC motoneurons versus an increase in $I_{\text{Nap}}$ in RP2 motoneurons. It seems unlikely, however, that these changes are brought about as a direct consequence of phenytoin either blocking $I_{\text{Nap}}$ or potentiating $I_{\text{Nat}}$ because the animals from which we recorded developed in the complete absence of drug. More likely is that the presence of phenytoin during neural development contained the hyperexcitability present in the sda mutation and this allowed the individual neuron types to set an appropriate $I_{\text{Nap}}/I_{\text{Nat}}$.

Perhaps the most striking observation that we reported is that $I_{\text{Nap}}$ increased in sda motoneurons, whereas $I_{\text{Nat}}$ remained unchanged. This is surprising because both components represent different gating modes of the same ion channel (Mee et al. 2004; Vais et al. 2000). The Drosophila voltage-gated Na$^+$ channel, encoded by just a single gene (Paralytic) exhibits extensive alternative splicing (Lin et al. 2009; Loughney et al. 1989). We have recently shown that splicing of two mutually exclusive membrane spanning exons located in domain III3–4 of the channel protein (termed exons K and L) directly influence the magnitude of $I_{\text{Nap}}$ but not $I_{\text{Nat}}$ (Lin et al. 2009). Splice variants containing exon K have a significantly reduced persistent current compared with variants containing exon L. Thus, we predict that a shift in splicing to favor the inclusion of exon L would result in an increase in $I_{\text{Nap}}$ whereas $I_{\text{Nat}}$ would remain unchanged. Verification of this hypothesis will require the isolation of splice variants from the sda CNS.

In summary, human epilepsy is complex to both diagnose and treat because it can arise from a multitude of causes, many of which remain unknown. This lack of understanding may well underlie the fact that a significant number of patients are refractory to drug intervention (Schmidt 2002). The use of mammalian animal models for the development of novel antiepileptics, while appropriate for modelling human epilepsy, has significant limitations. Chief among these are cost and the extensive infrastructure required to screen large numbers. A promising alternative is Drosophila. It has been estimated that...
the genome contains ~75% of all human disease genes (Bier 2005), which include a number of identified genes that, when mutated, display a lower seizure threshold (Song and Tanouye 2008). Additionally, Drosophila has long been used for high-throughput screens, and a recent study (Stilwell et al. 2006) has exploited this significant advantage to screen for novel antiepileptics. Our analysis of sda suggests that heightened seizures in this mutant are due, at least in part, to increased network activity and, in particular, increased $I_{Na}$. 

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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